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Victor R. Preedy
Rajkumar Rajendram *Editors*

L-Arginine in Clinical Nutrition

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Adrianne Bendich, PhD, FACN, SERIES EDITOR

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L-Arginine in Clinical Nutrition

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Preface

In humans, L-arginine is considered as a conditionally essential amino acid. As with many other analytes, it may be harmful in some conditions. For example, L-arginine has been shown to impart an immunomodulatory effect and protects cells against cellular stress. On the other hand, L-arginine depletion has been shown to reduce experimental depression and some cancers depend on L-arginine derived from extracellular pools. The science of L-arginine is also interlinked with nitric oxide metabolism, itself a specialized area of study. On the whole though L-arginine supplementation has been shown to be beneficial to a variety of cells and tissues including immune cells, cardiovascular tissues, liver, muscle, kidney, and bone. However, finding all the relevant and wide ranging information on L-arginine in a single source has hitherto been problematic as the material is so diverse. This is, however, addressed in L-Arginine *in Clinical Nutrition.*

L-*Arginine in Clinical Nutrition* is divided into seven major Parts

1. *Basic Processes at the Cellular Level*
2. L-*Arginine Metabolism and Functions*
3. L-*Arginine Status in Cells Related to Organ Damage and Disease*
4. L-*Arginine Status and Use in Healthy Individuals*
5. L-*Arginine and Diseases of the Gastrointestinal Tract (GI tract)*
6. *Therapeutic Uses of* L-*Arginine: Diabetes, Obesity, and Cardiovascular Diseases*
7. *Therapeutic Uses of* L-*Arginine: Cancer, Sickle Cell Disease, Wound Healing, and Infectious Disease*

The book addresses the science and understanding of L-arginine and coverage ranges from cells to whole organs. The role of L-arginine in healthy individuals and those with various clinical conditions is also described. Contributors are authors of international and national standing, leaders in the field, and trendsetters. Emerging fields of science and important discoveries are also incorporated in L-*Arginine in Clinical Nutrition.*

This book is designed for nutritionists and dietitians, public health scientists, doctors, epidemiologists, health-care professionals of various disciplines, policymakers, and marketing and economic strategists. It is designed for teachers and lecturers, undergraduates and graduates, researchers and professors.

London, UK

Vinood B. Patel
Victor R. Preedy
Rajkumar Rajendram

Series Editor Page

The great success of the Nutrition and Health Series is the result of the consistent overriding mission of providing health professionals with texts that are essential because each includes (1) a synthesis of the state of the science, (2) timely, in-depth reviews by the leading researchers and clinicians in their respective fields, (3) extensive, up-to-date fully annotated reference lists, (4) a detailed index, (5) relevant tables and figures, (6) identification of paradigm shifts and the consequences, (7) virtually no overlap of information between chapters, but targeted, inter-chapter referrals, (8) suggestions of areas for future research, and (9) balanced, data-driven answers to patient as well as health professionals' questions which are based upon the totality of evidence rather than the findings of any single study.

The series volumes are not the outcome of a symposium. Rather, each editor has the potential to examine a chosen area with a broad perspective, both in subject matter and in the choice of chapter authors. The international perspective, especially with regard to public health initiatives, is emphasized where appropriate. The editors, whose trainings are both research and practice oriented, have the opportunity to develop a primary objective for their book, define the scope and focus, and then invite the leading authorities from around the world to be part of their initiative. The authors are encouraged to provide an overview of the field, discuss their own research, and relate the research findings to potential human health consequences. Because each book is developed de novo, the chapters are coordinated so that the resulting volume imparts greater knowledge than the sum of the information contained in the individual chapters.

"L-Arginine in Clinical Nutrition", edited by Vinood Patel, Victor Preedy and Rajkumar Rajendram, is a very welcome addition to the Nutrition and Health Series and fully exemplifies the Series' goals. This volume extends the Series that the editors have developed that includes volumes that review branched chain amino acids and glutamine. L-Arginine belongs to the family of nonessential amino acids as it can be synthesized within the human body mainly in the cells of the liver and kidney. L-Arginine has numerous functions, including its role in removing excess ammonia from the body, and is required for cell division, wound healing, and immune function. L-Arginine is also a component of several important larger, more complex molecules such as proteins. L-Arginine is also often considered a conditionally essential amino acid and has a central role in the synthesis of nitric oxide that is essential for controlling the flow of blood throughout the body. L-Arginine is of critical importance in human physiology and metabolism in healthy individuals and is of great importance during the stresses of injury, inflammation, chronic diseases, and maternal and fetal health. This unique volume represents the first text to provide an integrated review of the biochemistry, metabolism, and roles of L-arginine in human health and disease. The volume includes balanced, data-driven discussions of the beneficial as well as potentially harmful effects of both deficiency and overproduction of nitric oxide. The explosion of clinical research over the last two decades warrants this 48-chapter tome. The volume is designed as an important resource for nutritionists and dietitians, research and public health

scientists, cardiologists, gastroenterologists, and related physicians and health-care professionals who interact with clients, patients, and/or family members. The volume provides objective, relevant information for professors and lecturers, advanced undergraduates and graduates, researchers and clinical investigators who require extensive, up-to-date literature reviews, instructive tables and figures, and excellent references on all aspects of L-arginine's role in human health and disease.

The editors of this volume are experts in their respective fields and represent the medical profession as well as the academic research community. Dr Rajkumar Rajendram, AKC, BSc (Hons), MBBS (Dist), MRCP (UK), FRCA, EDIC, FFICM, is an intensive care physician, anesthetist, and perioperative physician. He was trained in general medicine and intensive care in Oxford, during which period he attained membership of the Royal College of Physicians (MRCP) in 2004. Dr Rajendram then trained in anesthesia and intensive care in the Central School of Anesthesia, London Deanery, and became a Fellow of the Royal College of Anesthetists (FRCA) in 2009. He is one of the first intensivists to become a Fellow of the Faculty of Intensive Care Medicine (FFICM). Dr Rajendram recognized that nutritional support was a fundamental aspect of critical care, and as a visiting lecturer in the Nutritional Sciences Research Division of King's College London, he has published over 100 textbook chapters, review articles, peer-reviewed papers, and abstracts. Dr Vinood B. Patel, BSc, PhD, FRSC, is currently a Senior Lecturer in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in liver disease, particularly related to mitochondrial energy regulation and cell death. Dr Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his PhD in protein metabolism from King's College London. His postdoctoral work was carried out at Wake Forest University Baptist Medical School. Dr Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr Patel has edited biomedical books in the area of nutrition and health prevention, autism, and biomarkers and has published over 150 articles, and in 2014, he was elected as a Fellow to The Royal Society of Chemistry. Professor Victor Preedy is a senior member of King's College London where he is a Professor of Nutritional Biochemistry and Professor of Clinical Biochemistry at King's College Hospital. He is also Director of the Genomics Centre and a member of the School of Medicine. He is a member of the Royal College of Pathologists, a Fellow of the Society of Biology, the Royal College of Pathologists, the Royal Society for the Promotion of Health, the Royal Institute of Public Health, the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry.

Part I: Basic Processes at the Cellular Level

The 48 chapters in this comprehensive volume are organized into seven parts: Basic Processes at the Cellular Level; L-Arginine Metabolism and Functions; L-Arginine Status in Cells Related to Organ Damage and Disease; L-Arginine Status and Use in Healthy Individuals; L-Arginine and Diseases of the Gastrointestinal Tract; Therapeutic uses of L-Arginine: Diabetes, Obesity, and Cardiovascular Diseases; and lastly, Therapeutic Uses of L-Arginine: Cancer, Wound Healing. and Infectious Disease. The eight introductory chapters in the first part provide readers with the basics of L-arginine metabolism so that the more clinically related chapters can be easily understood. The chapters describe investigations into the mechanisms and factors affecting L-arginine transport into cells as well as its metabolism within these cells and its release from cells and/or degradation. Because L-arginine is the major source of nitric oxide (NO) and transport into the cells is a critical regulator of NO production, knowledge of the enzymes and mechanisms involved in the movement of L-arginine into cells is of great value. Several chapters review the recent findings that L-arginine transport is defective in a number of cardiovascular and metabolic diseases. Defective L-arginine transport has been linked to a higher risk of both cardiovascular and diabetes-related morbidity and mortality.

Because of the critical role of L-arginine in cell growth, there are a number of investigations into the potential of altering L-arginine availability in cancer cells, especially those that have lost the capacity to synthesize L-arginine. Descriptions of three novel biochemical analogue classes of potential therapeutics are reviewed, and each chapter contains excellent figures that help the reader to better understand the strategies undertaken to specifically deprive cancer cells of L-arginine while preserving the viability of healthy cells. The introductory chapters also provide reviews of the genetic factors and cellular metabolic processes that link L-arginine status in erythrocytes, lymphocytes, and other immune cells and pancreatic cells with the development of diseases discussed in the therapeutic sections of this volume.

Part II: L-Arginine Metabolism and Functions

Part II contains six chapters that continue the examination of the cellular functions of L-arginine and the factors that affect its metabolism. The first chapter reminds us that in preterm infants, proline, a nonessential amino acid that can cycle back to form L-arginine, and then be cycled to form proline, serves as a limiting precursor of L-arginine. In preterm and term infants, L-arginine is not considered nonessential as it is critical for growth. Adequate levels of proline must be provided to the preterm neonate as rapidly as possible following birth to help avoid L-arginine deficiency and consequent serious oxidative damage. The importance of L-arginine in the release of growth hormone is reviewed in a second chapter that informs us of the use of an L-arginine challenge to determine if there is a growth hormone deficiency in young children who are small for their age. The section also contains chapters that include in-depth discussions of the two major enzymes that utilize L-arginine as their substrate: nitric oxide synthase and arginase. There are also two chapters that review the importance of L-arginine in macrophages. Macrophages contain specific L-arginine transport systems, and the NO formed from L-arginine metabolism is important in killing of pathogens and signaling of other immune cells.

Part III: L-Arginine Status in Cells Related to Organ Damage and Disease

The intensity of research into the effects of L-arginine in different cell types from healthy and diseased tissues and overall effects on the related human organs has resulted in important data reviewed in the six chapters of the fourth part of this insightful volume. As mentioned earlier, L-arginine has been linked to many different functions within the body. Three chapters in this section describe the effects of L-arginine binding to genetic components, DNA, RNA binding proteins, and histones. In each case, L-arginine affects the functioning of these critical molecules and can alter the recognition of these molecules by the immune system, resulting in increased risk of certain autoimmune diseases. Some of the L-arginine-bound DNA molecules are photosensitive and can also increase the risk of adverse immune-mediated reactions. Two chapters examine L-arginine's role at the cellular level in the prevention of cardiovascular disease and stroke. The methylated analogue of L-arginine can compete with L-arginine for the enzyme forming NO and increase the risk of vascular constriction. Altering the balance between these two molecules is the focus of new research strategies. New, preliminary research findings concerning an endogenous amino acid—homoarginine—and its effects on the synthesis of creatine are also reviewed and link this amino acid's competition with L-arginine to increased risk of cardiovascular diseases. Both L-arginine uptake and the catabolic end products are examined as potential effectors of carcinogenesis in colon and breast cancer cells. These cancer cells have enhanced uptake of L-arginine and thereby reduce circulating levels. Potential therapeutic strategies involve the targeting of molecules used to synthesize and/or degrade L-arginine to starve tumor cells of L-arginine.

Part IV: L-Arginine Status and Use in Healthy Individuals

This comprehensive volume contains four parts that focus on the clinical aspects of L-arginine research. Two chapters in this section involve females and the requirements for L-arginine during pregnancy. The two other chapters in this part involve the role of L-arginine in muscle and exercise examined in males. L-Arginine is considered an essential amino acid during human pregnancy, and the lack of sufficient L-arginine is associated with significantly increased risks of low birth weight neonates as well as intrauterine growth retardation. There is also evidence for an increased risk of preeclampsia. Calculations of the increased requirement throughout singleton pregnancies in adult and adolescent pregnancies are provided as are data from women of different ethnic groups. All of the functions of L-arginine are essential to the normal development of the fetus as well as the maternal uterine and fetal placental tissues. The importance of L-arginine and its metabolites in the development of the blood vessels, cells, and other blood components, collagen for bones, and virtually all of the tissues in the developing fetus are reviewed in detail. The requirement for adequate L-arginine begins even before embryo implantation as L-arginine is normally secreted into the uterus during the normal menstrual cycle. L-Arginine's metabolite, NO, is involved in the embryo's implantation and development. The functions of L-arginine throughout gestation are described in detail. The major functions of L-arginine that are so vital for the normal development of the fetus are the same functions that make L-arginine a prime amino acid of interest to athletes and serious strength-training exercisers. Two chapters examine the data linking L-arginine status with skeletal muscle growth and energy production as well as removal of ammonia from the exercised body. The chapters provide objective evaluations of the studies that look at L-arginine, citrulline, and/or ornithine supplementation on exercise performance and improvements in strength.

Part V: L-Arginine and Diseases of the Gastrointestinal Tract (GI Tract)

The six chapters in Part V describe the numerous roles of L-arginine in the GI tract including the molecular interactions, cellular, tissue, and organ responses, and the important animal models used to examine these functions. Chapters review the importance of L-arginine status when the GI tract is exposed to pathogens, increased gastric acidity, inflammation from genetically linked autoimmune disease as well as preterm intestinal failure, imbalances in the microbiome, and ischemia–reperfusion injury. This section contains over 40 figures and tables that enhance the reader's understanding of the importance of L-arginine balance especially in the face of pathogens that can utilize L-arginine to further infect the host. L-Arginine also serves as a buffer in esophagus and stomach as well as a source of repair molecules. There is a chapter that reviews the effects of L-arginine transport defects in chronic inflammation. Further evidence is also provided concerning the provision of L-arginine in the preterm infants where L-arginine is an essential nutrient especially for the rapidly growing intestine. Lastly, L-arginine status can affect the balance between beneficial and harmful bacteria in the colon and reduce the adverse effects of reperfusion in the intestine that is seen following ischemia–reperfusion injury.

Part VI: Therapeutic Uses of L-Arginine: Diabetes, Obesity, and Cardiovascular Diseases

The nine chapters contained in Part VI have a common theme of examining the mechanisms of action previously described for L-arginine in the context of patients with aberrant cardiovascular functions. The first five chapters examine the importance of L-arginine in glucose control and insulin sensitivity

and we are reminded that L-arginine is critical for beta cell function. L-Arginine functions in both Type I and Type 2 diabetics, and laboratory animal models and cell culture studies of these diseases are described. Survey studies and clinical intervention trials are presented in helpful tables, and numerous figures are also included. Novel chapters explore the potential for specific foods, such as cod and related fish, and food engineering of apples to enhance L-arginine intakes. Studies with these products in patients with diabetes are reviewed. The specific effects of L-arginine in adipose tissue and the potential for L-arginine supplementation to be of benefit in weight loss are also reviewed. The chapter authors caution that dosage and duration of use as well as patient characteristics, including sex, age, and concomitant diseases, all impact the potential benefits seen with L-arginine and may in part account for the lack of consistent findings between studies. The next three chapters in this Part look at the important mechanisms of action of L-arginine related to cardiovascular disease. There is a unique chapter in this comprehensive volume that examines the potential for L-arginine to lessen the heart muscle damage associated with inherited defects in mitochondrial DNA. The use of the ratio of L-arginine to ornithine plus citrulline, which is also called the Global L-Arginine Bioavailability Ratio, has been used as an index of potential cardiovascular injury in patients with sickle cell anemia and both cardiovascular and coronary artery disease especially in light of the importance of NO in vascular functioning. However, this ratio may not be of value in hypertensive patients and another marker of L-arginine status, urinary orotic acid, is proposed. At present, there is a dearth of clinical research into the potential effects of L-arginine supplementation in hypertension. The last chapter in this Part reviews the significant adverse vascular effects of sickle cell disease and the potential for L-arginine to reduce the risks of intravascular embolisms and serious pain that may be caused by microvascular blockages. Sickle cell patients are often L-arginine deficient that is worsened during vascular stress. A partial explanation for the L-arginine deficiency especially during episodes is that hemoglobin released from sickled red blood cells destroys NO and may therefore reduce precursor (L-arginine) levels. L-Arginine supplementation has been provided to adults with sickle cell disease with some benefits.

Part VII: Therapeutic Uses of L-Arginine: Cancer, Sickle Cell Disease, Wound Healing, and Infectious Disease

The final Part of the volume includes nine chapters that describe the current state of the data on the potential for L-arginine to impact several major clinical outcomes. The final chapter provides readers with a comprehensive list of resources including relevant journals and volumes related to L-arginine. Five chapters look at the potential of modulating L-arginine concentrations in the patient or directly in tumor cells as a mechanism for controlling tumor growth as several types of tumor cells cannot synthesize L-arginine. Because these tumor cells require exogenous sources of L-arginine, the timing of administration and dose can have widely different effects. In head and neck cancer patients who have undergone surgery to remove tumors, circulating L-arginine levels are often quite low. These cancers may limit the ability to eat and the surgeries also can interfere with swallowing. L-Arginine supplementation has enhanced wound healing and immune responses in these patients, and both presurgery and postsurgery supplementation have been beneficial. Exploratory research studies that target the deprivation of L-arginine to tumors that are unable to synthesize L-arginine are being developed and include the use of albumin to carry L-arginine, the formation of polyarginines, and/or polyethylene glycol modifications that mask the presence of an enzyme for L-arginine metabolism (a foreign molecule) from the host's immune system. There is also new research on animal models of radiation damage to see if L-arginine or another amino acid, glycine, can protect adjacent normal host tissues from irradiation damage. A number of these modalities are in early stage clinical trials. Of importance are the studies of the responses of cancer cells when exposed to L-arginine depletion. In vitro studies document the induction of different pathways in these tumor cells that overcome the L-arginine deficiency. Combination therapies are being developed to respond to this resistance to L-arginine deprivation.

The next three chapters in this comprehensive volume examine the role of L-arginine and its effects on the host's immune system when it is challenged during wounding and bacterial infections. The complex cascade of events involved in wound healing are explained in detail, and L-arginine's function in the formation of proline, a component of collagen, and L-arginine's direct effects on T cells are reviewed. NO is a critical factor in the early stages of wound healing. NO is also a critical factor in the activation of a number of immune cells and is an important molecule used in the killing of bacterial pathogens. When pathogenic bacteria escape the gastrointestinal tract via bacterial translocation, there is an increased risk of sepsis. L-Arginine has been shown to enhance intestinal epithelial integrity and reduce bacterial translocation in animal models. Clinical studies are difficult once sepsis has been initiated and pretreatment of patients at risk is under study. The chapter on tuberculosis describes the complex life cycle of this bacteria and the importance of L-arginine sufficiency to help in the eradication of this disease. Tuberculosis is linked to malnourishment and L-arginine deficiency as well as reduced immune responses to the bacterial infection. Theoretically, L-arginine's numerous functions in the immune system should point to benefits with supplementation; however, clinical intervention trials have not shown these benefits consistently. Further research is needed as tuberculosis continues to be an important infection with no vaccination available. The final chapter contains helpful lists of websites for regulatory bodies and relevant professional organizations around the world; important journals and book volumes that will be helpful for the reader are also tabulated.

The above description of the volume's 48 chapters attests to the depth of information provided by the 145 well-recognized and respected editors and chapter authors. Each chapter includes complete definitions of terms with the abbreviations fully defined for the reader and consistent use of terms between chapters. Key features of the comprehensive volume include over 280 detailed tables and informative figures, an extensive, detailed index, and more than 2500 up-to-date references that provide the reader with excellent sources of worthwhile information. Moreover, the final chapter contains a comprehensive list of web-based resources that will be of great value to the health provider as well as graduate and medical students.

In conclusion, "L-Arginine in Clinical Nutrition", edited by Vinood Patel, Victor Preedy and Rajkumar Rajendram, provides health professionals in many areas of research and practice with the most up-to-date, well-referenced volume on the importance of L-arginine in maintaining the overall health of the individual especially in certain disease conditions. The volume will serve the reader as the benchmark in this complex area of interrelationships between dietary protein and individual amino acid intakes, the unique role of L-arginine in the synthesis of NO, polyamines, and proline for collagen formation, insulin and glucose modulation, and the functioning of major organ systems that are involved in the maintenance of the body's metabolic integrity. Moreover, the physiological, genetic, and pathological interactions between plasma levels of L-arginine and the functioning of the endothelium in the gastrointestinal tract, vascular system, and immune cells are clearly delineated so that students as well as practitioners can better understand the complexities of these interactions. Unique chapters examine the effects of genetic mutations at a number of points in the metabolism of L-arginine and the consequences during fetal development and birth, infancy, and through the aging process. The editors are applauded for their efforts to develop the most authoritative and unique resource on the amino acid, L-arginine, and its role in health and disease, and this excellent text is a very welcome addition to the Nutrition and Health Series.

Adrianne Bendich, Ph.D., F.A.C.N., F.A.S.N.
Series Editor

About the Series Editor

Dr Adrianne Bendich, PhD, FASN, FACN, has served as the "Nutrition and Health" Series Editor for 20 years and has provided leadership and guidance to more than 200 editors who have developed the 70+ well-respected and highly recommended volumes in the Series.

In addition to "L-*Arginine in Clinical Nutrition*", *edited by Rajkumar Rajendram, Vinood Patel, and Victor Preedy*, major new editions published in 2012–2015 and expected to be published shortly include:

1. *Preventive Nutrition: The Comprehensive Guide For Health Professionals, Fifth Edition*, edited by Adrianne Bendich, Ph.D. and Richard J. Deckelbaum, M.D., 2015
2. *Beverage Impacts on Health and Nutrition, Second Edition*, edited by Ted Wilson, Ph.D. and Norman J. Temple, Ph.D., 2015
3. *Nutrition in Cystic Fibrosis: A Guide for Clinicians*, edited by Elizabeth H. Yen, M.D., and Amanda R. Leonard, MPH, RD, CDE, 2015
4. *Glutamine in Clinical Nutrition*, edited by Rajkumar Rajendram, Victor R. Preedy and Vinood B. Patel, 2015
5. *Nutrition and Bone Health, Second Edition*, edited by Michael F. Holick and Jeri W. Nieves, 2015
6. *Branched Chain Amino Acids in Clinical Nutrition, Volume 2*, edited by Rajkumar Rajendram, Victor R. Preedy and Vinood B. Patel, 2015
7. *Branched Chain Amino Acids in Clinical Nutrition, Volume 1*, edited by Rajkumar Rajendram, Victor R. Preedy and Vinood B. Patel, 2015
8. *Fructose, High Fructose Corn Syrup, Sucrose and Health*, edited by James M. Rippe, 2014
9. *Handbook of Clinical Nutrition and Aging, Third Edition*, edited by Connie Watkins Bales, Julie L. Locher and Edward Saltzman, 2014

10. *Nutrition and Pediatric Pulmonary Disease*, edited by Dr. Youngran Chung and Dr. Robert Dumont, 2014
11. "*Integrative Weight Management*" edited by Dr. Gerald E. Mullin, Dr. Lawrence J. Cheskin and Dr. Laura E. Matarese, 2014
12. *Nutrition in Kidney Disease, Second Edition* edited by Dr. Laura D. Byham-Gray, Dr. Jerrilynn D. Burrowes and Dr. Glenn M. Chertow, 2014
13. *Handbook of Food Fortification and Health, volume I* edited by Dr. Victor R. Preedy, Dr. Rajaventhan Srirajaskanthan, Dr. Vinood B. Patel, 2013
14. *Handbook of Food Fortification and Health, volume II*edited by Dr. Victor R. Preedy, Dr. Rajaventhan Srirajaskanthan, Dr. Vinood B. Patel, 2013
15. *Diet Quality: An Evidence-Based Approach*, *volume I* edited by Dr. Victor R. Preedy, Dr. Lan-Ahn Hunter and Dr. Vinood B. Patel, 2013
16. *Diet Quality: An Evidence-Based Approach*, *volume II* edited by Dr. Victor R. Preedy, Dr. Lan-Ahn Hunter and Dr. Vinood B. Patel, 2013
17. *The Handbook of Clinical Nutrition and Stroke*, edited by Mandy L. Corrigan, MPH, RD Arlene A. Escuro, MS, RD, and Donald F. Kirby, MD, FACP, FACN, FACG, 2013
18. *Nutrition in Infancy*, *volume I* edited by Dr. Ronald Ross Watson, Dr. George Grimble, Dr. Victor Preedy and Dr. Sherma Zibadi, 2013
19. *Nutrition in Infancy*, *volume II* edited by Dr. Ronald Ross Watson, Dr. George Grimble, Dr. Victor Preedy and Dr. Sherma Zibadi, 2013
20. *Carotenoids and Human Health*, edited by Dr. Sherry A. Tanumihardjo, 2013
21. *Bioactive Dietary Factors and Plant Extracts in Dermatology*, edited by Dr. Ronald Ross Watson and Dr. Sherma Zibadi, 2013
22. *Omega 6/3 Fatty Acids*, edited by Dr. Fabien De Meester, Dr. Ronald Ross Watson and Dr. Sherma Zibadi, 2013
23. *Nutrition in Pediatric Pulmonary Disease*, edited by Dr. Robert Dumont and Dr. Youngran Chung, 2013
24. *Magnesium and Health*, edited by Dr. Ronald Ross Watson and Dr. Victor R. Preedy, 2012.
25. *Alcohol, Nutrition and Health Consequences*, edited by Dr. Ronald Ross Watson, Dr. Victor R. Preedy, and Dr. Sherma Zibadi, 2012
26. *Nutritional Health, Strategies for Disease Prevention, Third Edition*, edited by Norman J. Temple, Ted Wilson, and David R. Jacobs, Jr., 2012
27. *Chocolate in Health and Nutrition*, edited by Dr. Ronald Ross Watson, Dr. Victor R. Preedy, and Dr. Sherma Zibadi, 2012
28. *Iron Physiology and Pathophysiology in Humans*, edited by Dr. Gregory J. Anderson and Dr. Gordon D. McLaren, 2012

Earlier books included *Vitamin D, Second Edition*, edited by Dr Michael Holick; "*Dietary Components and Immune Function*" edited by Dr Ronald Ross Watson, Dr Sherma Zibadi, and Dr Victor R. Preedy; "*Bioactive Compounds and Cancer*" edited by Dr John A. Milner and Dr Donato F. Romagnolo; "*Modern Dietary Fat Intakes in Disease Promotion*" edited by Dr Fabien De Meester, Dr Sherma Zibadi, and Dr Ronald Ross Watson; "*Iron Deficiency and Overload*" edited by Dr Shlomo Yehuda and Dr David Mostofsky; "*Nutrition Guide for Physician*s" edited by Dr Edward Wilson, Dr George A. Bray, Dr Norman Temple, and Dr Mary Struble; "*Nutrition and Metabolism*" edited by Dr Christos Mantzoros; and "*Fluid and Electrolytes in Pediatrics*" edited by Leonard Feld and Dr Frederick Kaskel. Recent volumes include "*Handbook of Drug-Nutrient Interactions*" edited by Dr Joseph Boullata and Dr Vincent Armenti; "*Probiotics in Pediatric Medicine*" edited by Dr Sonia Michail and Dr Philip Sherman; " *Handbook of Nutrition and Pregnancy*" edited by Dr Carol Lammi-Keefe, Dr Sarah Couch, and Dr Elliot Philipson; "*Nutrition and Rheumatic Disease*" edited by Dr Laura Coleman; " *Nutrition and Kidney Disease*" edited by Dr Laura Byham-Grey, Dr Jerrilynn

Burrowes, and Dr Glenn Chertow; "*Nutrition and Health in Developing Countries*" edited by Dr Richard Semba and Dr Martin Bloem; "*Calcium in Human Health*" edited by Dr Robert Heaney and Dr Connie Weaver; and "*Nutrition and Bone Health*" edited by Dr Michael Holick and Dr Bess Dawson-Hughes.

Dr Bendich is President of Consultants in Consumer Healthcare LLC and is the editor of ten books including "*Preventive Nutrition: The Comprehensive Guide for Health Professionals, Fifth Edition*" coedited with Dr Richard Deckelbaum (www.springer.com/series/7659). Dr Bendich serves on the Editorial Boards of the Journal of Nutrition in Gerontology and Geriatrics, and Antioxidants, and has served as Associate Editor for "Nutrition" the International Journal, served on the Editorial Board of the Journal of Women's Health and Gender-based Medicine, and served on the Board of Directors of the American College of Nutrition.

Dr Bendich was Director of Medical Affairs at GlaxoSmithKline (GSK) Consumer Healthcare and provided medical leadership for many well-known brands including TUMS and Os-Cal. Dr Bendich had primary responsibility for GSK's support for the Women's Health Initiative (WHI) intervention study. Prior to joining GSK, Dr Bendich was at Roche Vitamins Inc. and was involved with the groundbreaking clinical studies showing that folic acid-containing multivitamins significantly reduced major classes of birth defects. Dr Bendich has coauthored over 100 major clinical research studies in the area of preventive nutrition. She is recognized as a leading authority on antioxidants, nutrition and immunity and pregnancy outcomes, vitamin safety, and the cost-effectiveness of vitamin/mineral supplementation.

Dr Bendich received the Roche Research Award, is a *Tribute to Women and Industry* Awardee, and was a recipient of the Burroughs Wellcome Visiting Professorship in Basic Medical Sciences. Dr Bendich was given the Council for Responsible Nutrition (CRN) Apple Award in recognition of her many contributions to the scientific understanding of dietary supplements. In 2012, she was recognized for her contributions to the field of clinical nutrition by the American Society for Nutrition and was elected a Fellow of ASN. Dr Bendich is Adjunct Professor at Rutgers University. She is listed in Who's Who in American Women.

Editor Biography

Vinood B. Patel, BSc, PhD, FRSC, is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in tissue pathology particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol and fatty acids in tissue pathology. Other areas of interest identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimers disease and gastrointestinal dysfunction in autism. Dr Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his PhD in protein metabolism from King's College London in 1997. His post-doctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr Patel is a nationally and internationally recognized liver researcher and was involved in several NIH funded biomedical grants related to alcoholic liver disease. Dr Patel has edited biomedical books in the area of nutrition and health prevention, autism, biomarkers and has published over 150 articles and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy, BSc, PhD, DSc, FRSPH, FRSC, FRSB, FRCPath, is a senior member of King's College London (Professor of Nutritional Biochemistry) and King's College Hospital (Professor of Clinical Biochemistry; Hon). He is attached to both the Diabetes and Nutritional Sciences Division and the Department of Nutrition and Dietetics. He is also Director of the Genomics Centre and a member of the Faculty of Life Sciences and Medicine. Professor Preedy graduated in 1974 with an

Honors Degree in Biology and Physiology with Pharmacology. He gained his University of London PhD in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993, he gained his second Doctoral degree for his contribution to the science of protein metabolism in health and disease. Professor Preedy was elected as a Fellow of the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then, he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health and Hygiene (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy worked at the National Heart Hospital (part of Imperial College London) and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, the USA and Germany. He is a leading expert on biomedical sciences and has a long-standing interest in how nutrition and diet affects well-being and health. He has lectured nationally and internationally. To his credit, Professor Preedy has published over five hundred articles, which includes peer-reviewed manuscripts based on original research, reviews and numerous books and volumes.

Rajkumar Rajendram, BSc, (Hons), MBBS (Dist), EDIC, is a clinician scientist whose focus is on anesthesia, intensive care, and perioperative medicine. Dr Rajendram graduated in 2001 with a distinction from Guy's, King's, and St. Thomas Medical School, in London. As an undergraduate he was awarded several prizes, merits, and distinctions in preclinical and clinical subjects.

Dr Rajendram began his postgraduate medical training in general medicine and intensive care in Oxford. He attained membership of the Royal College of Physicians (MRCP) in 2004 and completed specialist training in acute and general medicine in Oxford in 2010. Dr Rajendram also trained in anesthesia and intensive care in London and became a fellow of the Royal College of Anaesthetists (FRCA) in 2009. He has completed advanced training in regional anesthesia and intensive care. He became a fellow of the Faculty of Intensive Care Medicine (FFICM) in 2013 and obtained the European diploma of intensive care medicine (EDIC) in 2014.

Dr Rajendram returned to Oxford as a Consultant in General Medicine at the John Radcliffe Hospital, Oxford, before moving to the Royal Free London Hospitals as a Consultant in Intensive Care, Anesthesia, and Perioperative Medicine. He is currently a Consultant in Anesthesia and Intensive Care at King Saud University Medical City, Riyadh, Saudi Arabia.

Dr Rajendram recognizes that nutritional support is a fundamental aspect of critical care and perioperative medicine. As a clinician scientist he has therefore devoted significant time and effort into nutritional science research. As a visiting lecturer in the Nutritional Sciences Research Division of King's College London, he has published over 100 textbook chapters, review articles, peer-reviewed papers, and abstracts.

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Part I
Basic Processes at the Cellular Levels

Chapter 1
L-Arginine Uptake by Cells

Geoffrey P. Candy and Marietha J. Nel

Key Points

- Extracellular L-arginine regulates nitric oxide production in many cell types.
- Impaired uptake of L-arginine into cells has been demonstrated in various cardiovascular diseases.
- In endothelial cells, L-arginine uptake is mediated largely by low affinity/high capacity y^+ transport and high affinity/low capacity y^+L transport.
- Eadie Hofstee and other linear plots are not suited to determining kinetic constants for uptake of amino acids by more than one transport system and overestimate the rate of uptake.
- Non-linear methods include the concentration of the radiolabelled amino acid in the equation, allowing statistical analysis of the fit of the model to the data and the error of the calculated constants.
- The contribution of the individual transport to total uptake can be plotted and the type of inhibition, competitive, non-competitive, and uncompetitive, can be readily determined.
- Such methods, while in agreement with the literature, also provided further insight into the mechanisms by which sodium, leucine, and NEM affects uptake of cationic amino acids.
- BCH was found to strongly inhibit y^+L but not y^+ transport.
- Non-linear modelling of L-arginine uptake could be used to further investigate the effects of other factors, such as polarity, hyperglycaemia, and insulin, on L-arginine transport and NO synthesis.

Keywords L-Arginine transport • Non-linear modelling • Amino acid uptake • Endothelial cells • y^+ transport • y^+L transport

Abbreviations

CAA	Cationic amino acid
CAT	Cationic amino acid transport proteins
cts	Counts
Dep.	Dependent

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eNOS	Endothelial nitric oxide synthase enzyme
Eq	Equation
HATs	Heterometric amino acid proteins (HATs)
HUVEC	Human umbilical vein endothelial cells
$[I]$	Concentration of inhibitor
I_{50}	The concentration of inhibitor which inhibits substrate uptake by 50 %
Indep.	Independent
k_D	Diffusion constant
K_i	Inhibition constant
K_m	Michaelis constant or half saturation constant
K_m'	Apparent Michaelis constant or half saturation constant
min	Minute
µM	Micromolar (10^{-6} M)
mM	Millimolar (10^{-3} M)
Na	Sodium
NEM	*N*-ethylmaleimide (NEM
nM	Nanomolar (10^{-9} M)
NO	Nitric oxide
NOS	Nitric oxide synthase
$[S]$	Substrate concentration
SLC	Solute carrier genes
$[T]$	Concentration of radiolabelled trace amino acid
vs.	Versus
v_T	Total rate of uptake
$V_{\max}$	Maximum rates of uptake of substrate
$V_{\max}'$	Apparent maximum rates of uptake of substrate
y^+	Low affinity/high capacity transport
y^+L	High affinity/low capacity cationic transport

Introduction

Cardiovascular disease accounted for nearly 40 % of all deaths in the USA in 2000 [1]. Hypertension, heart failure, and metabolic diseases such as obesity, type 2 diabetes mellitus, and hypercholesterolaemia have impaired endothelial function as a common feature [2]. Endothelial dysfunction, arising from underlying reduced bioavailability of nitric oxide (NO), causes inflammation and oxidative stress to drive the atherosclerotic process [3, 4].

Nitric oxide is a key molecule, regulating vascular tone, vasodilation, and hence blood pressure, and also prevents platelet aggregation and smooth muscle cell proliferation [5]. Nitric oxide is synthesised from the amino acid, L-arginine, by the enzyme nitric oxide synthase (NOS). Extracellular L-arginine regulates NO production in many cell types [6–8], and supplemental L-arginine can decrease blood pressure in patients with hypertension [9, 10]; although benefit in other cardiovascular diseases has been demonstrated, such supplementation is controversial [11].

Therefore, the observation in patients with hypertension that fasting plasma L-arginine concentrations are increased, rather than diminished, is unexpected [12–15]. This can be explained if L-arginine uptake into cells were impaired, and indeed, this has been determined to be the case in various cardiovascular diseases including heart failure, hypertension, and end-stage renal failure [8, 16]. Therefore, the L-arginine transporters have been suggested as possible therapeutic targets in these cardiovascular diseases [8].

In such studies, measurement of the kinetics of cellular L-arginine uptake and determining kinetic constants of L-arginine uptake are essential. It can then be established whether L-arginine uptake is defective in cardiovascular disease and the effects of substances modifying uptake can be determined.

Cationic Amino Acid Transport

Uptake of cationic amino acids into various cell types and platelets has been reviewed previously [17–19]. Comparison of uptake and the value of kinetic constants between studies are complicated by use of different methodologies used to determine constants, the use of different cell types, and the expression of the transporters on different membranes [17–19].

In differentiated human endothelial cells, influx of L-arginine and other cationic amino acids is mediated largely by sodium independent transport. Uptake by low affinity/high capacity transport, termed y^+ transport, is mediated by cationic amino acid transport (CAT) proteins encoded by the solute carrier (SLC) genes. High affinity/low capacity cationic amino acid transport, termed y^+L and $b^{0,+}$ transport, is mediated by heterometric amino acid proteins (HATs), a complex of a glycoprotein (4F2hc) and a carrier protein (y^+LAT) [20, 21] (Table 1.1).

Importantly, neutral amino acids including leucine, methionine, and glutamine are taken up by the y^+L transporter in the presence of sodium, whereas these neutral amino acids are only transported by y^+ transport with low affinity [17, 18]. Independent uptake by y^+L transport can be observed at low concentrations of L-arginine (1–5 μM), when y^+L transport takes up most of the L-arginine, and in the presence of *N*-ethylmaleimide (NEM), which inhibits the low affinity/high capacity (y^+) transport. Differentiating the transport can be confirmed by determining L-arginine uptake in the presence of leucine, which inhibits y^+L uptake only in the presence, but not the absence of sodium (Fig. 1.1) [22, 23].

Measurement of L-Arginine Uptake

Uptake of amino acids by a single transport system generally follows Michaelis–Menten kinetics [24]. Therefore, when two transport systems and diffusion facilitate cationic amino acid influx into cells, the equation modelling influx would be:

$$v_T = \frac{V_{\max a} \times [S]}{(K_{ma} + [S])} + \frac{V_{\max b} \times [S]}{(K_{mb} + [S])} + k_D \times [S] \tag{1.1}$$

where v_T = total rate of uptake; $V_{\max a}$ and $V_{\max b}$ and K_{ma} and K_{mb} are the maximum rates of uptake of substrate S and half saturation constants for transporters a and b, respectively; and k_D is the diffusion constant.

Although kinetic constants have been determined using undiluted radiolabelled amino acids [24–27], most studies have used a trace of radioactively labelled amino acid (10 nM–1 μM) diluted with the unlabelled amino acid [28–31]. As the unlabelled amino acid directly competes for uptake of labelled amino acid, the rate of labelled amino acid uptake declines as the concentration of unlabelled amino acid increases (Figs. 1.2a and 1.3a).

The total rate of uptake has been determined by correcting for the dilution of the labelled amino acid by the unlabelled and plotted against the concentration of substrate as a Michaelis–Menten plot

Table 1.1 Summary of cationic membrane transporters, genes, proteins, and systems [17–21]

Gene	Protein	Transport	Sodium	Km (mM/L)	Neutral amino acids	NEM	pH	Cells	Notes
SLC									
SLC7A1	CAT-1	y^+	Indep.	0.1–0.16; 0.3–0.6	Some low affinity transport	Inhibited	Indep; 5.5–8.5	Many cell types, HUVEC, skin fibroblasts	
SLC7A2	CAT 2A		Indep.	2.15–5.2	No			Hepatocytes	
	CAT 2B	y^+	Indep.	0.19–0.25 (0.70)	No	Inhibited	pH 7.5 optimal	Macrophages, lymphocytes	
SLC7A3	CAT-3	y^+	Indep.	0.20–0.50	No	Inhibited	5.5–8.5		
SLC7A4	CAT-4	–	Indep.	–	–				
SLC7A14	KIAA1613								
HAT subfamily l*-type; LAT: 4F2hc*									
SLC3A2/ SLC7A7	4F2hc/ y^+LAT1	y^+L	Indep.	0.34; 0.01–0.02	+Na (leu, met, gln) inhibit CAA uptake	Not inhibited		HUVEC, erythrocytes, platelets	
SLC3A2/ SLC7A6/	4F2hc/ y^+LAT2	y^+L	Indep.	0.12–0.14	+Na			HUVEC, erythrocytes	
SLC3A1/ SLC7A9	rBAT/ $b^{0,+}$AT	$b^{0,+}$	Indep.	0.080–0.20	Na-indep; inhibit CAA uptake			Blastocysts, small intestine	Broad scope transport
SLC6A14	AT $B^{0,+}$	$B^{0,+}$	Dep. $+Cl^-$	0.10–0.15	+Na $+Cl^-$			Blastocysts, small intestine, brain kidney	Broad scope transport

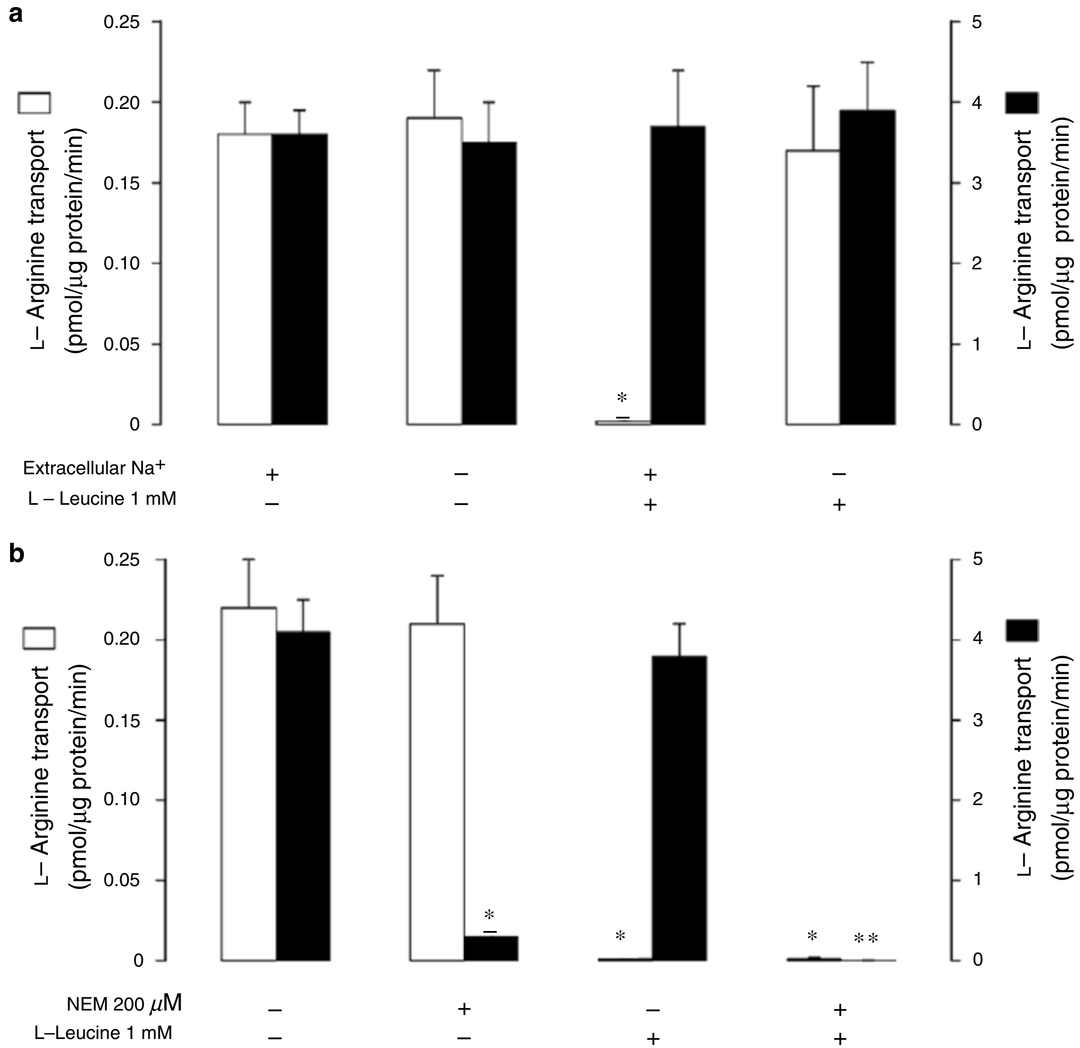

Fig. 1.1 Effect of Na^+ on L-leucine inhibition of L-arginine transport in human umbilical endothelial cells. Transport in 1.5 µM (*unfilled bars*) or 100 µM (*filled bars*) L-arginine (4 µCi mL^{-1} L-arginine, 1 min, 37 °C) was determined in Na^+-Krebs (see Methods [23]). (**a**) L-arginine transport in the absence or presence of L-leucine in Krebs solution with or without Na^+. (**b**) L-arginine transport in Na^+-Krebs in the absence or presence of L-leucine in cells pre-incubated (10 min) with 200 µM NEM. $^*p<0.05$ versus corresponding control values. $^{**}p<0.01$ versus corresponding value in the presence of NEM ($n=12$). Reproduced from Arancibia-Garavilla et al. [23] with permission from John Wiley & Sons, Inc.

(Total v vs. [S]; Figs. 1.2d and 1.3d). These data have been linearised, either as Lineweaver–Burk or Eadie–Hofstee plots (Figs. 1.2c and 1.3c). In the presence of more than one uptake transporter, the plots resolve into two linear components from which apparent kinetic constants, V_{max}' and K_m' for each component can be calculated (Figs. 1.2c and 1.3c). The discontinuity or change in slope of the graph results from the predominant uptake by high affinity/low capacity transport at low substrate concentrations, whereas at higher substrate concentrations the low affinity/high capacity transport predominates.

However, these are apparent and combined constants of total uptake, and if inserted into Eq. (1.1), and a theoretical rate of uptake, v_T, versus [S] is plotted, the theoretical uptake exceeds the

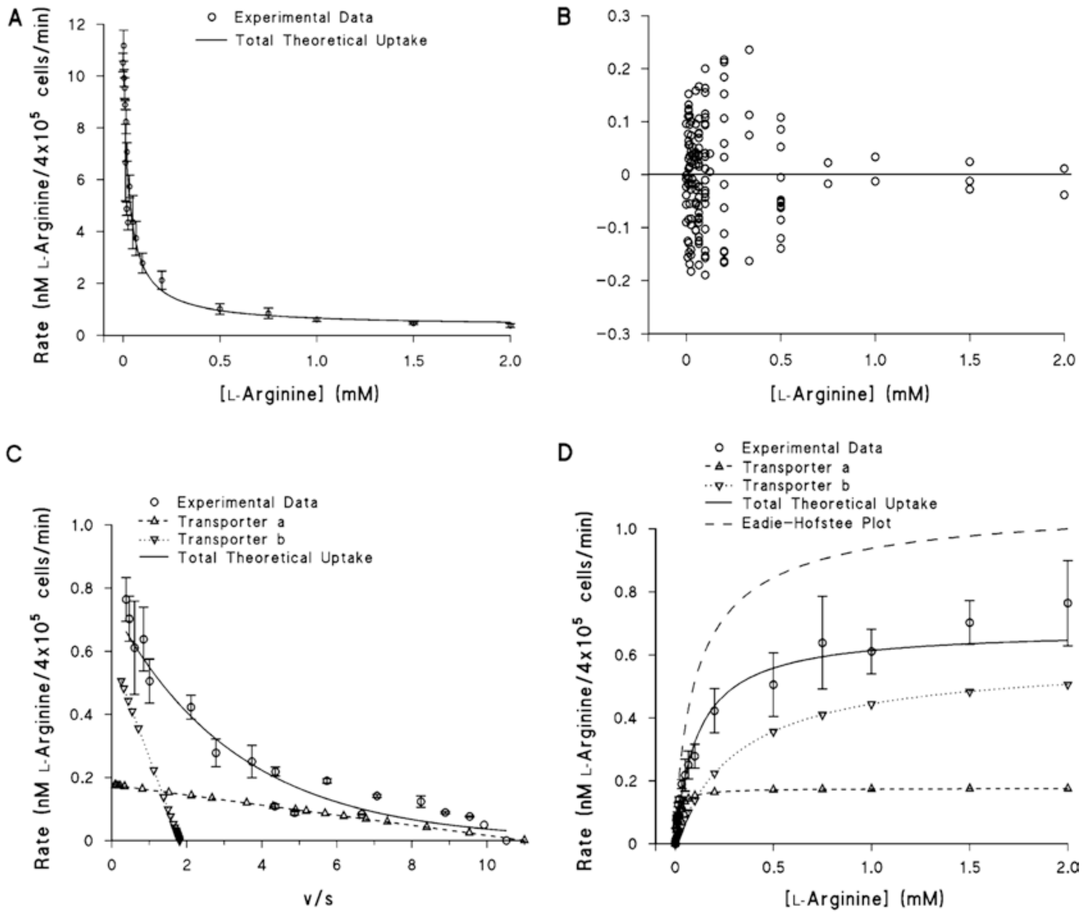

Fig. 1.2 Data for ECV_{304} to determine kinetic constants. Mean ± std of experimental data is shown with theoretical contributions of total uptake determined by non-linear modelling (the fitted curve) and theoretical contributions of individual transport (*stippled lines* for transport "*a*" (y^+L) and transport "*b*" (y^+). (**a**) Inhibition of uptake of 10 nM ^{3}H-arginine by unlabelled L-arginine; (**b**) Insert of residuals for graph (**a**): D'Agostino and Pearson omnibus $K2$: $p = 0.49$ for the difference between experimental L-arginine uptake and the theoretical model contributions; (**c**) Eadie–Hofstee plots calculated from (**a**); (**d**) Michaelis–Menten plots showing experimental dilution corrected total uptake calculated from (**a**), with theoretical uptake determined by non-linear modelling. Reproduced from Nel et al. [31] with permission from Springer Press

experimental data (Figs. 1.2d and 1.3d). Such methodologies overestimate the V_{max}, and the calculated K_m may not be accurate for both transport systems. Therefore, these methodologies are not suited to determine kinetic constants where uptake is facilitated by more than one transporter [31, 32]. Furthermore, in early studies [24], subtracting a first-order diffusion component may have resulted in the high affinity/low capacity (y^+L) transport being overlooked [17, 18]. Thus, other methods were required to determine the kinetic constants of two and more transport systems acting on one substrate.

Devés et al. [22] used a permeability ratio, which is a constant combining the K_m and V_{max} for each of the two transport systems. To calculate the kinetic constants, the ratio was determined in the presence of competitive substrates, including unlabelled L-arginine, as well as in the presence of an irreversible inhibitor of one of the transports. When calculating this ratio, leucine was taken to be a competitive inhibitor of L-arginine uptake and NEM used to inhibit the y^+ transport. It was by using this method that the presence of the high affinity/low capacity (y^+L) transport was demonstrated [22, 33].

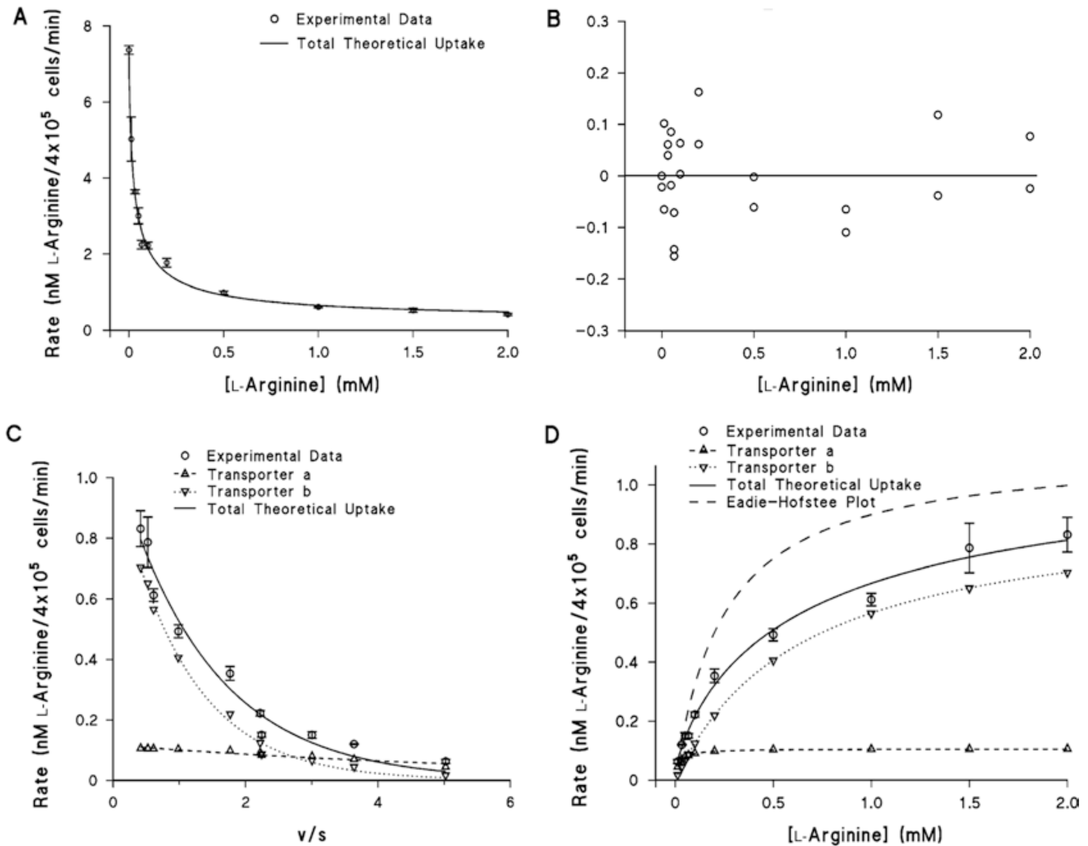

Fig. 1.3 Data for HUVEC to determine kinetic constants. Mean ± std of experimental data is shown with theoretical contributions of total uptake determined by non-linear modelling (the fitted curve) and theoretical contributions of individual transport (*stippled lines* for transport "*a*" (y^+L) and transport "*b*" (y^+). (**a**) Inhibition of uptake of 10 nM ^{3}H-arginine by unlabelled L-arginine; (**b**) Insert of residuals for graph (**a**): D'Agostino and Pearson omnibus $K2$: $p = 0.49$ for the difference between experimental L-arginine uptake and the theoretical model contributions; (**c**) Eadie–Hofstee plots calculated from (**a**); (**d**). Michaelis–Menten plots showing experimental dilution corrected total uptake calculated from (**a**), with theoretical uptake determined by non-linear modelling. Reproduced from Nel et al. [31] with permission from Springer Press

Other studies have calculated the I_{50}, the concentration at which the neutral amino acid competitively inhibits L-arginine uptake by 50 % [34–37]. When calculating the I_{50} to elucidate the effect of neutral amino acids on cationic amino acid transport, the fit of other models of inhibition, non-competitive and uncompetitive, should be tested to determine whether these equations better fit the data [38].

Non-linear Modelling of Uptake

Methods for calculating kinetic constants for single substrates using two transport systems (enzymes) have not been well described. Constants have been determined by iteratively adjusting the values of the constants to best fit a model of uptake to the experimental data [39]. Alternatively, non-linear methods have the major advantage in allowing statistical analysis of the fit of a mathematical model (Eq. 1.1) to the experimental data and to determine the error of the calculated constants [32, 40, 41].

Malo and Berteloot [32] used a fast sampling, rapid filtration apparatus to determine the uptake of glucose into the membrane vesicle and, importantly, accounts for the concentration of the radiolabelled amino acid relative to that of the unlabelled amino acid, and which determines the slope of the curve illustrated in Figs. 1.2a and 1.3a.

Modelling the rate of uptake (v_T) of trace labelled amino acid ([T]) in the presence of unlabelled amino acid ([S]) by two transport systems (denoted by the subscripts a and b), both following Michaelis–Menten kinetics and a diffusion component (k_D*[T]), can be described (Malo and Berteloot 1991) [32] by

$$v_T = \frac{V_{\max a} \times [T]}{(K_{ma} + [T] + [S])} + \frac{V_{\max b} \times [T]}{(K_{mb} + [T] + [S])} + k_D \times [T] \tag{1.2}$$

where the symbols are as for Eq. (1.1) and [T] is the concentration of the labelled amino acid. The equation can be modified to test for additional transporters to determine the presence of an inhibition or, indeed, activation of transport [42].

Equation (1.2) requires the calculation of four unknowns and the k_D. Constants estimated from Lineweaver–Burk and Eadie–Hofstee plots can provide useful initial estimates of V_{maxa} and V_{maxb} and K_{ma} and K_{mb} in the equation. The calculation of these data points requires >10 data points per determination to achieve a robust estimate of the constants and many more to achieve stability in the calculation, i.e. a small standard error in constants.

The rapid flow apparatus used for glucose uptake by more than one transporter allows many data points to be obtained in a very short time period [32, 43]. Amino acid uptake appears to be an order of magnitude slower [24] than glucose uptake, which with fewer transporters than glucose, makes the determination of constants easier.

Although Lineweaver–Burk and Eadie–Hofstee plots may indicate the type of inhibition present, these are appropriate for uptake by a single transport system. Using Eq. (1.2), the type of inhibition can be identified by determining changes in the kinetic constants as (where K_i is the inhibition constant):

- Only K_m changes ($K_m' = K_m(1 + [I]/K_i)$) with competitive inhibition
- The apparent V_{max}' becomes $V_{max}' = V_{max}/(1 + [I]/K_i)$ with non-competitive inhibition, and
- K_m'/V_{max}' (the slope of a Lineweaver–Burk plot $1/v$ versus $1/[S]$) would remain constant with uncompetitive inhibition, as ($K_m' = K_m/(1 + [I]/K_i)$) and $V_{max}' = V_{max}/(1 + [I]/K_i)$

Including the inhibition constants in the Eq. (1.2) would require the calculation of additional unknowns, and the equation would need to be modified and tested for the combinations of the possible types of inhibition of each transport system.

These unknowns can be calculated by best fit of the model to the experimental data Eq. (1.2) using programs such as GraphPadPrism® (Version 5, GraphPad Software Inc., La Jolla, California, USA). This program allows statistical comparison between the calculated kinetic constants and models to best fit the experimental data. Ideally, the units of K_m and V_{max} should be adjusted so that the numerical values of the constants are of the same order of magnitude. If not, the larger numerical value will be preferentially adjusted. The program allows an initial “robust” calculation of the kinetic constants to provide useful initial estimates of the constants. Finally, outliers in the dataset can be detected from residual plots (Figs. 1.2b and 1.3b).

It is useful to check the results by substituting the calculated constants obtained from Eq. (1.2) with the dilution-corrected data and re-calculating best fit values for the constants using Eq. (1.1). Similarly, in the presence of inhibitors, once the type of inhibition has been determined, Eqs. (1.1 and 1.2) can

be modified, and the value of the constants confirmed. Little change in the value of the constants provides confidence in the determination. Having determined the constants, the individual contributions of y^+L and y^+ transport can be resolved with the dilution corrected data, over a range of substrate concentrations with Michaelis–Menten v versus $[S]$ plots (Eq. 1.1; Figs. 1.2d and 1.3d) [31].

Validation of a Non-linear Method Determining L-Arginine Uptake

Using this non-linear approach, Nel et al. [31] determined the uptake of 10 nM ^{3}H-arginine in the presence of unlabelled L-arginine, into human umbilical vein endothelial cells (HUVEC) and "endothelial-like" ECV_{304} cells. Further details of methodology to determine the kinetic constants are described in this chapter [31].

In the absence of inhibitors (Table 1.2):

(a) Under the experimental conditions used, the error in the diffusion constant was large and a model without diffusion was preferred for both cell types. Given the low concentration of labelled L-arginine (10 nM) used in this study, the contribution of diffusion could not be determined accurately.
(b) The maximal rate of L-arginine uptake for both the high affinity/low capacity transport (y^+L) and low affinity/high capacity transport (y^+) was similar in both cell types.
(c) y^+L was responsible for most of the L-arginine uptake, up to a concentration of 100 µM L-arginine, and was maximal above 300 µM L-arginine. However, above a concentration of 100 µM, the low affinity/high capacity y^+ transport facilitated most of the L-arginine uptake.
(d) When the calculated constants were fitted into a plot of v_T versus $[S]$, the theoretical plot closely matched the experimental data (Figs. 1.2d and 1.3d).
(e) The contributions of the individual transport systems could be plotted (Figs. 1.2d and 1.3d).

Table 1.2 Comparison of kinetic constants obtained from non-linear modelling of L-arginine uptake into ECV_{304} and HUVEC cells

	ECV_{304}		HUVEC	
	Model without diffusion[a]	Model with diffusion	Model without diffusion[b]	Model with diffusion
V_{maxa} (×10^6 cts/4×10^5 cells/min)	3.133±0.635 (4.065)	2.715±0.169 (4.061)	1.844±0.443 (2.114)	1.577±0.644 (2.079)
K_{ma} (mM)	0.018±0.004 (0.021)	0.017±0.004 (0.021)	0.018±0.004 (0.020)	0.016±0.005 (0.020)
V_{maxb} (×10^6 cts/4×10^5 cells/min)	11.245±0.738 (11.256)	9.195±1.831 (11.175)	16.234±1.340* (15.686)	11.236±4.497 (14.610)
K_{mb} (mM)	0.358±0.106 (0.550)	0.239±0.125 (0.546)	0.646±0.170 (0.658)	0.401±0.264 (0.611)
k_D (diffusion constant)	–	1.142×10^6±1.152×10^6 (0.036×10^6)	–	1.804×10^6±1.864×10^6 (0.406×10^6)

Data from 10 experiments were combined for the calculation of kinetic constants for ECV_{304} cells and duplicate experiments were used for the HUVEC calculations. Results are the mean±SEM with robust values in *brackets*. The differences between ECV_{304} versus HUVEC without diffusion (*$p<0.05$) and with diffusion: not significant for all parameters. Abbreviation: *cts* counts. Reproduced from [31] with permission from Springer Publishers
a: lower capacity/higher affinity (y^+L) transport; *b*: higher capacity/lower affinity (y^+) transporter
[a]Preferred model in ECV_{304} [$F=0.536$ ($n=210$), $p=0.46$]; note the high SEM for k_D
[b]Preferred model in HUVEC [$F=0.863$ ($n=23$), $p=0.37$]; note the high SEM for k_D

Effect of Salt and Inhibitors on L-Arginine Uptake

Incubation of cells in the presence or absence of sodium and various substances inhibiting transport has allowed the distinction between y^+ and y^+L transport [22, 23, 37, 44]. To test the model described above, L-arginine uptake by ECV_{304} cells was determined in the presence of these substances.

Effect of sodium: Cationic amino acid transport is largely independent of sodium. In the presence of sodium, neutral amino acids can also be taken up by y^+L and inhibit cationic uptake (Table 1.1) [17–19]. The question arises as to how sodium facilitates neutral amino acid uptake? It was suggested that sodium occupies the site normally occupied by the ω-amino group of cationic amino acids and thus neutral amino acids may be transported [45]. Therefore, it follows that in the presence of sodium, the ω-amino group of cationic amino acids would have to displace this sodium in order to facilitate transport. Nel et al. [31] determined the effect of sodium alone on L-arginine uptake in:

- Krebs buffer (157 mM sodium),
- Krebs buffer with choline chloride (26 mM sodium) replacing sodium chloride, and
- Krebs buffer with all sodium salts replaced with the equivalent potassium salt and choline chloride (0 mM sodium) [23, 45, 46].

Only the absence of sodium reduced both the rate and the affinity (K_{ma}) of L-arginine uptake by y^+L transport. Inhibition was uncompetitive, as the ratio of K_{ma}/V_{ma} (slope of a Lineweaver–Burk plot) remained constant. Reducing sodium decreased the rate of uptake by y^+ transport (V_{mb}) with the affinity (K_{mb}) remaining constant, in keeping with non-competitive inhibition (Table 1.3). Using non-linear modelling, the data suggested that sodium ions are required for the functional integrity of the transport complexes or interact with the L-arginine-transport complex to facilitate uptake [31].

Effect of leucine: The inhibition of lysine uptake by leucine in erythrocytes led to the discovery of the y^+L transport system [22]. In the presence of sodium, neutral amino acids, including leucine, glutamine, methionine, and others, inhibit cationic amino acid uptake by y^+L transport [17, 24]. Such inhibition has been described as competitive in erythrocytes [33], HUVECs [23, 36], platelets [44], and human airways cells [37]. These studies did not determine the effect of low concentrations of leucine on cationic amino acid uptake.

Using the non-linear modelling approach, inhibition of L-arginine uptake by leucine was determined to be complex, affecting L-arginine uptake by y^+L transport through mixed inhibition. L-Arginine uptake by y^+ transport was inhibited in a competitive-like manner at leucine concentrations ≤200 mM [31]. As leucine did not completely abolish L-arginine uptake, it is possible that L-arginine uptake was facilitated by transport other than y^+L and y^+ [47], which was unaffected by the presence of leucine. Further studies are needed to elucidate the mechanism of inhibition by leucine, and indeed other neutral amino acids, of cationic amino acid uptake.

Table 1.3 Kinetic constants for ECV_{304} cells determined at various sodium concentrations

	Sodium 157 mM	Sodium 26 mM	Sodium 0 mM
V_{maxa} ($\times 10^6$ cts/4×10^5 cells/min)	2.581 ± 0.412 (3.185)	3.263 ± 0.612 (3.977)	1.710 ± 0.126$^{**,\wedge}$ (2.043)
K_{ma} (mM)	0.016 ± 0.002 (0.019)	0.017 ± 0.003 (0.021)	0.011 ± 0.001$^{*,\wedge\wedge}$ (0.012)
V_{maxb} ($\times 10^6$ cts/4×10^5 cells/min)	11.384 ± 0.348 (11.924)	5.625 ± 0.478*** (6.082)	6.249 ± 0.141*** (7.061)
K_{mb} (mM)	0.199 ± 0.025 (0.277)	0.190 ± 0.060 (0.347)	0.204 ± 0.020 (0.315)

Results as mean ± SEM and the robust values in *brackets*. Reproduced from [31] with permission from Springer Publishers

Sodium 157 mM versus 26 or 0 mM: $^{*}p<0.05$; $^{**}p<0.005$; $^{***}p<0.0005$; Sodium 26 mM versus 0 mM: $^{\wedge}p<0.005$; $^{\wedge\wedge}p<0.0005$; Abbreviation: *cts* counts

Table 1.4 Effect of pre-incubation of the inhibitors NEM (0.2 mM) and BCH (30 mM) on L-arginine uptake by ECV_{304} cells

	NEM		BCH	
	0 mM	0.2 mM	0 mM	30 mM
V_{maxa} ($\times 10^6$ cts/4×10^5 cells/min)	$2.66 \pm 0.53 \times 10^6$ (3.81×10^6)	$6.70 \pm 0.20 \times 10^6$ (16.71×10^6)	$3.53 \pm 0.31 \times 10^6$ (4.15×10^6)	$0.13 \pm 0.04 \times 10^{6***}$ (0.12×10^6)
K_{ma} (mM)	0.023 ± 0.004 (0.031)	0.062 ± 0.004 (~0.001)	0.027 ± 0.002 (0.030)	$0.002 \pm 0.001^{***}$ (0.002)
V_{maxb} ($\times 10^6$ cts/4×10^5 cells/min)	$4.94 \pm 0.39 \times 10^6$ (5.21×10^6)	$\sim 1.239 \times 10^{21***,a}$ (–)	$20.55 \pm 1.84 \times 10^6$ (1.003)	$23.87 \pm 1.16 \times 10^6$ (23.58)
K_{mb} (mM)	0.243 ± 0.078 (0.682)	$\sim 3.261 \times 10^{17***,a}$ (–)	0.665 ± 0.096 (1/026)	0.602 ± 0.048 (0.595)

[a]After pre-incubation with NEM, the best fit of V_{maxb} and K_{mb} defaulted to a large value (∞), with an ambiguous result. Hence, a single transport model with diffusion was preferred

Results as mean ± SEM and robust values in *brackets*. Reproduced from [31] with permission from Springer Publishers

$^{***}p < 0.0001$ versus without inhibitor. Abbreviation *cts* counts

NEM and BCH: The reagent for sulphydryl groups, *N*-ethylmaleimide (NEM), inhibits L-arginine uptake by y^+ transport, whereas 2-amino-2-norboranecarboxylic acid (BCH), a sodium-independent system-L transport-specific substrate, has little effect on cationic transport in human fibroblasts, Ehrlich cells [48], and erythrocytes [33].

In agreement with the literature, NEM (0.2 mM) inhibited y^+ transport in ECV_{304} cells, whereas y^+L transport was relatively unaffected (Table 1.4) [31]. In the presence of sodium, BCH (30 mM) almost completely abolished L-arginine uptake by y^+L transport (96 %; Table 1.4), in contrast to the finding, where 5 mM BCH decreased uptake by 16.5 ± 3.5 % [33]. BCH may thus be an inhibitor of L-arginine uptake by y^+L transport.

Kinetics of L-Arginine Transport and the L-Arginine Paradox

Plasma and intracellular L-arginine concentrations are significantly higher than the Michaelis–Menten constant for NOS (K_m = 2.9–10 μmol/L), yet extracellular L-arginine is required to increase NO synthesis by cells. Various mechanisms have been proposed to explain this phenomenon [2, 11].

L-Arginine appears to be compartmentalised within cells, with a second pool less accessible within the caveolae, in proximity of the CAT-1 transporter which has been shown to be co-localised with endothelial NOS (eNOS) enzyme. Thus extracellular L-arginine would be readily available for transport across the cell membrane to the eNOS for NO production [49]. However, several workers [46] have shown that increases in NO production were associated with y^+L transport and were unaffected when y^+ transport was inhibited by NEM. The finding that the K_m for y^+L transport is close [23, 31] to that of eNOS [18] has led to the suggestion that y^+L transport is important in NO production by eNOS, whereas y^+ transport is important for NO production by the inducible NO synthase [22].

Conclusions

Investigations of the mechanisms and factors affecting L-arginine transport into cells have determined that such transport is a critical regulator of NO production. Such transport is defective in a variety of cardiovascular and metabolic diseases with higher risk of both morbidity and mortality. In such

studies measurement of the kinetic constants of L-arginine uptake is essential before the effects of substances modifying uptake can be determined. However, methodologies using linear transformations of rate data, such as Lineweaver–Burk and Hofstee plots, are not suited to determine kinetic constants where uptake is facilitated by more than one transport system.

However, non-linear modelling of uptake allows uptake by more than one transport system to be determined and can include the concentration of the labelled amino acid used in experiments. Such modelling allows statistical analysis of both the fit of the model to the experimental data and the error in the calculated constants. Such methods, while in agreement with the literature, also provide further insight into the mechanisms by which sodium, leucine, and BCH affect uptake of cationic amino acids. They could be used to further investigate the effects of other factors, including polarity, hyperglycaemia, and insulin, on L-arginine transport and NO synthesis.

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The authors referred to and used GraphPadPrism® (Version 5, GraphPad Software Inc., La Jolla, California, USA) in their studies and found the software useful. However, reference to this product is not an endorsement of the software and the authors have no financial conflict of interest by referring to this product. Other software could be used to undertake the required calculations and obtain the results presented.

References

1. American Heart Association. Heart disease and stroke statistics—2003 update. Dallas, TX: American Heart Association; 2002.
2. Bode-Böger SM, Scalera F, Ignarro LJ. The L-arginine paradox: importance of the L-arginine/asymmetrical dimethylarginine ratio. Pharmacol Ther. 2007;114:295–306.
3. Tousoulis D, Kampoli A-M, Papageorgiou CTN, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012;10:4–18.
4. Vita JA. Endothelial function. Circulation. 2011;124:e906–12.
5. Qain J, Fulton D. Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. Front Physiol. 2013;4:1–11.
6. Sessa WC, Hecker M, Mitchell JA, Vane JR. The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. Proc Natl Acad Sci USA. 1990;87:8607–11.
7. Simmons WW, Closs EI, Cunningham JM, et al. Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes. J Biol Chem. 1996;271:11694–702.
8. Chin-Dusting JPF, Willems L, Kaye DM. L-arginine transporters in cardiovascular disease: a novel therapeutic target. Pharmacol Ther. 2007;116:428–36.
9. Siani A, Pagano E, Lacone R, et al. Blood pressure and metabolic changes during L-arginine supplementation in humans. Am J Hypertens. 2000;13:547–51.
10. Ast J, Jablecka A, Bogdanski P, et al. Evaluation of the antihypertensive effect of L-arginine. Med Sci Monit. 2010;16:CR266–71.
11. Dioguardi FS. To give or not to give? Lessons from the L-arginine paradox. J Nutrigenet Nutrigenomics. 2011;4:90–8.
12. Penttinen J, Pennanen S, Liesivuori J. Indicators of L-arginine metabolism and cardiovascular risk factors. A cross-sectional study in healthy middle-aged men. Amino Acids. 2000;18:199–206.
13. Moss MB, Brunini TMC, Soares de Moura R. Diminished L-arginine bioavailability in hypertension. Clin Sci. 2004;107:391–7.
14. Perticone F, Sciacqua A, Maio R, et al. Asymmetric dimethylarginine, L-arginine and endothelial dysfunction in essential hypertension. J Am Coll Cardiol. 2005;46:518–23.
15. Naidoo C, Cromarty AD, Snyman T, et al. Relationships between plasma amino acid concentrations and blood pressure in South Africans of African descent. SA Heart. 2009;6:142–7.
16. Mendes-Ribeiro AC, Brunini TMC, Ellory JC, Mann GE. Abnormalities in L-arginine transport and nitric oxide biosynthesis in chronic renal and heart failure. Cardiovasc Res. 2001;49:697–712.

17. Devés R, Boyd CAR. Transporters for cationic amino acids in animal cells: discovery, structure, and function. Physiol Rev. 1998;78:487–545.
18. Mann GE, Yudilevich DL, Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiol Rev. 2003;83:183–252.
19. Bröer S. Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev. 2008;88:249–86.
20. Closs EI, Boissel JP, Habermeier A, Rotmann A. Structure and function of cationic amino acid transporters (CATs). J Membr Biol. 2006;213(2):67–77.
21. Díaz-Pérez F, Radojkovic C, Aguilera V, et al. L-Arginine transport and nitric oxide synthesis in human endothelial progenitor cells. J Cardiovasc Pharmacol. 2012;60:439–49.
22. Devés R, Chavez P, Boyd CAR. Identification of a new transport system (y^+L) in human erythrocytes that recognizes lysine and leucine with high affinity. J Physiol. 1992;454:491–501.
23. Arancibia-Garavilla Y, Toledo F, Casanello P, Sobrevia L. Nitric oxide synthesis requires activity of the cationic and neutral amino acid transport system y^+L in human umbilical vein endothelium. Exp Physiol. 2003;88(6):699–710.
24. White MF, Christensen HN. The two-way flux of cationic amino acids across the plasma membrane of mammalian cells is largely explained by a single transport system. J Biol Chem. 1982;257:10069–80.
25. Durante W, Liao L, Iftikhar I, et al. Differential regulation of L-arginine transport and nitric oxide production by vascular smooth muscle and endothelium. Circ Res. 1996;78:1075–82.
26. Patel JM, Abeles AJ, Block ER. Nitric oxide exposure and sulfhydryl modulation alter L-arginine transport in cultured pulmonary artery endothelial cells. Free Radic Biol Med. 1996;20:629–37.
27. Sala R, Rotoli BM, Colla E, et al. Two-way arginine transport in human endothelial cells: TNF-α stimulation is restricted to system y^+. Am J Physiol Cell Physiol. 2002;282:C134–43.
28. Closs EI, Gräf P, Habermeier A, et al. Human cationic amino acid transporters hCAT-1, hCAT-2A, and hCAT-2B: three related carriers with distinct transport properties. Biochemistry. 1997;36:6462–8.
29. Speake PF, Glazier JD, Ayuk PT-Y, et al. L-Arginine transport across the basal plasma membrane of the syncytiotrophoblast of the human placenta from normal and pre-eclamptic pregnancies. J Clin Endocrinol Metab. 2003;88:4287–92.
30. Parnell MM, Chin-Dusting JPF, Starr J, Kaye DM. In vivo and in vitro evidence of Ach-stimulated L-arginine uptake. Am J Physiol Heart Circ Physiol. 2004;287:H3965–4000.
31. Nel MJ, Woodiwiss AJ, Candy GP. Modeling of cellular L-arginine uptake by more than one transporter. J Membr Biol. 2012;245:1–13.
32. Malo C, Berteloot A. Analysis of kinetic data in transport studies: new insights from kinetic studies of Na^+-D-glucose co-transport in human intestinal brush-border membrane vesicles using a fast sampling, rapid filtration apparatus. J Membr Biol. 1991;122:127–41.
33. Devés R, Angelo S, Chávez P. *N*-Ethylmaleimide discriminates between two lysine transport systems in human erythrocytes. J Physiol. 1993;468:753–66.
34. Angelo S, Irarrázabal C, Devés R. The binding specificity of amino acid transport system y^+L in human erythrocytes is altered by monovalent cations. J Membr Biol. 1996;153:37–44.
35. Dall'Asta V, Bussolati O, Sala R, et al. Arginine transport through system y^+L in cultured human fibroblasts: normal phenotype of cells from LPI subjects. Am J Physiol Cell Physiol. 2000;279:C1829–37.
36. Hardy TA, May JM. Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. Free Radic Biol Med. 2002;32:122–31.
37. Rotoli BM, Bussolati O, Sala R, et al. The transport of cationic amino acids in human airway cells: expression of system y^+L activity and transepithelial delivery of NOS inhibitors. FASEB J. 2005;19(7):810–2.
38. Cheng Y-C, Prusoff WH. Relationship between the inhibitor constant (*Ki*) and the concentration of inhibitor which causes 50 per cent inhibition (*I50*) of an enzymatic reaction. Biochem Pharmacol. 1973;22:3099–108.
39. Spears G, Sneyd JGT, Loten EG. A method for deriving kinetic constants for two enzymes acting on the same substrate. Biochem J. 1971;125:1149–51.
40. Leatherbarrow RJ. Using linear and non-linear regression to fit biochemical data. Trends Biochem Sci. 1990;15:455–8.
41. Coons DM, Boulton RB, Bisson LF. Computer-assisted nonlinear regression analysis of the multicomponent glucose uptake kinetics of *Saccharomyces cerevisae*. J Bacteriol. 1995;177:3251–8.
42. Walsh R, Martin E, Darvesh S. A versatile equation to describe reversible enzyme inhibition and activation kinetics: modeling β-galactosidase and butyrylcholinesterase. Biochim Biophys Acta. 2007;1770:733–46.
43. Berteloot A, Malo C, Breton S, Brunette M. Fast sampling, rapid filtration apparatus: principal characteristics and validation from studies of D-glucose transport in human jejuna brush-border membrane vesicles. J Membr Biol. 1991;122:111–25.
44. Signorello MG, Pascale R, Leoncini G. Transport of L-arginine and nitric oxide formation in human platelets. Eur J Biochem. 2003;270:2005–12.

45. Christensen HN, Antonioli JA. Cationic amino acid transport in the rabbit reticulocyte. J Biol Chem. 1969;244:1497–504.
46. Mendes-Ribeiro AC, Brunini TMC, Yaqoob M, et al. Identification of system y^+L as the high-affinity transporter for L-Arginine in human platelets: up-regulation of L-Arginine influx in uraemia. Pflügers Arch. 1999;438:573–5.
47. Rotmann A, Simon A, Martiné U, et al. Activation of classical protein kinase C decreases transport via systems y^+ and y^+L. Am J Physiol Cell Physiol. 2007;292:C2259–68.
48. White MF, Gazzola GC, Christensen HN. Cationic amino acid transport into cultured animal cells. I. Influx into cultured human fibroblasts. J Biol Chem. 1982;257:4443–9.
49. Rajapakse N, Mattson DL. Role of L-arginine in nitric oxide production in health and hypertension. Clin Exp Pharmacol Physiol. 2009;36:249–55.

Chapter 2
L-Arginine and the Expression of HSP70 and p53 Proteins

Agnieszka Pedrycz and Victor R. Preedy

Key Points

1. Heat shock protein 70 is a biomarker of cellular stresses such as those due to oxygen- or nitrogen-centred free radicals or environmental factors.
2. p53 protein is synthesised after DNA damage. It can prevent cells repairing their genetic material and can initiate apoptosis (also called programmed cell death).
3. Nitric oxide (NO) and its derivatives act as free radicals stimulating nitrosative stress, which leads to apoptosis.
4. We review how in preclinical studies L-arginine for 3 weeks increases apoptosis of renal tubular epithelial cells probably via the actions of L-arginine acting as a donor for the formation of NO. This remains to be elucidated however.
5. Expression of the heat shock protein 70 (HSP70), the p53 and caspase 3 increases in renal cells after L-arginine therapy.
6. Divergent effects are seen in hepatocytes compared to renal tubular epithelial cells in response to L-arginine treatment. In these conditions of L-arginine dosage, hepatic tissue appears to be refractory compared to renal tissue.

Keywords L-arginine • Heat shock protein 70 • p53 • Apoptosis • Caspase 3

Abbreviations

AIP1	Apoptosis inducing protein 1
APC	Antigen-presenting cells
17 AAG	Tanespimycin
ADR	Adriamycine
AEC + H	3-amino-9-ethylcarbazole + haematoxylin

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AIF	Apoptosis activating factor
Apaf 1	Apoptotic protease activating factor 1
APC cells	Antigen-presenting cells
Bcl-2	B-cell lymphoma 2
Caspase 3	Cysteinyl aspartic acid-protease-3
H+E	Haematoxylin and eosin
HSP	Heat shock protein
MHC complex	Major histocompatibility complex
NO	Nitric oxide
p53	Protein 53
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
HSPs	Small heat shock proteins

Introduction

L-arginine (2-amino-5-guanidinovaleric) was isolated in 1886 from lupin seedlings. Nine years later, it was also found in animal tissues. Examinations into its function and biochemical structure started in the 1930s. Since then, L-arginine has been described to be involved in numerous biochemical pathways and metabolic processes. For example, in the liver, L-arginine plays a role in the urea cycle for the removal of ammonia from the body. It is also a substrate for the biological synthesis of creatine (a precursor of creatinine) [1]. Although it can be synthesised endogenously, there are some conditions in which L-arginine cannot be synthesised adequately to meet metabolic demands, such as a consequence of physical injuries or renal insufficiency [2]. In other words, it can act as a *conditionally essential* amino acid.

The biochemical reactions whereby L-arginine is converted to nitric oxide (NO) open up many avenues which potentially can utilise L-arginine in a therapeutic manner. Essentially, nitric oxide synthases catalyse the oxidation of L-arginine to L-citrulline and nitric oxide. Endotheliocytes (also commonly called endothelial cells) synthesise NO using extracellular L-arginine from the blood plasma. The citrulline–nitric oxide cycle also is of importance as the enzyme argininosuccinate synthetase can catalyse the formation of arginosuccinate via the condensation of aspartate and citrulline: arginosuccinate can act as an immediate precursor of L-arginine [3].

Nitric oxide is a highly reactive and diffusible molecule [4]. Furthermore, because NO has a direct effect on vessels, its precursor L-arginine is used as an exogenous therapeutic agent in many pathological conditions and in preclinical and clinical studies. Arguably, its full potential has not been explored. L-arginine has hepatoprotective abilities, so it can be used clinically to treat liver dysfunction, such as when there are metabolic disturbances in the urea cycle, ammonia poisoning, or hypochloraemic alkalosis. In the former example, the mechanism of its action is to stimulate the urea cycle and remove toxic ammonia [5]. Exogenous NO can also be administered as a therapeutic agent to patients with coronary disease, pregnancy-induced hypertension, and venous atherosclerosis of the lower limbs.

Many tissues use L-arginine to synthesise nitric oxide such as hepatocytes, nerve endings, neurons, platelets, monocytes, neutrophils, macrophages, mast cells, and of course endothelial cells mentioned previously [6]. The liver and kidneys are the main organs which are responsible for L-arginine formation in the body.

Deficiencies of NO occur in many diseases, such as peripheral arterial occlusive disease, hypertension, hypercholesterolaemia, atherosclerosis, pulmonary hypertension, diabetes, chronic renal failure, preeclampsia, or hypotrophia of the foetus or glaucoma [7]. In contrast, overproduction of

endogenous NO occurs in other conditions such as inflammation, degenerative diseases of the nervous system, cancers, and autoimmune diseases [8]. At more detailed cellular and molecular levels, the mechanisms whereby NO participates in, or initiates, programmed cell death (apoptosis) are not fully known. The reactive forms of nitrogen (also called reactive nitrogen species RNS) include nitric oxide and derivatives arising as a result of the metabolism of nitric oxide: nitrosonium cation (NO+), nitroxyl anion (NO−), and peroxynitrite (ONOO−). Nitric oxide has the ability to react with molecular oxygen, metal cations, and superoxide anions to produce other reactive species which are damaging to molecules and subcellular organelles.

The overproduction of RNS which leads to cellular damage is called nitrosative stress—a phenomenon similar to, and closely linked with, the phenomena of oxidative stress caused by reactive oxygen species (ROS). Reactive nitrogen species react with proteins damaging their function by nitrosylation of amino acid residues, which may in turn stimulate an apoptotic response. Such a process has, for example, been described in macrophages [9]. Nitrosylation will occur with different classes of proteins, for example enzymes, regulatory proteins, or transport proteins [10]. Nitrosylation of DNA is responsible for neoplasm formation. Nitrosylation of cell organelles like mitochondria and membranes can cause increased permeability and depolarisation. It also decreases ATP production, impairs intracellular homeostasis of Ca^{2+}, and changes the antigenic properties of cells [11].

The generation of cellular free radicals is affected by the following conditions or scenarios: exposure to radiation and chemical compounds, diseases (such as diabetes), ageing, and exercise (increased oxygen use) [10,12]. Reactive nitrogen species produced in physiological conditions do not accumulate in the cells as they are inactivated by antinitrosative mechanisms, e.g. the enzymes produced by cells: SOD, catalase, reduced glutathione, dietary vitamins, and microelements such as selenium, copper, and zinc.

In pathological conditions, when antinitrosative mechanisms fail, nitrosative stress develops. Nitrosative stress and oxidative stress may be components of normative or constitutive metabolism. Such stresses are short-lasting and the excess free radical species are quickly "scavenged" by endogenous systems. In pathological nitrosative stress, the large amounts of free radicals accumulated in cells are likely to lead to cell degradation and death, e.g. damage to mitochondria then leads to apoptosis [13].

Heat Shock Proteins

Heat shock proteins, like HSP-70, protect cells against the damaging effects of proteins, which potentially can result in cellular dysfunction or death downstream. The protein p53 is the so-called guardian of the genome. And its expression increases as a result of an early damage to nucleic DNA. Caspase 3 is an executor of apoptosis. The analysis of this cohort of biomarkers (HSP70, p53, and caspase 3) is of benefit in understanding the putative effects of harmful and beneficial agents.

"Heat shock protein" was the term first used in 1974 by Tissieres and coauthors [14]. They described the protein in Drosophila exposed to high temperatures. The authors characterised heat shock proteins as molecules which prevent adverse effects caused by harmful factors. Heat shock proteins have subsequently been shown to regulate the functioning of numerous cellular processes [14]. In 1993, Lasky et al. named these protective proteins as "chaperones" [15]. It was also observed that these chaperones create non-covalent binding with aberrant polypeptides [16]. Chaperones are present in the blood plasma of healthy, young people. Their concentration decreases with age. The impaired activity of heat shock proteins may constitute or initiate various diseases, such as cancers, Parkinson's disease, strokes, Alzheimer's disease, cardiac infarction, immunological disorders, inflammatory diseases, autoimmune diseases, cataracts, cognitive impairment, and behavioural dysfunction [17]. Mukhopadhyay et al. in their research on Drosophila noticed that HSP70 can be used as a putative biomarker of cellular stress arising from a variety of environmental conditions [18].

Soti et al. observed that the correct functioning of heat shock proteins in a cell is essential for endogenous adaptations to stress [19]. Their synthesis increases when cells are exposed to stresses such as toxins, hypoxia, starvation, an increase of temperature, water deprivation, inflammation, UV radiation, ethanol, arsenic, and nitrogen deficiency [20].

According to their molecular weight (kDa), we can distinguish four families (groups) of heat shock proteins as follows [16]:

1. Low-molecular heat shock proteins
2. HSP70
3. HSP60
4. HSP90

There are also cofactors for these proteins: HSP10 for HSP60; HSP40 for HSP70.

The most important functions of heat shock proteins include:

1. Protection during replication of DNA and its transcription.
2. Participation in the translation of mRNA to the amino acid polypeptide chain.
3. Facilitating the correct folding of new proteins.
4. Protection of newly synthesised proteins during their transport from the cytoplasm to their cellular destination.
5. Their contribution to the proteolysis of proteins (degradation) and their oligomerisation [21].
6. Activation of receptors.
7. Contribution to the functioning of MHC (major histocompatibility complexes)–peptide complexes responsible for the activity of the immunological system.
8. Participation in the presentation of antigens to the immunological system [e.g. anticancer factor HSP90, 17 AAG (Tanespimycin)]. This ability is used in the production of anticancer vaccines [22].

HSPs also identify cancerous or infected cells and modify them to act as antigens. Thus, the protective proteins present the newly formed antigens to the immune system and supply HSP-peptide antigens cells to antigen-presenting cells (APC). APC cells, through the CD 91 receptor, present this antigen to T-lymphocytes. In response, T-lymphocytes multiply and locate these cells with the purpose of their destruction. HSP70 is similarly involved in the immune response and can alter components of cancer cells and introduce them to the immunological system. Thus, HSPs ensure genetic stability in the cellular milieu. HSP70 itself can maintain transcription factors and steroid receptors [21,22]. Chaperones can also act as proteases against denatured proteins when restoration is not possible [20]. Some heat shock proteins can prevent the formation of protein aggregates and some others can protect aggregates against disassociation [21]. For example, HSP60 prevents the aggregation of proteins in a high temperature environment [23]. Proteins of the HSP100 family are responsible for protein degradation and cooperation with DNA in the disaggregation of proteins [20].

Laskey et al. documented an interesting phenomena: healthy cells in unfavourable conditions condense protective proteins in the nucleus of the cell. In contrast, cancer cells keep them outside of the nucleus and often modify them with peptides [15]. The type of peptide depends on the cancer type and the tissue in which cancer is developing. In mice injected with a vaccine of peptide-tagged HSPs, a regression of metastases is observed. Tissues also become immune to the development of new cancers [24].

HSPSs may participate in exocytosis and endocytosis of proteins. Apart from their basic or group function, individual heat shock proteins have their own distinct tasks. HSPs 20 and 27, in cooperation with phospholipase, are involved in the relaxation and contraction of smooth muscles. HSP75 inhibits the development of free radicals, reduces oxidation of lipids, increases ATP levels, prevents activation of thrombocytes, and decreases apoptosis [24]. Potentially heat shock proteins can be used in the treatment of some diseases such as HSP90 usage in Alzheimer's, Parkinson's, and "mad cow" diseases [25]. Inhibitors of HSPs also can be used in putative treatment regimens. The HSP90 inhibitor geldamycin has an antiproliferative and anticancer effect and is potentially useful in the treatment of myeloma [17].

In some diseases, the expression of HSP proteins is intensified. They are detected on endothelial surfaces in hypertension and metabolic disorders. The level of antibodies against HSPs 60 and 65 in the blood serum correlates with the level of advancement of arteriosclerosis in cerebral, cephalic, carotid, and peripheral arteries [26].

Small heat shock proteins (sHSPs), as the name implies, have a small molecular weight in the order of 15–45 kDa. They possess one domain of l-crystallin composed of about 90 amino acids, participate in the regulation of apoptosis, and have been shown to have perturbed functions in cancer cells.

The most sensitive, and one of the most researched, chaperones that respond to cellular stress is HSP70. After a cell is exposed to various destructive factors, like oxygen stress, ethanol, viruses and bacteria, radiation, antibiotics, or fluctuations of pH, there is increased expression of genes encoding HSP70. Very simply, one of the main actions of HSP70 is protection against cell destruction, though other roles have been ascribed including as a chaperone in transport of proteins through cellular membranes and prevention of protein aggregation. HSP70 selects irregular proteins and directs them onto their route for photolytic destruction [15,16].

Importantly, HSP70 also protects cells against molecular signals that initiate cell death or apoptosis. For example, HSP70 inhibits cell death induced by cytochrome C (component of the intrinsic pathway of apoptosis), inhibits apoptosis activating factor (AIF), and prevents overexpression of caspase 3 (executor cysteine protease of apoptosis). HSP70 can be used as a biomarker of cellular stress.

Programmed Cell Death, p53 and Caspase 3

Programmed cell death is under genetic control and plays an important role in the homeostatic control of organisms, during both embryogenesis and, subsequently, individual life stages [**27**].

Many pathological factors can stimulate cell to apoptosis. They include DNA damage due to stress-inducing factors, such as hypoxia, free radicals, radiation, anticancer agents, and antibiotics [28]. Apoptosis also can be stimulated by activation of apoptosis cell membrane receptors (also called death receptors) via external signals [29]. Activation of intracellular receptors may cause damage to some cell organelles, such as the mitochondria or endoplasmic reticulum [30].

Two main pathways leading to apoptosis are described, namely: the intrinsic and extrinsic pathways [31].

The extrinsic pathway is induced by external signals which stimulate membranous death receptors. This pathway can be also stimulated by cytotoxic T-lymphocytes, which recognise damaged cells or those infected with viruses.

In the intrinsic pathway, the mitochondria or endoplasmic reticulum is involved. This pathway is activated by cellular stress and initiates apoptosis via direct or indirect involvement of mitochondria or the endoplasmic reticulum [32]. When DNA damage is first observed, it leads to the intense production and accumulation of p53 in the cell. As a consequence there is inhibition of the cell cycle. p53 stimulates the synthesis of BAX protein. This protein opens the channels in the mitochondrial membrane, leading to the release of cytochrome c. Cytochrome c complexes with apoptotic protease activating factor 1 (Apaf 1) which activates procaspase 9 which in turn activates the caspase cascade.

Apoptosis is divided into individual phases: induction, execution, and degradation [33].

The induction phase lasts from the generation of the apoptotic signals to the initiation of the caspase cascade. In this phase, a key role is played by p53, the so-called guardian of the genome. This is a phosphoprotein activated by damaged DNA. In response, p53 prevents the cell cycle in the G1 phase, allowing the cell to repair itself. In normal conditions, the cell synthesises small amounts of p53. In the cell, in which DNA is damaged or is compromised, level of p53 increases. Then, p53 stimulates the production of p21 which inhibits kinases required in the cell cycle, stopping in this way the cell cycle for several hours [34–36].

When cell repair is not possible, p53 initiates apoptosis. In this process, p53 stimulates production of the pro-apoptotic protein BAX (also called Bcl-2 like protein 4) and blocks expression of the Bcl-2 gene (also called B-cell lymphoma 2). Bcl-2 protein has antiapoptotic effects [33]. In cells where DNA is damaged, p53 stimulates production of another protein—namely apoptosis inducing protein 1 (AIP1) [37]. This protein attaches to mitochondrion which then releases molecules such as cytochrome c to activate caspases [34]. Due to its role in apoptosis, p53 is a very sensitive biomarker of DNA damage.

The execution phase of apoptosis is irreversible. Its base components are cysteine proteases from the interleukin-1-beta-converting enzyme family, also called the caspase family [38]. The caspases (cysteine-dependent asparaginian-specific proteases) degrade proteins behind asparaginian residues using their cysteine residues [39]. They activate one another. The last caspases are effectors (also called executioners). In this group, there are caspases 3, 6, and 7. Caspase 3 (cysteinyl aspartic acid-protease-3) activates enzymes responsible for the degradation of DNA and proteins. Caspase 3 is also capable of inducing cell death by itself. Detection ofincreased caspase 3 level in the cell is indicative that apoptosis has occurred or is occurring.

Previous Studies on L-Arginine

In previous studies, one of the authors (PA) of this review examined the ultrastructure of kidneys and liver of rats treated with L-arginine. To confirm destruction of cells, biomarkers of apoptotic pathways were detected. Immunohistochemical location of HSP70, p53, and caspase 3 was assessed. Animals received L-arginine in a dose similar to that used in clinics to treat preeclampsia in pregnant woman. Theoretically, this dose of 40 mg/kg of body weight should be safe for the mother and foetus on a dose/per unit body weight basis.

The experimental group consisted of young female rats to which were administered L-arginine (Argininum, Curtis Healthcare, Poland) every other day for 2 weeks (40 mg/kg of body weight) through a stomach tube. Controls were treated identically but without L-arginine. After 3 weeks, the experiment was terminated and post-mortem specimens of liver and kidneys were taken. Histological specimens were examined at the electron and light microscope level.

L-arginine treatment caused changes in the kidneys, observed at the light microscopic level. The lesions in the renal tubules were segmental and focal, observed in single tubules, or even single epithelial tubular cells which overall was characteristic of apoptosis (Fig. 2.1).

The lumen of some tubules was broadened and others narrowed. There were some isolated pyknotic nuclei in the lumen of the tubules of L-arginine treated animals, which were signs that cells were undergoing a cellular demise. Pyknotic nuclei, dark and small in diameter, were also observed in the epithelial tubular cells. The cytoplasm of single cells was lighter in intensity than the corresponding control specimens stained with haematoxylin and eosin. The presence of intense apoptosis in kidney cells from L-arginine treated animals compared to controls was confirmed by quantitative measures of an apoptotic index [40].

Confirmation of elevated apoptosis was also found at the electron microscopic level. The changes were focal as observed at the light microscopic level. The borders between renal epithelial tubular cells were blurred. Some apoptotic cells were noticed. In the cytoplasm pyknotic, flattened nuclei, with reduced volumes, were seen and nuclei were partially or completely destroyed. The lumen of tubules was widened with homogenous deposits inside. Inside the lumen, nuclei, mitochondria, and single epithelial cells were visible. The brush borders in the proximal convoluted tubules were focally destroyed [41].

The partially destroyed cells contained in their cytoplasm numerous vacuoles. The channels of rough endoplasmic reticulum were widened. The mitochondria were oedematous with light matrix

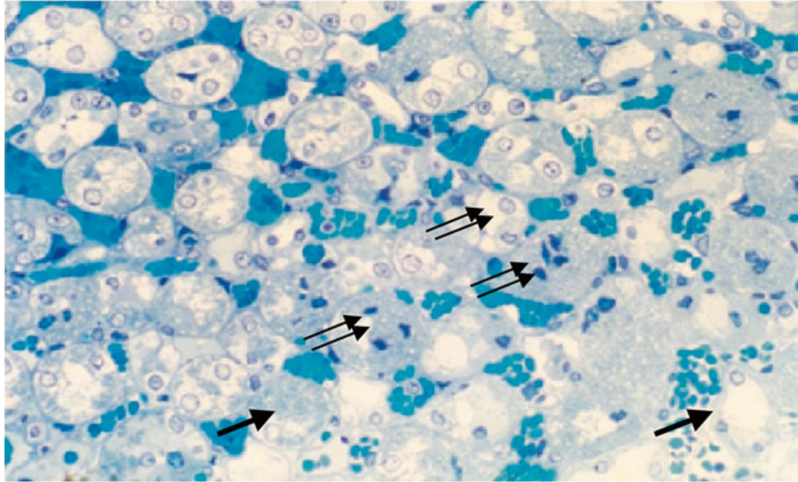

Fig. 2.1 A kidney section of the rat treated with L-arginine. Renal tubules with reduced lumen and destroyed epithelial cells of the tubular wall are visible (*arrows*). The micrograph shows apoptotic cells with pyknotic nuclei (*dark blue*), brightened (*blue*) cytoplasm with numerous vacuoles (*white*) with congestion (*double arrows*). The semi-thin specimen used methylene blue + Azure II staining. Magnification × 200. (copyright A. Pedrycz)

and destroyed crests. The peribasal striations, characteristic of proximal convoluted tubules of the nephron, were damaged. Many lysosomes and peroxysomes were observed in the cytoplasm of the epithelial cells. The nuclei of apoptotic cells were located in one of the cell poles. Their shapes were changed and smaller than the nuclei of normal cells. Condensation and peripherally location of chromatin were observed [41]. Moreover, the formation of apoptotic bodies was observed. This process is known as "cell boiling". The alveoli contained cellular nuclei and other organelles [41].

Specimens of the liver of rats treated with L-arginine were also examined. At the light microscopic level, images did not differ from those described in textbooks or treated controls. The architecture was clearly visible with consistently regularly contoured lobules. The cytoplasm of hepatocytes was stained pink (H + E staining) or blue (semi-thin specimens). In the cell centre, one or two large and round nuclei were located. One or two nucleoli were visible in the nucleus. In the sinusoids, hepatic macrophages were visible (Fig. 2.2).

The electron microscope images of hepatocytes in L-arginine treated rats were not different from the textbooks' norms nor controls. Distinct borders were visible with normative junctions (zonula occludens, adhesions, or desmosomes) in between cells from the L-arginine treated groups. In the central parts of the hepatocyte's cytoplasm, one or two round or oval large nuclei were observed with intact membranes. The mitochondria were mainly in the condensed, low-energy form. The well-developed Golgi complex, single lysosomes, and peroxysomes with dark, crystalline core were noticed in hepatocytes in both L-arginine treated and control animals.

Nitric oxide and its derivatives act as free radicals and initiate cellular stress. This stress is responsible for the induction of the intrinsic pathway of apoptosis. HSP70 (as a marker of nitrosative stress) and p53 (as an early marker of damage to the cell's genetic material, i.e. DNA) were immunolocalised in the kidneys and the liver.

Observation of kidneys at the light and electron microscopic levels showed the presence of apoptosis after L-arginine therapy. It was confirmed by an increased apoptotic index, but the apparent increase in immunohistochemical staining for caspase 3 in the renal tubular cells did not reach statistical significance (Fig. 2.3) [42].

In the liver, there were no changes in light and electron microscopic examinations. L-arginine given to rats did not influence the apoptotic index nor immunohistochemical staining for caspase (Fig. 2.4) [42].

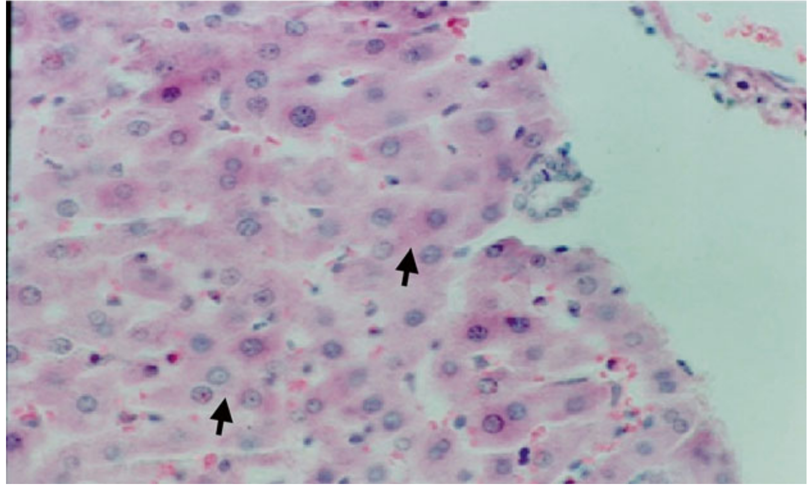

Fig. 2.2 A liver section of a rat treated with L-arginine. A fragment of portal area is visible. Normal architecture of the hepatic trabeculae is seen. Nuclei of hepatocytes are *round*, *bright violet*, and centrally located (*arrows*). H + E staining was used. Magnification × 200. (copyright A. Pedrycz)

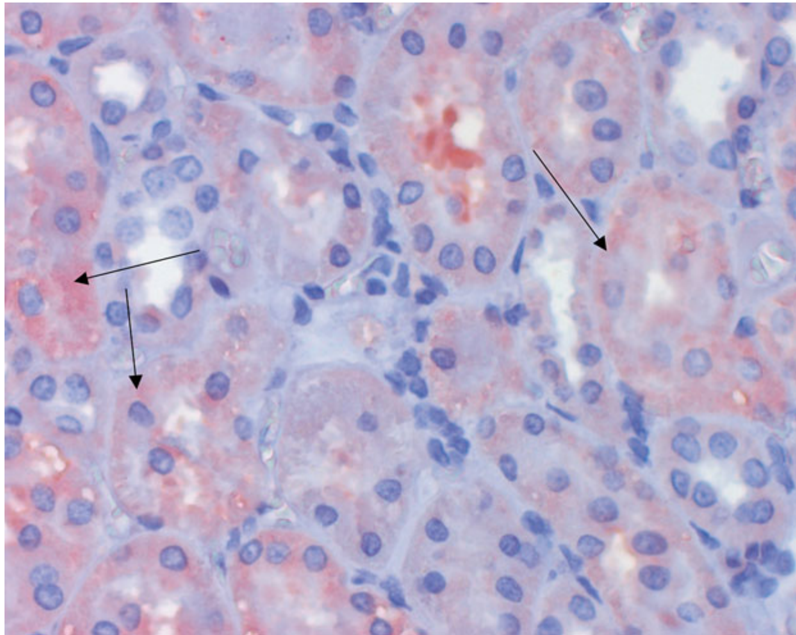

Fig. 2.3 A kidney section of the rat treated with L-arginine, showing caspase 3 immunohistochemical reaction of high intensity. A fragment of renal convoluted tubules. Immunohistochemical positive (*red*) reaction mostly seen in basal part of cytoplasm of renal tubular epithelial cells (*arrows*). AEC + H (-3-amino-9-ethylcarbazole + haematoxylin) staining. Magnification × 400. (copyright A. Pedrycz)

Qualitative evaluation of immunohistochemical reaction in the kidneys of rats treated with L-arginine showed focal p53 reactions. This was observed in the cytoplasm of epithelial cells of renal tubules. p53 positive reactions were observed in renal tubules and were not observed within the renal corpuscles. The quantitative evaluation showed the increased p53 reaction in comparison to the control.

The mean area occupied with a positive colour reaction for p53 was statistically greater than that observed in the control group (Fig. 2.5) [43].

Statistical analysis demonstrated that, in the kidneys, L-arginine administration increased immunoreactive HSP70 (Fig. 2.6) [44].

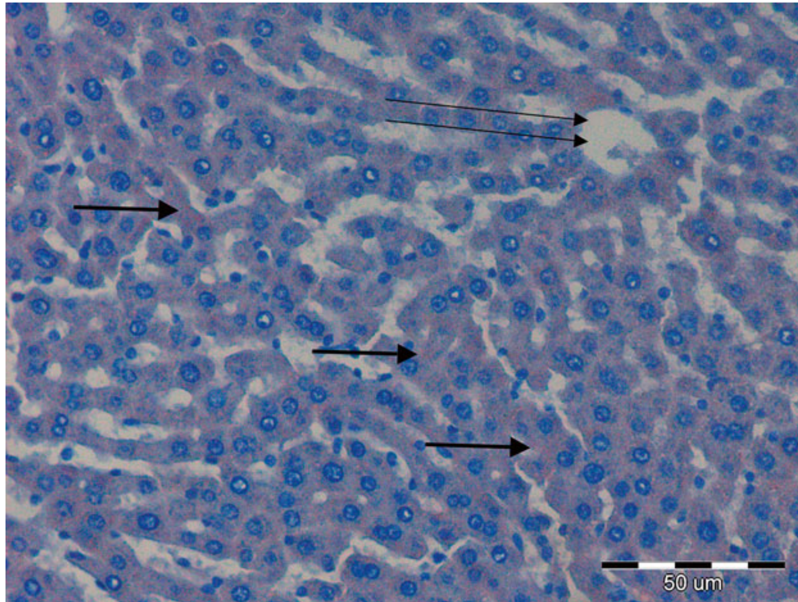

Fig. 2.4 A liver section of a rat treated with L-arginine, showing caspase 3 reaction of low intensity. Fragment of the rat's liver. Central vein and hepatic trabeculae with hepatocytes are seen (*double arrow*). Weak immunohistochemical red reaction for caspase 3 in cytoplasm of some hepatocytes is seen (*arrow*). Hepatocytes' nuclei are round and in the central part of the cell located. AEC + H (-3-amino-9-ethylcarbazole + haematoxylin) staining. (copyright A. Pedrycz)

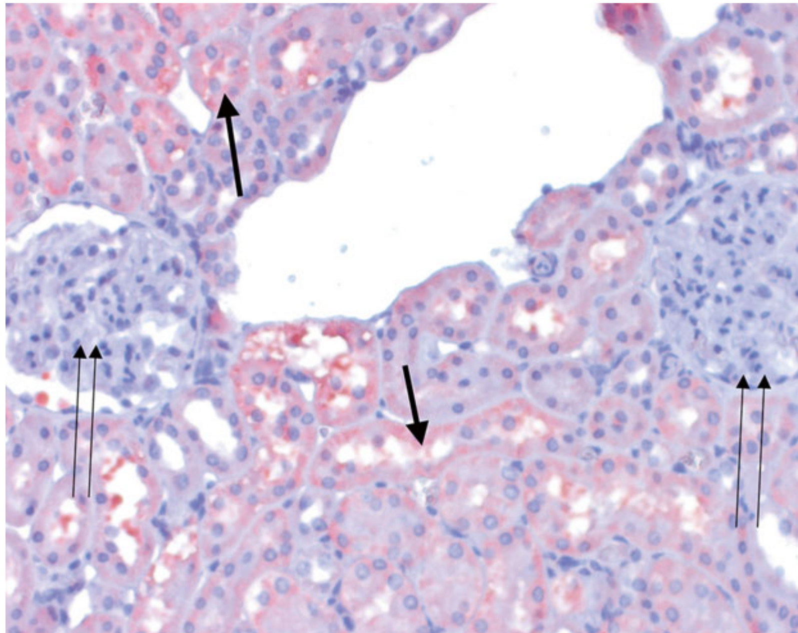

Fig. 2.5 The kidney section of the rat treated with L-arginine, showing p53 reaction of high intensity. Fragment of kidney's cortex with convoluted tubules and renal glomeruli. Positive, colour (*red*) immunohistochemical reaction for p53 in both basal and apical part of the renal tubular epithelial cells is seen. The positive reaction for p53 in brush border of proximal convoluted tubules is observed too (*arrow*). There is no immunohistochemical reaction for p53 in cells located in renal corpuscles (*double arrow*). AEC + H (-3-amino-9-ethylcarbazole + haematoxylin) staining. Magnification × 200. (copyright A. Pedrycz)

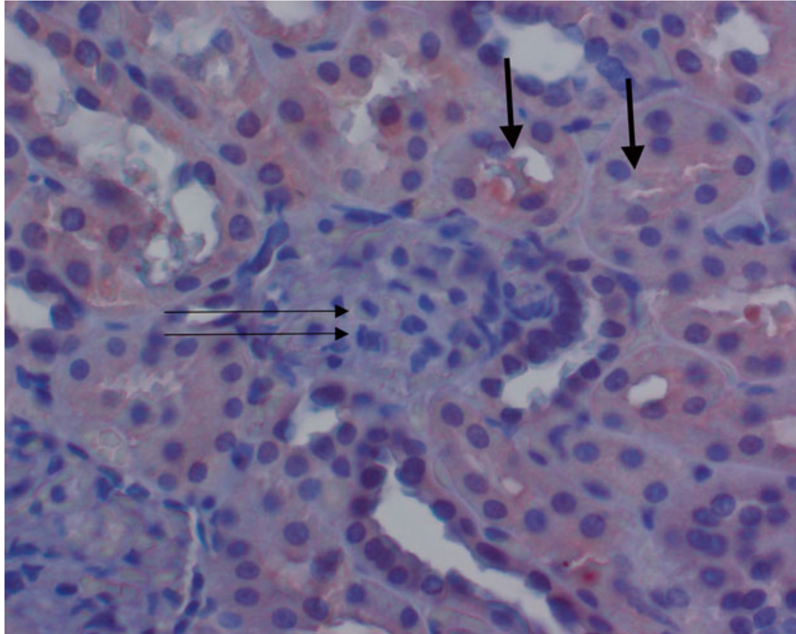

Fig. 2.6 The kidney section of the rat treated with L-arginine, showing HSP70 reaction of high intensity. Fragment of kidney with glomeruli, convoluted tubules, and collecting ducts. Positive immunohistochemical *red colour* reaction is visible in apical part of cytoplasm of renal tubular epithelial cells (*arrows*). There is no positive reaction for HSP70 in glomerular cells (*double arrows*). AEC + H (-3-amino-9-ethylcarbazole + haematoxylin) staining. Magnification × 400. (copyright A. Pedrycz)

L-arginine given to rats did not stimulate nitrosative stress in hepatocytes nor increase HSP70 level, destroy nuclear DNA, and furthermore did not increase apoptosis (Figs. 2.7 and 2.8) [45].

These observations have to be considered within the context that L-arginine may be cytotoxic to kidney tissue and thus its use requires caution. This is not the case for liver and there may or may not be other tissues that are refractory to the apoptotic effects of L-arginine. However, it is somewhat difficult to transcribe preclinical studies in the laboratory rat into the clinical situation without further studies.

Other studies have also shown that L-arginine stimulates HSPs, p53, caspases, or apoptosis. For example, L-arginine stimulates the L-arginine deiminase-induced inhibition of lymphoma cell growth via increases in p53 as well as NF-kBp65 [46]. In stomach cancer cells, L-arginine increases p53 together with concomitant decreases in Bcl-2 [47]. L-arginine supplementation has been shown to increase HSP70 in the liver of piglets, which contrasts with the observations in this study using rats [48]. It is difficult to make strict comparisons as the dosage per kg was not given, but the concentration of L-arginine in the aforementioned study was 6 g/kg feed [48]. In renal cells damaged by ascitic fluid, there is increased apoptosis [49]. In these conditions, L-arginine is protective [49]. A protective effect of L-arginine on apoptosis is also seen in intestinal cells where L-arginine reduced the apoptotic index compared to intestinal cells from animals subjected to experimental sepsis [50].

The involvement of L-arginine in apoptosis has also been reviewed previously where it was argued that nitric oxide-mediated apoptosis occurred via L-arginine recycling as well as by L-arginine directly [51]. Further evidence was provided by studies showing that induction of arginase II or expression by cDNA transfection inhibited apoptosis [51]. However, the situation is further complicated by the fact that low doses of nitric oxide protect cells from apoptosis, paradoxically by inducing HSP70 [51]. This leads to the idea that apparently discordant observations in biology have a rational explanation.

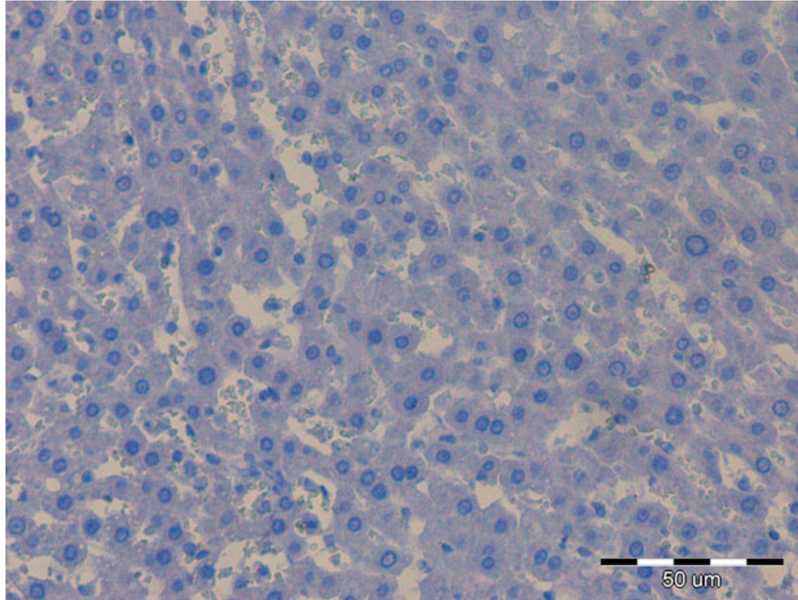

Fig. 2.7 The liver section of the rat treated with L-arginine, showing p53 reaction of low intensity. Weak, *red*, immunohistochemical reaction for p53 visible in cytoplasm of hepatocytes. AEC+H (-3-amino-9-ethylcarbazole+haematoxylin) staining. (copyright A. Pedrycz)

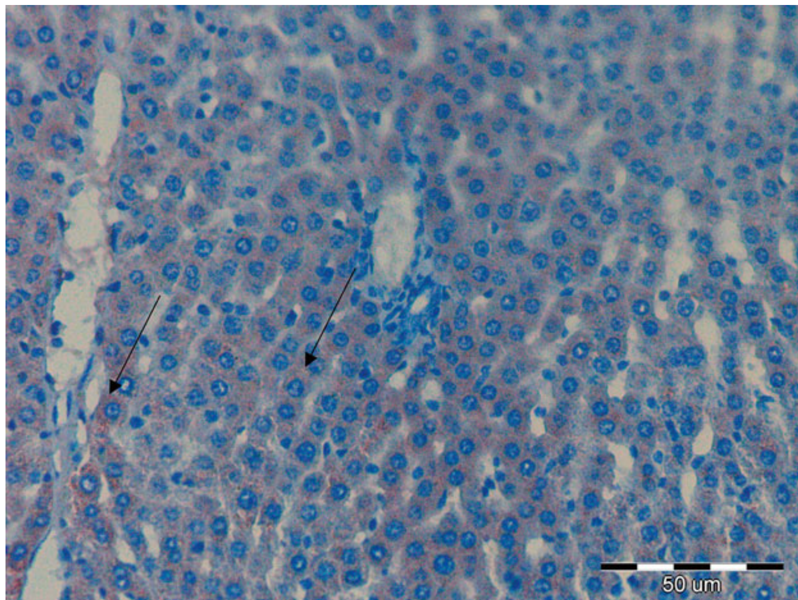

Fig. 2.8 A liver section of the rat treated with L-arginine, showing HSP70 reaction of medium intensity. Fragment of the liver. Hepatocytes with *round*, big nuclei and positive immunohistochemical *red colour*, granular reaction in cytoplasm (*arrows*) AEC+H (-3-amino-9-ethylcarbazole+haematoxylin) staining. (copyright A. Pedrycz)

For example, the period of timing for the administration of L-arginine in experimental situations where apoptosis is being examined is critical [52]. Furthermore, in the studies reported in this chapter, we do not know the concentrations of L-arginine in the cellular environment, nor the concentrations of nitric oxide, or even recycling enzymes such as argininosuccinate lyase and argininosuccinate synthetase.

Conclusions

L-arginine is a donor of exogenous NO and in this regard the expression of HPS70 (a biomarker of nitrosative stress) increases in the renal cells after L-arginine therapy. L-arginine does not stimulate nitrosative stress nor HSP70 levels in hepatocytes. L-arginine therapy destroys nuclear DNA and increases expression of p53 (genome guardian) in renal cells. These changes in the kidney are symptomatic of cellular toxicity. However, some cautionary note is required as such changes may be dose or time dependent. Furthermore, although the experiments were carried out in only two tissues, we do not know in such studies how this will be translated to the clinical situation in other tissues as one needs to consider not only the L-arginine dosage regimen but the cellular and cytoplasmic concentrations of L-arginine and its downstream products.

References

1. Wys M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80:1107–13.
2. Flynn NE, Meininger CJ, Haynes TE, et al. The metabolic basis of L-arginine nutrition and pharmacotherapy. Biomed Pharmather. 2002;56:427–38.
3. Husson A, Brasse-Lagnel C, Fairand A, et al. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. Eur J Biochem. 2003;270:1887–99.
4. Krzyżowska M. The regulatory function of nitric oxide in apoptosis. Post Biol Kom. 2005;32:633–46.
5. Kostka-Trąbka E. L-Arginine—common amino acid with new possibilities of acting in the clinic. Ordynator Leków. 2002;3:15–9.
6. Rytlewski K, Olszanecki R, Korbut R, et al. Effects of prolonged oral supplementation with L-arginine on blood pressure and nitric oxide synthesis in preeclampsia. Eur J Clin Invest. 2005;35:32–7.
7. Pardej A, Gryboś M, Kubicki J, et al. L-Arginine and citrulline in hypotrophy of foetus—part II. Gin Poł Med Project. 2009;1:11–6.
8. Rytlewski K, Zdebski Z. Nitric oxide as one of the protective mechanisms of pregnancy. Gin Poł Med Project. 2001;72:738–43.
9. Eu JP, Liu L, et al. Apoptotic model for nitrosative stress. Biochemistry. 2000;39:1040–7.
10. Golgfarb AH, Patrick SW, Bryer S, et al. Vitamin C supplementation affects oxidative-stress blood markers in response to a 30-minute run at 75% VO2max. Int J Sport Nutr Exerc Metab. 2005;15:279–90.
11. Augustyniak A, Michalak K, Skrzydlewska E. The action of oxidative stress induced by ethanol on the central nervous system (CNS). Postêpy Hig Dosw. 2005;59:464–71.
12. Heitzer T, Meinertz T. Prevention of coronary heart disease: smoking. Z Kardiol. 2005;94(Suppl):iii30–42.
13. Kovacic P, Pozos RS, Somanathan R, et al. Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. Curr Med Chem. 2005;12:2601–23.
14. Baehrecke EH. Autophagic programmed cell death in Drosophila. Cell Death Differ. 2003;10:940–5.
15. Laskey RA, Mills AD, Philpott A, et al. The role of nucleoplasmin in chromatin assembly and disassembly. Philos Trans R Soc Lond B Biol Sci. 1993;339:263–9.
16. Ellis RJ, Heminmingsen SM. The molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. Trends Biochem Sci. 1989;14:339–42.
17. Urbano A, Lakshmanan U, Choo PH, et al. AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. EMBO J. 2005;24:2815–26.
18. Mukhopadhyay I, Nazir A, Saxena DK, Chowdhuri DK. Toxicity of cypermethrin: HSp70 as a biomarker of response in transgenic Drosophila. Biomarkers. 2002;7:501–10.

19. Soti C, Nagy E, Giricz Z, et al. Heat shock proteins as emerging therapeutic targets. Br J Pharmacol. 2005;146:769–80.
20. Yoo JL, Janz DM. Tissue-specific HSP70 levels and reproductive physiological responses in fishes inhabiting a metal-contaminated creek. Arch Environ Contam Toxicol. 2003;45:110–20.
21. Evans TG, Yamamoto Y, Jeffery WR, et al. Zebrafish Hsp70 is required for embryonic lens formation. Cell Stress Chaperones. 2005;10:66–78.
22. Żylicz M, King FW, Wawrzynow A. HSP70 interaction with the p53 tumor suppressor protein. EMBO J. 2001;20:4634–8.
23. Gosh JC, Dohi T, Keng BH, Altieri CD. HSP60 regulation of tumor cell apoptosis. J Biol Cell. 2008;283:5188–94.
24. Ficus M. Chaperone for biophysics. Wiedza i życie. 2000;4:22–5.
25. Novoselova TV, Margulius BA, Novoselov SS, et al. Treatment with extracellular HSp70/HSC70 protein can reduce polyglutamine toxicity and aggregation. J Neurochem. 2005;94:597–606.
26. Gruber R, Lederer S, Bechtel U, Lob S, Riethmüller G, Feucht HE. Increased antibody titers against mycobacterial heat-shock protein 65 in patients with vasculitis and arteriosclerosis. Int Arch Allergy Immunol. 1996;110:95–8.
27. Lockshin RA, Zakeri Z. Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol. 2002;7:545–50.
28. Motyl T. Regulation of apoptosis: involvement of Bcl-2 related proteins. Reprod Nutr Dev. 1999;39:49–59.
29. Maśliñska D. Programmed cell death (apoptosis) in inflammation. Nowa Medycyna Reumatologia IV. 1999;12:34–41.
30. Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T, Alnemri ES. Caspase 2 induces apoptosis by releasing proapoptotic proteins from mitochondria. J Biol Chem. 2002;277:13430–7.
31. Bielak-Żmijewska A. The mechanisms of tumor cells' resistance to apoptosis. Kosmos Problemy nauk biologicznych. 2003;52:157–71.
32. Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood. 2001;98:2603–14.
33. Chmielewski M, Linke K, Zabel M, Szuber L. Apoptosis in the liver. Gastroenterol Pol. 2003;10:453–62.
34. Smith O. Cancer. Nota bene: the killer instinct of a p53 target. Science. 2000;290:67.
35. Hsu YL, Kuo PL, Tzeng WS, et al. Chalcone inhibits the proliferation of human breast cancer cell by blocking cell cycle progression and inducing apoptosis. Food Chem Toxicol. 2006;44:704–13.
36. Kaeser MD, Iggo RD. Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proc Natl Acad Sci USA. 2002;99:95–100.
37. Guicciardi ME, Gores GJ. AIP1: a new player in TNF signaling. J Clin Invest. 2003;111:1813–5.
38. Kiliańska ZM, Miskiewicz A. Caspases of vertebrates; their role in apoptosis. Post Biol Kom. 2003;30:129–35.
39. Sulejczak D. Apoptosis and methods of identification of thi phenomenon. Post Biol Kom. 2000;27:527–68.
40. Pedrycz A, Siermontowski P, Kaczerska D. Histological evaluation of influence of nitric oxide on rat's kidneys. Pol Hyperb Res. 2012;1:211–23.
41. Pedrycz A, Boratyński Z, Siermontowski P. Ultrastructure of renal tubular epithelial cells of rat's kidneys after administration of L-arginine. Bull Vet Inst Pulawy. 2013;57:113–7.
42. Pedrycz A, Boratyński Z, Krasowski A. Immunohistochemical assessment of the effects of L-arginine as a nitric oxide (NO) substrate on caspase 3 expression in rats' renal tubular cells. Bull Vet Inst Pulawy. 2010;54:441–3.
43. Pedrycz A, Kot K, Olesiński I. Immunohistochemical evaluation of caspase 3 expression in rats' hepatocytes after L-arginine therapy. Bull Vet Inst Pulawy. 2010;54:101–3.
44. Pedrycz A, Siermontowski P. Influence of L-arginine on expression of HSP70 and p53 proteins—early biomarkers of cellular danger in renal tubular cells. Immunohistochemical assessment. Arch Med Sci. 2013;9:719–23.
45. Pedrycz A, Boratyński Z, Orłowski M, et al. Assessment of L-arginine as an effect of exogenous nitric oxide (NO) on expression of markers of cellular stress in rats' hepatocytes. Bull Vet Inst Pulawy. 2012;56:89–92.
46. Shu XL, Liu XL, Zhong JX, Liu J. L-arginine enhances L-arginine deiminase induced human lymphoma cell growth inhibition through NF-kBp65 and p53 expression in vitro. Eur Rev Med Pharmacol Sci. 2014;18:2124–31.
47. Shu XL, Xu H, Yu TT, Zhong JX, Lei T. Regulation of apoptosis in human gastric cancer cell line SGC-7901 by L-arginine. Panminerva Med. 2014;56:227–31.
48. Wu X, Xie C, Yin Y, et al. Effect of L-arginine on HSP70 expression in liver in weanling piglets. BMC Vet Res. 2013;9:63.
49. Cheng R-C, Liu Q-Y, Su Y-J, Zhang J-M, Diao C, Luo H-Y. Curative effects of L-arginine on apoptosis of renal cells induced by pancreatitis associated ascitic fluid. World Chin J Digestol. 2007;15:1893–8.
50. Wu CT, Ren YF, Liu JF, Zhang JH, Lei ST. L-arginine reduces intestinal epithelial cell apoptosis in rats with severe abdominal infection. Nan fang yi ke da xue xue bao. 2007;27:1728–30.
51. Mori M. Regulation of nitric oxide synthesis and apoptosis by arginase and L-arginine recycling. J Nutr. 2007;137:1616S–20.
52. Liang F, Gao E, Tao L, et al. Critical timing of L-arginine treatment in post-ischemic myocardial apoptosis—role of NOS isoforms. Cardiovasc Res. 2004;62:568–77.

Chapter 3
AMP-Activated Protein Kinase and L-Arginine

Srinidi Mohan

Key Points

- The therapeutic benefits of L-Arginine (ARG), often clearly observed in short-term studies, are not evident after long-term use.
- Endothelial exposure to ARG triggers oxidative stress via superoxide overproduction.
- The reaction between superoxide and nitric oxide (NO) results in peroxynitrite synthesis, which promotes tetrahydrobiopterin (BH_4) oxidation and hence accumulation of 7,8-dihydrobiopterin (BH_2).
- The accumulated BH_2 binds with greater avidity than BH_4 to nitric oxide synthase (NOS) forming the complex, which initiates NOS uncoupling, oxidant formation, and perpetuating additional BH_4 oxidation, thereby initiating the vicious cycle favoring endothelial dysfunction.
- Diminished BH_4-to-BH_2 ratio is likely to be the fundamental molecular link between oxidative stress and endothelial dysfunction during long-term ARG supplementation.
- AMP-activated protein kinase acts as the functional modulating switch for determining ARG-mediated beneficial versus deleterious events and controls the downstream cascade of tolerance-sparing events.

Keywords L-Arginine • Superoxide • Tetrahydrobiopterin • Nitric oxide synthase • AMP-activated protein kinase • Tolerance

Introduction

L-Arginine (ARG) is a cationic, conditionally essential amino acid that is involved in numerous physiological processes [1]. It plays an important role not only in removing ammonia from the body but also in cell division, wound healing, immune function, and hormone release. L-Arginine also serves as the precursor for the synthesis of L-Ornithine, L-Proline, polyamines, agmantine,

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creatine, and protein [2, 3] (Fig. 3.1). But more significantly, ARG is known to be the exclusive substrate for nitric oxide synthase (NOS), which utilizes ARG to generate the signaling molecule, nitric oxide (NO) [4].

The identification of ARG as the endogenous substrate for NOS has allowed it to gain popularity as a dietary supplement. Because of the extensive involvement of NOS in many physiological systems, a wide variety of disease states [1–16] are expected to benefit from increased NO bioavailability through increased ARG supply. In the National Institute of Health website Medlineplus [5], the use of ARG in as many as 44 diseases and diagnoses was discussed and categorized according to the strength of scientific evidence supporting its use. Two indications with strong scientific evidence for ARG usefulness as dietary supplement are for growth hormone reserve test/pituitary disorder diagnosis and inborn errors of urea synthesis. Five indications with good scientific evidence for ARG usefulness as dietary supplement include coronary artery disease (angina), critical illness, heart failure, migraine headache, and peripheral vascular disease/claudication. Nearly 30 conditions with unclear scientific evidence have shown ARG usefulness as dietary supplement in treatment or preventive care, which includes diabetes, erectile dysfunction, myocardial infarction, preeclampsia, and wound healing, among others. The range of diseases that can be potentially benefited by ARG supplementation is therefore quite wide [6–11].

6 g ARG/day for 3 days in patients with stable angina pectoris has shown improvement in their exercise tolerance [12], and individuals supplemented with two food bars enriched with ARG per day for 2 weeks showed improved vascular function, exercise capacity, and quality aspects of life [13]. Patients with congestive heart failure were able to prolong their exercise duration after taking 9 g ARG/day for 7 days [14, 15]. In addition, ARG has been found to improve immunity [16–18], in patients under critical care [6, 19] and in sickle cell disease [11, 20]. Examples of commercial products include N.O.XPLODE®, NiteWorksTM, and ARG Extreme.

Of concern, recent studies revealed that the therapeutic benefits of ARG, often clearly observed in short-term studies, are not evident after long-term use [7, 14, 21, 22]. These recent studies have revealed the development of ARG tolerance (and possible toxicity) upon chronic dosing, thereby representing a major hindrance for the use of this important amino acid to benefit patients. Thus, there is a crucial need to understand the mechanistic relevance for ARG tolerance and toxicity to allow us to devise suitable alternative approaches that would extend the beneficial effects of ARG supplementation among patients in whom short-term therapeutic effects have already been demonstrated.

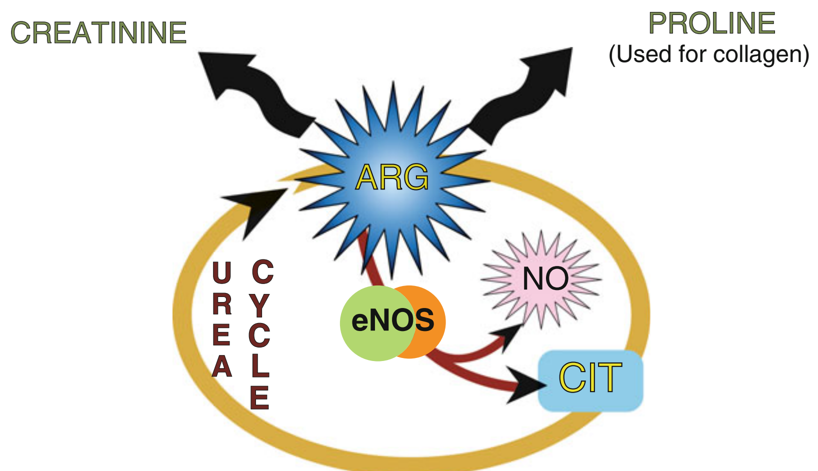

Fig. 3.1 Illustration on the biological utilization of L-Arginine (ARG). *NO* nitric oxide, *CIT* L-Citrulline, *eNOS* endothelial nitric oxide synthase

Intracellular ARG Does Not Determine NO Production

As a semi-essential amino acid, our body produces sufficient amounts of ARG, in millimolar quantities, while the metabolic requirements are in micromolar quantities. Thus, the fundamental question to ask is how the supplementation of this amino acid is primarily important to provide the beneficial effects over those produced endogenously. Studies have shown the relative contributory roles of extracellular versus intracellular ARG on cellular activation of NOS in human endothelial cells [23]. The study involved cellular exposure with different concentration of $^{15}N_4$-ARG (stable isotope of ARG), ARG, or ARG ethyl ester (ARG-EE). The study found that ARG-EE incubation increased cellular ARG concentration but no increase in nitrite/nitrate (that was observed as an end-point measurement for NO, due to the short half-life of NO), while ARG incubation increased both cellular ARG concentration and nitrite accumulation. Cellular nitrite/nitrate production did not correlate with cellular total ARG concentration. Reduced $^{15}N_4$-ARG cellular uptake in cationic amino acid transporter knockdown cells versus control was accompanied by reduced NOS activity, as determined by ^{15}N-nitrite, total nitrite, and $^{15}N_3$-L-Citrulline formation. These findings suggest that extracellular ARG, not intracellular ARG, is the major determinant of NO production in endothelial cells. It is likely that once transported inside the cell, ARG can no longer gain access to the membrane-bound NOS.

Dualistic Nature of NOS

While NOS is involved in ARG utilization for NO production, and the transport of ARG across the membrane (viz. ARG influx) seems to be the determinant factor for the beneficial effects of ARG supplementation, to determine the fundamental factor responsible for the tolerance development, we need to understand the dualistic nature of NOS. The NOS enzyme by itself consists of two domains: an oxygenase domain and a reductase domain [24]. The presence of two subunits for NOS favors the dualistic nature of NOS, producing both NO and superoxide (Fig. 3.2). In neuronal NOS, both the domains are proved to be capable of producing superoxide [25], while in vascular endothelial NOS, the situation is less clear, as the endothelial NOS is dually acylated. The acylation helps in the association of the endothelial NOS with the plasma membrane and the cationic transport mechanism. The membrane bound and association of endothelial NOS with the cationic transport mechanism are important for NOS activity. It has been recently identified that in contrast to neuronal NOS, the oxygenase domain is the exclusive source of superoxide production in endothelial NOS, while NO production occurs via the reductase domain [24]. The balance of NO to superoxide production by NOS seems to be controlled by tetrahydrobiopterin (BH_4, cofactor for NOS) [26].

Role of Oxidative Stress During ARG Continuous Supplementation

Since the NOS exhibits dualistic nature in producing NO and superoxide, it becomes important to analyze the importance of oxidative stress during continuous ARG supplementation. A recent study [27] shows that short-term (2 h) exposure to at least 50 μM ARG moderately increased endothelial NOS activity and intracellular glucose ($p < 0.05$), with no change in endothelial NOS mRNA or protein expression. In contrast, continuous ARG exposure suppressed endothelial NOS expression and activity. This was accompanied by an increase in glucose and superoxide accumulation. Co-incubation with 100 μM ascorbic acid, 300 U/ml polyethylene glycol-superoxide dismutase (PEG-SOD), 100 μM L-lysine, or 30 μM 5-chloro-2-(N-2,5-dichlorobenenesulfonamido)-benzoxazole (a

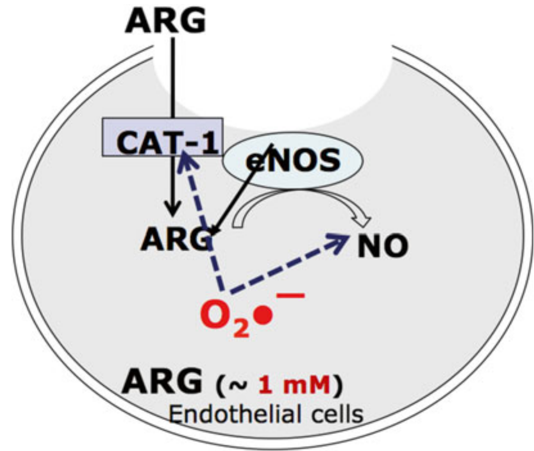

Fig. 3.2 Dualistic nature of NOS. *ARG* L-Arginine, *CAT-1* cationic amino acid transporter, *eNOS* endothelial nitric oxide synthase, *NO* nitric oxide and O2•– superoxide

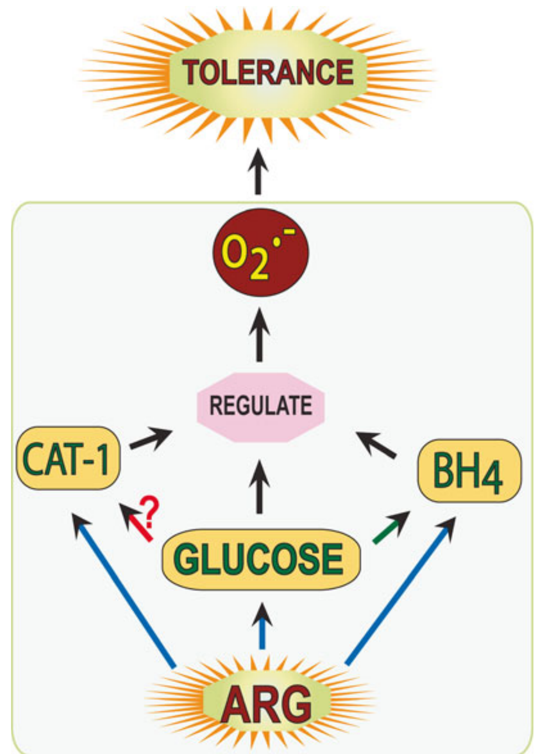

Fig. 3.3 Illustrates ARG-induced oxidative stress may be a primary causative factor for the development of cellular ARG tolerance

fructose-1,6-bisphosphatase inhibitor) prevented the occurrence of cellular ARG tolerance. Short-term co-incubation of ARG with PEG-SOD improved cellular nitrite accumulation without altering cellular ARG uptake. These findings suggest that ARG-induced oxidative stress may be a primary causative factor for the development of cellular ARG tolerance (Fig. 3.3). Several other studies in whole animals have also indicated ARG supplementation to be associated with oxidative stress [28–30], and the specific formation of superoxide from ARG is implicated [30].

Role of NOS Cofactor (BH_4) in Tolerance

The superoxide formed in association with continuous ARG exposure scavenges the available NO, resulting in the formation of peroxynitrite formation, thereby compromising NO bioactivity by promoting the oxidation of BH**4**, leading to eNOS uncoupling [31, 32]. Depletion in redox-sensitive tetrahydrobiopterin (BH**4**), an essential cofactor for eNOS, has caused uncoupling of eNOS from ARG and oxidation of NADPH and ferrous dioxygen species, thereby resulting in endothelial dysfunction via product switching from NO to superoxide [33]. Restoring BH**4** level through subsequent dosing has reduced the symptoms of endothelial dysfunction in chronic smokers and patients with diabetes [34, 35], hypercholesterolemia [26] or ischemia–reperfusion injury [36]. Treatment of deoxycorticosterone acetate salt-induced hypertensive mice with oral BH**4** attenuated vascular reactive oxygen species production, increased NO levels, and blunted hypertension compared with non-hypertensive control mice [37]. Besides supplementing BH**4**, cells exposed to antioxidants such as Glutathione, Vitamin C, or Vitamin E (which are capable of providing chemical stabilization to BH**4**) prevent BH**4** oxidation and increase cellular eNOS activity [38, 39]. These studies provide the initial evidence to suggest oxidation of BH**4** during ARG-induced superoxide generation to be the basis for eNOS uncoupling in vascular dysfunctions.

However, the functionally incompetent oxidized species of BH_4 (7,8-dihydrobiopterin, BH_2) is also known to bind to eNOS [40–42]. A recent study [43] shows the increase in the rate of BH2 binding to eNOS, alteration to intracellular BH_4 to BH_2 ratio, and the greater binding avidity of eNOS with BH2 than BH_4 as the key determinants in initiating the various tolerance-sparing events observed during continuous ARG supplementation, rather than simply a consequence of BH_4 oxidation (Fig. 3.4).

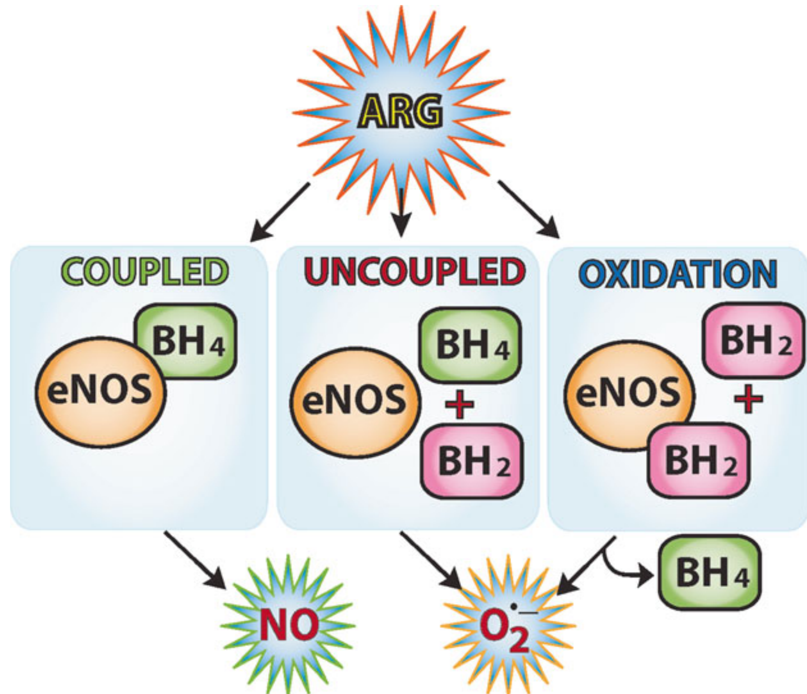

Fig. 3.4 Illustrates the role of BH_4 and BH_2 as key determinants in ARG-mediated oxidative stress

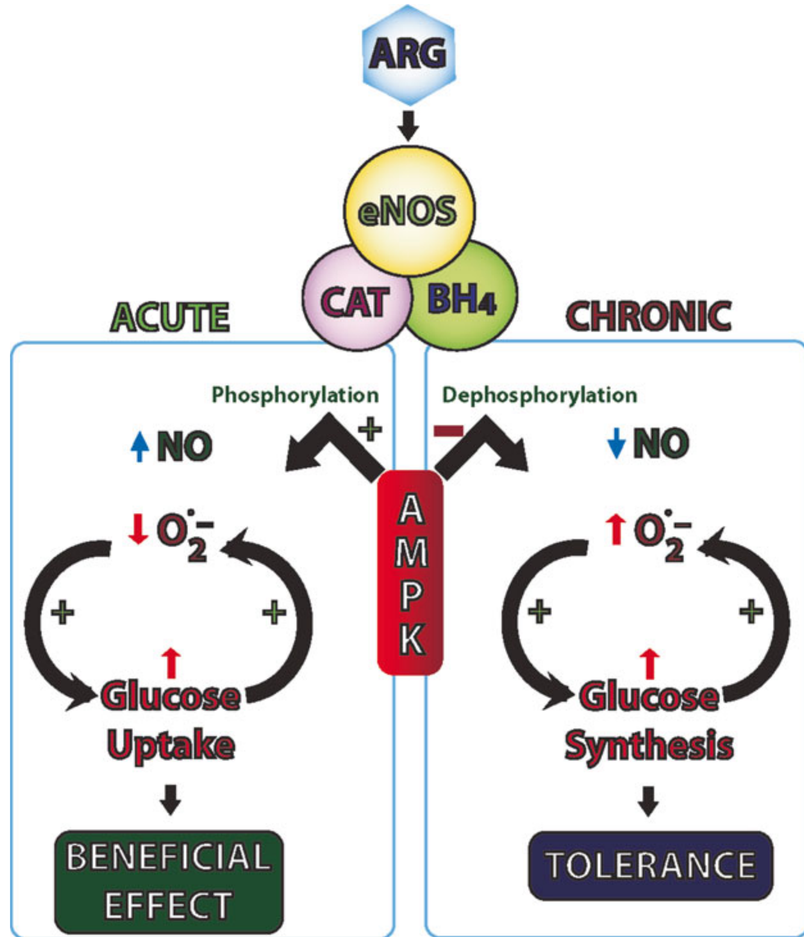

Fig. 3.5 AMP-activated protein kinase (AMPK) as the primary modulating factor in L-Arginine tolerance

Role of AMP-Activated Protein Kinase (AMPK) Towards ARG Responses

While studies have identified ARG tolerance after continuous supplementation of this amino acid to be mediated by endothelial NOS downregulation, secondary to oxidative stress, the fundamental mechanistic factor that controls ARG-mediated superoxide production that is responsible for initiating further downstream tolerance-sparing events remains unaddressed. Not much progress has been reached until recently, where AMPK has been suggested as the potential modulator of the events associated with ARG tolerance (and potential toxicity) [44, 45].

AMPK plays a significant role in energy-sensing/signaling system utilized by cells to detect and respond to changes in energy levels [46]. The NO generated by eNOS via ARG utilization is known to be required for the initial activation of AMPK [47], possibly via calmodulin-dependent protein kinase kinase [48, 49] or other mechanistic pathways [50, 51]. eNOS knockdown in mice or shear stress in endothelial cells [52] suppressed AMPK activity, emphasizing the importance of endogenous NO in AMPK activation and subsequent metabolism of energy substrates.

When AMPK is activated, processes of ATP-consumption, such as lipogenesis or gluconeogenesis, are switched off, whereas ATP-producing pathways like fatty acid and glucose oxidation are switched on. AMPK activation also increases NO synthesis by eNOS under various physiological and pathological conditions [53, 54]. While NO controls AMPK function through the activation of

guanylyl cyclase, peroxynitrite that is formed by the reaction between NO and superoxide can also impose its regulation on AMPK activation by impairing guanylyl cyclase [55–57]. The recent AMPK modulation study suggests that ARG mediated short-term therapeutic benefits to be initiated via the activation of AMPK, which stimulates downstream NO release by maintaining eNOS activity and allowing glucose to accumulate only via cellular transport (Fig. 3.5). The dysfunction in AMPK enzyme activity affected eNOS function, decreased glucose uptake from medium, and increased cellular glucose synthesis and oxidative stress. All of these events seen during AMPK dysfunction are concomitant with those reported to occur during continuous ARG supplementation [58].

Conclusion

We have addressed some of the potential factors that play an important role in developing tolerance during continuous ARG supplementation. AMPK has been shown as the fundamental modulating factor in the development of ARG tolerance and in controlling the downstream tolerance-sparing events. Oxidative stress or superoxide overproduction has been shown as the primary indicator in triggering tolerance-sparing events. The BH_4-to-BH_2 ratio serves as the molecular link between oxidative stress and endothelial dysfunction during continuous ARG supplementation.

References

1. Durante W, Johnson FK, Johnson RA. Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin Exp Pharmacol Physiol. 2007;34(9):906–11.
2. Barbul A. L-Arginine: biochemistry, physiology, and therapeutic implications. JPEN J Parenter Enteral Nutr. 1986;10(2):227–38.
3. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(Pt 1):1–17.
4. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988;333(6174):664–6.
5. Medlineplus. http://www.nlm.nih/gov/medlineplus/drugingo/natural/875.html. http://www.nlm.nih/gov/medlineplus/drugingo/natural/875.html; 2013.
6. Baydoun AR, Emery PW, Pearson JD, Mann GE. Substrate-dependent regulation of intracellular amino acid concentrations in cultured bovine aortic endothelial cells. Biochem Biophys Res Commun. 1990;173(3):940–8.
7. Bednarz B, Jaxa-Chamiec T, Maciejewski P, et al. Efficacy and safety of oral L-arginine in acute myocardial infarction. Results of the multicenter, randomized, double-blind, placebo-controlled ARAMI pilot trial. Kardiol Pol. 2005;62(5):421–7.
8. Kakoki M, Kim HS, Edgell CJ, Maeda N, Smithies O, Mattson DL. Amino acids as modulators of endothelium-derived nitric oxide. Am J Physiol Renal Physiol. 2006;291(2):F297–304.
9. Oka RK, Szuba A, Giacomini JC, Cooke JP. A pilot study of L-arginine supplementation on functional capacity in peripheral arterial disease. Vasc Med. 2005;10(4):265–74.
10. Pollock JS, Forstermann U, Mitchell JA, et al. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad Sci USA. 1991;88(23):10480–4.
11. Tangphao O, Grossmann M, Chalon S, Hoffman BB, Blaschke TF. Pharmacokinetics of intravenous and oral L-arginine in normal volunteers. Br J Clin Pharmacol. 1999;47(3):261–6.
12. Ceremuzynski L, Chamiec T, Herbaczynska-Cedro K. Effect of supplemental oral L-arginine on exercise capacity in patients with stable angina pectoris. Am J Cardiol. 1997;80(3):331–3.
13. Maxwell AJ, Anderson BE, Cooke JP. Nutritional therapy for peripheral arterial disease: a double-blind, placebo-controlled, randomized trial of HeartBar. Vasc Med. 2000;5(1):11–9.
14. Bednarz B, Jaxa-Chamiec T, Gebalska J, Herbaczynska-Cedro K, Ceremuzynski L. L-arginine supplementation prolongs exercise capacity in congestive heart failure. Kardiol Pol. 2004;60(4):348–53.
15. Hambrecht R, Hilbrich L, Erbs S, et al. Correction of endothelial dysfunction in chronic heart failure: additional effects of exercise training and oral L-arginine supplementation. J Am Coll Cardiol. 2000;35(3):706–13.

16. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood. 2007;109(4):1568–73.
17. Shang HF, Wang YY, Lai YN, Chiu WC, Yeh SL. Effects of L-arginine supplementation on mucosal immunity in rats with septic peritonitis. Clin Nutr. 2004;23(4):561–9.
18. Zhu H, Liu Y, Xie X, Huang J, Hou Y. Effect of L-arginine on intestinal mucosal immune barrier function in weaned pigs after Escherichia coli LPS challenge. Innate Immun. 2012;19:242–52.
19. Zhou M, Martindale RG. L-Arginine in the critical care setting. J Nutr. 2007;137(6 Suppl 2):1687S–92S.
20. Raghupathy R, Billett HH. Promising therapies in sickle cell disease. Cardiovasc Hematol Disord Drug Targets. 2009;9(1):1–8.
21. Chen J, Kuhlencordt P, Urano F, Ichinose H, Astern J, Huang PL. Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knockout mice. Arterioscler Thromb Vasc Biol. 2003;23(1):97–103.
22. Wilson AM, Harada R, Nair N, Balasubramanian N, Cooke JP. L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. Circulation. 2007;116(2):188–95.
23. Shin S, Mohan S, Fung HL. Intracellular L-arginine concentration does not determine NO production in endothelial cells: implications on the "L-arginine paradox". Biochem Biophys Res Commun. 2011;414(4):660–3.
24. Stroes E, Hijmering M, van Zandvoort M, Wever R, Rabelink TJ, van Faassen EE. Origin of superoxide production by endothelial nitric oxide synthase. FEBS Lett. 1998;438(3):161–4.
25. Miller RT, Martasek P, Roman LJ, Nishimura JS, Masters BS. Involvement of the reductase domain of neuronal nitric oxide synthase in superoxide anion production. Biochemistry. 1997;36(49):15277–84.
26. Stroes E, Kastelein J, Cosentino F, et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest. 1997;99(1):41–6.
27. Mohan S, Wu CC, Shin S, Fung HL. Continuous exposure to L-arginine induces oxidative stress and physiological tolerance in cultured human endothelial cells. Amino Acids. 2012;43(3):1179–88.
28. Chen J, Kuhlencordt P, Urano F, Ichinose H, Astern J, Huang PL. Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knockout mice. Arterioscler Thromb Vasc Biol. 2003;23(1):97–103.
29. Huang HS, Ma MC, Chen J. Chronic L-arginine administration increases oxidative and nitrosative stress in rat hyperoxaluric kidneys and excessive crystal deposition. Am J Physiol Renal Physiol. 2008;295(2):F388–96.
30. Simonet S, Rupin A, Badier-Commander C, Coumailleau S, Behr-Roussel D, Verbeuren TJ. Evidence for superoxide anion generation in aortas of cholesterol-fed rabbits treated with L-arginine. Eur J Pharmacol. 2004;492(2–3):211–6.
31. Bitar MS, Wahid S, Mustafa S, Al-Saleh E, Dhaunsi GS, Al-Mulla F. Nitric oxide dynamics and endothelial dysfunction in type II model of genetic diabetes. Eur J Pharmacol. 2005;511(1):53–64.
32. Schmidt TS, Alp NJ. Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. Clin Sci (Lond). 2007;113(2):47–63.
33. Veresh Z, Racz A, Lotz G, Koller A. ADMA impairs nitric oxide-mediated arteriolar function due to increased superoxide production by angiotensin II-NAD(P)H oxidase pathway. Hypertension. 2008;52(5):960–6.
34. Pieper GM, Jordan M, Dondlinger LA, Adams MB, Roza AM. Peroxidative stress in diabetic blood vessels. Reversal by pancreatic islet transplantation. Diabetes. 1995;44(8):884–9.
35. Shinozaki K, Nishio Y, Okamura T, et al. Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ Res. 2000;87(7):566–73.
36. Tiefenbacher CP, Chilian WM, Mitchell M, DeFily DV. Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. Circulation. 1996;94(6):1423–9.
37. Landmesser U, Dikalov S, Price SR, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest. 2003;111(8):1201–9.
38. Wolff DJ, Datto GA, Samatovicz RA. The dual mode of inhibition of calmodulin-dependent nitric-oxide synthase by antifungal imidazole agents. J Biol Chem. 1993;268(13):9430–6.
39. Yoshida A, Pozdnyakov N, Dang L, Orselli SM, Reddy VN, Sitaramayya A. Nitric oxide synthesis in retinal photoreceptor cells. Vis Neurosci. 1995;12(3):493–500.
40. Gross SS, Jaffe EA, Levi R, Kilbourn RG. Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by L-arginine analogs with a rank-order of potency characteristic of activated macrophages. Biochem Biophys Res Commun. 1991; 178(3):823–9.
41. Kwon NS, Nathan CF, Stuehr DJ. Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. J Biol Chem. 1989;264(34):20496–501.
42. Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. J Biol Chem. 1989;264(33):19654–8.

43. Mohan S, Patel H, Bolinaga J, Soekamto N, Achu L, Teklemariam K. Dihydrobiopterin (BH2): key determinant in influencing L-arginine mediated endothelial tolerance and dysfunction. Am J Biochem Biotechnol. 2012;8(2): 54–62.
44. Mohan S, Patel H, Bolinaga J, Soekamto N. AMP-activated protein kinase regulates L-arginine mediated cellular responses. Nutr Metab. 2013;10(1):40.
45. de Castro Barbosa T, Jiang LQ, Zierath JR, Nunes MT. L-Arginine enhances glucose and lipid metabolism in rat L6 myotubes via the NO/ c-GMP pathway. Metabolism. 2013;62(1):79–89.
46. Winder WW. Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. J Appl Physiol. 2001;91(3):1017–28.
47. Zhang J, Xie Z, Dong Y, Wang S, Liu C, Zou MH. Identification of nitric oxide as an endogenous activator of the AMP-activated protein kinase in vascular endothelial cells. J Biol Chem. 2008;283(41):27452–61.
48. Mount PF, Lane N, Venkatesan S, et al. Bradykinin stimulates endothelial cell fatty acid oxidation by CaMKK-dependent activation of AMPK. Atherosclerosis. 2008;200(1):28–36.
49. Stahmann N, Woods A, Carling D, Heller R. Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca2+/calmodulin-dependent protein kinase kinase beta. Mol Cell Biol. 2006;26(16):5933–45.
50. Wohlfart P, Malinski T, Ruetten H, et al. Release of nitric oxide from endothelial cells stimulated by YC-1, an activator of soluble guanylyl cyclase. Br J Pharmacol. 1999;128(6):1316–22.
51. Hwang TL, Hung HW, Kao SH, Teng CM, Wu CC, Cheng SJ. Soluble guanylyl cyclase activator YC-1 inhibits human neutrophil functions through a cGMP-independent but cAMP-dependent pathway. Mol Pharmacol. 2003;64(6):1419–27.
52. Chen H, Levine YC, Golan DE, Michel T, Lin AJ. Atrial natriuretic peptide-initiated cGMP pathways regulate vasodilator-stimulated phosphoprotein phosphorylation and angiogenesis in vascular endothelium. J Biol Chem. 2008;283(7):4439–47.
53. Reihill JA, Ewart MA, Hardie DG, Salt IP. AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. Biochem Biophys Res Commun. 2007;354(4):1084–8.
54. Fisslthaler B, Fleming I, Keseru B, Walsh K, Busse R. Fluid shear stress and NO decrease the activity of the hydroxy-methylglutaryl coenzyme A reductase in endothelial cells via the AMP-activated protein kinase and FoxO1. Circ Res. 2007;100(2):e12–21.
55. Weber M, Lauer N, Mulsch A, Kojda G. The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. Free Radic Biol Med. 2001;31(11):1360–7.
56. Munzel T, Daiber A, Mulsch A. Explaining the phenomenon of nitrate tolerance. Circ Res. 2005;97(7):618–28.
57. Stasch JP, Schmidt PM, Nedvetsky PI, et al. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. J Clin Invest. 2006;116(9):2552–61.
58. Mohan S, Wu CC, Shin S, Fung HL. Continuous exposure to L-arginine induces oxidative stress and physiological tolerance in cultured human endothelial cells. Amino Acids. 2011.

Chapter 4
Amidine-Based Compounds Affecting L-Arginine Metabolism

Cristina Maccallini, Marialuigia Fantacuzzi, and Rosa Amoroso

Key Points

- In the living organism, one of the main functions of L-Arg is the role as a precursor of NO, a free radical molecule considered one of the main regulators of cell metabolism.
- The amidine motif has emerged as highly representative of the alternative guanidine architecture in a number of L-Arg mimics, and several bioactive amidines were discovered as inhibitors of enzymes involved in L-Arg metabolism.
- In the research of non-amino acid NOS inhibitors, the amidino group has been revealed to be a pharmacophoric element useful for obtaining potent and highly selective iNOS and nNOS inhibitors.
- The amount of NO in human body is regulated by NO–ADMA–DDAH axis. Some alkylamidines are DDAH inhibitors, increasing ADMA and NMMA, with inhibition of nNOS and reduction of NO levels in pathological situations.
- The overproduction of citrullinated proteins by PAD enzyme results in autoimmune system attacks. A series of amidines containing an electron-withdrawing leaving group were selected as selective PAD inhibitors.
- The upregulation of $PRMT_1$ expression has been disclosed in heart disease and in various types of human cancers like prostate cancer, breast cancer, and leukemia. Therefore, the selective inhibition of $PRMT_1$ by acetamidino compounds would have beneficial effects on these pathologies.

Keywords Amidines • L-Arginine metabolism • Dimethylarginine dimethylaminohydrolase (DDAH) • Nitric oxide synthase (NOS) • Protein L-arginine methyltransferase 1 ($PRMT_1$) • Peptidylarginine deiminase (PAD)

Abbreviations

ADMA	N^{ϖ},N^{ϖ}-dimethyl-L-arginine, asymmetric dimethyl-L-arginine
BH_4	Tetrahydrobiopterin
DDAH	Dimethylarginine dimethylaminohydrolase

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eNOS or NOS III	Endothelial NOS
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
HNO	Nitroxyl
iNOS or NOSII	Inducible NOS
L-Arg	L-arginine
L-Cit	L-citrulline
L-Cl-NIO	N^5-(1-imino-2-chloroethyl)-L-ornithine
L-IPO	N^5-(1-iminoproyl)-L-ornithine
L-VNIO	N^5-(1-imino-3-butenyl)-L-ornithine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMMA	N^{ϖ}-methyl-L-arginine, N^{ω}-monomethyl-L-arginine
nNOS or NOS I	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
PAD	Peptidylarginine deiminase
PRMT	Protein L-arginine methyltransferase 1
SDMA	N^{ω},N'^{ω}-dimethyl-L-arginine, symmetric dimethyl-L-arginine

Introduction

The L-Arg availability or the production of end products can be altered in different pathological conditions, resulting in profound physiologic consequences, with disruption of normal homeostasis in the human body. In particular, in recent years great interest has been directed to therapeutic applications able to restore the metabolism of L-Arg, increasing substrate availability or influencing specific pathways. The development of L-arginine mimetics is one of the approaches used for treating diseases related to the altered metabolism of L-Arg; main L-Arg structural modifications refer to the α-*N* or α-*C* derivatization, the synthesis of constrained analogs, or the diversification of the guanidino moiety with a bioisotere. Among L-arginine mimetics, the amidino motif has proved to be the most important substitute of the guanidine architecture. In effect, amidines are derivatives of carboxylic acids, in which the hydroxyl group is replaced by an amino group and the carbonyl group by an azomethine double bond. They are strong organic bases due to the charge delocalization occurring over the two nitrogen atoms in the protonated form. This structure offers the possibility to establish specific interactions, mainly bidentate hydrogen bondings, with proteins, and the nature of the amidino substituent can modulate the structural protein recognition process [1].

Several bioactive amidines were discovered in the last years as inhibitors of four enzyme families, namely nitric oxide synthase (NOS), dimethylarginine dimethylaminohydrolase (DDAH), protein L-arginine methyltransferase (PRMT), and peptidylarginine deiminase (PAD). Here we review the research for potent and selective amidine compounds acting on the mentioned enzyme families.

Amidine-Based Inhibitors of Nitric Oxide Synthase

NO is a small, gaseous radical molecule, endowed with high diffusion rate and lipophilicity, resulting in tissue bioaccumulation. Due to its complex reactivity in cellular environments and to the availability of many reactive targets, NO plays complicated and sophisticated roles in mammals. Thus, NO may be reduced to nitroxyl (HNO), oxidized to higher oxides, or bound to iron centers with a number of distinctive biological consequences in each of these cases [2].

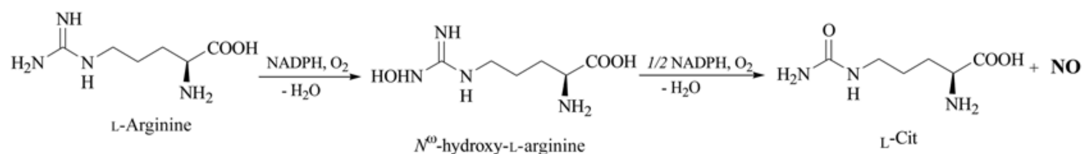

Fig. 4.1 Biosynthesis of NO

NO is in vivo synthesized from L-Arg by three distinct isoforms of the enzyme NOS, via a five-electron transfer process involving reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen as additional substrates, together with a series of cofactor and prosthetic groups, such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, and tetrahydrobiopterin (BH_4) (Fig. 4.1) [3]. Two of the NOS isoforms are expressed in a constitutive manner, and, according to the tissue where they were for the first time identified, they are reported as neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III). Under normal physiological conditions, these enzymes generate low, transient levels of NO. In particular, nNOS mainly catalyzes NO production in the Nervous System and it is involved in the neurotransmission and long-term potentiation; eNOS is essential in the regulation of the vascular tone, in platelet adhesion, and in the smooth muscle relaxation. The expression of the third isoform, referred as inducible NOS (iNOS or NOSII), is induced in response to endotoxins and/or cytokines and this enzyme generates high, sustained levels of local NO, important in the immune defense against pathogens.

Depending on the isoform involved in its biosynthesis and on the level of its production, NO may exert both positive and negative effects on physiological conditions and pathophysiological progression. Thus, a decreased NO production or a reduction of its bioavailability in the vasculature are key features of the endothelial dysfunction [4]. Moreover, NO in the vasculature enhances insulin signaling, and low levels of NO, as well as the generation of different reactive oxygen and nitrogen species by uncoupled eNOS are connected to diabetes progression [5]. On the other hand, also the uncontrolled overproduction of NO by iNOS and/or nNOS is detrimental and plays a role in a number of disease states, such as congestive heart failure, septic shock, migraine, asthma, cerebral ischemia, Parkinson's disease, and cancer [6]. Therefore, the inhibition of iNOS or nNOS, especially when an overexpression of these two isoforms occurs, could provide effective therapeutic approaches, although it is imperative that NOS inhibitors do not affect eNOS, as this will lead to unwanted side effects in the cardiovascular system [7].

The high level of structural homology between the three isoforms, in particular in the binding site oxygen domain, explains the difficulty in finding selective NOS inhibitors. Structural features responsible for the selective inhibition over eNOS mainly rely on the inhibitors' ability to give specific interactions at the periphery of iNOS or nNOS catalytic domain, where the major differences among the three isoforms are localized. Anyway, key features are also the different size of the heme ligand binding cavity or the conformational flexibility in the active site, that allow the adoption of different conformations in response to interactions with inhibitors. In the research of selective, non-amino acid NOS inhibitors, the amidino group has been revealed to be a pharmacophoric element useful for obtaining potent and highly selective iNOS inhibitors. Indeed the amidino group provides useful interactions within the NOS catalytic domain, establishing a bidentate interaction with a conserved glutamic acid, and one hydrogen bond with the carbonyl group of a tryptophan residue (Fig. 4.2) [8].

Several acetamidines were disclosed in the past years, such as the *N*-(3-(aminomethyl)benzyl) acetamidine (**1**, 1400W, Fig. 4.3), which is one of the most potent inhibitors of the human iNOS (IC_{50} < 0.2 μM) [9].

Bioactive acetamidines are supposed to act as irreversible inhibitors, affecting the heme prosthetic group of NOS and leading to protein degradation [10]. Recently, new benzyl- and *N*-substituted-aminomethyl-benzyl acetamidines (**2–4**, Fig. 4.4), structurally related to 1400W, were synthesized; these molecules inhibit iNOS or nNOS with a very high degree of selectivity toward eNOS (best IC_{50} 0.1 μM for iNOS and 0.2 μM for nNOS) [11–13]. In particular, in the benzyl-acetamidine series, the

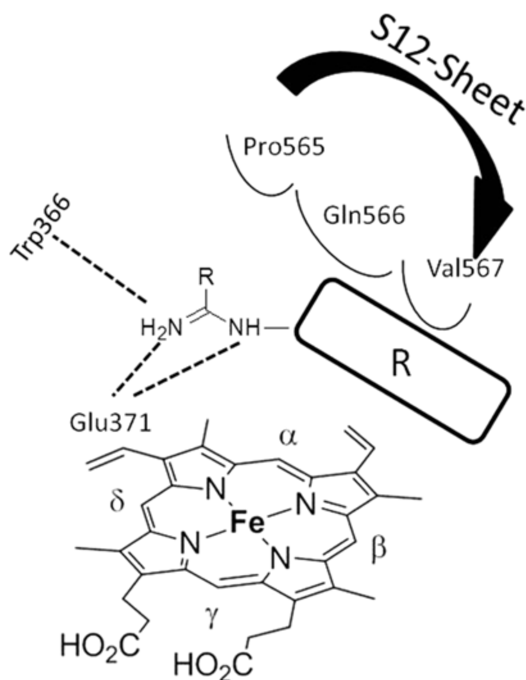

Fig. 4.2 Amidine-based inhibitors into the catalytic pocket of NOS

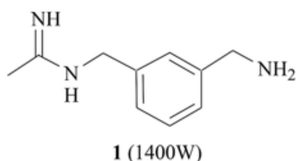

Fig. 4.3 1400W

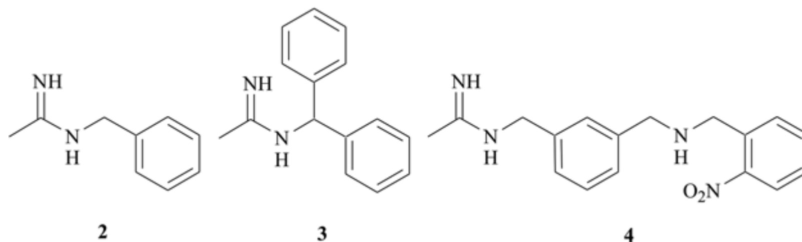

Fig. 4.4 N- substituted-aminomethyl-benzyl acetamidines

bulky substituents on the benzylic carbon, connecting the aromatic core to the acetamidine nitrogen, seem to play a role in promoting the binding at the nNOS catalytic site. Indeed they might furnish positive contacts with the hydrophobic residues Pro565 and Val567 of the S12 sheet, which is positioned atop the heme cavity.

In the last years, Pharmacia patented several selective acetamidine iNOS inhibitors (**5–8**), based on a rigid alkenyl or thio-alkyl α-amino acid scaffold (Fig. 4.5). For these molecules, best IC_{50} values on

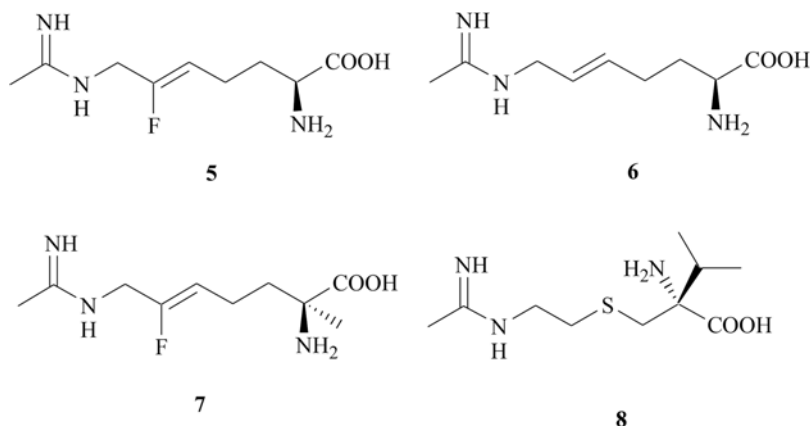

Fig. 4.5 Pharmacio-patented selective iNOS inhibitors

human iNOS are comprised between 0.4 and 0.9 μM, with a selectivity ratio toward human eNOS between 60- and 100-fold [14]. These iNOS inhibitors were found to be highly active in the human cartilage explant assay, a model for osteoarthritis, demonstrating also to be less able to penetrate other organs in test system.

The thiophene-2-carboximidamido group conjugated to an aromatic system was discovered to be an important pharmacophoric moiety in the research of nNOS inhibitors (Fig. 4.6). The fact that these compounds are selective inhibitors of nNOS, while *N*-substituted acetamidines mainly inhibit the iNOS, could be probably due to the larger size of the heme binding cavity of nNOS with respect to eNOS and iNOS, which appear to be less able to fit the steric hindered heterocyclic amidines [15]. Initially a series of 5- and 6-amidino indoles were patented by Neuraxon, and the representative compounds **9–11** showed nNOS IC_{50} ranging between 0.2 μM (**11**) and 12 μM (**10**) and high selectivity toward eNOS and iNOS (>100-folds) [16]. These inhibitors also showed efficacy in several models of neuropathic-like pain states. New series of 3,5-indole and 1,6-indoline substituted thiophene-2-carboximidamides (**12–14**) were later disclosed, with selective inhibition of nNOS and efficacy in visceral and neuropathic pain (best nNOS IC_{50} 0.26 μM for compound **12**) [17, 18]. Other series of thiophene-2-carboximidamido derivatives developed by Neuraxon included quinolones, benzoxazines, and benzothiazines (**15–17**) [19]. Recently, Silverman and coworkers patented a series of potent nNOS inhibitors, including compound **18**, featuring a double thiophene carboximidamide moiety properly spaced by different linkers, exhibiting excellent efficacy in melanoma cell line [20].

Other examples of bioactive nNOS inhibitors are aminopyridine or indazole derivatives such as compound **19** (Fig. 4.7), in which the amidino group is included into an aromatic ring [21].

Amidine-Based Inhibitors of Human $DDAH_1$

The amount of NO available in the human body is regulated not only by the levels of intracellular L-Arg but also by endogenous inhibitors of NOS, the methylarginines, such as N^{ϖ},N^{ϖ}-dimethyl-L-arginine (asymmetric dimethyl-L-arginine, ADMA) and N^{ϖ}-methyl-L-arginine (N^{ω}-monomethyl-L-arginine, NMMA). ADMA and NMMA are produced by posttranslational methylation of protein-bound L-Arg residues, and they are mainly degraded by cysteine hydrolase DDAH into L-Cit and the corresponding alkylamines (Fig. 4.8) [22]. They inhibit all of the three NOS isoforms with *Ki* values in the low μM range.

9 R = N(CH$_3$)$_2$

10 R = Ph

11 R =

12

13

14

15

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17

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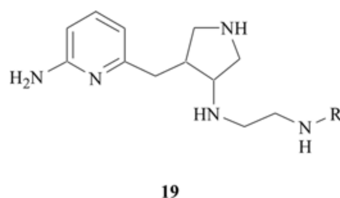
Fig. 4.6 Thiophene carboximidamide derivatives developed as nNOS inhibitors



19

Fig. 4.7 Aminopyridine scaffold of nNOS inhibitors

In recent years, considerable attention was focused on methylarginines, because they can indirectly modulate NO bioavailability. Increased levels of ADMA are associated with inhibition of eNOS and decrease in NO bioavailability, leading to endothelial dysfunction, with consecutive increased systemic vascular resistance, elevated blood pressure, and risk for cardiovascular events. On the other hand, reduced ADMA levels are associated with NO overproduction by iNOS, resulting in elevate oxidative stress and several pathophysiological conditions. Since the plasma levels of ADMA are mainly regulated by its catabolism, the degrading enzyme DDAH represents an interesting target for pharmacological interventions [23].

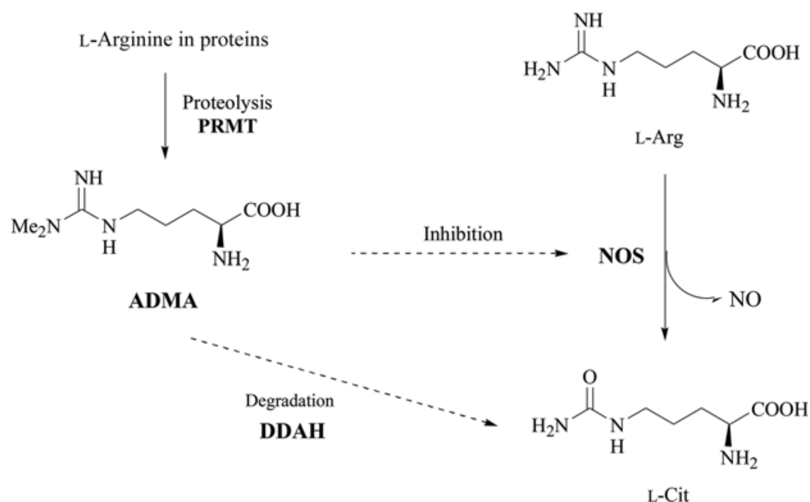


Fig. 4.8 Overview on the NO–NOS–DDAH pathway

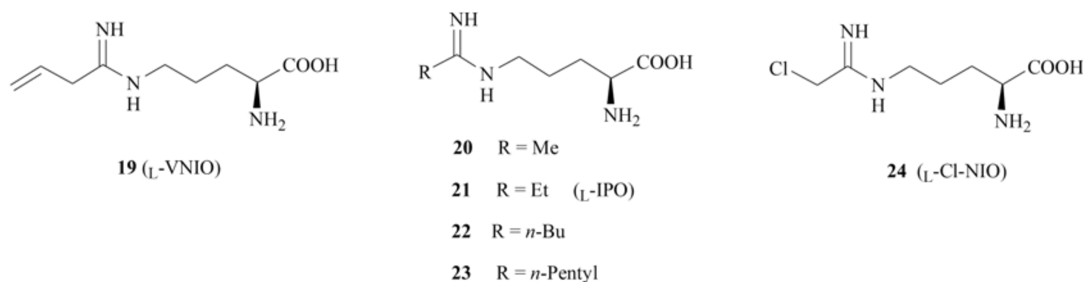


Fig. 4.9 Amidine-based inhibitors of DDAH

The crystallographic structure of DDAH shows that the active site contains a Cys–His–Glu/Asp catalytic triad. Binding of ADMA leads to deprotonation of the Cys; the resulting thiolate attacks the guanidine group of ADMA, releasing dimethylamine and forming a stable thiouronium intermediate, which releases L-Cit upon hydrolysis. Two isoforms of DDAH have been discovered so far, sharing 62 % sequence similarity, with different tissue expression patterns: $DDAH_1$ is mainly localized in the brain, pancreas, and liver, while $DDAH_2$ is highly expressed in the vascular endothelial cells, kidneys, and placenta. Although there is a very complicated relationship between DDAH and NOS, depending on cell type and tissue, as well as pathophysiological conditions, $DDAH_1$ is connected with nNOS, while $DDAH_2$ predominates in tissues expressing eNOS, indicating an isoform-specific regulation of NOS activity [24].

The inhibition of DDAH would increase the concentrations of ADMA and NMMA, with inhibition of NOS and reduction of NO levels in pathological situations where NO is overproduced, through the axis NO–ADMA–DDAH. However, the inhibition of the $DDAH_2$ isoform should be carefully avoided, as it results in a NO deficiency in the cardiovascular system, where it plays an essential role [25].

Recently, some inhibitors of DDAH were designed, replacing the guanidino moiety of L-Arg with a bioisosteric amidine (Fig. 4.9). In a context of a study on the binding pocket of the guanidino moiety of ADMA, a series of N^5-(1-iminoalk(en)yl)-L-ornithines were obtained and tested as inhibitors of $DDAH_1$ [26]. The N^5-(1-imino-3-butenyl)-L-ornithine (**19**, L-VNIO) showed 97 % inhibition at 1 mM with an IC_{50} of 13 µM, when assayed against human $DDAH_1$. Analogs of L-VNIO, with shorter side

chains and without double bonds, showed reduced potency. Since some amidines are also known to be selective inhibitors of NOS, studies following the discovery of L-VNIO concerned the development of dual targeted inhibitors. NOS and DDAH share the same substrate, the ADMA, and selective inhibitors on both nNOS and $DDAH_1$ could be very useful therapeutic agents in the control of NO production in several diseases.

The alkylamidine scaffold was selected for exploration of the chemical space occupied by ligands of NOS, DDAH, or both, showing that simple alterations in the chain length resulted in a different activity: nNOS was inhibited by amidines with shorter alkyl chain (compounds **20**–**22**, R = Me, Et, *n*-Bu, respectively), whereas the compound having an iminopentyl side chain **23** (R = *n*-pentyl) inhibits $DDAH_1$ [27]. Comparison of inhibitor-bound structures of both $DDAH_1$ and NOS revealed some peculiarities, explaining the different enzymatic affinities: the active site of $DDAH_1$ is slightly larger than NOS, thereby allowing longer substituents. In contrast, the smaller active site of NOS limits the size of ligand by steric hindrance. The alkylamidines with intermediate side chains can be inserted in the overlapping portion of the two enzymes. Therefore, the choice of the appropriate alkyl chain could achieve a dual inhibition of both enzymes. The inhibition of $DDAH_1$ by alkylamidine N^5-(1-iminoproyl)-L-ornithine (**21,** L-IPO) was studied in detail: an X-ray crystal structure of L-IPO:$DDAH_1$ complex indicated that the inhibitor binds in a manner similar to that of related DDAH substrates. The amidino carbon forms a tetrahedral intermediate with Cys274 in the active site; kinetic studies showed this covalent adduct to be in rapid equilibrium with the parent compound and enzyme, indicating a reversible competitive inhibition.

To rank the inhibitory potency of *N*-alkylamidines in live mammalian cell culture, a click chemistry-based activity probe was constructed. The IC_{50} values were measured in living HEK293T cells, showing the same trend observed in vitro. The specific binding interactions of L-VNIO with DDAH were determined by site-directed mutagenesis of the active-site Cys residue, combined with X-ray crystallography and isothermal titration calorimetry [28]. L-VNIO forms favorably a tetrahedral adduct with DDAH, but this covalent bond does not make it quite potent as compared to other reversible inhibitors. This behavior is probably due to the inability of covalent adduct to establish the key binding interactions normally made by substrates during turnover, such as the interaction with His173 in the active site. The stabilization of covalent adduct, through the insertion of additional favorable interactions into the enzyme binding site, is the basis for the development of more potent $DDAH_1$ inhibitors as NO-blocking therapeutics.

In a recent study, a new alkylamidine, the N^5-(1-imino-2-chloroethyl)-L-ornithine (**24**, L-Cl-NIO), was shown to be a potent and selective inhibitor of human $DDAH_1$ (IC_{50} 6.6 μM) [29]. A screen of diverse melanoma cell lines revealed an upregulation of $DDAH_1$ relative to normal melanocyte control lines. Treatment of the melanoma A375 cell line with L-Cl-NIO showed a decrease in NO production mediated by DDAH–methylarginine–NO axis.

Amidine-Based Inhibitors of PAD

The deimination of peptidyl L-arginine residues in peptidyl citrulline is another posttranslational modification, referred as citrullination, catalyzed by a family of enzymes named PAD (Fig. 4.10). The citrullination is implicated in a number of physiological processes, including gene regulation, embryonic development, terminal differentiation of epidermis, and apoptosis [30]. In humans, the PAD family consists of five calcium-dependent isozymes ($PAD_{1,2,3,4,6}$), each encoded by a distinct gene clustered on chromosome 1 (1p35-36), possessing high degree of sequence homology (~50 %). There is a tissue-specific expression of the isozymes: PAD_2 is widely expressed in every tissue and cell type, while the other PADs are quite restricted, $PAD_{1,3,4,6}$ being most predominant in skin, hair follicles, immune cells, and oocytes, respectively [31]. Noteworthy,

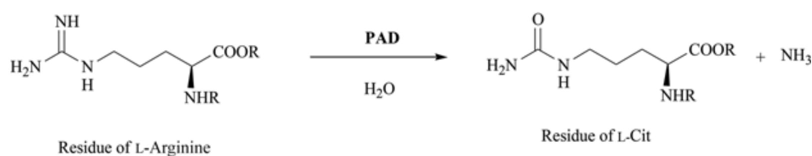


Fig. 4.10 PAD hydrolyzes the guanidinium group of a peptidyl-Arg in a peptidyl-Cyt and ammonium

all PADs are localized in the cell cytoplasm, with the exception of PAD_4 that is localized in the nucleus; even though, very recent data suggest that PAD_4 could be present in granules and PAD_2 in mitochondria. PAD_4, and partly PAD_2, has been implicated in the histone deimination with a consequent effect on overall chromatin function, as for example in gene transcription, but many other PAD substrates like fibrinogen, filaggrin, and actin exist, and, more specifically, PAD_4 is responsible for the citrullination of other proteins such as p300, ING4, RPS2, lamin C, and nucleophosmin [32].

PAD contains a Ca^{2+}-binding motif and its activity depends on the presence of high levels of Ca^{2+}, achieved by influx from intracellular Ca^{2+} stores or extracellular space (apoptosis, oxidative stress), while the intracellular concentration of Ca^{2+} is too low for PAD activation. Although different efforts were made to elucidate the structures of each isozyme, PAD_4 is the most investigated. The *3D* PAD structure could be divided into a *C*-terminal domain, where the catalytic reaction takes place, a *N*-terminal domain, folded into β-sheets, and five calcium binding sites, two of which are involved in the bridge between *N* and *C* domains, while the others are located in the *N*-domain [33]. Conformational changes of the enzyme that allow the exposition of catalytic triad Cys–His–Glu/Asp in the active site, are caused by the binding of Ca^{2+}. The substrate binding mode is similar to other L-arginine-converting enzyme, like DDAH: a Cys residue is critical for catalysis, acting as a nucleophile, and binding the carbon atom of the guanidino group of peptidyl-arginine. The resulting thiouronium intermediate is subsequently hydrolyzed to form peptidyl-citrulline [34].

Protein citrullination has been related to several human diseases, since citrullinated proteins activate the immune system against its own tissues, causing many autoimmune diseases such as rheumatoid arthritis, demyelinating disease (in particular multiple sclerosis), psoriasis, and systemic lupus erythematosus. Furthermore, elevated citrullinated proteins were found in other human diseases including ulcerative colitis, ankylosing spondylitis, osteoarthritis, Crohn's disease, glaucoma, and cancer [35].

The development of PAD-selective inhibitors is essential to understand the precise role of each PAD isozyme in human diseases and to realize the possible use of a single or multiple isozymes inhibitor as therapeutics. The substitution of the guanidino group of the L-Arg residue with an acetamidino group allows the inhibitor to make *H*-bond contacts with both Asp350 and Asp473 (PAD_4), two important residues for the catalytic process and substrate recognition. In this way, PAD inhibitors/inactivators could react with the catalytic nucleophile Cys to form a tetrahedral intermediate, which subsequently collapses to form a thioether product via two proposed mechanisms (Fig. 4.11). All the PAD inhibitors are haloacetamidines, constituted by an amidine warhead containing an electron-withdrawing leaving group, a polymethylene linker, and a specific backbone crucial to target the active site (Fig. 4.12). The *F*-amidine **25** (IC_{50} 21.6 μM) and *Cl*-amidine **26** (IC_{50} 5.9 μM) were the first irreversible pan-PAD inhibitors discovered [34]. A consecutive SAR study performed on the same molecular scaffold and the active site of the enzyme led to the discovery that many modifications on the side chain do not result in potency enhancement, while the introduction of a carboxylate at the *orto* position on the benzoyl ring both increases potency for all of the PADs and addresses selectivity mainly toward PAD_1 (**27**, *o-F*-amidine) or $PAD_{1,4}$ (**28**, *o-Cl*-amidine) [36].

Mechanism 1

Mechanism 2

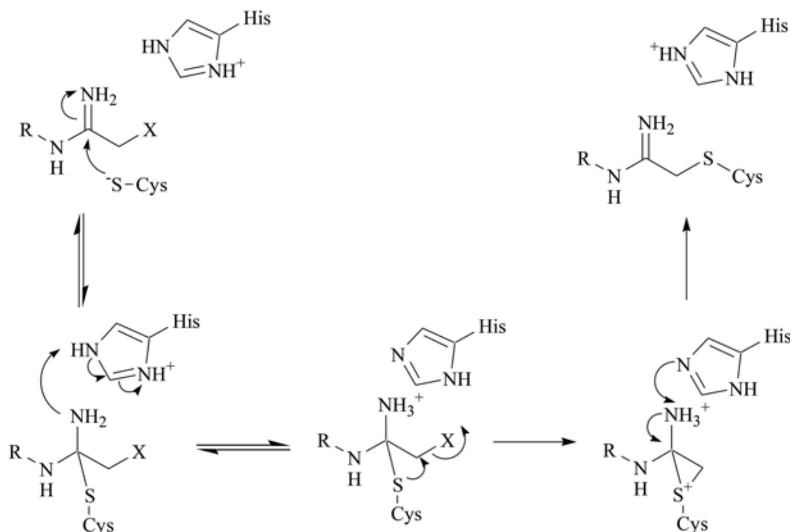


Fig. 4.11 Proposed mechanisms of PAD inactivation

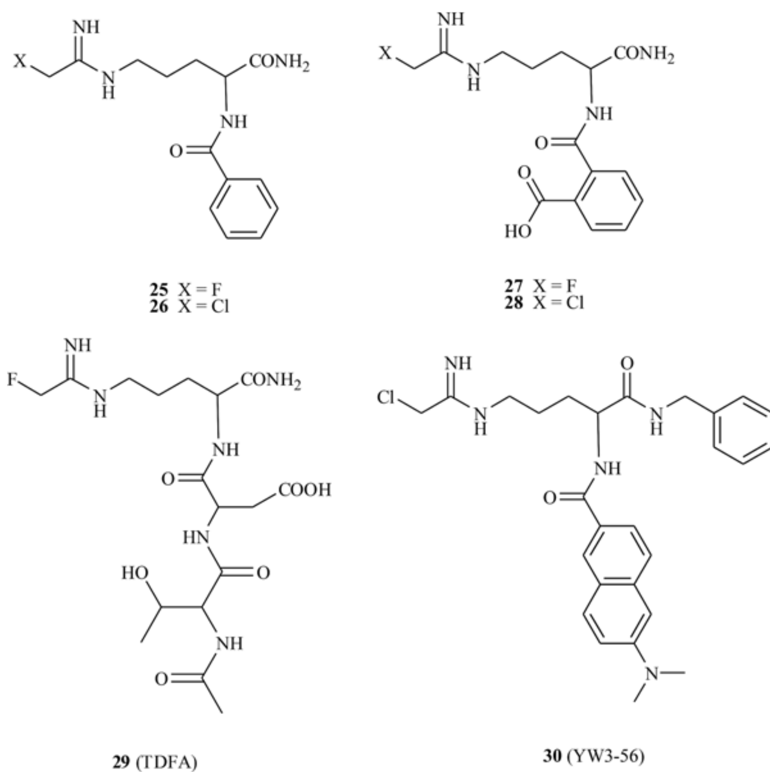


Fig. 4.12 Structures of the haloacetamidine-based inhibitors

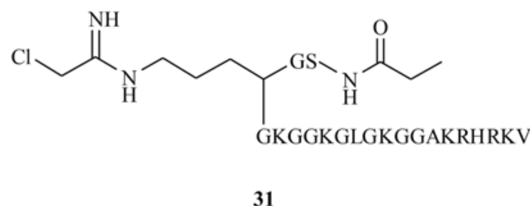


Fig. 4.13 PRMT$_1$ inhibitor chemical structure

A library of 264 tripeptides containing the fluoroacetamidine head was synthesized and tested with the aim to identify a more isoform-specific inhibitor. Compound **29** (TDFA) ensued a highly selective (up to 65-fold) PAD$_4$ inhibitor with excellent in vivo potency. Moreover, TDFA was added to a biotin moiety in the *N*-terminus and transformed in a PAD$_4$-selective activity-based proteomic probe [37].

Recently, Wang et al. starting from *Cl*-amidine **26** as lead compound synthesized a library of 23 compounds modifying the *N*-α and *C*-α positions of the ornithine backbone to develop more potent inhibitors for PAD$_4$ and cancer cell killing. Compound **30** (YW3-56) showed PAD$_4$ and PAD$_2$ inhibitor activity (IC$_{50}$ 1–2 μM and 0.5–1 μM, respectively), and furthermore an improved bioavailability, due to the introduction of more hydrophobic substituents (dimethylamide-naphthalene and benzylamide groups) [38].

L-Arginine-Based Inhibitors of PRMT$_1$

L-Arginine methylation is an important posttranslational modification, and deregulation of this process contributes to the onset and/or the progression of multiple human pathologies. PRMT catalyzes the addition of one or two methyl groups to a L-Arg residue in proteins, leading to the synthesis of three types of methylarginine species: ADMA, NMMA, and *N*$^{\omega}$,*N'*$^{\omega}$-dimethyl-L-arginine (symmetric dimethyl-L-arginine, SDMA). PRMT includes nine isozymes that are expressed ubiquitously and controls significant cellular processes involved in cell growth, proliferation, and differentiation [39]. PRMT$_1$, the predominant mammalian isozyme and the major asymmetric L-arginine methyltransferase, is implicated in the transcription activation, signal transduction, RNA splicing, and DNA repair. The upregulation of PRMT$_1$ expression has been disclosed in heart disease and in various types of human cancers like prostate, breast cancer, and leukemia [40]. Therefore, the selective inhibition of PRMT$_1$ could explicate beneficial effects on these pathologies. Also in this case, the substitution of the guanidino moiety of the L-Arg with the acetamidine led to compounds with inhibitory activity on the enzyme. The only potent and selective PRMT$_1$ inhibitor described to date is the chloroacetamidine **31** (Fig. 4.13), which acts also as a PAD inhibitor. The possible mechanisms of PRMT inhibition are the same showed for PAD inhibitors [41].

Conclusions

During the progression of several diseases, L-Arg signaling and metabolism often appear to be impaired. New therapeutic efforts to reset them are directed to the selective inhibition of four enzymatic families: NOS, DDAH, PRMT, and PAD. Here we reviewed the research for potent and

selective inhibitors of the named enzymes, all of them containing the amidino motif as the core structural element able to anchor the molecule into the catalytic pocket of the enzyme. As for NOS, the amidino moiety is able to establish key interactions with a conserved Glu residue, although substituents' specific sizes and interactions are responsible for selectivity between NOS isoforms. Alkyl-substituted amidines appear to be potent inhibitors of the DDAH. PAD inhibitors or inactivators contain a specific backbone to target the active site while the haloacetamidine warhead targets Cys residue of the catalytic triad determining PADs inactivation. The presence of a carboxylate on the backbone amide benzoyl ring and the halogen direct the selectivity over PAD_1 and/or $_4$. The biological activities' spectra exhibited by amidines are quite broad, and, among the others, they are also antimicrobial, antinflammatory, and anticancer agents. For this reason, medicinal chemists should carefully consider possible wanted or unwanted multiple actions in the design of new amidino compounds.

References

1. Maccallini C, Fantacuzzi M, Amoroso R. Amidine-based bioactive compounds for the regulation of arginine metabolism. Mini Rev Med Chem. 2013;13:1305–10.
2. Hughes MN. Chemistry of nitric oxide and related species. Methods Enzymol. 2008;436:3–19.
3. Li H, Poulos TL. Structure-function studies on nitric oxide synthases. J Inorg Biochem. 2005;99:293–305.
4. de Mel A, Murad F, Seifalian AM. Nitric oxide: a guardian for vascular grafts? Chem Rev. 2011;9:5742–67.
5. Wang H, Wang AX, Aylor K, Barrett EJ. Nitric oxide directly promotes vascular endothelial insulin transport. Diabetes. 2013;62:4030–42.
6. Hirst DG, Robson T. Nitric oxide physiology and pathology. Methods Mol Biol. 2011;704:1–13.
7. Tinker AC, Wallace AV. Selective inhibitors of inducible nitric oxide synthase: potential agents for the treatment of inflammatory diseases? Curr Top Med Chem. 2006;6:77–9.
8. Ji H, Li H, Flinspach M, et al. Computer modeling of selective regions in the active site of nitric oxide synthases: implication for the design of isoform-selective inhibitors. J Med Chem. 2003;46:5700–11.
9. Garvey EP, Oplinger JA, Furfine ES, et al. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J Biol Chem. 1997;272:4959–63.
10. Zhu Y, Nikolic D, Van Breemen RB, Silverman RB. Mechanism of inactivation of inducible nitric oxide synthase by amidines. Irreversible enzyme inactivation without inactivator modification. J Am Chem Soc. 2005;127:858–68.
11. Maccallini C, Patruno A, Besker N, et al. Synthesis, biological evaluation, and docking studies of N-substituted acetamidines as selective inhibitors of inducible nitric oxide synthase. J Med Chem. 2009;52:1481–5.
12. Maccallini C, Patruno A, Lannutti F, et al. N-substituted acetamidines and 2-methylimidazole derivatives as selective inhibitors of neuronal nitric oxide synthase. Bioorg Med Chem Lett. 2010;20:6495–9.
13. Fantacuzzi M, Maccallini C, Lannutti F, et al. Selective inhibition of iNOS by benzyl- and dibenzyl derivatives of N-(3-aminobenzyl)acetamidine. ChemMedChem. 2011;6:1203–6.
14. Pharmacia Corp US196118 and WO2007203823, 2007
15. Fedorov R, Hartmann E, Ghosh DK, Schlichting I. Structural basis for the specificity of the nitric-oxide synthase inhibitors W1400 and N^{ω}-propyl-L-arg for the inducible and neuronal isoforms. J Biol Chem. 2003;278:45818–25.
16. Neuraxon Inc US20060258721, 2006; WO2007118314, 2007; WO2009062318 and WO2009062319, 2009, WO2010132072, 2010
17. Mladenova G, Annedi SC, Ramnauth J, et al. First-in-class, dual-action, 3,5-disubstituted indole derivatives having human nitric oxide synthase (nNOS) and norepinephrine reuptake inhibitory (NERI) activity for the treatment of neuropathic pain. J Med Chem. 2012;55:3488–505.
18. Annedi SC, Ramnauth J, Maddaford SP, et al. Discovery of cis-N-(1-(4-(methylamino)cyclohexyl)indolin-6-yl) thiophene-2-carboximidamide: a 1,6-disubstituted indoline derivative as a highly selective inhibitor of human neuronal nitric oxide synthase (nNOS) without any cardiovascular liabilities. J Med Chem. 2012;55:943–55.
19. Ramnauth J, Renton P, Dove P, et al. 1,2,3,4-Tetrahydroquinoline-based selective human neuronal nitric oxide synthase (nNOS) inhibitors: lead optimization studies resulting in the identification of N-(1-(2-(methylamino) ethyl)-1,2,3,4-tetrahydroquinolin-6-yl)thiophene-2-carboximidamide as a preclinical development candidate. J Med Chem. 2012;55:2882–93.
20. Huang H, Li H, Yang S, et al. Potent and selective double-headed thiophene-2-carboximidamide inhibitors of neuronal nitric oxide synthase for the treatment of melanoma. J Med Chem. 2014;57:686–700.

21. Xue F, Kraus JM, Labby KJ, et al. Improved synthesis of chiral pyrrolidine inhibitors and their binding properties to neuronal nitric oxide synthase. J Med Chem. 2011;54:2039–48.
22. Pope AJ, Karuppiah K, Cardounel AJ. Role of the PRMT-DDAH-ADMA axis in the regulation of endothelial nitric oxide production. Pharmacol Res. 2009;60:461–5.
23. De Gennaro CV, Bianchi M, Pascale V, et al. Asymmetric dimethylarginine (ADMA): an endogenous inhibitor of nitric oxide synthase and a novel cardiovascular risk molecule. Med Sci Monit. 2009;15:RA91–101.
24. Palm F, Onozato ML, Luo Z, Wilcox CS. Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems. Am J Physiol Heart Circ Physiol. 2007;293:H3227–45.
25. Schade D, Kotthaus J, Clement B. Modulating the NO generating system from a medicinal chemistry perspective: current trends and therapeutic options in cardiovascular disease. Pharmacol Ther. 2010;126:279–300.
26. Kotthaus J, Schade D, Muschick N, et al. Structure-activity relationship of novel and known inhibitors of human dimethylarginine dimethylaminohydrolase-1: alkenyl-amidines as new leads. Bioorg Med Chem. 2008;16:10205–9.
27. Wang J, Mozingo AF, Hu S, et al. Developing dual and specific inhibitors of dimethylarginine dimethylaminohydrolase-1 and nitric oxide synthase: toward a targeted polypharmacology to control nitric oxide. Biochemistry. 2009;48:8624–35.
28. Lluis M, Wang J, Mozingo AF, et al. Characterization of C-alkyl amidines as bioavailable covalent reversible inhibitors of human DDAH-1. ChemMedChem. 2011;6:81–8.
29. Wang Y, Hu S, Gabisi Jr AM, et al. Developing an irreversible inhibitor of human DDAH-1, an enzyme upregulated in melanoma. ChemMedChem. 2014;9:792–7.
30. Wei L, Wasilewski E, Chakka SK, et al. Novel inhibitors of protein arginine deiminase with potential activity in multiple sclerosis animal model. J Med Chem. 2013;56:1715–172.
31. Vossenaar ER, Zendman AJW, van Venrooij WJ, Pruijn GJM. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays. 2003;25:1106–18.
32. Jang B, Shin HY, Choi JK, et al. Subcellular localization of peptidylarginine deiminase 2 and citrullinated proteins in brains of scrapie-infected mice: nuclear localization of PAD2 and membrane fraction-enriched citrullinated proteins. J Neuropathol Exp Neurol. 2011;70:116–24.
33. Arita K, Hashimoto H, Shimizu T, et al. Structural basis for Ca2+–induced activation of human PAD4. Nat Struct Mol Biol. 2004;11:777–83.
34. Luo Y, Arita K, Bhatia M, et al. Inhibitors and inactivators of protein L-arginine deiminase 4: functional and structural characterization. Biochemistry. 2006;45:11727–36.
35. Gyorgy B, Toth E, Tarcsa E, et al. Citrullination: a posttranslational modification in health and disease. Int J Biochem Cell Biol. 2006;38:1662–77.
36. Causey CP, Jones JE, Slack JL, et al. The development of N-α-(2-carboxyl)benzoyl-N5-(2-fluoro-1-iminoethyl)-L-ornithine amide (o-F-amidine) and N-α-(2-carboxyl)benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide (o-Cl-amidine) as second generation protein L-arginine deiminase (PAD) inhibitors. J Med Chem. 2011;54:6919–35.
37. Jones JE, Slack JL, Fang P, et al. Synthesis and screening of a haloacetamidine containing library to identify PAD4 selective inhibitors. ACS Chem Biol. 2012;7:160–5.
38. Wang Y, Li P, Wang S, et al. Anticancer peptidylarginine deiminase (PAD) inhibitors regulate the autophagy flux and the mammalian target of rapamycin complex 1 activity. J Biol Chem. 2012;287:25941–53.
39. Cha B, Jho E-H. Protein L-arginine methyltransferases (PRMTs) as therapeutic targets. Expert Opin Ther Targets. 2012;16:651–64.
40. Yang Y, Bedford MT. Protein L-arginine methyltransferases and cancer. Nat Rev Cancer. 2013;13:37–50.
41. Obianyo O, Causey CP, Osborne TC, et al. A chloroacetamidine-based inactivator of protein L-arginine methyltransferase 1: design, synthesis, and in vitro and in vivo evaluation. Chembiochem. 2010;11:1219–23.

Chapter 5
Oxy- and Sulfoanalogues of L-Arginine

Tatyana Dzimbova and Tamara Pajpanova

Key Points

- L-Arginine is a semi-essential amino acid, vital for normal growth and development of organisms.
- Canavanine, a natural L-arginine antimetabolite, may act as a substrate of any enzymes from the L-arginine cycle, and thus affects strongly normal metabolism.
- By analogy with canavanine oxy- and sulfoarginine analogues were synthesised.
- Oxy- and sulfoarginine analogues have potent biological effects, including analgesic, cytotoxic, and antibacterial activity.
- Oxy- and sulfoarginine analogues are successful substrates of different enzymes participating in L-arginine metabolism. This results in seriouws disturbances in normal cell development.
- If incorporated in biologically active peptides in place of L-arginine, oxy- and sulfoanalogues enhance the effects of the native peptides, due to their high enzyme stability and their ability to bind strongly to the important macromolecules.
- When incorporated into a variety of proteins using mRNAs templates, changes in protein conformation occur, which result in altered biological functions.

Keywords Canavanine • L-Arginine analogues • Norcanavanine • Sulfoarginine • Cytotoxicity

Abbreviations

ADC	L-Arginine decarboxylase
ADI	L-Arginine deiminase
AGAT	L-Arginine-glycine amidinotransferase
ARG	Arginase
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase

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Boc	tert.-Butyloxycarbonyl
Bzl	Benzyl
DEAD	Diethyl azodicarboxylate
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
Et_3N	Triethylamine
EtOH	Ethanol
HBTU	O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate
HOBT	Hydroxybenzotriazole
Me	Methyl
Mes	Methylsulfonyl (mesyl)
NOS	Nitric oxide synthase
NsArg	Norsulfoarginine
RNA	Ribonucleic acid
sArg	Sulfoarginine
SLE	Systemic lupus erythematosus
TFA	Trifluoroacetic acid
Tos	p-Toluolsulfonyl (tosyl)
TPP	Triphenylphosphine
tRNA	Transfer RNA
Trt	Trityl
Z	Benzyoxycarbonyl

Introduction

L-Arginine (L-Arg) is a natural cationic amino acid, containing a guanidinium group. This group is positively charged at neutral pH and is involved in a variety of physiological and pathological processes. L-Arginine plays an important role in the normal growth and development of organisms. L-Arginine depletion is associated with serious cell and organ functional impairment, ultimately lethal to the body. Elevated L-arginine levels can influence cellular functions, too; they may cause cell death or cell proliferation. The biological role of L-arginine involves several aspects. L-Arginine in the peptide chain is important in the regulation of peptide synthesis. Usually, peptides contain several L-arginine residues, and this repeat signals the end of peptide chain in the biosynthesis process. This is particularly important in neuropeptides, an essential class of molecules involved in cell signalling. Neuropeptides are synthesised as long precursor molecules, containing multiple copies of the active species. They are known as “pro-peptides” and are functionally inactive. They can be conveniently stored in the cells in this inactive form, until they will be needed. The individual neuropeptide copies in the precursor molecule are separated by L-arginine-rich regions. These rich in L-arginine domains serve not only for recognising the active peptide fragments, but are a source of L-arginine as well. Most amino acids inside the cells are known to be stored in a bonded form, much the same as proteins. Cells unable to synthesise L-arginine *de novo* supply their free amino acids by proteolysis. L-Arginine is involved in many metabolic pathways in the human body. It is a precursor for the biosynthesis of peptides and proteins, but also of ornithine, polyamines, nitric oxide, proline, glutamic acid, glutamine, creatine, agmatine and dimethyl-L-arginines. In mammals, L-arginine is a substrate of the five different enzyme systems, including nitric oxide synthases, arginase, L-arginine: glycine amidinotransferase, L-arginine decarboxylase and L-arginine deiminase [1]. The latter enzyme is not expressed in mammalian cells, but takes part in L-arginine metabolism when expressed by pathogens. Once inside the mammalian cells, pathogens strongly affect the metabolism of L-arginine in the host

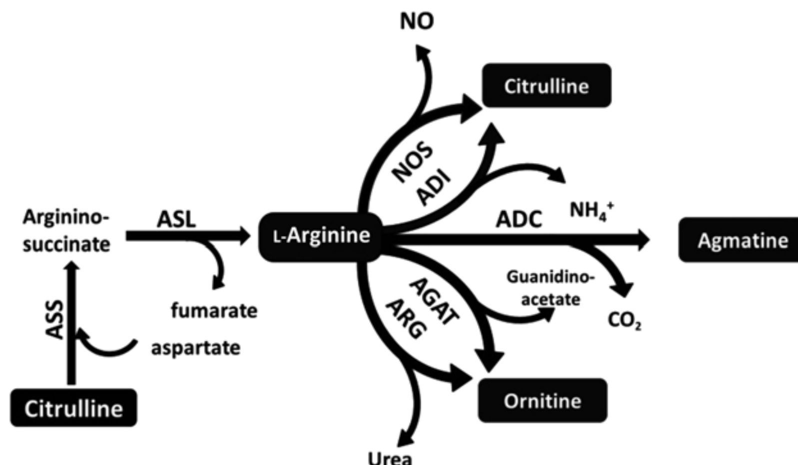


Fig. 5.1 Biosynthesis and metabolic pathways of L-arginine: *ASS*, argininosuccinate synthase; *ASL*, argininosuccinate lyase; *NOS*, nitric oxide synthase; *ADI*, arginine deiminase; *ADC*, arginine decarboxylase; *AGAT*, arginine-glycine amidinotransferase, *ARG*, arginase

(Fig. 5.1). L-Arginine is a semi-essential amino acid in mammals, because it can be synthesised from citrulline. The process is catalysed by argininosuccinate synthase, one of two enzymes, responsible for converting L-arginine into citrulline. The biosynthesis of L-arginine from citrulline is catalysed by the cytosolic fraction of L-arginine succinate synthase and argininosuccinate lyase.

Carcinogenesis is an area with an increased interest, and the role of L-arginine in the growth of the neoplastic cells was proven. The effect of L-arginine is mainly because of its end product, nitric oxide. It has been shown that the L-arginine/nitric oxide pathway plays an important role in tumour development. Recent studies suggest that nitric oxide produced from L-arginine can affect angiogenesis factors, including vascular permeability, prevascular recovery, vascular remodelling and maturation. Furthermore, this pathway can activate various genes involved in proliferation, metastasis and apoptosis. Interestingly, those processes impact both tumorigenesis and tumour degradation. On the other hand, it has been well established that a variety of tumour cells are auxotrophic [2] with respect to L-arginine: breast cancer, pancreatic cancer cells, certain types of melanoma cells, cervical carcinoma cells, hepatocellular carcinoma, lymphoblastic leukaemia, etc. ASS is either not expressed or is expressed at very low levels in the above-mentioned cell types. This fact is the rationale for the so-called “deprivation therapy”, which is very effective in the treatment of certain cancers. There are other cancer treatments that are based on blocking the enzymes, responsible for L-arginine metabolism in order to stop tumour growth.

Since L-arginine is a readily ionisable amino acid, it is most commonly found on the surface of proteins [3]. L-Arginine residues are important in a variety of physiological processes, including regulation of conformation or redox potentials, virus envelope formation, of static interactions, establishing a voltage across the lipid bilayer, proton transport and peptide translocation through the bilayer. L-Arginine residues play a key role in protein–protein interactions in enzyme active sites [4, 5] and in various transport channels [6].

L-Arginine is one of the most important residues in the catalytic site of many enzymes. It is third most common in the catalytic site, which equals about 11 % of all catalytic residues [4]. L-Arginine is more common than other basic residues, e.g. lysine, because there is a group at its side chain containing three nitrogen atoms. Each of these nitrogen atoms can participate in electrostatic interactions (Fig. 5.2). The L-arginine side chain has a geometry that favours the stabilisation of the oxygen pair of atoms in the phosphate group, common in biological molecules [3]. L-Arginine in the catalytic site may be involved in a variety of interactions, such as electrostatic, hydrogen bonding, the transition state stabilisation, the activation of a water molecule and the activation of the substrate molecule.

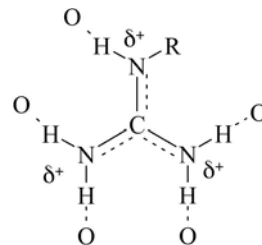


Fig. 5.2 A diagram of hydrogen bond formed by guanidinum group with 5 different oxygen atoms

It is well known that metabolic processes are of great importance for the existence of living organisms. Food is a source of various substances that are processed by the body to give suitable building blocks for making its own proteins, DNA, and various cell structures. A metabolite is any compound that is synthesised, modified or degraded in the cell. Metabolites perform various important biological functions in the cell, from building blocks to a variety of regulatory molecules in the body. A variety of compounds of both natural and synthetic origin, which are structural analogues of metabolites, are known. Due to their structural similarity to metabolites, they can replace the corresponding natural metabolites as substrates in different metabolic processes, but because of the differences between them, they cannot perform their biological function.

Antimetabolites are compounds which have been designed to be structurally similar to a natural metabolite and able to hinder a specific metabolic process, particularly for the treatment of various disorders, such as infection caused by various microorganisms, genetic diseases, etc. Antimetabolites are targeted at disrupting metabolic processes in microorganisms, which in turn will lead to microbial death.

One of the best definitions for the term "antimetabolites" is as follows: ***Antimetabolites are called those compounds, which resemble in their chemical structure a natural metabolite, and therefore can replace it in the corresponding metabolic process, but because of certain structural differences, they cannot perform its biological role*** [7].

Another reason for designing and synthesising structural analogues of a natural metabolite is to increase its biological activity. Changes in the molecules of various biologically active peptides are known to have resulted in peptides having greater stability and enhanced biological effect, particularly when the modifications are with non-proteinogenic amino acids resistant to proteolytic enzyme attack.

The term "mimetic" refers to a substance whose chemical structure is different from that of the natural compound, but has identical or enhanced biological effects. The modified compounds have a different structure, and therefore, the enzyme–substrate reaction, which is very specific, may not occur. It was found that even non-specific enzymes are practically unable to affect the modified analogues. Another important property of mimetics is their conformational stability. It is known that the interaction between the molecule of the biologically active substance and the receptor requires a specific complementation. The actual interaction is a dynamic process that is carried out through induced conformational changes. Therefore, conformationally stable analogues of natural bioactive substances are good agonists.

Since L-arginine is involved in many important biological processes, much research is focused on the synthesis of various analogues. Hundreds of analogues have been synthesised in order to achieve an enhanced specific inhibitory action for various L-arginine utilising enzymes.

Biological Role of Canavanine, a Natural Oxy-L-arginine Analogue

Nature itself has created a natural antimetabolite of L-arginine, known by the names canavanine, L-Canavanine or L-2-amino-4-(guanidinooxy) butyric acid, which is a non-proteinogenic amino acid. L-Canavanine was discovered in 1928, when first isolated from the seeds of legumes [8]. It is synthesised by leguminous plants of the *Lopoidea* family, subfamily *Leguminosae* [9] (Fig. 5.3). In plants, it has two

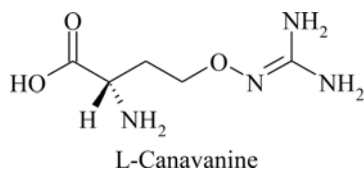


Fig. 5.3 The structure of L-canavanine

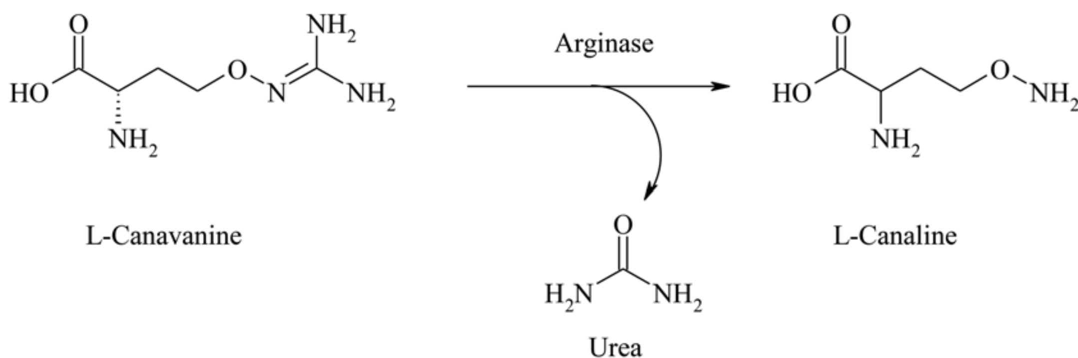


Fig. 5.4 Arginase-catalysed biotransformations of canavanine

main functions: as a metabolite for keeping the nitrogen [10–12] and as an integral part of the chemical defence system of plants [13]. Many canavanine-synthesising legumes accumulate significant amounts of canavanine, including members of the genus *Canavalia,* in which canavanine accounts for 3–4 % of the dry content of seeds, and legume plant *Dioclea megacarpa,* which produces so much canavanine in the seeds that the synthesis of this single metabolite utilises more than 95 % of all nitrogen atoms, necessary for the synthesis of all other free amino acids. *Colutea arborescens*, *Caragana arborescens*, *Vicia gigantea*, *Robinia pseudoacacia* and *Wisteria floribunda* are sources of canavanine containing 6–13 % dry weight of canavanine.

Canavanine protects plants from insects and herbivores. It can greatly influence the growth and development of organisms feeding on canavanine-containing plants. For example, tobacco budworm, *Manduca sexta*, will absorb L-canavanine, wherein its development will stop, and its growth becomes attenuated in both the pupa and adult insect [13].

L-Canavanine shows a remarkable structural analogy with L-arginine, in the molecule of which a terminal methylene group is replaced by oxygen.

Oxygen is significantly more electronegative than carbon, which facilitates deprotonation and reduces the pKa value of the oxyguanidino group to about 7.04 [14], much lower than 12.48, pKa of the guanidine group in L-arginine [15]. Under physiological conditions, L-arginine, being a strongly basic amino acid, is up to 99 % protonated, while only about 30 % of canavanine is protonated under such conditions. The destabilising effect of the oxygen atom reduces the electron density at the guanidium group. In addition to the decreased basicity of the guanidium group, the presence of a δ-oxygen atom determines the tautomeric form of the guanidium group. As seen in Fig. 5.4, the guanidium group in canavanine can exist in the imino and in an amino form. Crystallographic studies on a single L-canavanine crystal have shown that it exists in an amino tautomeric form and is uncharged [14]. That same study indicated that, like the other amino acids, L-canavanine forms a zwitterion via proton transfer from the carboxyl group of the α-nitrogen atom. L-Arginine preferably exists in the imino form (imino: amino, 2:1), which was also evidenced by crystallography. Despite the structural similarity of L-canavanine to L-arginine, the presence of δ-oxygen atom causes significant differences in these analogues. First, the state of the

R-group of canavanine, compared to that of L-arginine, distorts important group R-protein interactions and have a strong impact on its conformation, and hence, the behaviour of the protein. Based on its structural similarity with L-arginine, an L-arginine antimetabolite can serve as a substrate for any enzyme-catalysed reaction, which preferably uses L-arginine as a substrate.

Canavanine participates in the urea cycle, as L-arginine does, and the enzymes involved in this cycle will successfully use it as substrate and catalyse the biosynthesis of the following compounds: canaline, O-ureidohomoserine, canavanine succinate and canavanine [11].

As a structural analogue of L-arginine, canavanine can participate in all enzyme-catalysed reactions whose substrate is L-arginine [16]. This analogue of L-arginine could block the synthesis of nitric oxide, and acts as selective inhibitor nitric oxide synthase. Canavanine strongly affects the release of ammonia.

As a structural analogue of L-arginine, canavanine can be a substrate of the enzyme arginase and decrease its activity. This reaction will yield L-canaline and urea (Fig. 5.4). Factors affecting the decrease in enzyme activity are not fully understood [17], but our assumption is that probably canavanine hydrolysis by arginase is much slower, which in turn results in a temporary block at the active site of the enzyme and, thereby, to a decrease in its activity.

Some canavanine-resistant organisms, such as *Heliothis virescens*, have developed a new enzyme called canavanine-hydrolase, which catalyses the hydrolysis of L-canavanine to L-homoserine and hydroxyguanidine (Fig. 5.5) [18].

There is evidence that L-canavanine acts as a weak substrate inhibitor of L-arginine deiminase [19]. The reaction catalysed by this enzyme is shown in Fig. 5.6. The results of the study showed that the substitution of a methylene group with an oxygen atom decreased the covalent intermediate formation rate. Therefore, this step significantly slowed down the hydrolysis step, and hence, the enzyme was temporarily inactivated.

Canavanine is a substrate of the enzyme L-arginine decarboxylase and the enzyme action yields CO_2 and γ-guanidinooxypropylamine [20].

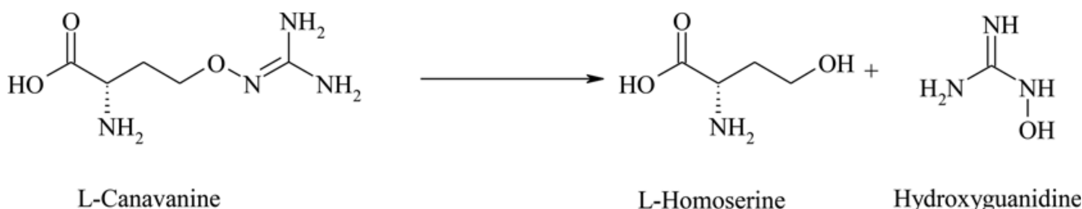


Fig. 5.5 Canavanine-hydrolase biotransformation of canavanine

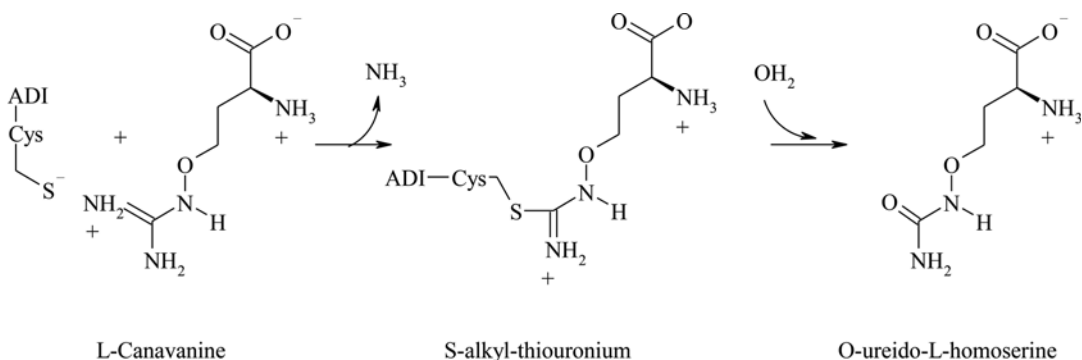


Fig. 5.6 Arginine deiminase biotransformation of canavanine

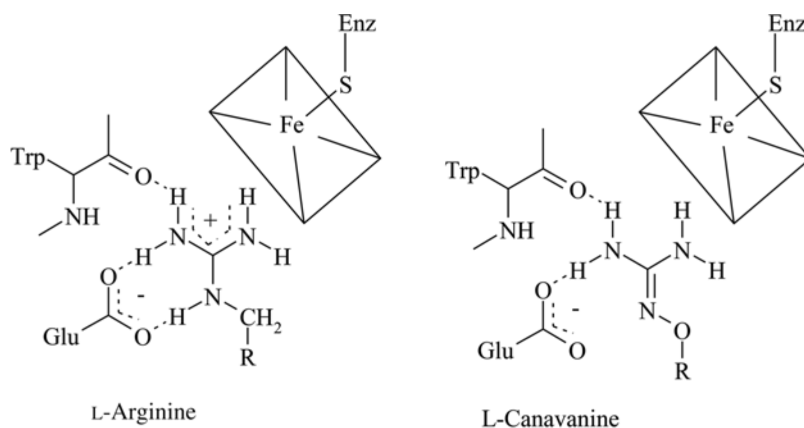


Fig. 5.7 Binding interactions of L-arginine and L-canavanine with the NOS active site

L-Arginine: glycine amidotransferase catalyses the conversion of ornithine to L-arginine with the release of guanidinoacetate. On the other hand, this enzyme catalyses the opposite reaction also [21]. As far back as 1956, it was found that amidinotransferase can catalyse the reaction *canavanine* + *ornithine* = *canaline* + *L-arginine* in kidney [22].

Another well-known effect of canavanine is its selective inhibitory activity on the inducible nitric oxide synthase [23]. The guanidinium groups in L-arginine and canavanine are different in structure and so are the interactions with the active site of the enzyme. The guanidinium group in L-arginine is fully protonated under physiological conditions and binds by giving off a proton to heme-bound O_2, which improves the conversion of O_2 to water and the oxo-heme species required for the hydroxylation of the substrate. The NOH-Arg formed has a lower pKa value (about 7), thus remaining unprotonated, and promotes the reaction with the next heme-bound O_2. The reaction results in the release of nitric oxide. However, the presence of an oxygen atom attached to the nitrogen in canavanine will shift the double bond to the side chain and protonation of the nitrogen atom will be impossible. Protonation will be accomplished with one of the important hydrogen bonds in the active site and will thus block enzymatically catalysed reactions [24] (Fig. 5.7).

Perhaps, the most deleterious effect of canavanine results from its activation and aminoacylation to the relevant $tRNA^{Cav}$ by arginyl-tRNA-synthase in canavanine-sensitive organisms [25]. When incorporated in the polypeptide chain, the decreased basicity of canavanine compared to that of the L-arginine residues will interfere with the interaction between the amino acid residues and distort the tertiary and/or quaternary structure of the protein. Detailed biochemical studies focused on the structurally modified canavanine-containing enzymes have clearly shown that canavanine utilisation led to changes in protein conformation, which affect adversely their biological functions and their biochemical activities [26–28]. Canavanine was reported to prevent normal reproduction of arthropods [27] and rodents [29]. In addition, canavanine was shown to cause a condition, resembling systemic lupus erythematosus in primates [30, 31], to increase anti-nuclear antibody levels, and to facilitate SLE-like lesions in autoimmune susceptible mice [32].

Synthesis and Biological Effects of Synthetic Oxy- and Sulfoarginine Analogues

Nature's concept of replacing a methylene group in the side chain of L-arginine for an oxygen atom was utilised in the design of analogues with shortened side chain and an oxygen atom and with a substituted oxygen atom for a sulfo group [33–35]. Thus, a series of oxy- and sulfoarginine analogues was produced (Fig. 5.8).

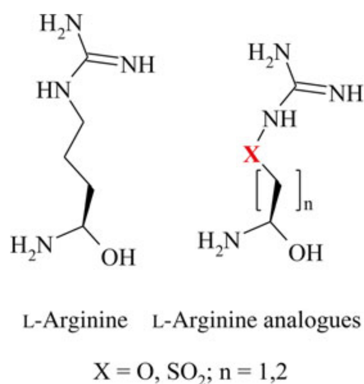


Fig. 5.8 The general formula of oxy- and sulfoarginine analogues

Sulfonamide derivatives are known to have a potent biological effect, and this was enshrined in the idea of a sulfoanalogue of L-arginine: on the one hand, it mimics the structure of the natural amino acid; on the other hand, it exhibits high potency through the sulfo group. The sulfo group is more electronegative than the methylene group and the sulfoguanidinium group is expected to be preferably in its amino-tautomeric form, as in canavanine. Although the resulting compounds are S-(aminoiminomethyl) amides of cysteic (homocysteic) acid, if regarding them as analogues of norarginine (L-arginine) with substituted methylene group, they may be properly named—norsulfoarginine (NsArg) and sulfoarginine (sArg).

L-Arginine analogues with a shorter side chain and an oxygen atom directly bound to the guanidinium group exhibit properties similar to those of canavanine, and the shorter the chain, the stronger the hindrance of normal metabolism.

Various synthetic protocols have been used for the synthesis of these analogues. The synthesis of sulfonyl analogues of L-arginine started from the main compound—an ester of Nα-benzyloxycarbonyl-protected sulfonylchloride of the S-cysteine (homocysteine) sulfonic acid. Sulfoanalogues were prepared with reaction of the sulfochloride with guanidine, followed by enzyme-catalysed hydrolysis of the ester, and removing the amino-protecting group (Fig. 5.9).

For the synthesis of oxy-analogues, L-serine (homoserine) was used as the starting material, which was converted into norcanaline (canaline) and after guanylation gave norcanavanine (canavanine) [36] (Fig. 5.10).

Various approaches for the preparation of norcanavanine were used. The amino group of serine was protected using different protecting groups, e.g. benzyloxycarbonyl and trityl groups (a). The protection of the carboxyl group of the serine was carried out by esterification to a methyl or a benzyl ester (b). Mesyl and tosyl esters (c) were used for the activation of the hydroxyl group, which were easily cleaved to give the phthalimide derivative of the protected serine or its oxyamine analogue (d). Amino- or carboxyl group-protected norcanaline was obtained by the removal of the phthalimide group, or the protective group at the oxyamino group (e). Guanylation was performed by various guanylating reagents, typically di-protected 1H-pyrazole –1-carboxamidine or N,N′-protected thiourea (f). After saponification of the ester (g) and removal of the amino protecting group (h), norcanavanine was obtained.

An original method for the synthesis of oxy- and sulfoanalogues is presented in Fig. 5.11.

The synthesis of oxy- and sulfoanalogues used the same starting compound, L-serine. The first steps of this synthetic route were the same up to the mesylation of the fully protected serine. A novel approach for the synthesis of NsArg has been used, obtaining the sulfonylchloride from a sulfonic acid [38] by a conventional method in solution, or in solution with microwave irradiation. In the latter case, the reaction time was shortened from 24 h to 5 min.

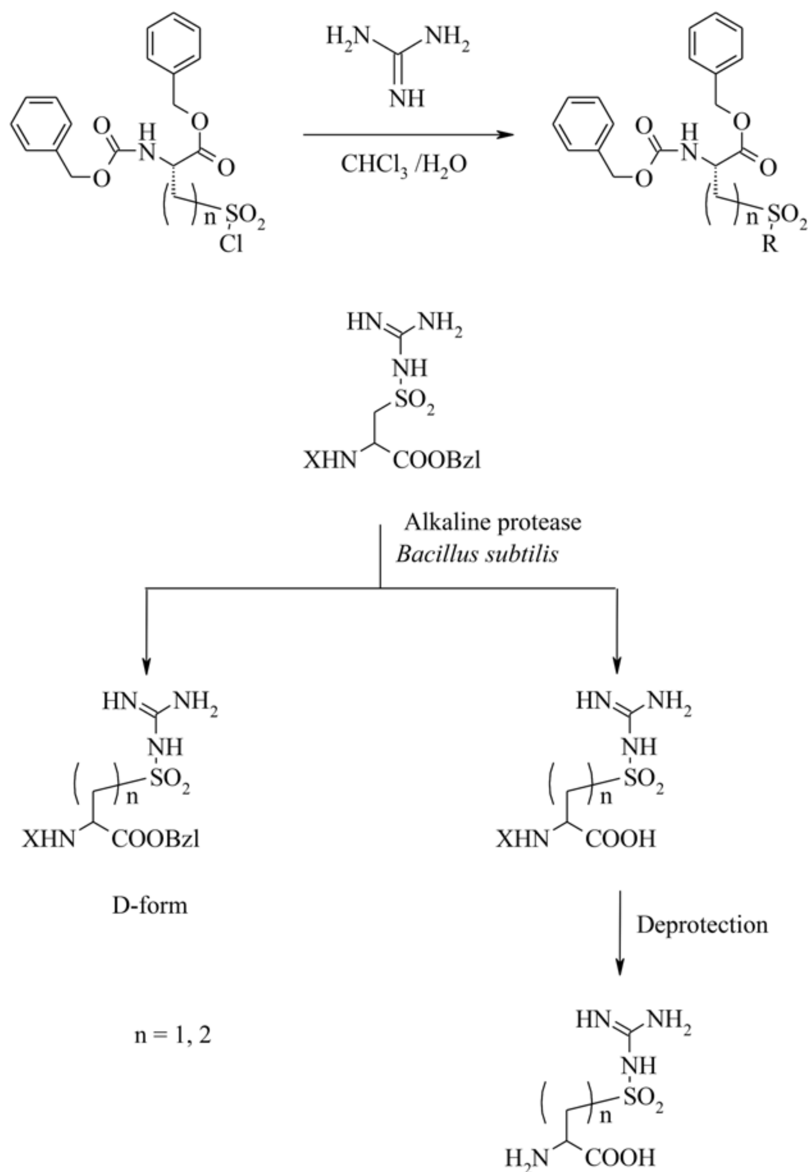


Fig. 5.9 Synthesis of sulfoarginine analogues

NCav was synthesised using two approaches. The first one is based on the conventional methods in solution (Fig. 5.10) and the second one is solid phase organic synthesis (Fig. 5.12). Microwave radiation was applied in some steps of both synthetic schemes. In microwave-assisted solid phase synthesis, the first and the second steps were carried out at 40° Centigrade and microwave irradiation for 2 min. The introduction of a guanidinium group also was performed under microwave irradiation at 40° Centigrade for 5 min [39].

These analogues were studies using different biological assays and were embedded in various biologically active peptides in place of L-arginine.

Initial analgesic activity tests of canavanine have been carried out. Canavanine was found [40] to have a strong naloxone reversible activity. The new oxy- and sulfoarginine analogues also had analgesic effects, which were longer lasting, due to improved bioavailability and enzymatic stability.

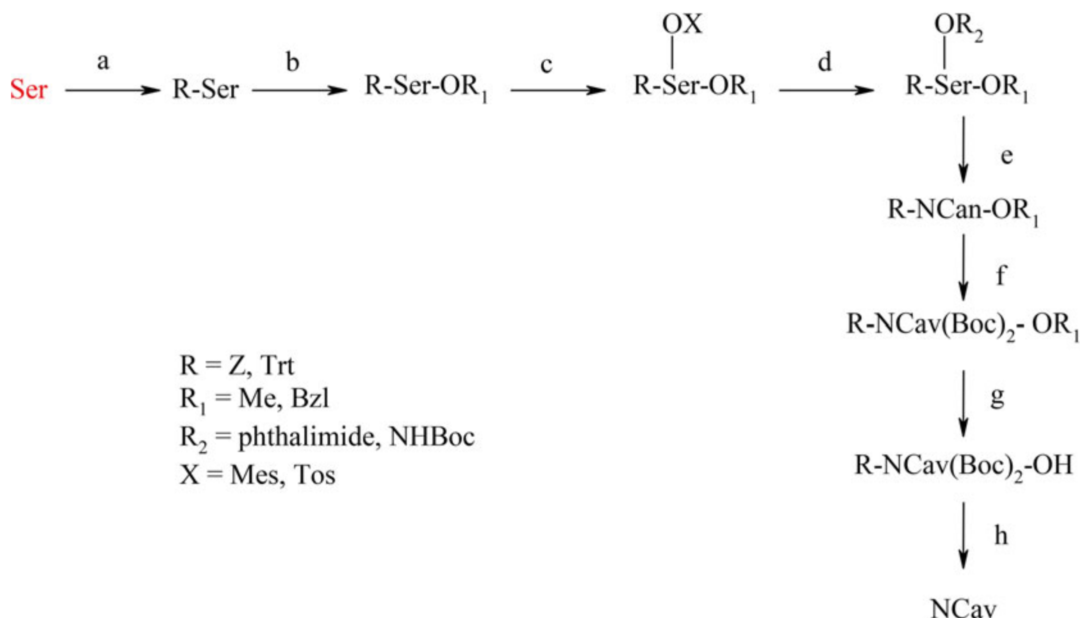


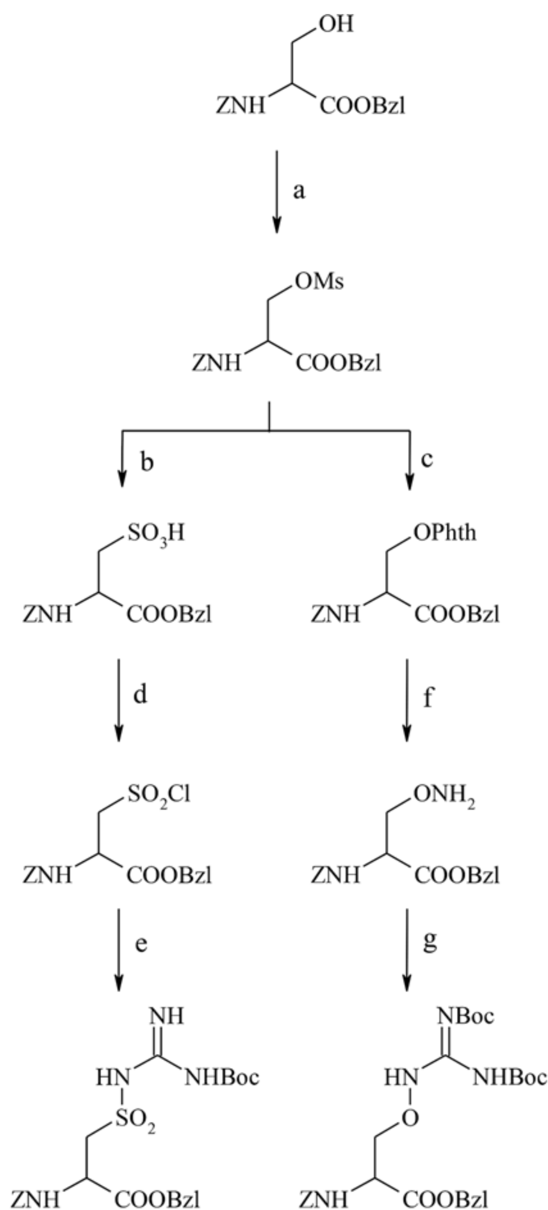
Fig. 5.10 General scheme for the synthesis of NCav

The presence of a new binding site in the molecule may be proposed to confer their stronger binding to the opioid receptors, and this probably is also associated with a prolonged effect [41, 42]. Substitution of an L-arginine residue in bioactive peptides with oxy- and sulfoarginine analogues ultimately led to an enhanced biological activity. Previous studies had shown that the substitution of this amino acid position in the kyotorphin molecule yielded analogues with a more potent and prolonged analgesic effect [43] (Fig. 5.13). This effect is due to the stronger binding of the newly synthesised molecules to the receptor.

It may be suggested that the effect is due to the enzymatic stability of kyotorphin analogues and failure to cross cell membranes via oligopeptide transporters.

Antibacterial studies indicated [35] that norcanavanine derivatives exhibited cytotoxic effect against Gram (+) and Gram (−) bacteria.

The cytotoxicity of oxy- and sulfoanalogues for HepG2 cells and 3T3 was examined. The analogues affected the growth and the development of tumour line cells, while their effects on normal cells were significantly weaker [38, 44–46]. In order to clarify the mechanism of action of these analogues, docking studies were conducted, targeting enzymes involved in L-arginine metabolism [47], and their ability to act as substrates for the arginyl-tRNA transferase enzyme [48]. Analysing the interaction of the oxy- and sulfoanalogues with enzymes of L-arginine metabolism has been established that they are not substrates of ARG, ADC, and ADI, but all bind strongly to eNOS, in this way able to act as its inhibitors. The sulfoanalogues sArg and NsArg interact strongly with iNOS yielding stable enzyme–substrate complexes. Oxy- and sulfoanalogues having a shorter chain, NCav and NsArg, interacted with AGAT and blocked its activity. All analogues acted as inhibitors of ASS (Fig. 5.14). NCav was not an effective inhibitor of the enzymes of L-arginine cycle, because it did not bind strongly enough to the active site of the enzymes. For their part, sulfoanalogues had an additional centre in their molecule, which enabled strong interactions with the active site of the enzymes and gave strong bonds with them, blocking their activity. These assays provide an explanation for the cytotoxic effect of oxy- and sulfoarginine analogues. The putative mechanism of action of the oxy- and sulfoanalogues of L-arginine was explained by the docking studies' results and they confirmed the relationships observed with the in vitro assays.



a) CH_3SO_2Cl/DIPEA/DMF; b) Na_2SO_3/H_2O; c) N-Hydroxyphthalimide/NaH/DMF;
d) SO_2Cl/CH_2Cl_2; e) mono-Boc-Guanidine/Et_3N/DMF; f) $N_2H_4{*}H_2O$/EtOH;
g) Boc_2-pyrazole/CH_3CN/DIPEA

Fig. 5.11 Original scheme for the synthesis of oxy- and sulfoanalogues of L-arginine y [37]

Furthermore, canavanine is recognised as a substrate of arginyl-tRNA transferase, yielding canavanine containing proteins in place of L-arginine. The docking studies have shown that norcanavanine and sulfoarginine also can act as substrates of this enzyme. The enzyme recognised the analogue as substrates, bound to them at the active site, and activated it to provide the corresponding product—$tRNA^{analogue}$. This makes it possible for these analogues to be incorporated into a variety of

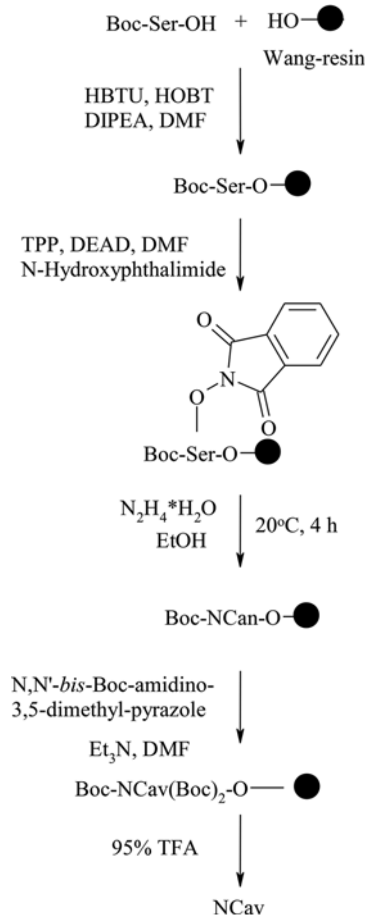


Fig. 5.12 Solid phase synthesis of an oxyanalogue

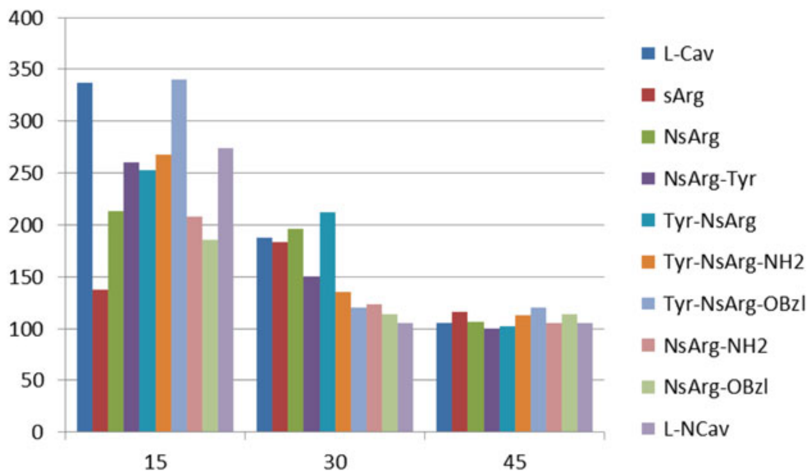


Fig. 5.13 Effects of i.p. administration of (all at a dose of 5 mg/kg) assessed by the paw-pressure test. Data are presented as mean ± S.E.M

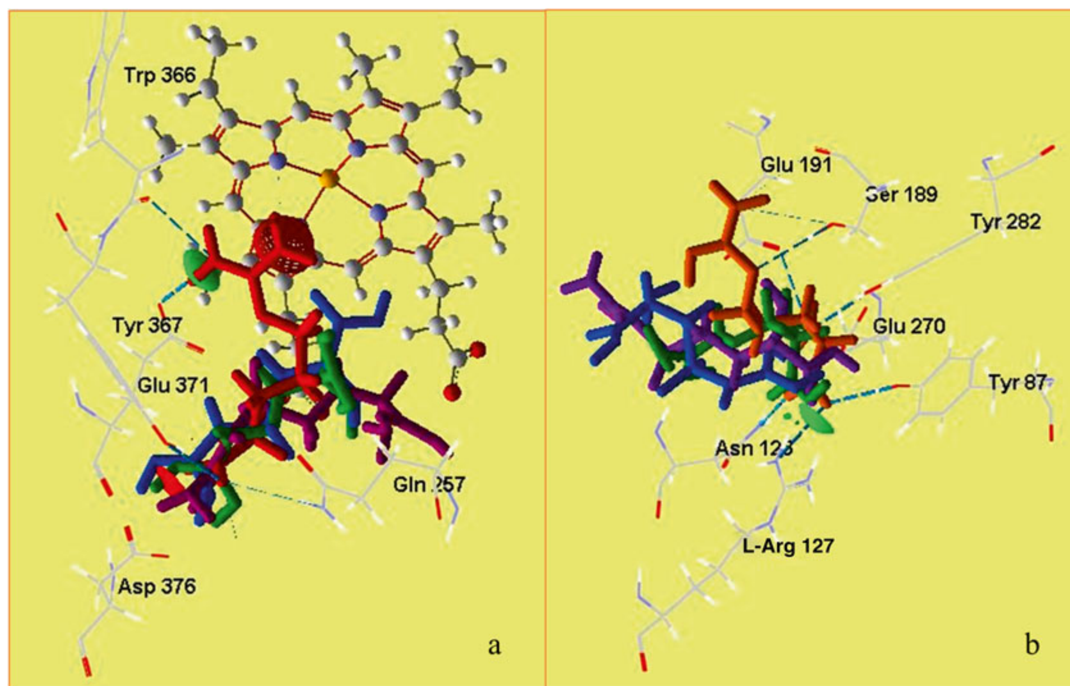


Fig. 5.14 (**a**) Superposition in the active sites of eNOS and ASS, (**b**) Arg—*red*, Cit—*orange*, NCav—*green*, NsArg—*blue*, and sArg—*purple*

biologically important peptides and proteins. Since these analogues have modified structures of their side chains and reduced basicity, as well as an additional binding site in the molecule, an oxygen atom and a sulfo group, this would result in a change in the structure of the resulting peptide or protein, and hence—in its biological activity. A similar effect was observed in a docking study as well, using an enzyme (ADK) with incorporated oxy- or sulfoanalogue. In mutant adenylate kinase, changes were made in the active centre. As a result of these mutations, the conformation of the enzyme active site was changed and activities were blocked (Figs. 5.15 and 5.16). This led to metabolic disorders and, consequently, to cell death. Two key L-arginine residues, Arg138 and Arg175, were consistently replaced and the effects of the analogue on the conformation of the enzymes were detected. Arg138 is essential for the recognition and binding of the substrate to a guanidinium group, and its replacement with an analogue yielded a mutant enzyme, which had a weaker binding to substrates, such as oxy-guanidinium, and the sulfoguanidium group was of reduced basicity. Such a mutation had an overall effect on the conformation of the enzyme and on a sequence, such as the GAGKG (residues 25–29), which is a conserved sequence responsible for reacting with the phosphate oxygens of the ADP molecule. This sequence was strongly affected by the change.

The second mutation (Arg175) had no influence on the ability of the enzyme to bind to the substrate, but due to the strong conformational changes in original GAGKG, it had no contribution in the enzyme-catalysed reaction.

The ADK is one of many examples of enzymes with L-arginine residues in their active sites. Incorporation of oxy- and sulfoanalogues in different enzymes could lead to serious metabolic disorders and, hence, to cell death.

Fig. 5.15 (**a**) Superposed active sites of ADK and Arg138 mutated ADK with *bis*(adenosine)-5′-tetraphosphate, (**b**) focus on Arg138 residue in Arg138 mutated enzymes, (**c**) superposed active sites of ADK and Arg175 mutated ADK with bis(adenosine)-5′-tetraphosphate

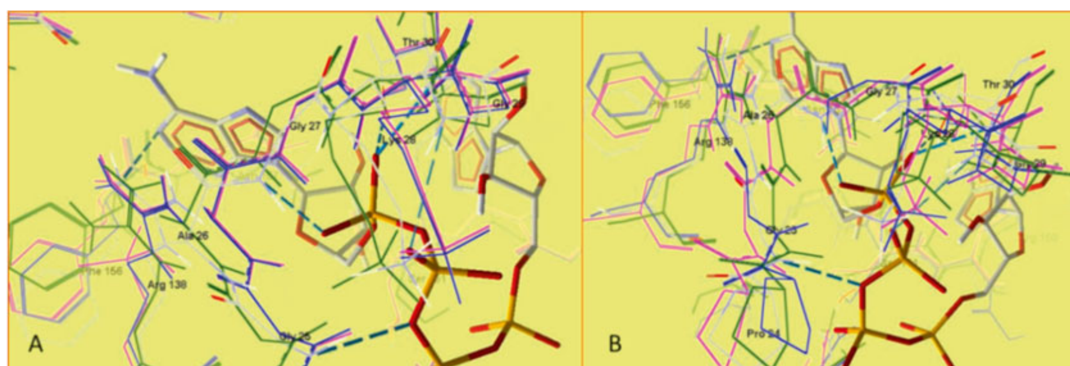


Fig. 5.16 Superposition of the sequences GAGKG (25–29). (**a**) ADK and ADK mutated with Cav138 (*blue*), NCav138 (*green*), and sArg138 (*purple*), (**b**) ADK and ADK mutated with Cav175 (*blue*), NCav175 (*green*), and sArg175 (*purple*)

Conclusion

Nature is our best source of learning and everyone can draw on its knowledge. It has given to plants the ability to cope with pests, and we will benefit from learning to combat severe human diseases. Using the example, provided by nature, for compounds that successfully mimic the molecule of L-arginine, and combining them with modern methods for the design of biologically active compounds, a whole new class of its antimetabolites has been created. Oxy- and sulfoarginine analogues can successfully replace L-arginine in the metabolic transformations, but they are incapable to perform its functions, and these result in severe functional impairment. Analogue incorporation in the structure of biologically active peptides in place of the L-arginine resulted in analogues with an increased biological activity and enzyme stability. Studies with these compounds are in their very beginning, but it may be anticipated that their targeted use could be of benefit in the treatment of various diseases, including cancer and infections.

References

1. Morris Jr SM. Recent advances in L-arginine metabolism: role and regulation of the arginases. Br J Pharmacol. 2009;157:922–30.
2. Dillon BJ, Prieto VG, Curley SA, Ensor CM, Holtsberg FW, Bamalaski JS, et al. Incidence and distribution of argininosuccinate synthetase deficiency in human cancers. Cancer. 2004;100:826–33.

3. Borders Jr CL, Broadwater JA, Bekeny PA, Salmon JE, Lee AS, Eldridge AM, et al. A structural role for L-arginine in proteins: multiple hydrogen bonds to backbone carbonyl oxygens. Protein Sci. 1994;3:541–8.
4. Bartlett GJ, Porter CT, Borkakoti N, Thornton JM. Analysis of catalytic residues in enzyme active sites. J Mol Biol. 2002;324:105–21.
5. Pednekar D, Tendulkar A, Durani S. Electrostatic-defying interaction between L-arginine termini as a thermodynamic driving force in protein-protein interaction. Proteins. 2009;74:155–63.
6. Crowley PB, Golovin A. Cation-pi interactions in protein-protein interfaces. Proteins. 2005;59:231–9.
7. Golovinsky EV. Biochemie der antimetabolite, Sofia: prof. Marin-Drinov-Verlag der Bulgarishen Academie der Wissenschaften; 2002.
8. Kitagawa M, Tomiyama T. A new amino-compound in the jack bean and a corresponding new ferment (I). J Biochem (Tokyo). 1929;11:265–71.
9. Bell EA, Lackey J, Pohill RM. Systematic significance of canavanine in the Papilionoideae (faboideae). Biochem Syst Ecol. 1978;6:201–12.
10. Rosenthal GA, Nkomo P. The natural abundance of L-canavanine, an active anticancer agent, in Alfalfa, Medicago Sativa (L.). Pharm Biol. 2000;38:1–6.
11. Rosenthal GA. An ontogenetic study of canavanine formation in the fruit of Jack Bean, Canavalia ensiformis (L.) DC. Plant Physiol. 1971;47:209–11.
12. VanEtten CH, Miller RW, Wolf IA, Jones Q. Nutrients in seed meals, amino acid composition of twenty-seven selected seed meals. J Agric Food Chem. 1961;9:79–82.
13. Rosenthal GA. The protective action of a higher plant toxic product: biochemical studies reveal how the allelochemical L-canavanine disrupts insect development. BioScience. 1988;38:104–9.
14. Boyar A, Marsh RE. L-Canavanine, a paradigm for the structures of substituted guanidines. J Am Chem Soc. 1982;104:1995–8.
15. Greenstein JP, Winitz M. Chemistry of the amino acids, vol. 1–3. New York: Wiley; 1963.
16. Rosenthal GA. The biological effects and mode of action of L-canavanine, a structural analogue of L-arginine. Q Rev Biol. 1977;52:155–78.
17. Michelangeli C, Vargas RE. L-Canavanine influences feed intake, plasma basic amino acid concentrations and kidney arginase activity in chicks. J Nutr. 1994;124:1081–7.
18. Michelangeli C, Rosenthal GA, Dalman DL. The biochemical basis for L-canavanine tolerance by tobacco budworm Heliothis virescens (Noctuide). Proc Natl Acad Sci USA. 1997;94:2255–60.
19. Lu X, Li L, Feng X, Wu Y, Dunaway-Mariano D, Engen JR, et al. L-canavanine is a time-controlled mechanism-based inhibitor of Pseudomonas aeruginosa L-Arginine deiminase. J Am Chem Soc. 2005;127:16412–3.
20. Giles TN, Graham DE. Characterization of an acid-dependent L-arginine decarboxylase enzyme from Chlamydophila pneumoniae. J Bacteriol. 2007;187:7376–83.
21. Humm A, Fritsche E, Steinbacher S, Huber R. Crystal structure and mechanism of human L-arginine:glycine amidinotransferase: a mitochondrial enzyme involved in creatine biosynthesis. EMBO J. 1997;16(12):3373–85.
22. Walker JB. Biosynthesis of L-arginine from canavanine and ornithine in kidney. J Biol Chem. 1956;218:549–56.
23. Suzuki N, Sakamoto A, Ogawa R. Effects of L-canavanine, an inhibitor of inducible nitric oxide synthase, on myocardial dysfunction during septic shock. J Nippon Med Sch. 2002;69:13–8.
24. Badu BR, Frey C, Griffith O. L-Arginine binding to nitric-oxide synthase: the role of H-bonds to the non-reactive guanidinium nitrogens. J Biol Chem. 1999;274:25218–26.
25. Crine P, Lemieux E. Incorporation of canavanine into rat pars intermedia proteins inhibits the maturation of pro-opiomelanocortin, the common precursor to adrenocorticotropin and beta-lipotropin. J Biol Chem. 1982;257:832–8.
26. Rosenthal GA, Lambert J, Hoffmann D. Canavanine incorporation into the antibacterial proteins of the fly, Phormia terranovae (Diptera), and its effect on biological activity. J Biol Chem. 1989;264:9768–71.
27. Rosenthal GA, Reighart JM, Hoffman JA. L-canavanine incorporation into vitellogenin and macromolecular conformation. J Biol Chem. 1989;264:13693–6.
28. Rosenthal GA, Dahlman DL. Studies of L-canavanine incorporation into insectan lysozyme. J Biol Chem. 1991;266:15684–7.
29. Brown DL. Reproductive toxicity and increased longevity in mice fed L-canavanine. J Anim Sci. 1994;72 Suppl 1:145.
30. Malinow MR, Bardana EJ, Pirofski B, Craig S, McLaughlin P. SLE-like syndrome in monkeys fed alfalfa sprout: role of a non-protein amino acid. Science. 1982;216:415–7.
31. Montanaro A, Bardana EJ. Dietary amino acid-induced systematic lupus erythematosus. Rheum Dis Clin N Am. 1991;17:323–33.
32. Prete PE. Effects of L-canavanine on immune function in normal and autoimmune mice: disordered B-cell function by a dietary amino acid in the immunoregulation of autoimmune disease. Can J Physiol Pharmacol. 1985; 63:843–54.

33. Dzimbova TA, Pajpanova TI, Golovinsky EV. Synthesis of some sulfoguanidino group-containing amino acids. Collection Symp Ser. 2003;6:12–4.
34. Dzimbova TA, Pajpanova TI, Bocheva AI, Golovinsky EV. Oxyamino- and sulfoguanidino containing amino acids. Synthesis and incorporation into physiologically active peptides. Amino Acids. 2005;29:67.
35. Dzimbova T, Pajpanova T, Tabakova S, Golovinski E. In: Cordopatis PA, Manessi-Zoupa E, Pairas GN, editors. 5th Hellenic forum on bioactive peptides. Patras, Greece: Typorama; 2007. p 223–7.
36. Dzimbova T, Pajpanova T. Synthesis of different types of PNAs, containing chiral pseudopeptide backbone. Trakia J Sci. 2010;8:98–101.
37. Carson KG, Schwender CF, Shroff HN, Cochran NA, Gallant DL, Briskin M. Sulfonopeptide inhibitors of leukocyte adhesion. Bioorg Med Chem Lett. 1997;7:711–4.
38. Dzimbova T, Miladinova E, Mohr S, Decheva R, Balacheva A, Schmid MG, et al. Sulfo- and oxy-analogues of L-arginine: synthesis, analysis and preliminary biological screening. Croat Chem Acta. 2011;84:447–53.
39. Brakadanska T, Pajpanova T, Bocheva A, Petkov V, Golovinsky E. Effects of some non-proteinogenic amino acids on nociception. Bull Chem Commun. 2001;33:79–82.
40. Dzimbova T, Djambazova E, Bocheva A, Pajpanova T, Golovinsky E. In: Lankinen H, editor. Peptides 2008. Helsinki: Helsinki University Press; 2008. p 232–3.
41. Bocheva A, Dzambazova-Maximova E, Dzimbova T, Pajpanova T, Golovinsky E. Analgesic action of norsulfoarginine kyotorphin analogues in rats. C R Acad Bulg Sci. 2006;59:219–22.
42. Dzimbova T, Bocheva A, Pajpanova T. Kyotorphin analogues containing unnatural amino acids: synthesis, analgesic activity and computer modeling of their interactions with μ-receptor. Med Chem Res. doi:10.1007/s00044-014-0953-9. Accessed Feb 2014.
43. Dzimbova T, Iliev I, Georgiev K, Detcheva R, Balacheva A, Pajpanova T. In vitro assessment of the cytotoxic effects of sulfo-L-arginine analogues and their hydrazide derivatives in 3T3 and HepG2 cells. Biotechol Biotechnol Eq. 2012;26:180–4.
44. Georgiev K, Dzimbova T, Iliev I, Balacheva A, Detcheva R, Schmid M, et al. Growth inhibition of HepG2 cell line by canavanine, norcanavanine and their hydrazide derivatives. Der Pharma Chemica. 2014;6:360–9.
45. Dzimbova T, Milanov P, Pajpanova T. An explanation of cytotoxic potential of L-arginine mimetics containing sulfo- and oxy-groups in their side chains. J Comput Methods Mol Des. 2013;3:10–8.
46. Dzimbova T, Milanov P, Pajpanova T. Long-lasting effects of oxy- and sulfoanalogues of L-arginine on enzyme action. J Amino Acids. doi:10.1155/2013/407616. Accessed 15 Sep 2013.
47. Dzimbova T, Iliev I, Detcheva R, Balacheva A, Pajpanova T. In vitro assessment of the cytotoxic effects of hydrazide derivatives of sulfoarginines in 3T3 and HepG2 cells. Collection Symp Ser. 2011;13:34–6.

Chapter 6
Regulation of Expression and Activity of L-Arginine Transporters by Nutrients and Hormones: A Focus in Transcriptional Mechanisms Regulated by Glucose and Insulin

Marcelo González

Key Points

- L-Arginine transport is essential for nitric oxide synthesis.
- The major transporter for L-arginine is the human cationic amino acids transporter 1 (hCAT-1).
- The gene that codified hCAT-1 is the solute carrier family 7 member 1 (*SLC7A1*).
- Transcriptional regulation of *SLC7A1* is determinant for hCAT-1 expression.
- Insulin increases the transcription of *SLC7A1* through specificity protein 1 (Sp1) activity.
- There is a positive feedback between L-arginine and insulin secretion.
- High extracellular concentrations of D-glucose increase the transcription of *SLC7A1*.
- Insulin restores the L-arginine transport and oxidative stress induced by high D-glucose.

Keywords Nitric oxide • hCAT-1 • *SLC7A1* • Insulin • D-glucose • Oxidative stress • Cardiovascular

Abbreviations

3′-UTR	Three prime untranslated region
CAT	Cationic amino acid transporter
EC_{50}	Half-maximal effective concentration
EDCFs	Endothelium-derived contracting factors
EDRFs	Endothelium-derived relaxing factors
eNOS	Endothelial nitric oxide synthase
H_2O_2	Hydrogen peroxide
HAEC	Human aortic endothelial cells
hCAT	Human cationic amino acid transporter
HUVEC	Human umbilical vein endothelial cells
K_m	Michaelis–Menten constant
MAPK	Mitogen-activated protein kinase

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miRNA	MicroRNA
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite
PI3K	Phosphatidylinositol 3-phosphate
PKC	Protein kinase C
sGC	Soluble guanylate cyclase
SLC7A	Solute carrier family 7 subfamily A
Sp1	Specificity protein 1
V_{max}	Maximal velocity
VSMC	Vascular smooth muscle cells

Introduction

L-Arginine transport is essential for nitric oxide (NO) synthesis and regulation of vascular health in mammals. This semi-essential amino acid is transported into the cells by several proteins grouped into families of transporters, with different expression and kinetic characteristics. In human vascular cells, specifically in endothelial cells, the properties and expression of two main L-arginine transporters, called human Cationic Amino acid Transporter 1 (hCAT-1) and 2 (hCAT-2), were described. Between them, the kinetic parameters and role of hCAT-1 are well characterized in human umbilical vein endothelial cells (HUVEC). Also, the transcriptional regulation of solute carrier family 7 type 1 (*SLC7A1,* coded gene for hCAT-1) has been partially elucidated under different physiological or pathological stimuli such as insulin, high extracellular concentrations of D-glucose, or amino acid deprivation. Evidence shows that changes in the extracellular environment regulate the expression and activity of L-arginine transporters in order to maintain amino acid uptake, especially for the fine-tuning of NO bioavailability. In other words, the regulation of L-arginine transporters could be a key factor in the feedback mechanism that allows the autoregulation of cellular metabolism for homeostasis. In this chapter, we expose the main evidence related to the regulation of L-arginine transporters at functional and transcriptional levels.

L-Arginine Transporters and Endothelial Function

The endothelium (or tunica intima) is a unique layer of specialized epithelial cells (endothelial cells) that coat the inner surface of blood vessels, showing a remarkable heterogeneity with regard to structure and function. At present, the endothelium is considered an organ that has a fundamental role in the regulation of cardiovascular function, especially in vascular tone, blood cell trafficking, hemostatic balance, vascular permeability, vascular cell proliferation, and innate and acquired immunity [1]. There are different physiological stimuli (i.e., physical forces, hormones, autacoids, prostaglandins, peptides, etc.) with the capacity to induce changes in endothelium-dependent vascular tone through the release of molecules grouped as endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs). EDRFs and EDCFs act as physiological antagonists, inducing opposite effects in endothelial and vascular smooth muscle cells (VSMC) for the regulation of vascular tone, vascular resistance, and blood flow. Some examples of EDRFs are prostacyclin (**PGI2**), NO,

endothelium-derived hyperpolarizing factor (EDHF), and C-type natriuretic peptide (CNP), among others [2]. Between them, NO has been documented as the most important physiological vasodilator, and the release of this gaseous molecule from the endothelium is one of the main mechanisms for vascular health maintenance. NO synthesized in the endothelium and diffuses (has not yet been established whether it is simple or facilitated diffusion) to the VSMC to increase the activity of the soluble guanylate cyclase (sGC) receptor, which in turn increases levels of cyclic guanosine monophosphate (cGMP). cGMP is a second messenger that activates protein kinase G (PKG) and decreases intracellular calcium (Ca^{2+}) levels, evoking the relaxation of blood vessels [3] (Fig. 6.1). As its central role, NO inhibits VSMC proliferation, platelet aggregation, the activity of the vasoconstrictor peptide endothelin-**1**, and the adhesion and migration of macrophages [4].

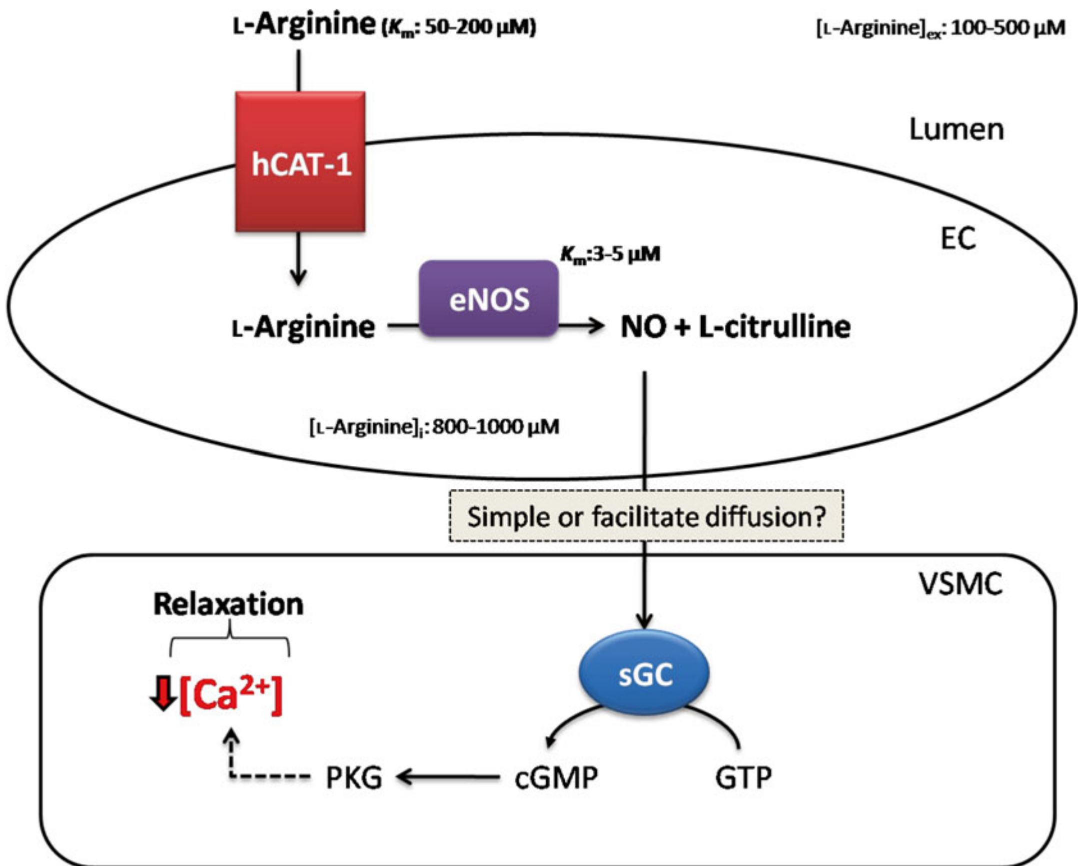

Fig. 6.1 L-Arginine/NO pathway. In blood vessel wall, the interaction between endothelial cells (EC) and smooth muscle cells (SMC) maintains the vascular physiology and the regulation of the vascular tone, mainly through the mechanism that induces the nitric oxide (NO) synthesis. The human cationic amino acid transporter 1 (hCAT-1) is associated with endothelial NO synthase (eNOS) for supplementation of L-arginine from extracellular space toward the enzyme. This mechanism is necessary although the intracellular concentration of the amino acid overloads the enzymatic capacity of eNOS. The Michaelis–Menten constant of hCAT1 is in the range of plasma concentration of L-arginine; therefore, the changes in food intake and diet affect the L-arginine/NO pathway by deterioration or increases in L-arginine transport. Once synthesized, NO diffuses to the smooth muscle cells for activating the soluble guanylate cyclase receptor (sGC) for enhancing the cyclic GMP (cGMP) levels, activation of protein kinase G (PKG), and lowering the intracellular calcium. The mechanism of NO diffusion still is not well characterized and some publications reveal the role of hemichannels in this process

NO is synthesized from the semi-essential cationic amino acid L-arginine and molecular oxygen (O_2) in a reaction catalyzed by NO synthases (NOS) in which NO and the neutral amino acid L-citrulline are released (Fig. 6.1). NOS is a family of enzymes constituted by three proteins codified in different genes and is classified into two categories: constitutive NOS (cNOS), which includes neuronal NOS (nNOS) and endothelial NOS (eNOS); and inducible NOS (iNOS) [5, 6]. In physiological conditions, endothelial cells express mainly eNOS, highly abundant in enriched caveolae domains of plasma membrane. In order to the activity of this enzyme to take place, several cofactors are required such as the Ca^{2+}/calmodulin (Ca^{2+}/CaM) complex, tetra-hydro-biopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) [7, 8]. The physiological response of eNOS induced by different vasoactive agonists includes the increase of intracellular Ca^{2+} as a previous step for NO synthesis, in a mechanism that involves the uptake of L-arginine from extracellular space through plasma membrane proteins.

In endothelial cells, the uptake of L-arginine is mediated by the plasma membrane systems y^+, y^+L, $b^{0,+}$, and $B^{0,+}$. However, the main transport system involved in endothelial NO synthesis is the y^+ system (~85 % of L-arginine used for NO synthesis is transported by this system) [9, 10]. The members of this system are CAT-1, CAT-2A, CAT-2B, CAT-3, and CAT-4 [9, 11], coded by the genes *SLC7A1*, *SLC7A2* (hCAT-2A and hCAT-2B), *SLC7A3*, and *SLC7A4*, respectively [12]. CAT-1 is ubiquitously expressed, while CAT-2A and CAT-3 are constitutively expressed in the liver and brain, respectively. CAT-2B is induced by pro-inflammatory cytokines in different cell types (including T cells and macrophages) and its activity is associated with iNOS rather than eNOS. CAT-4 derives from a sequence with a 41–42 % similarity to the other members of the CAT family, but still there is no transport activity associated with this protein. CAT-1, CAT-2B, and CAT-3 are Na^+-independent transporters, with high affinity for the substrate (Km ~ 50–200 μM), whereas CAT-2A has a relatively low affinity for cationic amino acids (Km ~ 2–5 mM) [9, 13]. Interestingly, just two members of the CAT family have been detected and characterized in primary cultures of HUVEC: hCAT-1 and hCAT-2B, without data showing activity or expression of other members of the CAT family in this cell type [14, 15] (Table 6.1). In summary, L-arginine transporters are of great relevance for vascular system physiology. This is especially true for endothelial cells, because L-arginine uptake is a key step for the synthesis of NO—the most important endogenous vasodilator. In this regard, the most relevant L-arginine transporter in human endothelial cells is the hCAT-1 transporter (Fig. 6.1).

Controversy still exists about the physiological relevance of L-arginine transport for cellular metabolism, mainly because the intracellular concentration of total L-arginine is ~1000 μM, 10-fold higher than the K_m of hCAT-1 (~100 μM) and more than 300-fold higher that the K_m of eNOS (~3 μM). These data, along with evidence that the blood concentration of L-arginine is 100–500 μM, constitutes the "L-arginine paradox," a condition that still is not fully documented by current literature. However, there is agreement that endothelial cells, at least, incorporate L-arginine from extracellular space for NO synthesis in a carrier-mediated mechanism. Shin et al. [10] reported that the CAT-1 knockdown expression decreased L-arginine transport and NO synthesis in endothelial cells. More importantly,

Table 6.1 Classification of cationic amino acid transporters

Name	Gene	K_m (μM)	Main expression	Presence in human endothelium
hCAT-1	*SLC7A1*	70–150	Ubiquitous	Yes
hCAT-2A	*SLC7A2*	2000–5000	Liver	No
hCAT-2B	*SLC7A2*	150–250	Brain, macrophages	Yes
hCAT-3	*SLC7A3*	200–500	Thymus, uterus, brain	No
hCAT-4	*SLC7A4*	Not reported	Brain, testis, placenta	No

The family of human cationic amino acid transporters (hCAT) is classified into different proteins codified by a family of genes called solute carrier family 7 subfamily A (*SLC7A*). Main characteristics of cationic amino acid transporters family, focused on kinetics parameters (K_m) and tissue distribution. Just hCAT-1 and hCAT-2B have been detected and studied in human endothelium [10–20].

this study has demonstrated that if endothelial cells incorporate L-arginine in a simple diffusion mechanism (with modified L-arginine), there is no increase of NO or L-citrulline synthesis. This gives strong evidence that L-arginine uptake from the extracellular space is necessary for endothelial function, and the mechanism involved is a type of facilitated diffusion system [10]. Furthermore, several studies show that it is possible to determine the expression and kinetic of L-arginine transport in the endothelium [16–19], showing the importance of hCAT-1 activity for vascular physiology regulation. Thus, it is possible to asseverate that every change in environmental parameters that affects the plasma concentration of amino acids could possess high relevance in the L-arginine/NO pathway and vascular health.

Transcriptional Regulation of CAT-1

CAT-1 is recognized as homologous to the ecotropic murine leukemia virus (MuLV) receptor. CAT-1 is described as a 70 kDa protein (gp70) with the ability to provide infectious capacity to the virus in nonpermissive cell lines transfected with a chimeric gene that expresses the protein [20]. In human genomics, the gene that expresses hCAT-1 is called *SLC7A1* (GenBank accession numbers NM_003045 for mRNA, NT_009799 for genomic sequence) localized in the chromosome 13q12–q14. The open reading frame of *SLC7A1* is constituted by 11 exons and 10 introns with 2 untranslated exons (−2, −1) at the 5′-end. The hCAT-1 and hCAT-2 genes have a high sequence homology, which is related to similarities between the activity of hCAT-1 and hCAT-2B [21]. CAT-1 genes from mice, rats, and humans have common characteristics: the transcriptional activity depends on a promoter without TATA-box (TATA-less promoter), with several binding sites for specificity protein 1 (Sp1) and a large 3′-untranslated region (3′-UTR) that could have important functions on the regulation of mRNA stability or degradation [22–25]. In rats, the physiological stress induced by amino acid deprivation increases mRNA expression of rCAT-1 by a mechanism that involves higher mRNA stability [22] that is related to a regulatory region inserted in the 3′-UTR of the gene [23]. This increased expression could be connected to the presence of a specific region regulated by amino acid response elements in the 3′-UTR. Other assays demonstrate that the effects of amino acid deprivation on rCAT-1 expression depend on transcriptional [24] and posttranscriptional [26] mechanisms. This evidence shows that the activity and expression of CAT-1 is a compensatory mechanism that responds when cells are exposed to several stress conditions, such as amino acid deprivation. In this condition, cells activate a survival mechanism that includes a high expression of CAT-1 in the plasma membrane with the purpose to maintain cellular metabolism and function, especially NO signaling pathways in different cells.

On the other hand, in studies using DNA from subjects with hypertension whose parents had a similar condition, a single-nucleotide polymorphism (SNP) was detected in the 3′-UTR of *SLC7A1* (nucleotide 2178) [27]. The relevance of the 3′-UTR in regulation of rCAT-1 and hCAT-1 has been well documented and is related to the activity of microRNA 122 (miR122) and binding of an AU-rich element binding protein (HuR) [28]. The finding of this SNP in 3′-UTR of *SLC7A1* could be related to results showing a reduction in hCAT-1 expression in myocardial samples from patients with congestive heart failure [29], as well as lower L-arginine uptake in peripheral blood mononuclear cells (PBMC) from hypertensive subjects [30]. These new findings, combined with the information we mentioned about the importance of L-arginine transport for vascular and endothelial function, reveal that the capacity of endothelial cells for adaptation to different physiological conditions (blood flow, hormones, nutrients, oxygen levels, among others) must at least in part be determined through the regulation of activity and expression of hCAT-1. For this reason, studies of transcriptional mechanisms underlying regulation of hCAT-1 expression in different conditions associated with vascular function and dysfunction are fundamental in understanding the progression of cardiovascular diseases and proposing therapeutic options.

Regulation of *SLC7A1* by Insulin

Stimulation of hCAT-1-mediated L-arginine transport by insulin has been correlated with the improvement of vascular function [31, 32]. The same phenomena, induced by high concentrations of D-glucose or diabetes, have been related to endothelial dysfunction and cardiovascular disease [31]. The molecular and cellular explanation for this paradox is still unclear. Kohlhaas et al. [33] demonstrated that insulin increases L-arginine transport by human aortic endothelial cells (HAEC) under normal glycemic conditions. However, in a hyperglycemic medium (25 mM D-glucose; 48 h) insulin restored the elevated L-arginine transport via an hCAT-1 activity regulation mechanism. This shows that the effects of insulin on L-arginine transport could be different if the cellular environment changes from a physiological condition to a stress condition, such as hyperglycemia or amino acid deprivation. In the following section, we will describe the effects of insulin on L-arginine metabolism in a physiological state, focused on molecular mechanisms that increase the hCAT-1 expression at transcriptional level.

In human endothelial cells, incubation with physiological concentration of insulin (0.01–10 nM) increases L-arginine transport, with a maximal stimulation after eight hours of incubation [18]. This insulin effect is related to the increase of maximal velocity of transport (V_{max}) from 2.4 ± 0.3 (pmol/μg protein/min) to 9.2 ± 1 and 9.9 ± 0.9 (pmol/μg protein/min) with 1 and 10 nM of insulin, respectively [32]. Notably, it has been reported in healthy volunteers that insulin plasma levels reach concentrations of between 1.0 nM and 12.5 nM in postprandial state after a protein-rich or carbohydrate-rich meal, respectively [34]. This similitude in insulin concentrations shows that insulin probably induces the increase of L-arginine transport in endothelial cells, increasing the synthesis of NO, vascular relaxation, and increased blood flow to several vascular beds. This is supported by the finding that 1 nM of insulin induces relaxation of umbilical vein rings in ex vivo assays. The vascular relaxation induced by insulin is completely abolished when the endothelial cells are removed, or when the vessels are incubated with the general y^+ system inhibitor, N-ethylmaleimide (NEM). This also occurs under incubation with L-lysine, the competitive inhibitor of L-arginine transport [32]. This data has been obtained in endothelial cell culture and tissue isolated from umbilical and chorionic veins from human placenta, where both receptors for insulin, isoforms A and B, are expressed [35].

Considering special characteristics of fetoplacental tissues, some recent theories propose that changes in placental features affect the intrauterine environment, fetal programming, and the post-pregnancy life of women [36–38]. Globally, the most significant alterations in pregnant women are problems coming from overnutrition, poor diet quality, and bad food habits. One of the main mechanisms affected by these nutritional alterations is the insulin–glucose axis, causing insulin resistance and the progression of metabolic diseases such as diabetes mellitus type 2 or metabolic syndrome. It is currently accepted that fetal hyperinsulinemia is the key factor for phenotypic modulation in newborns from gestational diabetic mothers, including an imbalance in amino acid metabolism in fetal tissues [39]. There is a positive feedback between L-arginine in diet, insulin secretion, and L-arginine transport that could collaborate with adequate control of cardiovascular health. Conversely, an imbalance of these parameters could contribute to the progression of cardiovascular diseases, especially in intrauterine life when genetic programming is important for early or later childhood development (Fig. 6.2).

Regarding the insulin effect on L-arginine transporter expression, the main mechanism already studied is the transcriptional regulation of *SLC7A1* (hCAT-1) expression. The stimulation of human endothelial cells with insulin for 6 or 8 h induces an important increase of L-arginine transport mediated by the y^+ system with a Michaelis–Menten constant (K_m) between 103 and 204 μM [32]. Is important to note that this K_m is similar to the previously reported constant of hCAT-1 and hCAT-2B for L-arginine [9]. Using the trans-stimulation assay with L-lysine, it is possible to demonstrate that the main protein involved in this mechanism is hCAT-1, at least in physiological conditions [18]. The effect of insulin on L-arginine transport is blocked with pharmacological inhibitors of

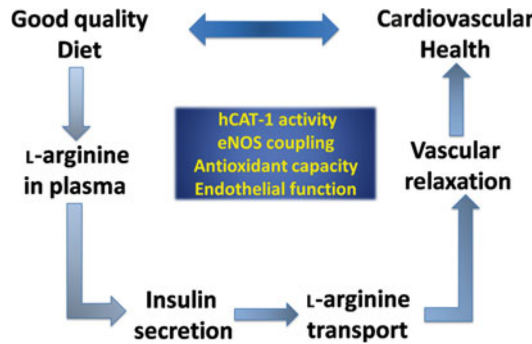

Fig. 6.2 Positive feedback between diet quality and cardiovascular health. The consumption of a balanced diet with enough content of L-arginine allows adequate levels of L-arginine in plasma and positive feedback between plasma concentrations of L-arginine and insulin secretion from pancreatic beta cells. The insulin signaling in vascular cells induces NO synthesis and regulation of vascular tone for maintenance of blood pressure and integrity of blood vessels. For good performance of this virtuous circle, the endothelial function is essential, supported by adequate activity of hCAT-1, eNOS coupling, and antioxidant capacity

phosphatidylinositol 3 kinase (PI3K) and p42/p44 mitogen-activated protein kinase ($p42/p44^{mapk}$). This suggests activation of classic insulin pathways for the induction of L-arginine uptake, NO synthesis, and vasodilatation [18, 32]. However, the temporality of these phenomena and the direct effect of NO on hCAT-1 activity are still unclear. Our hypothesis is that in physiological conditions, there is positive feedback, between NO and *SLC7A1* expression or directly between NO and hCAT-1 activity. The direct effect of NO on hCAT-1 activity could be related to posttranslational modifications, such as nitration of important amino acids for the transporter activity, and it is necessary to clarify this point with further research. Transcriptional mechanisms induced by insulin include nuclear expression and higher activity of the transcriptional factor Sp1, a protein related to basal transcription and regulation of genes without TATA-box, like *SLC7A1* [31, 40]. In summary, the stimulation of vascular cells with insulin (0.1–10 nM) activates the MAPK pathway that leads to nuclear expression and activation and binding of Sp1 to the proximal region of the *SLC7A1* promoter [31] and higher expression of hCAT-1 (Fig. 6.3). This molecular response is evoked by physiological concentrations of insulin after 6–8 h of incubation in a controlled, in vitro environment. The similitude between concentration and temporality of the effects of insulin on vasculature suggests that its postprandial effects include stimulation of D-glucose transport (glycemia regulation) together with the regulation of vascular tone and blood flow mediated by L-arginine transport and NO synthesis in endothelial cells. The lack of this insulin capacity could therefore be correlated to the progression of cardiovascular diseases in patients with insulin resistance or type 2 diabetes.

Regulation of *SLC7A1* by D-glucose

Expression and regulation of hCAT-1 in stress conditions is a key mechanism related to the effects of oxidative and nitrosative stress on vascular health and cellular distress [41]. One of the most relevant oxidative stimuli for cardiovascular diseases is hyperglycemia associated with insulin resistance and diabetes. Using this connection, it is important to remark that the most relevant pathologies in developed and middle-income countries are noncommunicable diseases such as cardiovascular diseases, metabolic syndrome, obesity, and diabetes mellitus. The causes of this reality are mainly associated with changes in lifestyle and nutrition quality of Western civilization [42–44]. In this regard, studies using human cells and controlled stress conditions such as high extracellular concentrations of

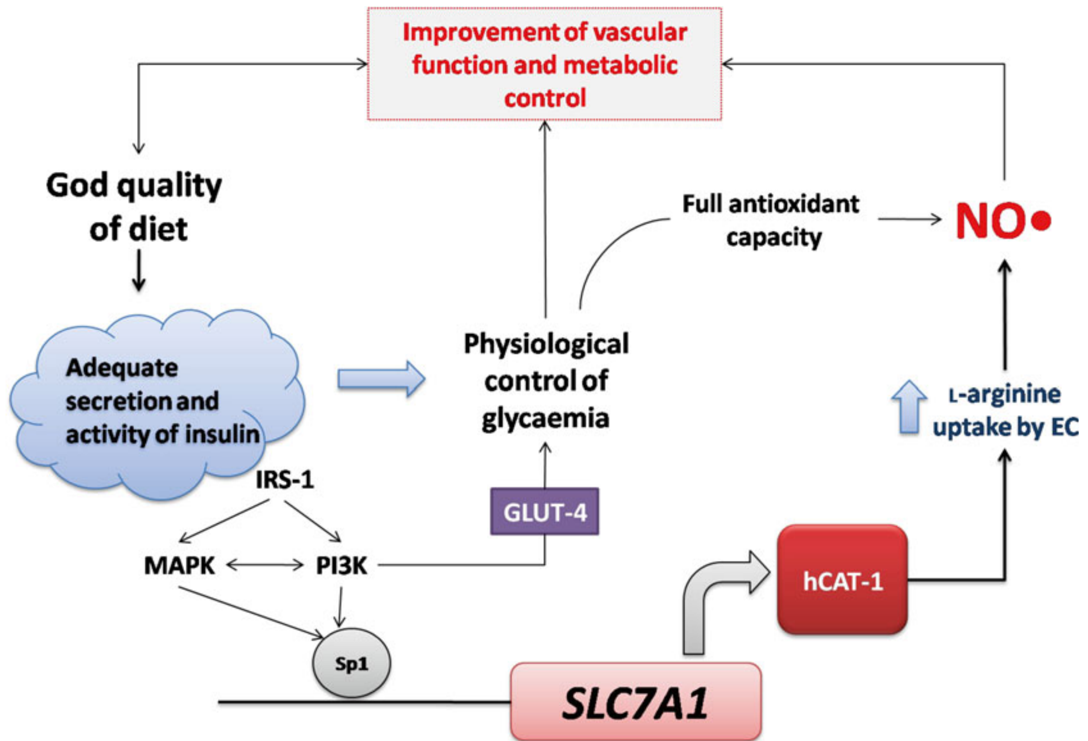

Fig. 6.3 Transcriptional regulation of *SLC7A1* by insulin in physiological state. Besides the metabolic effects of insulin, the hormone regulates the vascular health through the regulation of expression and activity of L-arginine transporters, key proteins in the mechanism of nitric oxide synthesis. The insulin signaling (PI3K) increases the glucose transporter 4 (GLUT-4) mobilization from cytoplasm to plasma membrane for glucose uptake. On the other hand, the activation of MAPK cascade (Ras-Raf-MEK-ERK) induces the activation and migration to the nucleus of specificity protein 1 (Sp1). Sp1 have several binding sites in the proximal (between −176 and −105 bp from transcription start site). The activity of this transcription factor allows the expression of hCAT-1 and L-arginine transport. Also the antioxidant control for superoxide regulation is key for improving the NO availability and vascular function

D-glucose are an effective way to analyze the mechanisms associated with the progression of these pathologies. Thereby, long-term incubation (24 or 48 h) of human endothelial cells with 15–25 mM of D-glucose is useful for understanding the deleterious effect of hyperglycemia on vasculature. By applying these types of protocols, it is possible to determine that high extracellular concentrations of D-glucose increase L-arginine transport and cyclic GMP (cGMP) accumulation, in a similar way that occurs in endothelial cells from gestational diabetes. This is the first evidence that the L-arginine/NO system is activated [16, 17] in stress conditions related to hyperglycemia and could be associated with oxidative stress [19]. Short-term endothelial cell incubation (two minutes) with increasing concentrations of D-glucose increases L-arginine transport with a half-maximal effective concentration (EC_{50}) of 11 mM D-glucose [15]. Long-term (24 h) or short-term (2 min) effects of 25 mM D-glucose are related to hCAT-1 activity in endothelial cells, but it is still not possible to discard the role of hCAT-2B in these phenomena [15, 19]. Concentrations that are usually used in these protocols are similar to the postprandial plasma concentrations of D-glucose found in patients with insulin resistance or diabetes. This demonstrates that the stimulation of this mechanism in isolated cells could be translated to etiological mechanisms of cardiovascular diseases in diabetic patients.

Elevated L-arginine transport in response to long-term incubation with high D-glucose, or in gestational diabetes, has been related to elevated levels of hCAT-1 mRNA and eNOS activity in HUVEC [45]. In other cell types, such as rat aorta endothelial cells, incubation with 25 mM D-glucose, for 1 week, induces lower nitrite levels associated with decreased expression and activity of eNOS [46]. In

HUVEC, it has been reported that chronic incubation with high D-glucose increases eNOS expression [47], an effect associated with the activity of PI3K and protein kinase B (PKB/Akt). Also, it has been shown that in bovine aortic endothelial cells (BAEC), there is a lower synthesis of NO induced by insulin when the cells are incubated with high extracellular concentrations of D-glucose. This effect of insulin is dependent on a signaling pathway that involves the insulin receptor 1 (IR1), PI3K, and the inhibitor of kinase subunit of nuclear factor kappa B (IKKB). Furthermore, high cGMP synthesis induced by high D-glucose in HUVEC is blocked through 1 nM insulin incubation [17]. In the same cell type, it has been shown that 1 nM of insulin (8 h) reverts the effect of high D-glucose on adenosine transport, an important vasoactive nucleoside [48, 49]. Thus, it can be concluded that high extracellular concentrations of D-glucose, the main problem associated with hyperglycemia, induce the expression and activity of L-arginine transporters (mainly hCAT-1) and NO synthesis. However, this fact is not logical if we recognize that hyperglycemia is associated with endothelial and vascular dysfunction. This controversy is solved if we contemplate the oxidative stress associated with the deleterious effect of D-glucose. In fact, high extracellular concentrations of D-glucose induce synthesis of superoxide ($O_2^{\bullet -}$) dependent on the activity of NADPH oxidase, increasing oxidative stress, lowering levels of BH_4, uncoupling of eNOS, and deteriorating NO. This pathophysiological response impacts the vascular reactivity of blood vessels, which is reflected in high contraction of human umbilical veins exposed to thromboxane A2 [15]. Figure 6.4 depicts the relationship between poor diet quality and hyperglycemia that leads to insulin resistance and diabetes. These conditions provide a cellular environment that triggers oxidative stress. This is mainly because $O_2^{\bullet -}$ reacts quickly with NO to generate peroxynitrite ($ONOO^-$), a very harmful molecule that induces damage to DNA, protein modifications, and metabolic changes that induce cellular dysfunction. Concomitantly, hyperglycemia is associated with high activity of protein kinase C (diacylglycerol-dependent isoforms) and

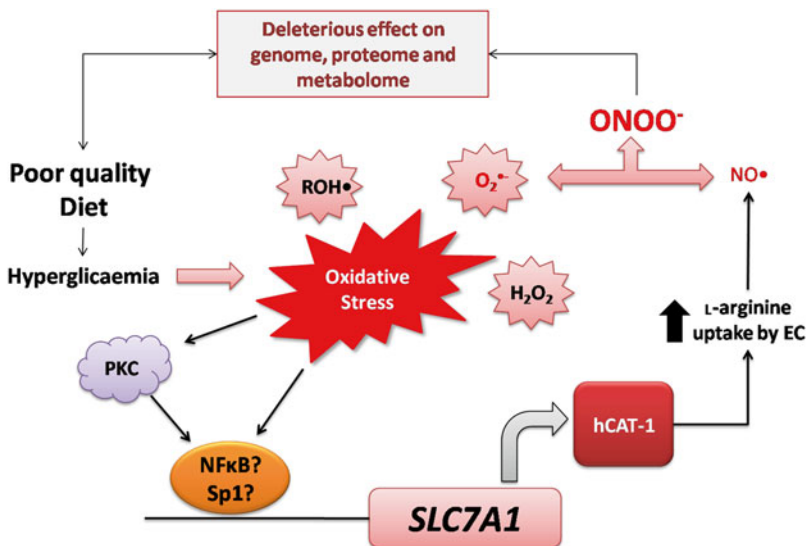

Fig. 6.4 Implications of poor quality diet on L-arginine regulation and oxidative stress. Unbalanced diet and sedentarism are associated with cardiovascular and metabolic diseases, being an important factor in these phenomena the deleterious cellular effects of elevated plasma concentrations of glucose and the resistance to the actions of insulin. Hyperglycemia, from diet with high content of carbohydrates, alters the antioxidant capacity of cells and leads to oxidative stress condition in vascular cells. This environment increases activity of signaling pathways that evokes the activation of transcription factors like nuclear factor kappa B (NFkB) or Sp1. NFkB have a binding site in promoter of *SLC7A1* (between −323 and −305 bp from transcription start site) and the hCAT-1 expression is linked with oxidative stress for increase of synthesis of peroxynitrite ($ONOO^-$), a molecule very dangerous for DNA and proteins integrity of vascular cells

high activity of nuclear factor kappa B (NFκB), a transcription factor with a binding site in the proximal promoter of *SLC7A1* [31]. We cannot discard a role of Sp1 in the transcriptional regulation of *SLC7A1* mediated by D-glucose, but presumably the main target in this stress condition could be NFκB and transcription of hCAT-1 (Fig. 6.4).

In gestational diabetes, a specific form of diabetes associated with fetoplacental vascular dysfunction, L-arginine transport is associated with elevated activity of protein kinase C. Also, it is associated with lower adenosine transport, molecule with vasoactive properties that is associated with vasodilatation through L-arginine/NO pathway: The accumulation of adenosine in the extracellular space activates A2a purinoceptor-dependent signaling, contributing to high L-arginine transport and NO synthesis [45]. Thus, in gestational diabetes and hyperglycemia, there is a vicious cycle in which the signaling pathways mediated by PKC and A2a purinoceptor activate the L-arginine/NO pathway for NO synthesis, but the activation of concomitant mechanisms for oxidative stress reduces the bioavailability of NO, as we mentioned above [41]. In fact, in patients with type 2 diabetes mellitus, the effect of insulin administration on forearm blood flow (the hormone induces relaxation of blood vessels) is diminished, and this phenomenon is related to a lower conversion of L-arginine to L-citrulline (L-arginine clearance) in these patients [50]. Previously we mentioned that Kohlhaas et al. [33] showed that in hyperglycemic conditions, L-arginine transport is higher than controls in HAEC, an effect that is reversed by insulin. Very recently, our group found similar results, showing that in HUVEC, the increase of L-arginine transport induced by high D-glucose is reverted through co-incubation with insulin. Furthermore, through this co-incubation, the V_{max} of L-arginine transport is decreased, in an effect resulting from the transcriptional regulation of hCAT-1. Is possible that these effects of insulin on L-arginine transport in hyperglycemic conditions could be related to oxidative stress regulation related to NADPH oxidase overactivity in HUVEC [19]. In Fig. 6.5, we diagram the proposal mechanism for regulation of L-arginine transport by insulin in hyperglycemic conditions. High extracellular concentrations of D-glucose increase L-arginine

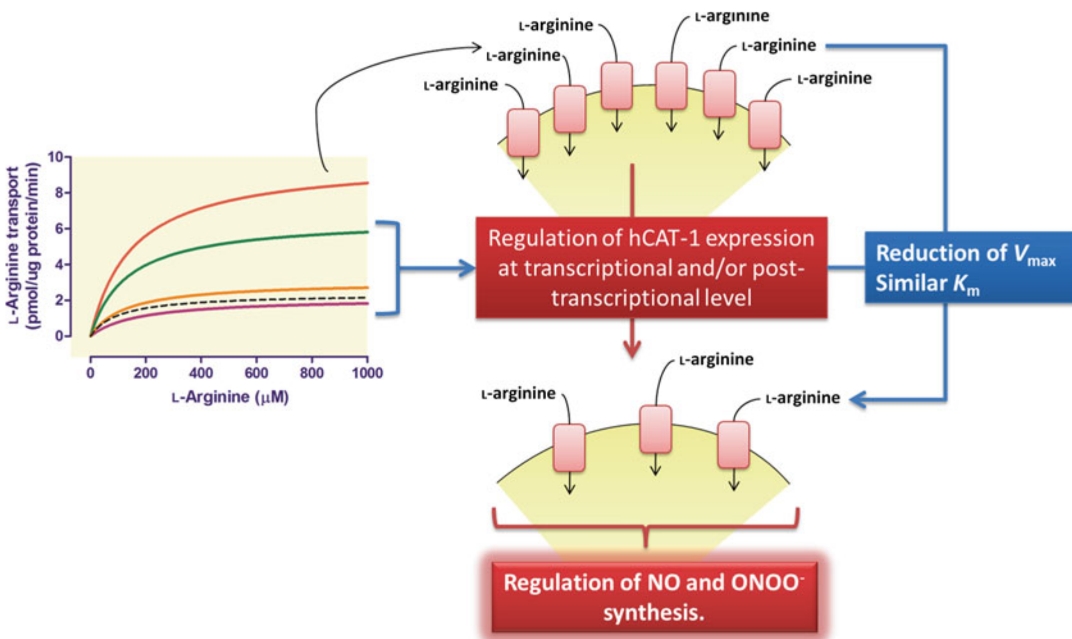

Fig. 6.5 Reversion of D-glucose effects on L-arginine transport by insulin. Insulin is able to restore the increased L-arginine transport in a cellular environment with high concentrations of glucose, like in diabetes or insulin resistance. The incubation of endothelial cells with high extracellular concentration of D-glucose increases the L-arginine transport

transport through higher expression of hCAT-1 in the plasma membrane of endothelial cells. In this setting, insulin decreases L-arginine transport by a reduction of V_{max} without changes in the K_m. These findings shows that the most likely mechanism for L-arginine transport is the regulation of hCAT-1 expression, especially the expression of this protein in plasma membrane and caveolae.

Conclusions

Control of L-arginine transport is a key mechanism for the maintenance of endothelial function and cardiovascular regulation. On the other hand, major changes in lifestyle and nutrition quality in Western civilization are affecting the cardiovascular health of the world population. Hence, the molecular mechanisms that regulate the endothelial function are targets for metabolic and hormonal changes from overweightness and obesity (also undernutrition).

First, regarding undernutrition, a lower availability of amino acids in diet, especially L-arginine, has important repercussions in cellular metabolism associated with the reduction in L-arginine uptake from extracellular space due to the lower concentration of L-arginine in the blood. L-arginine transport is highly dependent on L-arginine concentration in plasma, especially considering the interaction between L-arginine transporters and eNOS activity for NO synthesis, a molecule with pleiotropic properties in different cell types. In these conditions, the cells activate certain mechanisms with the objective of restoring the capacity for L-arginine uptake. These mechanisms are related to changes of hCAT-1 expression at different levels: transcriptional or posttranscriptional regulation of *SLC7A1*. The regulation of hCAT-1 expression by D-glucose and insulin is related to the activity of the proximal promoter of *SLC7A1*. In this region (~1600 base pairs upstream of start codon), there are several binding sites for Sp1 and one binding site for NFκB, both transcription factors which have been demonstrated to be regulated by insulin and D-glucose, respectively. Pathological concentrations of D-glucose in plasma affect the metabolism of endothelial cells, increasing the transcription of *SLC7A1* and hCAT-1 expression along with oxidative stress associated with activity of pro-oxidant enzymes like NADPH oxidase. Our most relevant findings show that under these conditions, physiological concentrations of insulin could regulate the metabolism alteration, decreasing the hCAT-1 expression and superoxide synthesis to normalize endothelial function and vascular relaxation.

Although the detailed mechanism that underlies the insulin regulation of L-arginine transport in hyperglycemia still is unknown, evidence shows that cardiovascular problems associated with obesity, insulin resistance, metabolic syndrome, or diabetes are related with impaired insulin signaling in endothelial cells, affecting the capacity of this hormone to regulate L-arginine/NO and NADPH oxidase/$O_2^{\bullet-}$ pathways. In further research, it will be important to describe the proteins involved in transcriptional regulation of *SLC7A1* in hyperglycemia or hyperinsulinemia, focusing efforts on the transcription factors with binding sites in the *SLC7A1* promoter. More focus should also be placed on the role of miR122 (or other miRs) which can be targets for diagnosis and intervention.

Fig. 6.5 (continued) through increases of maximal velocity (V_{max}) in transport mechanism (*red curve* in the plot showed in *left panel*). This effect is correlated with high abundance of hCAT-1 in plasma membrane, induced by transcriptional activity of *SLC7A1* promoter. In these cellular conditions, insulin decreases the L-arginine transport, diminishing the V_{max} and without changes in K_m: In the *left panel*, *green*, *orange*, and *purple lines* show effects of 0.1, 1, and 10 nM of insulin, respectively, in cells incubated with high concentrations of D-glucose. *Dotted line* shows the L-arginine transport in unstimulated cells. These evidences show that the regulation by insulin occurs at transcriptional levels, decreasing the abundance of hCAT-1 without altering the expression or activity of other L-arginine transporters

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References

1. Aird W. Phenotypic heterogeneity of the endothelium. II representative vascular beds. Circ Res. 2007;100:174–90.
2. Tang EH, Vanhoutte PM. Endothelial dysfunction: a strategic target in the treatment of hypertension? Pflugers Arch. 2010;459:995–1004.
3. Ignarro L. Physiology and pathophysiology of nitric oxide. Kidney Int Suppl. 1996;55:s2–5.
4. Vanhoutte PM, Shimokawa H, Tang EH, et al. Endothelial dysfunction and vascular disease. Acta Physiol. 2009;196:193–222.
5. Alderton W, Cooper C, Knowles G. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357:593–615.
6. Miranda LE, Tirapelli LF, Ramos SG, et al. Nitric oxide synthase in heart and thoracic aorta after liver ischemia and reperfusion injury: an experimental study in rats. Exp Clin Transplant. 2012;10:43–8.
7. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994;298:249–58.
8. Sessa WC. The nitric oxide synthase family of proteins. J Vasc Res. 1994;31:131–43.
9. Devés R, Boyd CAR. Transporters for cationic amino acids in animal cells: discovery, structure and function. Physiol Rev. 1998;78:487–545.
10. Shin S, Mohan S, Fung HL. Intracellular L-arginine concentration does not determine NO production in endothelial cells: implications on the "L-arginine paradox". Biochem Biophys Res Commun. 2011;414:660–3.
11. Casanello P, Escudero C, Sobrevia L. Equilibrative nucleoside (ENTs) and cationic amino acid (CATs) transporters: implications in foetal endothelial dysfunction in human pregnancy diseases. Curr Vasc Pharmacol. 2007;5:69–84.
12. Verrey F, Closs EI, Wagner CA, et al. CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch. 2003;447:532–42.
13. Mann GE, Yudilevich DL, Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiol Rev. 2003;83:183–252.
14. Casanello P, Sobrevia L. Intrauterine growth retardation is associated with reduced activity and expression of the cationic amino acid transport systems y+/hCAT-1 and y+/hCAT-2B and lower activity of nitric oxide synthase in human umbilical vein endothelial cells. Circ Res. 2002;91:127–34.
15. Flores C, Rojas S, Aguayo C, et al. Rapid stimulation of L-arginine transport by D-glucose involves p42/44(mapk) and nitric oxide in human umbilical vein endothelium. Circ Res. 2003;92:64–72.
16. Sobrevia L, Nadal A, Yudilevich DL, et al. Activation of L-arginine transport (system y+) and nitric oxide synthase by elevated glucose and insulin in human endothelial cells. J Physiol. 1996;490:775–81.
17. Sobrevia L, Yudilevich DL, Mann GE. Activation of A2-purinoceptors by adenosine stimulates L-arginine transport (system y+) and nitric oxide synthesis in human fetal endothelial cells. J Physiol. 1997;499:135–40.
18. González M, Flores C, Pearson JD, et al. Cell signalling-mediating insulin increase of mRNA expression for cationic amino acid transporters-1 and -2 and membrane hyperpolarization in human umbilical vein endothelial cells. Pflugers Arch. 2004;448:383–94.
19. González M, Rojas S, Avila P, et al. Insulin reverses D-glucose–increased nitric oxide and reactive oxygen species generation in human umbilical vein endothelial cells. PLoS One. 2015;10, e0122398.
20. Albritton LM, Tseng L, Scadden D, et al. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell. 1989;57:659–66.
21. Hammermann R, Brunn G, Racké K. Analysis of the genomic organization of the human cationic amino acid transporters CAT-1, CAT-2 and CAT-4. Amino Acids. 2001;21:211–9.
22. Aulak KS, Liu J, Wu J, et al. Molecular sites of regulation of expression of the rat cationic amino acid transporter gene. J Biol Chem. 1996;271:29799–806.
23. Aulak KS, Mishra R, Zhou L, et al. Post-transcriptional regulation of the L-arginine transporter Cat-1 by amino acid availability. J Biol Chem. 1999;274:30424–32.
24. Fernandez J, Lopez AB, Wang C, Mishra, et al. Transcriptional control of the L-arginine/lysine transporter, cat-1, by physiological stress. J Biol Chem. 2003;278:50000–9.
25. Hatzoglou M, Fernandez J, Yaman I, et al. Regulation of cationic amino acid transport: the story of the CAT-1 transporter. Annu Rev Nutr. 2004;24:377–99.

26. Lopez AB, Wang C, Huang CC, et al. A feedback transcriptional mechanism controls the level of the L-arginine/lysine transporter cat-1 during amino acid starvation. Biochem J. 2007;402:163–73.
27. Yang Z, Venardos K, Jones E, et al. Identification of a novel polymorphism in the 3'UTR of the L-arginine transporter gene SLC7A1: contribution to hypertension and endothelial dysfunction. Circulation. 2007;115:1269–74.
28. Bhattacharyya SN, Habermacher R, Martine U, et al. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell. 2006;125:1111–24.
29. Kaye DM, Parnell MM, Ahlers BA. Reduced myocardial and systemic L-arginine uptake in heart failure. Circ Res. 2002;91:1198–203.
30. Schlaich MP, Parnell MM, Ahlers BA, et al. Impaired L-arginine transport and endothelial function in hypertensive and genetically predisposed normotensive subjects. Circulation. 2004;110:3680–6.
31. Sobrevia L, González M. A role for insulin on L-arginine transport in fetal endothelial dysfunction in hyperglycaemia. Curr Vasc Pharmacol. 2009;7:467–74.
32. González M, Gallardo V, Rodríguez N, et al. Insulin-stimulated L-arginine transport requires SLC7A1 gene expression and is associated with human umbilical vein relaxation. J Cell Physiol. 2011;226:2916–24.
33. Kohlhaas CF, Morrow VA, Jhakra N, et al. Insulin rapidly stimulates L-arginine transport in human aortic endothelial cells via Akt. Biochem Biophys Res Commun. 2011;412:747–51.
34. Erdmann J, Töpsch R, Lippl F, et al. Postprandial response of plasma ghrelin levels to various test meals in relation to food intake, plasma insulin, and glucose. J Clin Endocrinol Metab. 2004;89:3048–54.
35. Westermeier F, Salomón C, González M, et al. Insulin restores gestational diabetes mellitus-reduced adenosine transport involving differential expression of insulin receptor isoforms in human umbilical vein endothelium. Diabetes. 2011;60:1677–87.
36. Fowden AL, Forhead AJ, Coan PM, et al. The placenta and intrauterine programming. J Neuroendocrinol. 2008;20:439–50.
37. Fowden AL, Moore T. Maternal-fetal resource allocation: co-operation and conflict. Placenta. 2012;33:e11–15.
38. Brennan LJ, Morton JS, Davidge ST. Vascular dysfunction in preeclampsia. Microcirculation. 2014;21:4–14.
39. Desoye G, van Poppel M. The feto-placental dialogue and diabesity. Best Pract Res Clin Obstet Gynaecol. 2015;29:15–23.
40. Samson SL, Wong NC. Role of Sp1 in insulin regulation of gene expression. J Mol Endocrinol. 2002;29:265–79.
41. Klandorf H, Van Dyke K. Oxidative and nitrosative stresses: their role in health and disease in man and birds. In: Lushchak V, Semchyshyn HM, editors. Oxidative stress-molecular mechanisms and biological effects. 1st ed. Shanghai: InTech Editorial; 2012. p. 47–60.
42. Kopp W. Role of high-insulinogenic nutrition in the etiology of gestational diabetes mellitus. Med Hypotheses. 2005;64:101–3.
43. Zhang C, Ning Y. Effect of dietary and lifestyle factors on the risk of gestational 1018 diabetes: review of epidemiologic evidence. Am J Clin Nutr. 2011;94:1975S–9S.
44. Pan A, Malik VS, Hu FB. Exporting diabetes mellitus to Asia: the impact of western-style fast food. Circulation. 2012;126:163–5.
45. Vásquez G, Sanhueza F, Vásquez R, et al. Role of adenosine transport in gestational diabetes-induced L-arginine transport and nitric oxide synthesis in human umbilical vein endothelium. J Physiol. 2004;560:111–22.
46. Srinivasan S, Hatley ME, Bolick DT, et al. Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. Diabetologia. 2004;47:1727–34.
47. Vásquez R, Farías M, Vega JL, et al. D-glucose stimulation of L-arginine transport and nitric oxide synthesis results from activation of mitogen-activated protein kinases p42/44 and Smad2 requiring functional type II TGF-beta receptors in human umbilical vein endothelium. J Cell Physiol. 2007;212:626–32.
48. Muñoz G, San Martín R, Farías M, et al. Insulin restores glucose inhibition of adenosine transport by increasing the expression and activity of the equilibrative nucleoside transporter 2 in human umbilical vein endothelium. J Cell Physiol. 2006;209:826–35.
49. San Martín R, Sobrevia L. Gestational diabetes and the adenosine/L-arginine/nitric oxide (ALANO) pathway in human umbilical vein endothelium. Placenta. 2006;27:1–10.
50. Rajapakse NW, Chong AL, Zhang WZ, et al. Insulin-mediated activation of the L-arginine nitric oxide pathway in man, and its impairment in diabetes. PLoS One. 2013;8, e61840.

Chapter 7
L-Arginine, Pancreatic Beta Cell Function, and Diabetes: Mechanisms of Stimulated Insulin Release and Pathways of Metabolism

Philip Newsholme, Kevin N. Keane, Mina Elahy, and Vinicius Fernandes Cruzat

Key Points

- L-arginine is a potent insulinotropic agent on acute exposure to β-cells.
- L-arginine can promote insulin secretion through a variety of mechanisms including membrane depolarisation, metabolism, and enhancement of antioxidant status.
- L-arginine conversion to NO, via nitric oxide synthase activity, may be regulated by the action of PEDF in β-cells.
- L-arginine supplementation may improve blood flow and endothelial function at rest.
- L-arginine supplementation may aid antioxidant status in athletes.

Keywords L-arginine • Insulin secretion • Insulin action • Metabolism • Antioxidants

Introduction

L-Arginine ($C_6H_{14}N_4O_2$) is nutritionally classified as a conditional essential amino acid that can be commonly found in the protein component of both plants and animal foods. Over the past two decades, studies have described its role as a mediator of multiple biological processes including the release of several hormones, collagen synthesis during wound healing, antitumor activity, and immune cell responses. Typically endogenous synthesis accounts for approximately 20 % of the daily expenditure, and normal levels of L-arginine in the blood range from 40 to 100 μmol/L, which may decrease by up to 20 % in diabetes. Moreover, L-arginine is an amino acid that, through its metabolism, can impact blood flow and blood pressure, especially in relation to the production of nitric oxide ($NO^•$).

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L-arginine has pronounced glucoregulatory and insulinotropic effects, stimulating insulin secretion acutely but reducing beta cell secretory function and proliferation following chronic exposure. The effect of reducing L-arginine concentration in vivo may have profoundly negative effects on the beta cell as discussed in this chapter. In addition, pigment epithelium-derived factor (PEDF) may be considered as a novel modulator of L-arginine metabolism and nitric oxide generation in the beta cell. Lastly, the effects of L-arginine supplementation in sport and exercise are considered.

Molecular Mechanisms of L-Arginine-Induced Insulin Release

It is well established that pancreatic β-cells are nutrient-sensing cells which produce and secrete insulin in response to elevated carbohydrate and fatty acid levels in blood. However, amino acids are also an important nutrient class that can regulate insulin release from β-cells. Several amino acids have been reported to positively and/or negatively modulate insulin release, and in vitro studies have demonstrated that combinations of amino acids at physiological concentrations, or administered individually at supra-physiological concentrations, can enhance insulin secretion from isolated pancreatic islets and β-cell lines, but the stimulatory effect was dependent on the duration of exposure and amino acid type (reviewed expertly in [1, 2]). For example, alanine, glutamine, leucine, taurine, and L-arginine are considered to positively influence glucose-stimulated insulin secretion (GSIS), while sulphur-containing amino acids such as cysteine and homocysteine are suggested to decrease GSIS [1]. Generally, the mechanisms by which amino acids regulate insulin release include (1) generating ATP via provision of substrates for TCA cycle and/or redox shuttles, (2) direct depolarisation of the plasma membrane during transport via amino acid transporters, and (3) indirect depolarisation of the plasma membrane during transport causing an influx of Na^+ ions along with the amino acid [3]. Consequently, this nutrient class can impact on both the triggering and amplification pathways of insulin exocytosis in the β-cell [2].

Interestingly, L-arginine has been reported to play a dual role in mediating insulin release, and some investigations suggest that acute exposure promotes insulin secretion, while chronic stimulation impedes insulin secretion [4, 5]. L-Arginine is a cationic amino acid and cellular uptake is mediated by the electrogenic mCAT2A transporter in the β-cell [6]. Entry and accumulation of L-arginine via mCAT2A directly stimulates plasma membrane depolarisation, leading to opening of voltage-gated Ca^{2+} channels with a subsequent influx of cytosolic Ca^{2+}, causing exocytosis of insulin-containing granules [7]. This particular mechanism of L-arginine-induced insulin release is entirely dependent on the extracellular Ca^{2+} concentration and withdrawal of Ca^{2+} abrogates the response [7]. These acute insulinotropic effects of L-arginine have been demonstrated in a variety of β-cell line models, such as BRIN-BD11, 1.1B4, and MIN6 [5, 8, 9], and also in ex vivo rodent islets [10]. Typically, the concentration of L-arginine used is supra-physiological at 10–30 mM, and the acute time of exposure is 20–60 min. However, the detrimental effects of chronic L-arginine exposure (48 h at 10 mM) on islet GSIS have been recently been described [4]. We reported that extended L-arginine exposure reduced insulin secretion and cell proliferation, as determined by 5-ethynyl-2′-deoxyuridine incorporation, in rat islets. Furthermore, prolonged L-arginine levels increased the number of apoptotic cells, while also increasing the expression of genes associated with endoplasmic reticulum (ER) stress, including DNA damage-inducible transcript 3 (CHOP) and activating transcription factors 3 and 4 (ATF3 and 4) [4]. Interestingly, these negative effects appeared to be independent of $NO^•$ production as no significant change in $NO^•$ levels was detected.

The dual role of L-arginine appears to centre and pivot on its ability to act as a substrate for $NO^•$ generation. As outlined briefly above, NOS enzymes convert L-arginine to citrulline and $NO^•$ (Fig. 7.1). $NO^•$ is a free radical but acts as a signalling molecule that regulates several metabolic pathways and physiological responses such as vasodilatation, inflammation, and insulin secretion

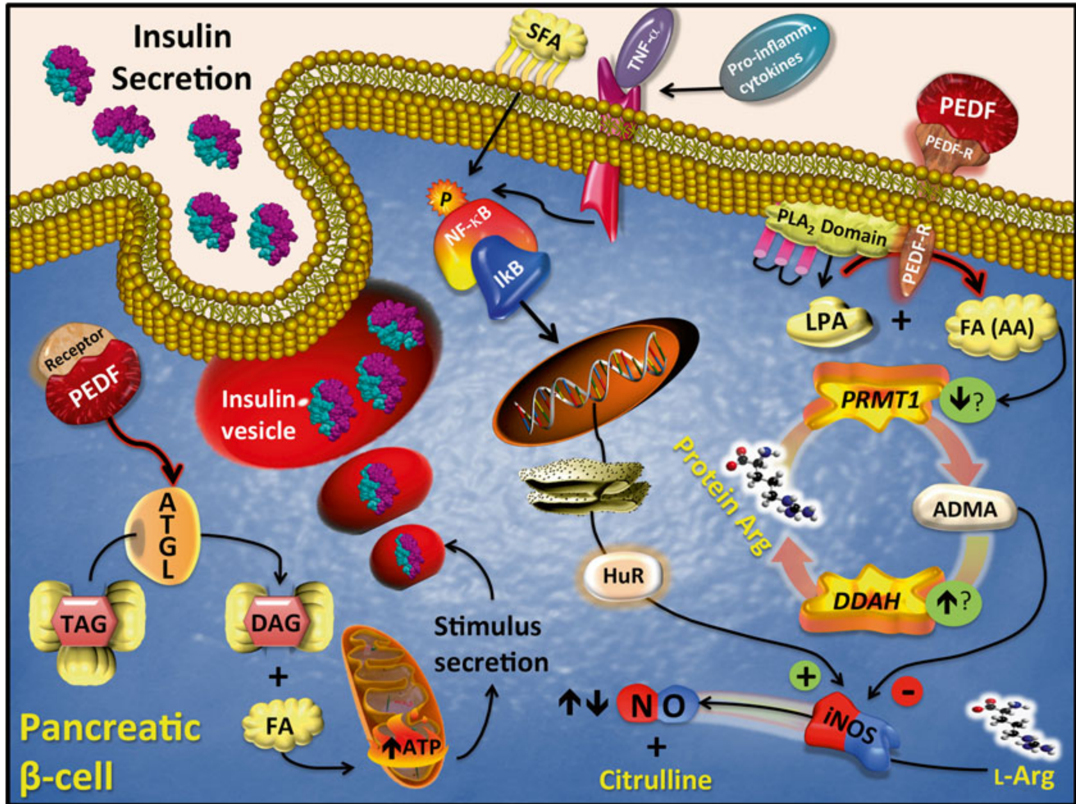

Fig. 7.1 Potential mechanisms of saturated fatty acid (SFA), cytokine, and PEDF action on L-arginine metabolism and regulation of β-cell insulin secretion. Saturated fatty acids (SFA) and pro-inflammatory cytokines, including TNFα, may activate NFKB and promote translocation to the nucleus, so enhancing the transcription of the RNA-binding protein HuR, which stabilises target transcripts including inducible nitric oxide synthase (iNOS). Plasma membrane PEDF receptor-associated phospholipase A_2 activity may generate arachidonic acid and so influence the expression of two key enzymes involved in asymmetric dimethylarginine (ADMA) synthesis, protein L-arginine methyltransferase (PRMT-1) and dimethylarginine dimethylaminohydrolase 2 (DDAH-2), decreasing the levels of PRMT-1 and increasing the levels of DDAH-2. The overall result will be to decrease the generation of ADMA, an endogenous nitric oxide synthase (NOS) inhibitor, resulting in higher NOS activity and elevating NO generation, which at moderate levels is stimulatory for insulin secretion. PEDF is transported into cells and co-localises with ATGL at lipid droplets [53]. Thus, PEDF may stimulate the intracellular generation of FA which could subsequently be oxidised, leading to stimulation of insulin secretion

[11–13]. β-cell lines such as BRIN-BD11 and INS-1 are known to express NOS isoforms including neuronal (nNOS), cNOS, and iNOS [11, 14, 15]), and it is entirely plausible that L-arginine-mediated NO• production facilitates insulin secretion. Eloquently designed experiments revealed that NO• was in fact a regulator of β-cell insulin release, and its action was mediated through the guanylate cyclase/guanosine 3′,5′-cyclic monophosphate (GC/cGMP) pathway in INS-1 cells [13], which is a common NO•-dependent signalling pathway observed in endothelial-muscle cell communication and vasodilation [16]. Furthermore, in the same study it was demonstrated that glucose metabolism was also important for endogenous NO• production [13]. Additional studies have shown that administration of pharmacological inhibitors of NOS, such as the non-selective asymmetric ω-N^G,N^G-dimethylarginine (ADMA), reduced NO• levels and consequently attenuated L-arginine-induced insulin secretion in INS-1 β-cells [17]. Moreover, homocysteine, which negatively impacts β-cell insulin secretion [1], is elevated in diabetic patients with renal complications and is an indirect substrate for ADMA synthesis, possibly contributing to reduced NO• levels and

impaired insulin release [16]. Therefore, in addition to its impact on plasma membrane depolarisation, L-arginine-mediated insulin secretion may partly result from its metabolism by NOS and subsequent production of "signalling" $NO^{\bullet}$.

Importantly, it is the delicate balance between reactive oxygen/nitrogen species (ROS/RNS) formation and clearance that significantly affects signalling and/or detrimental outcomes in the β-cell, which in turn is closely correlated with, and dependent on, endogenous antioxidant production. The excessive generation of ROS/RNS including $NO^{\bullet}$ can be induced by pro-inflammatory stimuli such as cytokines like tumour necrosis factor-α (TNFα), interferon-γ (INFγ), and interleukin-1 (IL-1) [18]. A plethora of studies have demonstrated that these cytokines enhance the expression of iNOS via activation of NFκB in immune and β-cells, which may subsequently utilise available L-arginine to produce $NO^{\bullet}$ (reviewed in [18]). Given that the β-cell is a metabolically active cell type that couples high nutrient metabolism and electron transport chain (ETC) activity to insulin secretory output, it is not surprising that elevated nutrient loads/metabolism and elevated inflammatory stimuli, both observed in diabetes, converge and yield high ROS/RNS levels that may possibly contribute to β-cell dysfunction, cytotoxicity, and death. Superoxide ($O_2^{\bullet-}$) derived from the ETC can react with $NO^{\bullet}$ to form peroxynitrate ($ONOO^-$), which is a highly reactive free radical, with a greater cytotoxicity than $O_2^{\bullet-}$ or $NO^{\bullet}$ individually [19]. Generation of excess ROS/RNS can lead to protein, membrane, or DNA damage via lipid, protein, or nucleic acid oxidation and peroxidation. Consequently, β-cell antioxidants such as GSH are extremely important for neutralising ROS/RNS generated by metabolism. It has been documented that the β-cell has a limited antioxidant capacity, including catalase levels [18]. However, the L-arginine pool could possibly be harnessed to contribute to the synthesis of GSH, as outlined earlier, through the action of arginase and subsequent production of glutamate from ornithine. High GSH levels may aid β-cell metabolism and increase the generation of ATP from the ETC, ultimately promoting greater insulin release.

L-Arginine-derived glutamate may also contribute directly to insulin secretion. However, the role of glutamate in β-cell insulin release remains contested and unclear due to the presence of conflicting findings in the literature. Some studies have shown that elevated cellular glutamate levels correlated with GSIS in β-cells [20], while others have failed to observe similar responses [21]. In an anaplerotic capacity, L-arginine-derived glutamate may possibly act as an intermediate in the TCA cycle via its conversion to α-ketoglutarate and consequently may influence ATP levels and insulin release. In addition, L-arginine can be metabolised through reactions of the urea cycle (active but incomplete in the β-cell), via the conversion of other amino acids into intermediates in the latter pathway via aminotransferase activity [18]. Taken together, it appears that a co-ordinated system regulates L-arginine metabolism in β-cells, balancing the requirement for NO generation and/or amino acid and antioxidant production.

In conclusion, it is clear that L-arginine exposure can alter insulin secretion and some studies have shown that supplementation improves insulin sensitivity, endothelial function, and glucose metabolism in vivo [22]. However, some other studies have shown that L-arginine exposure is detrimental to β-cell function [4]. Consequently, more research is required to fully understand the precise mechanisms of L-arginine metabolism, its influence on insulin secretion, and whether this amino acid can be utilised for the novel therapeutic treatment of diabetes.

A Potential Role for PEDF in Modulating Beta Cell L-Arginine and Lipid Metabolism: Impact on Insulin Secretion

Pigment epithelium-derived factor (PEDF; encoded by the gene *SERPINF*1, also known as EPC1 or caspin) is a serpin that has multiple biological actions. PEDF-focused research began approximately 25 years ago following discovery that PEDF is a differentiation factor for retinoblastoma cells [23, 24]. The PEDF protein was originally isolated from media that was conditioned by cultured retinal pigment epithelial cells. Expression of *SERPINF*1 has been shown to be increased in quiescent young

fibroblasts and PEDF levels decline during ageing. About 15 years ago, PEDF was reported to be a potent inhibitor of angiogenesis, leading to the identification of PEDF as a major antagonist to angiogenic factors (such as vascular endothelial growth factor (VEGF), its antitumorigenic and antimetastatic activities, and its potential for use as a biomarker in cancer management. PEDF is a member of the serine protease inhibitor (serpin) superfamily. Conformational transition between stressed and relaxed stages is a characteristic of catalytically active serpins; however, PEDF does not undergo this transition and hence does not exhibit common serpin inhibitory activity [25–27]. However, human PEDF structure and its three-dimensional folding have been confirmed as similar to serpins using X-ray crystallography [28]. PEDF is a protein with a molecular size of 50 kDa made up of 418 amino acids that include an amino-terminal secretion signal peptide, one N-glycosylation site at Asn285 (in the sequence NLT), and a serpin signature sequence YHLNQPFIFVL that ends at amino acid 398 [29]. PEDF is widely expressed in most body tissues but at a higher level in the eye, fetal and adult liver tissue, adult testis, ovaries, placenta, and the pancreas with a dramatic decrease in senescent/ageing cells [30]. PEDF has several biological activities and properties including anti-proliferative [31], pro-differentiation [32, 33], neuroprotective [34], anti-inflammatory [35], anti-oxidative [36], and anti-tumour [37, 38]. The PEDF–receptor interaction occurs at the C-terminal component of the molecule while the N-terminal region is involved in anti-angiogenic properties and neurotrophic activities of the protein [39, 40]. Evaluating the expression of different PEDF receptors (PEDF-Rs) could help to determine the specific biological responses of PEDF. Studies show that there are at least two different PEDF-Rs, specific to neural or endothelial cells, respectively [41]. The signalling pathways activated by PEDF regulate a number of key transcription factors including nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) [42], nuclear factor of activated T-cells (NFAT) [43], peroxisome proliferator-activated receptor (PPAR-γ) [44], and the potent pro-migratory urokinase-type plasminogen activator (uPA)/receptor (uPAR) system [37, 38].

PEDF is upregulated in individuals with the metabolic syndrome [45, 46] and patients with type 2 diabetes [47, 48]. Recent work has implicated PEDF in the development of obesity-related insulin resistance [49, 50]. PEDF induced inflammatory responses, increased adipocyte lipolysis, and promoted lipid accumulation in muscle and liver, events normally associated with insulin resistance [49]. Of relevance to this article a receptor that possesses phospholipase activity was reported in retinal pigment epithelial cells [51]. When the PEDF receptor is localised at the plasma membrane, phospholipase A_2 activity can be stimulated, possibly generating arachidonic acid [52]. Arachidonic acid can influence the expression of two key enzymes involved in asymmetric dimethylarginine (ADMA) synthesis, protein L-arginine methyltransferase (PRMT-1) and dimethylarginine dimethylaminohydrolase 2 (DDAH-2), decreasing the levels of PRMT-1 and increasing the levels of DDAH-2; see Fig. 7.1. The overall result will be to decrease the generation of ADMA, an endogenous nitric oxide synthase (NOS) inhibitor, resulting in higher NOS activity and elevating NO generation, which at moderate levels is stimulatory for insulin secretion. In addition, the PEDF receptor was reported to interact with adipose triglyceride lipase (ATGL), a triacylglycerol lipase that is critical for the maintenance of lipid and glucose homeostasis. There are other reports that have described that recombinant PEDF is transported into cells and co-localises with ATGL at lipid droplets [53]. Thus, PEDF may stimulate the intracellular generation of FA which could subsequently be oxidised, leading to stimulation of insulin secretion; see Fig. 7.1.

L-Arginine Supplementation in Sport, Exercise, and Diabetes

Nitric oxide ($NO^{•}$) plays an essential function in several metabolic pathways in the body, such as vasodilatation regulation and blood flow, inflammation and immune system activation, insulin secretion and sensitivity [11, 12], mitochondrial function, and neurotransmission. First identified as

an endothelium-derived relaxing factor (EDRF), and later as a free radical, $NO^{\bullet}$ is produced by several types of cells, especially endothelial cells, neurons, and monocytes. The synthesis of $NO^{\bullet}$ occurs through the enzyme nitric oxide synthase (NOS). This synthase exists as a constitutive nitric oxide synthase (cNOS), which is dependent on Ca^{2+} requirements, while the expression of the inducible form (iNOS) is promoted by inflammatory cytokines, bacterial endotoxins, oxidative stress, and other types of insults or toxins. The amino acid L-arginine is the main precursor of $NO^{\bullet}$, especially via iNOS activity (see above), and the availability of this amino acid may modulate endogenous $NO^{\bullet}$ production [16].

It is believed that increased $NO^{\bullet}$ will improve blood flow [54] and this could potentially be beneficial for individuals performing exercise [55]. Accordingly, an elevation in blood flow (by a rise in $NO^{\bullet}$ production) could improve exercise performance by increasing nutrient and O_2 delivery and/or removal of waste products, such as ammonia, from exercising skeletal muscles [55]. Although L-arginine, $NO^{\bullet}$ donors, and NOS inhibitors induce effects on blood pressure, heart rate, and blood flow at rest [56], several studies have shown that these agents have no effect during exercise [56–59]. A possible explanation can be attributed to the fact that under physiological conditions or exercise, the blood flow diverted to the active muscles and other organs involved in substrate supply, such as the liver, is sufficient to satisfy the tissue demands. Although L-arginine contributes to the synthesis of $NO^{\bullet}$, its supplementation has no ergogenic effect. Furthermore, endogenous L-arginine, mainly synthesised by the kidneys, appears to be sufficient to satisfy demand.

L-Arginine supplementation has the ability to increase the synthesis of creatine and growth hormone (GH), which may impact protein synthesis, especially if associated with strength training. Exercise training stimulates the cholinergic system, which in turn may potentiate GH secretion by suppressing somatostatin (SRIF), hence stimulating the secretion of growth hormone releasing hormone (GHRH). Since GH release induced by exercise is mediated by suppression of somatostatin, and L-arginine has a similar mechanism of action, the combination of both could be able to potentiate the impact of GH on protein synthesis. Although some nutritional intervention studies have shown that L-arginine alone or in combination with physical exercise may promote an increase in GH secretion, especially during the recovery period [60], there is no additional effect on protein synthesis [58, 60].

Several studies have been performed to establish critical roles for dietary protein and amino acids in the maintenance of health, including L-arginine supplementation. Since L-arginine is a non-essential (but conditional essential) amino acid, its exclusion from the diet may not alter nitrogen balance in healthy subjects.

The tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine) is the most important non-enzymatic soluble intracellular antioxidant, contributing to protective and metabolic function in specific cells including attenuation of oxidative stress in islet β-cells [1] and other organs and tissues [2, 61]. L-Arginine could be a precursor of glutathione (GSH) via glutamate generation. Increasing L-arginine availability allows for an increase in metabolic flux that produces glutamate from L-arginine by coupling production of ornithine to glutamate formation via pyrroline-5-carboxylate dehydrogenase and ornithine aminotransferase [12, 16]. This latter pathway may be important when glutamine or alanine availability is compromised, such as in exhaustive exercise [62]. Thus, the redox status of the cell can be reduced, given the index of intracellular concentration of oxidised (GSSG) and GSH [2, 12, 61]. This is particularly important in inflammatory conditions as superoxide ($O_2^{\bullet-}$) production evoked by pro-inflammatory stimulation can be detrimental to cell survival but may be attenuated by increased L-arginine availability [12].

In inflammatory situations, acute or chronic diseases, the role of Heat Shock Protein (HSP) responses has recently been investigated. An increase in HSP expression provides “stress tolerance”, protecting against many chronically and acutely stressful conditions that cause cell death and/or impair cell homeostasis, [2, 61]. It has been proposed that HSP’s expression can be modulated by L-arginine [12]. This novel finding may be via glutamate generation from both L-arginine and glucose (via 2-oxoglutarate formation and transamination of glutamate). Altering energy and nutrient metabolism in the intracellular environment, nutrient sensors such Sirtuin 1 (SIRT1), HuR, and

AMP-activated protein kinase (AMPK) initiate the activation of the main heat shock transcription factor, HSF-1, and heat shock elements (HSEs), modulating HSP responses [2].

Interestingly, it has been eported that a reduced plasma concentration of L-arginine may be associated with metabolic abnormalities in diabetes [63]. Since obesity, metabolic syndrome, and insulin resistance are associated with a pro-inflammatory state, together with the adverse effects of hyperglycaemia and hyperlipidaemia, they may lead to the progressive dysfunction and demise of pancreatic β-cells, resulting in reduced insulin secretion [16]. Thus, it is possible that L-arginine supplementation may help in diabetic patients. Interestingly, there is increasing evidence in the literature to show that L-arginine supplementation can be a powerful insulin secretagogue acting through membrane depolarisation and NO• synthesis, which increases intracellular Ca^{2+} flux and opens voltage-gated Ca^{2+} channels, promoting a physiological release of insulin in the β-cells [12] (see above).

L-Arginine administration may also enhance NO• production in the peripheral tissues, which improves insulin-mediated vasodilatation effects [1], hemodynamic function, and macrophage-mediated cell cytotoxicity [16, 63]. There is evidence that normal vascular endothelium plays an important role in maintaining vessel wall homeostasis, synthesising substances such as prostacyclins, which modulate vascular tone, reduce the risk of thrombosis, and influence smooth muscle growth.

High doses of L-arginine supplementation in some studies (between 30 and 100 g/day of the amino acid) and lower doses between 5 and 7 g/day (equivalent to about 90 mg/kg body weight in a 70 kg individual) have demonstrated varied effects of L-arginine supplementation. The case for nutritional intervention needs further evaluation, especially regarding the frequency, optimal doses, and pathways involved.

Conclusion

L-arginine is considered a conditionally essential amino acid as the body normally supplies sufficient quantities to match demand. However, supplementation may be required in specific conditions such as malnutrition, metabolic diseases including diabetes, excess circulating ammonia, burn injury, inflammation, rapid growth, disorders of urea synthesis, and/or sepsis.

Physiological concentrations of L-arginine in healthy individuals are enough to saturate endothelial NOS (eNOS), which has a Km of approximately 2–3 μmol/L. It is unlikely therefore that supplementary L-arginine should result in increased enzyme activity, elevating NO production and thus vasodilation. However, in specific situations intracellular L-arginine levels may be compromised (due, for example, to competing metabolic pathway requirements) and in these situations L-arginine supplementation may be beneficial. L-arginine supplementation may help individuals at increased risk, for example in conditions such as atherosclerosis and hypertension, diabetes mellitus, kidney failure, smoking, and advanced ageing, as these conditions are associated with reduced NO biosynthesis. Plasma levels of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, are increased two- to threefold in cardiovascular disease and may also be elevated in diabetes which can be detrimental to beta cell function and insulin secretion (as described in this chapter). Therefore, individuals with problems related to reduced NO synthesis are likely to benefit from L-arginine supplementation, after careful assessment of the appropriate dose.

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Author declaration

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References

1. Newsholme P, Abdulkader F, Rebelato E, et al. Amino acids and diabetes: implications for endocrine, metabolic and immune function. Front Biosci (Landmark Ed). 2011;16:315–39.
2. Newsholme P, Cruzat V, Arfuso F, Keane KN. Nutrient regulation of insulin secretion and action. J Endocrinol. 2014;221:R105–20.
3. Newsholme P, Gaudel C, McClenaghan NH. Nutrient regulation of insulin secretion and beta-cell functional integrity. Adv Exp Med Biol. 2010;654:91–114.
4. Mullooly N, Vernon W, Smith DM, Newsholme P. Elevated levels of branched-chain amino acids have little effect on pancreatic islet cells, but L-arginine impairs function through activation of the endoplasmic reticulum stress response. Exp Physiol. 2014;99(3):538–51.
5. McCluskey JT, Hamid M, Guo-Parke H, McClenaghan NH, Gomis R, Flatt PR. Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion. J Biol Chem. 2011;286(25):21982–92.
6. Smith PA, Sakura H, Coles B, Gummerson N, Proks P, Ashcroft FM. Electrogenic L-arginine transport mediates stimulus-secretion coupling in mouse pancreatic beta-cells. J Physiol. 1997;499(Pt 3):625–35.
7. Sener A, Best LC, Yates AP, et al. Stimulus-secretion coupling of L-arginine-induced insulin release: comparison between the cationic amino acid and its methyl ester. Endocrine. 2000;13(3):329–40.
8. Cheng K, Delghingaro-Augusto V, Nolan CJ, et al. High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. PLoS One. 2012;7(7):e40868.
9. McClenaghan NH, Barnett CR, O'Harte FP, Flatt PR. Mechanisms of amino acid-induced insulin secretion from the glucose-responsive BRIN-BD11 pancreatic B-cell line. J Endocrinol. 1996;151(3):349–57.
10. Thams P, Capito K. L-arginine stimulation of glucose-induced insulin secretion through membrane depolarization and independent of nitric oxide. Eur J Endocrinol. 1999;140(1):87–93.
11. Krause M, Rodrigues-Krause J, O'Hagan C, et al. Differential nitric oxide levels in the blood and skeletal muscle of type 2 diabetic subjects may be consequence of adiposity: a preliminary study. Metabolism. 2012; 61(11):1528–37.
12. Krause MS, McClenaghan NH, Flatt PR, de Bittencourt PIH, Murphy C, Newsholme P. L-arginine is essential for pancreatic β-cell functional integrity, metabolism and defense from inflammatory challenge. J Endocrinol. 2011;211(21784771):87–97.
13. Smukler SR, Tang L, Wheeler MB, Salapatek AM. Exogenous nitric oxide and endogenous glucose-stimulated beta-cell nitric oxide augment insulin release. Diabetes. 2002;51(12):3450–60.
14. Lajoix AD, Reggio H, Chardes T, et al. A neuronal isoform of nitric oxide synthase expressed in pancreatic beta-cells controls insulin secretion. Diabetes. 2001;50(6):1311–23.
15. Beffy P, Lajoix AD, Masiello P, et al. A constitutive nitric oxide synthase modulates insulin secretion in the INS-1 cell line. Mol Cell Endocrinol. 2001;183(1–2):41–8.
16. Newsholme P, Homem De Bittencourt PI, O'Hagan C, De Vito G, Murphy C, Krause MS. Exercise and possible molecular mechanisms of protection from vascular disease and diabetes: the central role of ROS and nitric oxide. Clin Sci (Lond). 2009;118(5):341–9.
17. Galal O, Podlogar J, Verspohl EJ. Impact of ADMA (asymmetric dimethylarginine) on physiology with respect to diabetes mellitus and respiratory system BEAS-2B cells (human bronchial epithelial cells). J Pharm Pharmacol. 2013;65(2):253–63.
18. Krause Mda S, de Bittencourt Jr PI. Type 1 diabetes: can exercise impair the autoimmune event? The L-arginine/glutamine coupling hypothesis. Cell Biochem Funct. 2008;26(4):406–33.
19. Crow JP, Beckman JS. The role of peroxynitrite in nitric oxide-mediated toxicity. Curr Top Microbiol Immunol. 1995;196(7634825):57–73.
20. Broca C, Brennan L, Petit P, Newsholme P, Maechler P. Mitochondria-derived glutamate at the interplay between branched-chain amino acid and glucose-induced insulin secretion. FEBS Lett. 2003;545(12804769):167–72.
21. MacDonald MJ, Fahien LA. Glutamate is not a messenger in insulin secretion. J Biol Chem. 2000; 275(10967090):34025–7.
22. Monti LD, Casiraghi MC, Setola E, et al. L-arginine enriched biscuits improve endothelial function and glucose metabolism: a pilot study in healthy subjects and a cross-over study in subjects with impaired glucose tolerance and metabolic syndrome. Metabolism. 2013;62(2):255–64.
23. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J. Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. Proc Natl Acad Sci U S A. 1993;90(4):1526–30.
24. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. Exp Eye Res. 1991;53(3):411–4.
25. Becerra SP. Focus on molecules: pigment epithelium-derived factor (PEDF). Exp Eye Res. 2006;82(5):739–40.
26. Filleur S, Nelius T, De Riese W, Kennedy RC. Characterization of pedf: a multi-functional serpin family protein. J Cell Biochem. 2009;106(5):769–75.

27. Silverman GA, Whisstock JC, Bottomley SP, et al. Serpins flex their muscle: I. Putting the clamps on proteolysis in diverse biological systems. J Biol Chem. 2010;285(32):24299–305.
28. Simonovic M, Gettins PGW, Volz K. Crystal structure of human PEDF, a potent anti-angiogenic and neurite growth-promoting factor. Proc Natl Acad Sci U S A. 2001;98(20):11131–5.
29. Tombran-Tink J, Pawar H, Swaroop A, Rodriguez I, Chader GJ. Localization of the gene for pigment epithelium-derived factor (PEDF) to chromosome 17p13.1 and expression in cultured human retinoblastoma cells. Genomics. 1994;19(2):266–72.
30. Samkharadze T, Erkan M, Reiser-Erkan C, et al. Pigment epithelium-derived factor associates with neuropathy and fibrosis in pancreatic cancer. Am J Gastroenterol. 2011;106(5):968–80.
31. Broadhead ML, Becerra SP, Choong PFM, Dass CR. The applied biochemistry of PEDF and implications for tissue homeostasis. Growth Factors. 2010;28(4):280–5.
32. Tombran-Tink J, Barnstable CJ. Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of angiogenesis and matrix remodeling in the bone. Biochem Biophys Res Commun. 2004;316(2):573–9.
33. Elahy M, Baindur-Hudson S, Dass CR. The emerging role of PEDF in stem cell biology. J Biomed Biotechnol. 2012. doi:10.1155/2012/239091
34. Sanchez A, Tripathy D, Yin X, Luo J, Martinez J, Grammas P. Pigment epithelium-derived factor (PEDF) protects cortical neurons in vitro from oxidant injury by activation of extracellular signal-regulated kinase (ERK) 1/2 and induction of Bcl-2. Neurosci Res. 2012;72(1):1–8.
35. Wang Y, Subramanian P, Shen D, Tuo J, Becerra SP, Chan CC. Pigment epithelium-derived factor reduces apoptosis and pro-inflammatory cytokine gene expression in a murine model of focal retinal degeneration. ASN Neuro. 2013;5(5):e00126.
36. Nakashima S, Matsui T, Yamagishi S. Pigment epithelium-derived factor (PEDF) blocks high glucose-induced inflammatory reactions in endothelial cells through its anti-oxidative properties. Int J Cardiol. 2013;168(3):3004–6.
37. Dass CR, Choong PF. uPAR mediates anticancer activity of PEDF. Cancer Biol Ther. 2008;7(8):1262–70.
38. Broadhead ML, Dass CR, Choong PF. In vitro and in vivo biological activity of PEDF against a range of tumors. Expert Opin Ther Targets. 2009;13(12):1429–38.
39. Yasui N, Mori T, Morito D, et al. Dual-site recognition of different extracellular matrix components by anti-angiogenic/neurotrophic serpin, PEDF. Biochemistry. 2003;42(11):3160–7.
40. Sánchez-Sánchez F, Aroca-Aguilar JD, Segura I, et al. Expression and purification of functional recombinant human pigment epithelium-derived factor (PEDF) secreted by the yeast Pichia pastoris. J Biotechnol. 2008;134(1–2):193–201.
41. Orgaz JL, Ladhani O, Hoek KS, et al. Loss of pigment epithelium-derived factor enables migration, invasion and metastatic spread of human melanoma. Oncogene. 2009;28(47):4147–61.
42. Yabe T, Wilson D, Schwartz JP. NFκB activation is required for the neuroprotective effects of pigment epithelium-derived factor (PEDF) on cerebellar granule neurons. J Biol Chem. 2001;276(46):43313–9.
43. Zaichuk TA, Shroff EH, Emmanuel R, Filleur S, Nelius T, Volpert OV. Nuclear factor of activated T cells balances angiogenesis activation and inhibition. J Exp Med. 2004;199(11):1513–22.
44. Ho TC, Chen SL, Yang YC, Liao CL, Cheng HC, Tsao YP. PEDF induces p53-mediated apoptosis through PPAR gamma signaling in human umbilical vein endothelial cells. Cardiovasc Res. 2007;76(2):213–23.
45. Yamagishi S, Adachi H, Abe A, et al. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. J Clin Endocrinol Metab. 2006;91(6):2447–50.
46. Wang P, Smit E, Brouwers MC, et al. Plasma pigment epithelium-derived factor is positively associated with obesity in Caucasian subjects, in particular with the visceral fat depot. Eur J Endocrinol. 2008;159(6):713–8.
47. Ogata N, Matsuoka M, Matsuyama K, et al. Plasma concentration of pigment epithelium-derived factor in patients with diabetic retinopathy. J Clin Endocrinol Metab. 2007;92(3):1176–9.
48. Jenkins A, Zhang SX, Gosmanova A, et al. Increased serum pigment epithelium derived factor levels in type 2 diabetes patients. Diabetes Res Clin Pract. 2008;82(1):e5–7.
49. Crowe S, Wu LE, Economou C, et al. Pigment epithelium-derived factor contributes to insulin resistance in obesity. Cell Metab. 2009;10(1):40–7.
50. Famulla S, Lamers D, Hartwig S, et al. Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells. Int J Obes (Lond). 2011;35(6):762–72.
51. Notari L, Baladron V, Aroca-Aguilar JD, et al. Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. J Biol Chem. 2006;281(49):38022–37.
52. Becerra SP, Notario V. The effects of PEDF on cancer biology: mechanisms of action and therapeutic potential. Nat Rev Cancer. 2013;13(4):258–71.
53. Chung C, Doll JA, Gattu AK, et al. Anti-angiogenic pigment epithelium-derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). J Hepatol. 2008;48(3):471–8.

54. Barbul A. L-Arginine: biochemistry, physiology, and therapeutic implications. JPEN J Parenter Enteral Nutr. 1986;10(2):227–38.
55. Wax B, Kavazis AN, Webb HE, Brown SP. Acute L-arginine alpha ketoglutarate supplementation fails to improve muscular performance in resistance trained and untrained men. J Int Soc Sports Nutr. 2012;9(1):17.
56. Fayh AP, Krause M, Rodrigues-Krause J, et al. Effects of L-arginine supplementation on blood flow, oxidative stress status and exercise responses in young adults with uncomplicated type I diabetes. Eur J Nutr. 2013;52(3): 975–83.
57. Willoughby DS, Boucher T, Reid J, Skelton G, Clark M. Effects of 7 days of L-arginine-alpha-ketoglutarate supplementation on blood flow, plasma L-arginine, nitric oxide metabolites, and asymmetric dimethyl L-arginine after resistance exercise. Int J Sport Nutr Exerc Metab. 2011;21(4):291–9.
58. Liu TH, Wu CL, Chiang CW, Lo YW, Tseng HF, Chang CK. No effect of short-term L-arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. J Nutr Biochem. 2009;20(6):462–8.
59. Castell LM, Burke LM, Stear SJ. A-Z of nutritional supplements: dietary supplements, sports nutrition foods and ergogenic aids for health and performance Part 2. Br J Sports Med. 2009;43(11):807–10.
60. Kanaley JA. Growth hormone, L-arginine and exercise. Curr Opin Clin Nutr Metab Care. 2008;11(1):50–4.
61. Cruzat VF, Pantaleao LC, Donato Jr J, de Bittencourt Jr PI, Tirapegui J. Oral supplementations with free and dipeptide forms of L-glutamine in endotoxemic mice: effects on muscle glutamine-glutathione axis and heat shock proteins. J Nutr Biochem. 2014;25(3):345–52.
62. Cruzat VF, Rogero MM, Tirapegui J. Effects of supplementation with free glutamine and the dipeptide alanyl-glutamine on parameters of muscle damage and inflammation in rats submitted to prolonged exercise. Cell Biochem Funct. 2010;28(1):24–30.
63. Basu HN, Liepa GU. L-Arginine: a clinical perspective. Nutr Clin Pract. 2002;17(4):218–25.

Chapter 8
Erythrocytes By-Products of L-Arginine Catabolism

Martha Lucinda Contreras-Zentella and Rolando Hernández-Muñoz

Key Points

- L-Arginine is a relative abundant amino acid in the blood, particularly in the case of the erythrocytes.
- In the erythrocytes, there are effective transporters for this dibasic amino acid which can determine the rate of L-arginine catabolism.
- Erythrocytes possess enzymes capable of metabolizing L-arginine, with an arginase which classification is still in debate well characterized, and possibly with a nitric oxide synthase activity similar to that found in the endothelium.
- L-Arginine is readily catabolized to ornithine, urea, citrulline, and nitrites (NO) in isolated erythrocytes, which is augmented in cells obtained from diabetic patients.
- Diabetes Mellitus drastically diminishes L-arginine levels in the erythrocytes, apparently in order to maintain serum L-arginine within the normal range.
- **Blood** levels for L-arginine in either serum and erythrocytes seem to be controlled by the NAD/NADH redox potential, being more evident in the cellular fraction (erythrocytes) of the blood.

Keywords Transport of L-arginine • Metabolism • Nitric oxide • Citrulline • Ornithine • Red blood cells • Cell redox state

Abbreviations

ASS1	Argininosuccinate synthetase-1
CATs	Cationic amino acid transporters
DM	Diabetes mellitus

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eNOS	Endothelial nitric oxide synthase
GD	Gestational diabetes
HbA1C	Glycosylated hemoglobin
HCC	Hepatocellular carcinoma
iNOS	Inducible nitric oxide synthase
IUGR	Intrauterine growth restriction
LATs	L-type amino acid transporters
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
RBC	Red blood cells

Introduction

L-Arginine is not only used as a precursor for protein synthesis, but it also functions as building stone for the synthesis of nitric oxide (NO), urea, ornithine, citrulline, creatinine, agmatine, glutamate, proline, and polyamines [1]. L-Arginine is relatively abundant in the blood in its "free" form, and its blood concentration is mainly affected by food intake, by protein turnover, as well as by L-arginine supply via the kidney [1]. This dibasic amino acid is conditionally essential during growth and is included in many pharmacological and nutritional formulations [2]. In the urea cycle, L-arginine is derived from arginosuccinate and is further metabolized to produce urea and the amino acid ornithine [1]. De novo biosynthesis of L-arginine uses citrulline as a precursor which, in turn, can be supplied from intestinal glutamine metabolism [2]. Besides dietary intake, several factors affect the bioavailability of dietary L-arginine, such as the levels of lysine, manganese, n-3 fatty acids in the diet, and circulating hormones including cortisol, growth hormone, leptin, cytokines, endotoxins, as well as other biomolecules, such as creatine, lactate, ornithine, and methylarginine [3]. Here, citrulline is converted to L-arginine in the body, and pharmacokinetic studies indicate that citrulline is better absorbed and, hence, has a greater systemic bioavailability than L-arginine [4]. Dietary citrulline is also capable of increasing blood levels of L-arginine and NO without affecting urea output [5].

After oral administration, an important part of the ingested L-arginine does not enter the systemic circulation in adult humans or some animals, because around 40 % is catabolized through first-pass metabolism by arginase located in the small intestine [6], which contracts in neonatal organisms, in whom the activity of this enzyme is scarce, resulting in an almost complete L-arginine absorption by enterocytes, entering the portal vein bloodstream. Since L-arginine is an important precursor of many bioactive nitrogen molecules, all the aforementioned considerations should be taken into account when using L-arginine or its derivative, citrulline, in pharmacological approaches. L-Arginine stimulates physiological processes acting as a regulatory molecule; L-arginine increases collagen synthesis and production of growth hormone, thus intervening in wound repair [2]. Moreover, L-arginine seems to regulate insulin release from pancreatic β-cells [7], as well as lymphocyte function, especially T cell development [2]. Remarkable is also the role of L-arginine in lymphocyte's memory following antigenic exposure [2].

Blood concentrations of L-arginine seem to depend not only on the ingested amount and on its first-pass metabolism in the gastrointestinal tract but also on the transport and further metabolism of this amino acid that occurs in several tissues.

Transport of L-Arginine

Specialized transporters exist that mediate the uptake of amino acids across the plasma membranes. The SLC7 family of amino acid transporters is divided into two subfamilies, the cationic amino acid transporters (CATs) and those known as the L-type amino acid transporters (LATs), which can also transport polyamines and organic cations. Most CATs function as facilitated diffusers mediating the entry and efflux of cationic amino acids, playing an important role in the delivery of L-arginine to certain cells [8].

In mammals, CAT-mediated L-arginine transport resembles that of the y + amino acid transport system. There are six CAT family members, CAT1, CAT2A, CAT2B, CAT3, which carry L-arginine, L-lysine, and L-ornithine, and CAT4 and CAT14, whose functions are not well known yet [9]; in addition, CAT members mediate sodium-independent transport of cationic L-amino acids [8]. These transporters have an apparent *Km* in the range of 40–450 μM for L-arginine, which is quite similar to that for ornithine and lies within the range of concentration found in plasma for both cationic amino acids [10]. Indeed, system y+ transporters could be considered as the major entry pathway for L-arginine and ornithine in non-epithelial cells, therefore being a relevant factor in the regulation of cell metabolism [11]. CAT-1 is restricted to the basolateral membrane in transfected epithelial cells and seems to be the major system y^+ transporter in most cells, including nitric oxide-producing cells [8]. The two CAT-2 splice variants (CAT-2A and CAT-2B) have a more defined expression pattern than CAT-1 [8]. CAT-2A is most abundant in the liver, but is also weakly expressed in cardiomyocytes, cardiac microvascular endothelial cells, the pancreas, and both skeletal and vascular smooth muscle, and it is expressed higher under pro-inflammatory conditions [12]. Lung CAT-2B seems to provide L-arginine for the synthesis of NO in macrophages. CAT-2B is induced together with the L-arginine-metabolizing enzymes, inducible nitric oxide synthase (iNOS) and arginase, in the classical and alternative activation of macrophages [13].

Evidence exists on the interconnections between function of L-arginine transporters and the activities of L-arginine-metabolizing enzymes. CAT-2A and CAT-2B are the cationic carriers relevant for macrophages and are produced by the alternative splicing of the same gene [11]. CAT-2B is responsible for the influx of L-arginine in response to cytokines produced during Th1 or Th2 immune responses [9]. Interestingly, CAT-2B blockage leads to undetectable levels of iNOS and arginase activity, suggesting that the intracellular L-arginine concentration is important for its metabolism by both enzymes [9], and the respiratory burst in macrophages can be regulated by cross-competition for L-arginine between arginase and iNOS [14].

L-Arginine Transport in Erythrocytes

It has been suggested that plasma rather than erythrocytes is the vehicle for amino acids' exchange among tissues [15]. The slow equilibration time of amino acid transport across erythrocyte membranes might indicate that these cells have little significance in the interorgan transfer of amino acids [16]. However, observations on glutamate flux in intact humans suggest a dynamic role for erythrocytes in the transport of this amino acid to the muscle [15]. In addition, studies in dogs indicate that erythrocytes and plasma may play independent and occasionally opposing roles in the exchange of several amino acids between the liver and gut [15].

Most amino acids seem to be similarly distributed between plasma and erythrocytes; however, 11 amino acids show a concentration gradient between plasma and erythrocytes, leading to an unequal

distribution of a number of basic amino acids, including L-arginine, asparagine, aspartic acid, histidine, lysine, ornithine, phosphoserine, among others [17]. This mechanism may involve co-transport with other ions, specific binding to erythrocyte macromolecules, or a process unique to the passage of erythrocytes through a vascular bed [17]. Analysis of the interorgan transport for alanine, threonine, serine, methionine, leucine, isoleucine, tyrosine, and citrulline led to conclude that data obtained from whole-blood analysis was indeed greater than that observed with plasma determination [15]. In fact, human erythrocytes show two components of L-arginine and L-lysine uptake that seem to be independent of sodium entry [18]. These transport systems are characterized by the inhibition of an amino acid uptake by other(s). Both L-arginine and L-ornithine are capable of inhibiting L-lysine uptake, whereas L-leucine can partially block transport of L-arginine, into the red blood cells (RBC) [18].

In addition, in the RBC, where cationic amino acid transport has been reported to occur through a single saturable pathway [19], very high concentrations of L-leucine (50 mM) produce an inhibition of L-arginine uptake by human erythrocytes, although its inhibition kinetics has not been clearly elucidated [18]. Moreover, L-arginine showed its familiar Na+-independent mode of uptake as a cation throughout the erythroid cell's differentiation. An exceptionally high-affinity Na+-dependent component of L-arginine uptake (Km = 0.03 mM) emerged after day 14, peaked at day 18, and then disappeared along further maturation of the erythroid cell [20]. Mature mammalian RBC possess specific, but not concentrative, amino acid transport systems, consisting of a single facilitated diffusion-type mechanism of transport [20].

L-Arginine translocation through RBC membranes is carrier mediated with simple Michaelis–Menten kinetics, with a high affinity, but with low capacity for transporting the amino acid [21]. Indeed, for some amino acids, erythrocyte transport sometimes exceeds that of serum and significant correlation coefficients show that strong serum–erythrocyte relationships exist for L-arginine and ornithine [21]. Therefore, both serum and RBC are physiologically involved in the blood transport of amino acids in humans.

Tissue L-Arginine Metabolism

Enzymes Involved in L-Arginine Metabolism

L-Arginine turns over rapidly in mammals with a half-life ranging from 0.65 to 1.10 h, because, once inside the cells, there are multiple pathways for L-arginine degradation to produce NO, ornithine, urea, polyamines, proline, glutamate, creatine, and/or agmatine [1]. These pathways are initiated by arginases, three isoforms of NOS, an L-arginine decarboxylase, as well as the L-arginine:glycine amidinotransferase. Among them, it could be considered that both arginase and NOS exert the main degrading actions on L-arginine, thus influencing its metabolism largely.

Arginase Activities

Arginase, the enzyme that hydrolyzes L-arginine, is a trimeric metalloenzyme depending on manganese per subunit for full activity [9] and exerts regulatory roles. It modulates L-arginine availability in the cells that express this enzyme and regulates polyamine synthesis due to the production of its precursor, namely, ornithine, which is essential for cell replication [1]. Arginase is widely distributed in mammalian tissues, being found in kidney, breast, testis, erythrocytes, and leukocytes [22]. Mammals have two arginase genes that encode two distinct isoforms, type I and II, which are similar

in their enzymatic properties; however, these enzymes have distinct subcellular locations, tissue distribution, expression patterns, and immunological reactivities [9]. Arginase I is cytosolic and is highly expressed in the liver, where it participates in the urea cycle, whereas arginase II is localized in mitochondria and is expressed in the brain, kidneys, mammary glands, intestines, and macrophages [1]. Some cell types express both isoforms, such as aorta endothelial cells in rats and murine macrophages [1]. Moreover, Hangenfeldt et al. [23] proposed the existence of an arginase in RBC, assumed to be type II, which was based on a negative relationship between concentrations and RBC/plasma ratios for L-arginine and ornithine.

Activity of NOS

In mammals, three nitric oxide synthase (NOS) isoenzymes are encoded by three different genes [9]. The human loci are defined as NOS1 (Gene ID 4842) coding for the neuronal nitric oxide synthase (nNOS), NOS2 (Gene ID 4843) for the inducible nitric oxide synthase (iNOS) and NOS3 (Gene ID: 4846) for endothelial nitric oxide synthase (eNOS) [24]. The iNOS is highly induced in macrophages by endotoxins and inflammatory cytokines [1], and the main effect of NO produced by this pathway is antiproliferative [9]. The constitutively expressed isoforms, nNOS and eNOS, which are Ca^{2+}/calmodulin-dependent enzymes, are lower NO output systems that are important for physiological processes such as neuronal signaling, inhibition of the hemostatic system, vasodilation, and blood pressure control [24]. NO synthases are heme-containing proteins catalyzing the five-electron oxidation of the guanidino nitrogen of L-arginine to NO and citrulline. This process requires oxygen and a number of cofactors including calcium, calmodulin, NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and tetrahydrobiopterin [24]. The proposed reaction mechanism involves electron transfer from the flavin-binding site via calmodulin to the heme group, where the oxidation of one of the guanidino nitrogens of L-arginine to the intermediate product, *N*-hydroxy-L-arginine, takes place [24].

The concentrations of L-arginine in extracellular fluids and within cells are considered to be in excess of the saturation points of the NOS enzymes [5]. Serum L-arginine concentrations in normal humans are approximately 100 μM [5] and maybe higher inside the cells, far in excess of the Km values for L-arginine of purified NOS [5]. After intravenous L-arginine supplementation, levels of nitric oxides are increased and vasodilatory responses can be restored to normal, suggesting physiologic regulation of cellular NOS activities by upper micromolar concentrations of serum L-arginine.

The NO produced by activity of NOS constitutes an important molecular signaling that regulates vasodilation of blood vessels and vascular permeability [2]. Elevated NO levels are observed in septic shock where hypotension, cardiac insufficiency, and increased tissue and endothelial permeability may precede organ failure [2]. In contrast to iNOS, the other myeloid enzyme, arginase, converts L-arginine to ornithine and shunts available L-arginine away from NO production [2]. Arginase is expressed in Th2-supporting macrophages, following stimulation by anti-inflammatory cytokines [2]. The ornithine produced by arginase can be used to synthesize polyamines and proline, which are needed for wound healing; however, only the limitation in NO production by the respective NOS could be underlying a possible participation of arginase at the Th2 response [2].

Regarding RBC, a number of recent reports have suggested the existence of NOS in neutrophils, platelets, and RBC. Various isoforms in circulating cells have been proposed to participate in the control of blood flow. Chen et al. [25] demonstrated the presence of an NOS-like enzyme in human neutrophils, showing that traditional NOS inhibitors merely reduced the uptake of tritiated L-arginine rather than inhibiting its conversion, suggesting that these analogs had no specific inhibitory effect on NOS activity in the neutrophil's cytosol. NO produced in the neutrophils may be relevant

in scavenging free radicals produced during states of oxidative stress [26]. However, other investigators could not show the presence of NOS mRNA or protein in either circulating or stored human neutrophils. Other studies have shown the presence of eNOS protein and activity in human platelets [26]; the presence of mRNA for eNOS and nNOS isoforms has been confirmed in human platelets and neutrophils [26].

Pathways for L-Arginine Catabolism

In the Vascular Endothelium

The endothelium, the largest organ in the body, is located between the bloodstream and the vessel wall. The importance of the endothelial function for vascular homeostasis has been increasingly recognized [27]. The initial state of endothelial dysfunction is also considered as an early stage of atherosclerosis, eventually leading to clinical manifestations like coronary artery disease [27]. NO is a signaling/regulatory product of the endothelium that is critically important for vascular health [24] and has been recognized to play a fundamental role in the control of vascular tone and blood flow [24]. In healthy blood vessels, eNOS-derived NO from L-arginine contributes to the regulation of blood flow and blood pressure and is an inhibitor of platelet activation and aggregation, as well as of leukocyte adhesion and migration [24]. Mice genetically deficient in endothelial nitric oxide synthase ($eNOS^{-/-}$) are hypertensive and have lower circulating nitrite levels, demonstrating the importance of constitutively produced NO for blood pressure regulation and vascular homeostasis [24].

In the RBC

Type-I arginase is expressed abundantly in hepatocytes [22] and, to a limited extent, in extrahepatic cells, including RBC from primates [22]. RBC from healthy subjects can synthesize urea apparently through an arginase-like activity, and the linear rate of urea synthesis along time suggests that extracellular and intracellular L-arginine equilibrate rapidly in blood cells [22]. Nonetheless, the significance of extrahepatic urea synthesis is not clear, since RBC contribution to urea synthesis has been estimated to be 1–3 % of the total urea production [22]. Based on the aforementioned facts, we hypothesized that fluctuations in serum levels of metabolites are influenced by RBC, and this putative "buffering" property of RBC for removing and/or releasing different metabolites from or into the serum can be altered largely by hyperglycemia and glycosylated by-products, disturbing structure and/or function of the RBC [22].

Our results show that in isolated RBC, levels of nitrites were equally distributed in serum and RBC in both healthy subjects and patients with type 2 diabetes mellitus (DM); citrulline predominated in serum, whereas urea, L-arginine, and ornithine were found mainly in RBC, in control subjects. On the other hand, blood samples from patients with DM showed hyperglycemia, increased glycosylated hemoglobin (HbA1C), and increased levels of the tested metabolites, except for L-arginine, significantly correlating with blood glucose levels. RBC were observed to be capable of catabolizing L-arginine to ornithine, citrulline, and urea, which was increased in RBC from DM patients, and correlated with an increased affinity for L-arginine in the activities of putative RBC arginase ($Km = 0.23 \pm 0.06$ vs. 0.50 ± 0.13 mM, in controls) and nitric oxide synthase ($Km = 0.28 \pm 0.06$ vs. 0.43 ± 0.09 mM, in controls). These data led us to conclude that DM alters metabolites' distribution between the serum and RBC, demonstrating that RBC regulate serum levels of metabolites that affect nitrogen metabolism, not only by transporting them but also by metabolizing amino acids such as L-arginine. Moreover, we confirmed that urea can be produced also by human RBC besides hepatocytes, being much more evident in RBC from patients with type 2 DM. These events are probably involved in the specific physiopathology of this disease, i.e., endothelial damage and dysfunction [21].

L-Arginine-derived NO has been implicated in the vascular dysfunction of diabetic patients, in whom this pathological process is characterized by impaired endothelial cell production of the vasodilator and antiplatelet adhesion factor and/or decreased NO bioavailability [21]. Moreover, increased arginase I activity and expression are associated with diabetes-induced increases in oxidative stress and with initiating the feedforward cycle of diminished NO levels and oxidative stress [21].

As to the NOS activity, this activity in RBC has been a matter of controversy for some time, and doubts about its functional significance, isoform identity, and disease relevance have been put forward by different authors [24]. RBC maturation is associated with loss of enzymatic functions. These cells have been considered almost exclusively as a transporter of metabolic gases and nutrients to the tissues. It is, therefore, possible and likely that earlier RBC forms do produce NO. Whether this occurs at the level of erythroblasts or later (or at all) requires more study on subpopulations of erythrocytes. If erythroblasts do in fact have the potential to generate NO, the function of this potential has not been defined. It is also possible that human RBC do not contain NOS activity and that the protein remains as an evolutionary vestige from prior times. These mysteries of residual but nonfunctional or minimally functional NOS within RBC surely will be a worthy cause of study for years to come [26].

In addition, RBC consistently convert [^{3}H]L-arginine to [^{3}H]L-citrulline in a Ca^{2+}-dependent fashion, and this conversion is inhibited by two different specific NO synthase inhibitors [28]. Suspension of RBC reduced platelet aggregation, and the RBC-mediated inhibition of platelet aggregation was blocked by pretreatment of RBC with an NO synthase inhibitor and potentiated by pretreatment with superoxide dismutase. Indeed, western analysis with a specific mouse monoclonal antibody provided direct evidence for the presence of human endothelium-type constitutive NO synthase with a molecular mass of approximately 140 kDa in the RBC cytosol. These observations suggest that RBC possess endothelium-type NO synthase and may regulate platelet function, at least in part, by in situ release of NO [28]. These results have been, however, challenged by other findings: it has been concluded that the enzymatic hydrolysis of L-arginine is not caused by NOS but is a result of the action of the enzyme arginase, which abounds in the RBC. However, proteins interacting with antibodies to the endothelial and inducible isoforms of NOS have been detected in human RBC by immune blot, indicating that human RBC possess proteins that react with monoclonal antibodies to the inducible and endothelial isoforms of NOS, but the proteins are without catalytic activity [29].

Another factor, through which RBC can control L-arginine metabolism, resides in these cells that can reversibly bind, transport, and release NO within the cardiovascular system [30]. These concepts were initially based mainly on the very potent NO scavenging ability of hemoglobin [30] as a central mechanism affecting NO release from RBC, through binding NO to oxyhemoglobin in the form of *S*-nitrosohemoglobin with NO being released when hemoglobin is deoxygenated in tissues with lower oxygen partial pressure [30], as well as through reduction of nitrites by hemoglobin [30]. In this regard, it is an accepted dogma that RBC take up and inactivate endothelium-derived NO via rapid reaction with oxyhemoglobin to form methemoglobin and nitrate, thereby limiting NO availability for vasodilatation. Yet, it has also been shown that RBC not only act as "NO sinks," but also exert an *erythrocrine function*—i.e., an endocrine function of RBC—by synthesizing, transporting, and releasing NO metabolic products and ATP, thereby potentially controlling systemic NO bioavailability and vascular tone; hemoglobin plays a central role in these biochemical processes [24]. Under hypoxic conditions, in particular, RBC induce NO-dependent vasorelaxation. Mechanisms of release and potential sources of NO in RBC are still a matter of debate, but candidates include iron–nitrosyl–hemoglobin, *S*-nitrosohemoglobin, and nitrite [24]. The latter may form NO either via deoxyhemoglobin (deoxyHb, Fe^{II}Hb) or xanthine oxido-reductase-mediated reduction or via spontaneous and/or carbonic anhydrase-facilitated disproportionation [24]. In addition, RBC are thought to contribute to the regulation of systemic NO bioavailability by releasing ATP when subjected to hypoxia or shear stress, which seems to be dependent on the activation of erythrocytic pannexin-1 channels inducing eNOS-dependent vasorelaxation and an increase in blood flow [24].

Recent data implicate a critical function for hemoglobin and the erythrocyte in regulating the activity of NO in the vascular compartment. Intravascular hemolysis releases hemoglobin from the RBC into plasma (cell-free plasma hemoglobin), which is then able to scavenge endothelium-derived NO 600-fold faster than erythrocytic hemoglobin, thereby disrupting NO homeostasis [31]. This may lead to vasoconstriction, decreased blood flow, platelet activation, increased endothelin-1 expression (ET-1), and end-organ injury, thus suggesting a novel disease mechanism for hereditary and acquired hemolytic conditions such as sickle cell disease and cardiopulmonary bypass. In addition to providing an NO scavenging role in the physiological regulation of NO-dependent vasodilation, hemoglobin and the erythrocyte may deliver NO as the hemoglobin deoxygenates [31]. While this process has previously been ascribed to S-nitrosated hemoglobin, recent data suggest that deoxygenated hemoglobin reduces nitrite to NO and vasodilates the human circulation along the physiological oxygen gradient. This newly described role of hemoglobin as a nitrite reductase is discussed in the context of blood flow regulation, oxygen sensing, and nitrite-based therapeutics [31]. In addition to maintaining basal vasodilator tone, NO tonically inhibits platelet aggregation, leukocyte adhesion, and smooth muscle proliferation, modulates respiration, and exerts antioxidant and anti-inflammatory activity. Under physiological conditions, reactions of vascular-derived NO with hemoglobin are thought to be the most important pathway for limiting NO bioactivity. As described in more detail elsewhere in this review series, reaction of the iron-containing heme groups of oxy- and deoxy-hemoglobin with NO produces methemoglobin and nitrate ions and iron–nitrosyl–hemoglobin, respectively [31]. Therefore, the compartmentalization model of hemoglobin allows for the existence of a sufficient diffusional gradient for NO between the endothelium and the smooth muscle to allow local paracrine activity (endothelium to smooth muscle) but limit distant endocrine bioactivity [31].

Effects of Redox State on Plasma and RBC L-Arginine Concentrations

Patients with DM, especially with microangiopathy, have augmented purine degradation during exercise and semi-ischemic tests, which occurs accompanied by elevated blood lactate and plasma ammonia [32, 33], which could result from the endothelial dysfunction occurring in DM [34, 35]. Increased NO production might be involved in vascular dysfunction and diabetic nephropathy [36]. Elevated glucose causes an increased NADH/NAD ratio, probably leading to ischemic nephropathy and retinopathy [37, 38], and causing increased production of mitochondria-derived ROS [39]. Increased formation of ROS leads to metabolic stress, resulting in changes of energy metabolism and of levels of inflammatory mediators and antioxidant systems [40]. Increases of blood lactic acid and ketone bodies are common features in diabetic patients [41, 42]. We have found that patients with type 2 DM show high levels of blood lactate and pyruvate; however, there was no lactic acidosis in these patients apparently due to two factors: (1) lactate was predominantly located in RBC, where levels of L-arginine were abruptly decreased, and (2) pyruvate “escaped” from RBC and increased in serum, lowering the lactate/pyruvate ratio in this blood compartment (Figs. 8.1 and 8.2). The fact that serum L-arginine level was maintained in detriment of its concentration in RBC led to find the existence of a direct correlation between the NAD/NADH ratio and L-arginine levels, as illustrated in Figs. 8.3 and 8.4. These data strongly suggest that the redox state (specifically the one occurring in RBC) could control the rate of L-arginine catabolism. In this context, diabetic rats course with decreased free mitochondrial and cytosolic NAD/NADH ratios and undergo activation of alternative pathways, such as that of sorbitol [43], which could contribute to increased vascular permeability [44], leukocyte adhesion to the endothelial wall [45], accelerated pericytes, endothelial cell apoptosis, and formation of pericyte ghosts and acellular capillaries [46]. Therefore, data would suggest a net and effective exchange of certain blood metabolites between the RBC and the serum.

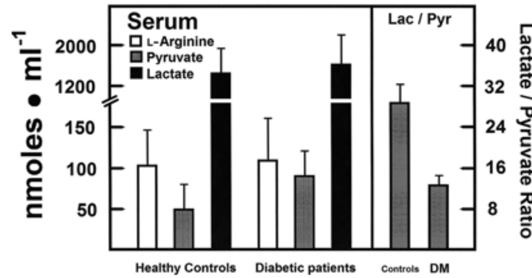

Fig. 8.1 Serum levels of L-arginine, pyruvate, lactate, and its relationship in samples from control subjects and patients with type 2 diabetes mellitus. The results are expressed as the mean ± SD (indicated by the *vertical bar*) for levels of L-arginine, pyruvate, lactate, and the lactate/pyruvate ratio in healthy control volunteers ($n=30$) and in patients with type 2 DM ($n=30$). These metabolites were determined through enzymatic methods, as previously described in detail [21]. *Symbols* for each experimental group are indicated at the *top* of the figure. Whereas the serum L-arginine level was not significantly affected by DM, it did promote an increased level of pyruvate, leading to a diminished lactate/pyruvate ratio

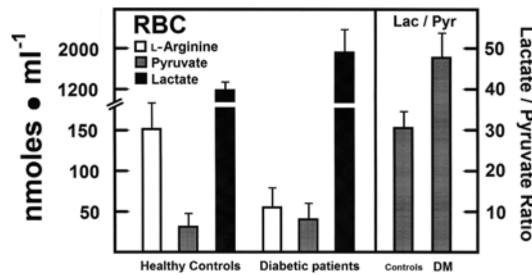

Fig. 8.2 RBC levels of L-arginine, pyruvate, lactate, and its relationship in samples from control subjects and patients with type 2 diabetes mellitus. The results are expressed as the mean ± SD (indicated by the *vertical bar*) for levels of L-arginine, pyruvate, lactate, and the lactate/pyruvate ratio in healthy control volunteers ($n=30$) and in patients with type 2 DM ($n=30$). *Symbols* for each experimental group are indicated in Fig. 8.1. In an opposite manner, onset of DM induced very low levels of RBC L-arginine, which was accompanied by an increased lactate/pyruvate ratio

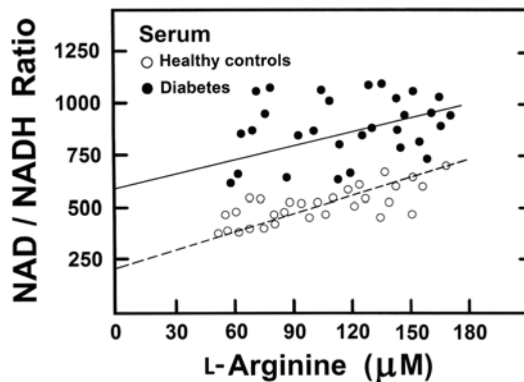

Fig. 8.3 Correlation between serum levels of L-arginine with the NAD/NADH redox potential. The levels of L-arginine from healthy control volunteers ($n=30$) and patients with type 2 DM ($n=30$) were taken from Fig. 8.1. The NAD/NADH ratio was calculated from data shown in Fig. 8.1 (lactate and pyruvate) by using the equilibrium constant for lactate dehydrogenase (1×10^{-4} M). *Symbols* for each experimental group are indicated at the *top* of the figure. In a opposite manner, onset of DM induced very low levels of RBC L-arginine, which was accompanied by an increased lactate/pyruvate ratio. Both control subjects and patients with DM had a straight relationship between NAD/NADH ratio and L-arginine levels, indicating that a more oxidized redox state favors L-arginine production and/or accumulation

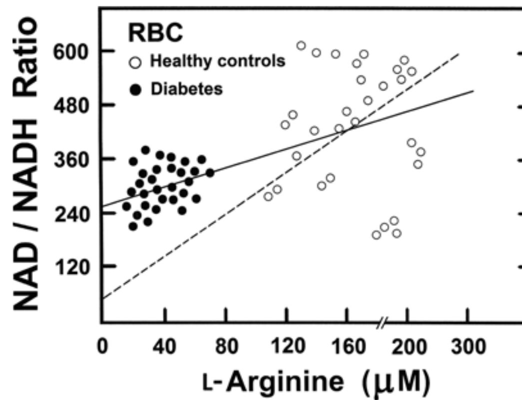

Fig. 8.4 Correlation between RBC levels of L-arginine with the NAD/NADH redox potential. The levels of L-arginine from healthy control volunteers ($n=30$) and patients with type 2 DM ($n=30$) were taken from Fig. 8.2. The NAD/NADH ratio was calculated as indicated in Fig. 8.3. *Symbols* for each experimental group are pointed out in Fig. 8.3. Here, the significant correlation between cell redox state (as assessed by the NAD/NADH redox potential) and the cellular levels for L-arginine was more evident

Pathologies Possibly Related with RBC Catabolism of L-Arginine

In Diabetes Mellitus

Diabetes mellitus (DM) is a worldwide disease characterized by metabolic disturbances, frequently associated with high risk of atherosclerosis and renal, nervous system, and ocular damage. Oxidative damage is involved in diabetes and its complications, and reactive oxygen species (ROS) have been implicated in the pathogenesis of DM, presenting frequently vascular endothelial dysfunction, associated with hypercholesterolemia, and nitric oxide (NO) deficiency is a major factor contributing to endothelial dysfunction, as has been evidenced in hypertension, tobacco smoking, and malaria [47, 48].

Recent findings emphasize the potential key role of amino acid metabolism early in the pathogenesis of diabetes, probably constituting an aid in diabetes risk assessment [49]. In this context, we have recently shown that RBC L-arginine metabolism is altered in patients with type 2 DM, producing increased by-products from L-arginine catabolism, therefore altering the mechanisms governing this apparent exchange of molecules among organs, blood cells, and serum [21]. Also we confirmed that RBC host the enzymatic machinery to metabolize amino acids, such as L-arginine, besides having efficient transport systems. Moreover, RBC could participate in the equilibrium between L-arginine metabolism and NO production, and the altered L-arginine catabolism found in cells from patients with type 2 DM could be involved in endothelial dysfunction, mainly regarding the direct interaction between RBC and endothelial cells [21].

Endothelial Dysfunction

Functional integrity of the vascular endothelium is of fundamental importance for normal vascular function, and a key factor, regulating endothelial function, is the bioavailability of NO. Recently, the enzyme arginase has emerged as an important regulator of NO production by competing for L-arginine, which is a substrate for both arginase and NO synthase. Increased activity of arginase may reduce the availability of

L-arginine for NO synthase, thus reducing NO production, increasing formation of ROS, and leading ultimately to endothelial dysfunction. Increased activity and expression of arginase have been demonstrated in several pathological cardiovascular conditions, including hypertension, pulmonary arterial hypertension, atherosclerosis, myocardial ischemia, congestive heart failure, and vascular dysfunction in DM [50].

Therefore, endothelial formation of NO by the endothelial NO synthase (eNOS) is a critical determinant of endothelial function. The NO availability can be limited by enhanced formation of superoxide anions, producing peroxynitrate, thus reducing the amount of available NO. This can significantly induce TNF-α mRNA and procoagulant activity in blood. Moreover, LDL cholesterol is a significant predictor of both endothelial dysfunction and oxidative stress. LDL cholesterol and oxidized LDL cholesterol can affect the trafficking of eNOS to the caveolae, increasing superoxide production and inducing NAD(P)H oxidase [27].

Gestational diabetes (GD, characterized by abnormal D-glucose metabolism) and intrauterine growth restriction (IUGR), a disease associated with reduced oxygen delivery (hypoxia) to the fetus, induce fetal endothelial dysfunction with implications in adult life and increase the risk of vascular diseases. Indeed, the synthesis of NO and uptake of L-arginine (the NO substrate) and adenosine (a vasoactive endogenous nucleoside) by the umbilical vein endothelium is altered in pregnancies with either GD or IUGR. These data, focusing on the role of altered vascular endothelial function in these pregnancy diseases, emphasize the epithelial and endothelial placental functions for normal fetal development and growth [51]. In this context, these newborns can exhibit L-arginine deficiency, compromising normal growth and development, and low levels of plasma L-arginine are often found in low birth weight and preterm infants, who become susceptible to intestinal inflammation and necrotizing enterocolitis [5].

Liver Diseases

Blood arginase has been considered a possible marker for liver damage, as it correlates well with serum activity of alanine aminotransferase (ALT). Since L-arginine is metabolized to ornithine and urea by arginase activity, decreased blood L-arginine has been well correlated with enhanced ornithine levels and higher blood arginase activity in several models of hepatic injury. The latter has led to suggest that arginase could play an important role in L-arginine metabolism, which could indeed be altered in the erythrocytes from patients with liver fibrosis [2].

Hemolytic Diseases

Recent data implicate a critical function for hemoglobin and the erythrocyte in regulating the activity of NO in the vascular compartment. Intravascular hemolysis releases hemoglobin from the red blood cell into plasma, which is then able to scavenge endothelium-derived NO. Furthermore, provided with an NO scavenging role in the physiological regulation of NO-dependent vasodilation, hemoglobin and the erythrocyte may deliver NO as the hemoglobin deoxygenates [31].

In Cancer

There exists an interesting approach for cancers that depend on L-arginine especially during the expression of urea cycle enzymes, mainly focused on the inactivation of argininosuccinate synthetase 1 (ASS1) in a range of malignancies, including melanoma, hepatocellular carcinoma (HCC),

sarcomas, and lymphomas. The idea is to exploit these key enzymatic pathways making the tumoral tissue dependent on exogenous L-arginine. In fact, clinical trials of several L-arginine depletors are ongoing, including pegylated L-arginine deiminase (ADI-PEG20, Polaris Group) and bioengineered forms of human arginase. However, resistance pathways to L-arginine deprivation require further study to optimize L-arginine-targeted therapies in the oncology clinic [52].

Neurodegenerative Diseases

Since its discovery, NO has been shown to be a regulator of important physiological processes in addition to vasodilation, including effector functions in the cardiovascular, immune, and nervous systems. In the central nervous system (CNS), NO functions as a diffusible chemical messenger and its actions are associated with cognitive function, synaptic plasticity, and pain perception. It is also involved in the regulation of sleep–wake cycles, appetite, body temperature, and neurosecretion, promoting optimal cerebral blood flow, consolidating memory processes, facilitating long-term potentiation, among other actions [5]. However, under specific conditions, NO appears to be produced in excess and in such cases may play a causative role in the development of inflammatory neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and other disorders of the CNS. Since physiologic levels of NO appear to be neuroprotective whereas higher concentrations are decidedly neurotoxic, it is of particular importance to understand the potential dietary regulation of the levels of L-arginine and NO in the circulation and the CNS. In fact, the effects of L-arginine-enriched animal and vegetable foods have been tested as a therapeutic approach for these diseases [5].

Concluding Remarks

The increased formation of by-products from L-arginine by RBC from patients with type 2 DM might have a negative impact on the functionality of vascular endothelial cells. In these patients, blood urea is drastically increased, predominating in RBC, and is associated with low intracellular levels for L-arginine. These would suggest that RBC from diabetic patients exert altered transport and metabolic functions for these compounds, which are associated with elevated levels of arginase and impaired NO synthesis by endothelial cells. The fact that oral administration of L-citrulline normalizes circulating levels of L-arginine and total leukocyte counts, improving the well-being in patients with sickle cell disease, strengthens these statements. The altered RBC L-arginine catabolism might contribute to the pathogenesis of DM, since L-arginine decreases serum levels of glucose, homocysteine, fatty acids, and triglycerides and improves insulin sensitivity in obese humans with type 2 DM. Thus, since citrulline or L-arginine supplementation delays the progression of atherosclerosis in obese rabbits, these findings indicate that L-arginine plays a role on insulin action; although the mechanism is not known, it is quite possible to be ascribed to NO formation. NO deficiency is a major factor contributing to endothelial dysfunction, which occurs in a variety of metabolic disorders, including diabetes. Dysregulation of L-arginine-produced NO is involved in endothelial dysfunction and endothelium-dependent relaxation, leading to oxidative stress, vascular oxidative damage, enhanced platelet adherence and aggregation, leukocyte adherence, and increased proliferation of vascular smooth muscle cells. Our data [21] indicate that RBC could participate in the equilibrium between L-arginine metabolism and NO production and that altered L-arginine catabolism found in cells from patients with type 2 DM could be involved in endothelial dysfunction, mainly regarding the direct interaction between RBC and endothelial cells. Therefore, DM promotes a characteristic pattern of disturbances in the blood levels of the tested metabolites by affecting still unknown properties of RBC. These seem to be linked to metabolite transport systems, putative metabolic pathways, and enzymes, such as arginase, depleting important substrates

or enhancing production of molecules potentially harmful, such as urea. The disturbed capacity of RBC to maintain "normal levels" of serum metabolites could be attributed to chronic exposure of blood cells to high levels of glucose, eliciting a type 2 DM-induced characteristic pattern of blood nitrogen metabolites, which is probably involved in the specific physiopathology of this disease.

References

1. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(Pt 1):1–17.
2. Saitoh W, Yamauchi S, Watanabe K, Takasaki W, Mori K. Metabolomic analysis of L-arginine metabolism in acute hepatic injury in rats. J Toxicol Sci. 2014;39:41–50.
3. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE, Yin Y. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
4. Cynober L. Pharmacokinetics of L-arginine and related amino acids. J Nutr, 6th Amino acid assessment workshop. 2007. p. 1646S–9S.
5. Virarkar M, Alappat L, Bradford PG, Awad AB. L-Arginine and nitric oxide in CNS function and neurodegenerative diseases. Crit Rev Food Sci Nutr. 2013;53(11):1157–67.
6. Castillo L, Chapman TE, Yu YM, Ajami A, Burke JF, Young VR. Dietary L-arginine uptake by the splanchnic region in adult humans. Am J Physiol Endocrinol Metab. 1993;265:E532–9.
7. Krause MS, McClenaghan NH, Flatt PR, de Bittencourt PI, Murphy C, Newsholme P. L-arginine is essential for pancreatic β-cell functional integrity, metabolism and defense from inflammatory challenge. J Endocrinol. 2011;211(1):87–97.
8. Fotiadis D, Yoshikatsu K, Palacín M. The SLC3 and SLC7 families of amino acid transporters. Mol Aspects Med. 2013;34(2-3):139–58.
9. Da Silva MFL, Floeter-Winter LM. Arginase in Leishmania. In: Santos ALS, Branquinha MH, D'Avila-Levy CM, Kneipp LF, Sodré CL, editors. Proteins and proteomics of Leishmania and Trypanosoma: subcellular biochemistry, vol. 74. Dordrecht: Springer; 2014. p. 103–17.
10. Kavanaugh MP. Voltage dependence of facilitated L-arginine flux mediated by the system y+ basic amino acid transporter. Biochemistry. 1993;32(22):5781–5.
11. Closs EI, Boissel JP, Habermeier A, Rotmann A. Structure and function of cationic amino acid transporters (CATs). J Membr Biol. 2006;213(2):67–77.
12. Rothenberg ME, Doepker MP, Lewkowich IP, Chiaramonte MG, Stringer KF, Finkelman FD, MacLeod CL, Ellies LG, Zimmermann N. Cationic amino acid transporter 2 regulates inflammatory homeostasis in the lung. Proc Natl Acad Sci U S A. 2006;103(40):14895–900.
13. Yeramian A, Martin L, Serrat N, Arpa L, Soler C, Bertran J, McLeod C, Palacin M, Modolell M, Lloberas J, Celada A. Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. J Immunol. 2006;176(10):5918–24.
14. Hrabak A, Bajor T, Temesi A, et al. The inhibitory effect of nitrite, a stable product of nitric oxide (NO) formation, on arginase. FEBS Lett. 1996;390(2):203–6.
15. Felig P, Wahren J, Räf L. Evidence of inter-organ amino-acid transport by blood cells in humans. Proc Natl Acad Sci U S A. 1973;70(6):1775–9.
16. Winter CG, Christensen HN. Migration of amino acids across the membrane of the human erythrocyte. J Biol Chem. 1964;239:872–8.
17. Drewes LR, Conway WP, Gilboe DD. Net amino acid transport between plasma and erythrocytes and perfused dog brain. Am J Physiol Endocrinol Metab. 1977;233:E320.
18. Young JD, Jones SEM, Ellory JC. Amino acid transport in human and in sheep erythrocytes. Proc R Soc Lond B. 1980;209:355–75.
19. Harvey C, Ellory JC. Identification of amino acid transporters in the red blood cell. In: Fleischer S, Fleischer B, editors. Methods in enzymology. New York: Academic; 1989. p. 122–60.
20. Vadgama JV, Castro M, Christensen HN. Characterization of amino acid transport during erythroid cell differentiation. J Biol Chem. 1987;262(27):13273–84.
21. Ramírez-Zamora S, Méndez-Rodríguez ML, Olguín-Martínez M, Sánchez-Sevilla L, Quintana-Quintana M, García-García N, Hernández-Muñoz R. Increased erythrocytes by-products of L-arginine catabolism are associated with hyperglycemia and could be Involved in the pathogenesis of type 2 diabetes mellitus. PLoS One. 2013;8(6):e66823.
22. Moore WT, Rodarte J, Smith Jr LH. Urea synthesis by hemic cells. Clin Chem. 1964;10(2):1059–65.
23. Hagenfeldt L, Arvidsson A. The distribution of amino acids between plasma and erythrocytes. Clin Chim Acta. 1980;100:133–41.

24. Cortese-Krott MM, Kelm M. Endothelial nitric oxide synthase in red blood cells: key to a new erythrocrine function? Redox Biol. 2014;2:251–8.
25. Chen LY, Mehta JL. Variable effects of L-arginine analogs on L-arginine-nitric oxide pathway in human neutrophils and platelets may relate to different nitric oxide synthase isoforms. J Pharmacol Exp Ther. 1996;276:253–7.
26. Mehta JL, Mehta P, Li D. Nitric oxide synthase in adult red blood cells: vestige of an earlier age or a biologically active enzyme? J Lab Clin Med. 2000;135(6):430–1.
27. Muller G, Goettsch C, Morawietz H. Oxidative stress and endothelial dysfunction. Hamostaseologie. 2007;27(1):5–12.
28. Chen LY, Mehta JL. Evidence for the presence of L-arginine-nitric oxide pathway in human red blood cells: relevance in the effects of red blood cells on platelet function. J Cardiovasc Pharmacol. 1998;32(1):57–61.
29. Kang ES, Ford K, Grokulsky G, Wang YB, Chiang TM, Acchiardo SR. Normal circulating adult human red blood cells contain inactive NOS proteins. J Lab Clin Med. 2000;135(6):444–51.
30. Kleinbongard P, Keymel S, Kelm M. New functional aspects of the L-arginine-nitric oxide metabolism within the circulating blood. Thromb Haemost. 2007;98(5):970–4.
31. Gladwin MT, Crawford JH, Patel RP. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. Serial review: biomedical implications for hemoglobin interactions with nitric oxide. Free Radic Biol Med. 2004;36(6):707–17.
32. Cylwik D, Mogielnicki A, Buczko W. L-arginine and cardiovascular system. Pharmacol Rep. 2005;57:14–22.
33. Kosaka H, Hisatome I, Ogino K, Tanaka Y, Osaki S, Kitamura H, et al. Excess purine degradation in muscle of chronic hemodialysis patients. Nephron. 1993;64:481–2.
34. Tanaka Y, Hisatome I, Kinugaqa T, Tanaka H, Tomikura Y, Ando F, et al. Excessive purine degradation during semiischemic forearm test in patients with diabetes mellitus. Intern Med. 2003;42:788–92.
35. Rexroth W, Hageloch W, Isgro F, Koeth T, Manzl G, Weicker H. Influence of peripheral arterial occlusive disease on muscular metabolism. Part 1: changes in lactate, ammonia and hypoxanthine concentration in femoral blood. Klin Wochenschr. 1989;67:576–82.
36. Srinivasan S, Hatley ME, Bolick DT, Palmer LA, Edelstein D, Brownlee M, Hendrick CC. Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. Diabetologia. 2004;47:1727–34.
37. Hartnett ME, Stratton RD, Browne RW, Rosner BA, Lanham RJ, Armstrong D. Serum markers of oxidative stress and severity of diabetic retinopathy. Diabetes Care. 2000;23:234240.
38. Salceda R, Vilchis C, Coffe V, Hernández-Muñoz R. Changes in the redox state in the retina and brain during the onset of diabetes in rats. Neurochem Res. 1998;23:893–7.
39. Memisoğullari R, Taysi S, Bakan E, Capoglu I. Antioxidant status and lipid peroxidation in type II diabetes mellitus. Cell Biochem Funct. 2003;21:291–6.
40. Misbin RI, Green L, Stadel BV, Gueriguian JL, Gubbi A, Fleming GA. Lactic acidosis in patients with diabetes treated with metformin. N Engl J Med. 1998;338:265–6.
41. Fulop M, Murthy V, Michilli A, Nalamati J, Qian Q, Saitowitz A. Serum β-hydroxybutyrate measurement in patients with uncontrolled diabetes mellitus. Arch Intern Med. 1999;159:381–4.
42. Obrosova IG, Drel VR, Kumagai AK, Szábo C, Pacher P, Stevens MJ. Early diabetes-induced biochemical changes in the retina: comparison of rat and mouse models. Diabetologia. 2006;49:2525–33.
43. Cheung AK, Fung MK, Lo AC, Lam TT, So KF, Chung SS, Chung SK. Aldose reductase deficiency prevents diabetes-induced blood-retinal barrier breakdown, apoptosis, and glial reactivation in the retina of db/db mice. Diabetes. 2005;54:3119–25.
44. Cruz JW, Oliveira MA, Hohman TC, Fortes ZB. Influence of tolrestat on the defective leukocyte-endothelial interaction in experimental diabetes. Eur J Pharmacol. 2000;391:163–74.
45. Miwa K, Nakamura J, Hamada Y, Naruse K, Nakashima E, Kato K, et al. The role of polyol pathway in glucose-induced apoptosis of cultured retinal pericytes. Diabetes Res Clin Pract. 2003;60:1–9.
46. Dagher Z, Park YS, Asnaghi V, Hoehn T, Gerhardinger C, Lorenzi M. Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy. Diabetes. 2004;53:2404–11.
47. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes. 1991;40:405–11.
48. Wu G, Meininger CJ. L-Arginine nutrition and cardiovascular function. J Nutr. 2000;130:2626–9.
49. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17:448–53.
50. Pernow J, Jung C. Arginase as a potential target in the treatment of cardiovascular disease: reversal of L-arginine steal? Cardiovasc Res. 2013;98(3):334–43.
51. Casanello P, Escudero C, Sobrevia L. Equilibrative nucleoside (ENTs) and cationic amino acid (CATs) transporters: implications in foetal endothelial dysfunction in human pregnancy diseases. Curr Vasc Pharmacol. 2007;5(1):69–84.
52. Phillips MM, Sheaff MT, Szlosarek PW. Targeting L-arginine-dependent cancers with L-arginine-degrading enzymes: opportunities and challenges. Cancer Res Treat. 2013;45(4):251–62.

Part II
L-Arginine Metabolism and Functions

Chapter 9
L-Arginine Synthesis from Enteral Proline

Christopher Tomlinson, Ronald O. Ball, and Paul B. Pencharz

Key Points

- Proline is a significant precursor for L-arginine in humans throughout the life cycle.
- Proline and L-arginine are co-indispensable in neonates.
- Proline is the sole dietary precursor for L-arginine in neonates.

Keywords Proline • Enterocyte • Intragastric • Intraportal • Precursor

Abbreviation

P5C-synthase Pyrroline 5 carboxylate synthase

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Introduction

The importance of L-arginine synthesis in humans throughout the life cycle is clear. The experiments of Young et al. with an L-arginine- and precursor-free diet for 4 weeks in healthy adults demonstrate that these amino acids are truly non-essential in the diet [1]. Thus, efficient L-arginine synthesis is implied. In newborn humans, the amount of L-arginine in breast milk is inadequate to meet the needs of growth and metabolism, again implying effective mechanisms of L-arginine synthesis [2, 3]. Yet in both adults and newborns, L-arginine may become essential under certain conditions where rates of synthesis are inadequate. The most significant example is that of parenteral nutritional where an L-arginine-free parenteral diet leads to life-threatening hyperammonemia [4, 5].

In adult humans the synthesis of L-arginine starts in the enterocyte with formation and then release of citrulline; this is then taken up by various tissues but largely the proximal tubule of the kidney where it is converted to L-arginine [6]. From the schematic overview of L-arginine synthesis (Fig. 9.1), we can see that L-arginine may be synthesized from three dietary amino acids: proline, glutamate and glutamine. In this chapter, we will review the evidence that proline makes a significant contribution to L-arginine synthesis throughout the human life cycle but is the sole precursor in newborns.

Proline is unique in that it is the only one of the 20 proteogenic amino acids where the amino nitrogen is secondary as it is part of a pyrrolidine ring. It is this ring structure which gives proline its physical properties that enable it to be an integral component of collagen, especially after posttranslational modification to hydroxyproline. This structural function of proline is its main role, and it had previously been thought to have few metabolic properties. However, it is now clear that proline can function as a neurotransmitter [7] and that proline oxidase has been implicated as a regulator of apoptosis in carcinogenesis [8].

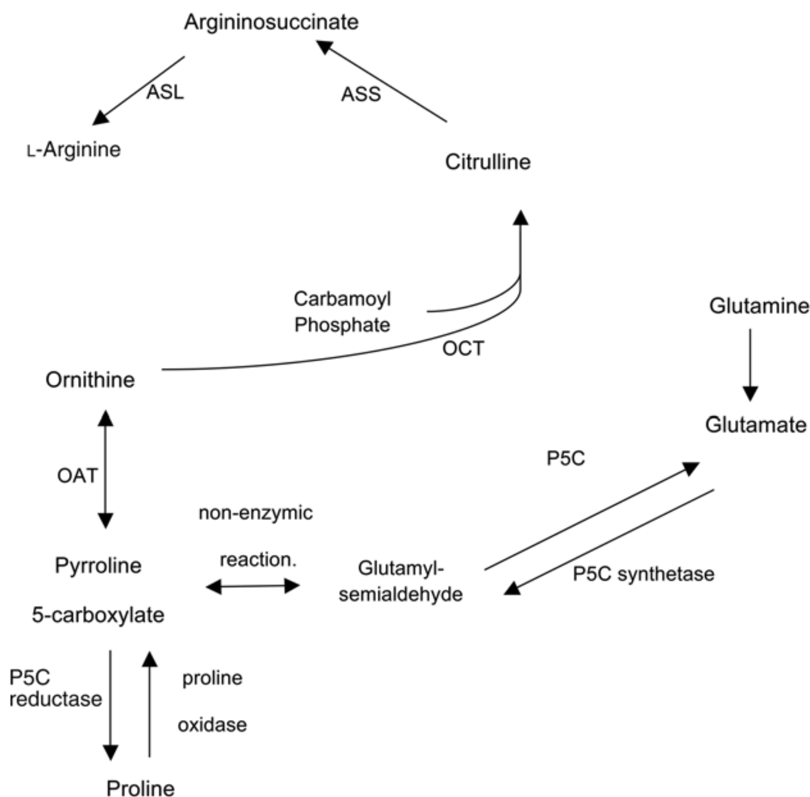

Fig. 9.1 Outline of L-arginine synthesis. *ASL* argininosuccinate lyase (EC—4.3.2.1), *ASS* argininosuccinate synthase (EC—6.3.4.5), *OAT* ornithine aminotransferase (2.1.6.13), *OCT* ornithine carbamoyl transferase (2.1.3.3), P5C reductase (1.5.1.2), Proline oxidase (1.5.99.8), P5C dehydrogenase (1.5.1.12), P5C synthetase (EC—not assigned)

One of its roles may be as a dietary precursor for other amino acids, specifically L-arginine. We, and others, have investigated this role of proline using a piglet model [9–16], in human adults [17], and in newborns [18].

In Vitro Studies

L-Arginine synthesis has been extensively studied in enterocytes by Wu et al. This group investigated the precursor for L-arginine in a series of studies using isolated enterocytes obtained from piglets at various ages designed to highlight the changes occurring after birth and during weaning. They used two outcome measures, enzyme levels and enzyme activity derived from precursor conversion to product. In light of the in vivo studies, we will focus on the enzyme activity results.

In the first of these studies, the group demonstrated that citrulline was not synthesized from glutamine in piglets preweaning (14–21 days) but that there was significant synthesis when weaned piglets were used (29–58 days) [16]. However, when younger animals were investigated there was considerable synthesis in the first few days after birth which fell over the first week [19]. This in vitro work would suggest then that there are considerable changes in the ability of the piglet enterocyte to metabolize glutamine to L-arginine from birth to postweaning. The group went on to show that these changes were reflected in rate of enzyme expression with P5C synthase being moderate at birth, falling and then rising again to much higher levels after weaning [15].

Given these developmental changes in the ability of glutamine to act as a precursor for L-arginine, the group went on to investigate proline as a potential precursor [12]. Using the same model, the group assessed changes in proline oxidase activity with age in newborn piglet enterocytes. The enzyme was highest in newborn animals (0–2 days), falling to a nadir by day 7–14 before rising again around weaning. However, in contrast to P5C synthase, the activity was only half of that in the weaning piglets compared to newborns [12]. The group went on to show that between 80 and 90 % of metabolized proline appeared as ornithine. The ability of the cells to synthesize L-arginine, citrulline, and ornithine was dependent on the concentration of proline but also required the presence of glutamine. This would suggest that the carbon skeleton of L-arginine is provided by proline, but the additional nitrogen atoms may come from glutamine with the carbon skeleton of glutamine being used for energy through oxidation.

Therefore, this series of experiments suggests that in newborn animals, proline is the precursor for the carbon skeleton of L-arginine with little or no synthesis from glutamine. At the time of weaning, this changes and then both glutamine and proline may contribute to L-arginine synthesis.

In Vivo Studies: Piglet Model

Our group has extensively studied proline and L-arginine metabolism in piglets in vivo. The first series of studies by Murphy et al. evaluated whether L-arginine or glutamate could act as precursors for proline when piglets were fed a proline-deficient diet [20]. The authors demonstrated that there was no synthesis of proline from intravenous glutamate, and although there was synthesis when the glutamate was given enterally it was not enough to meet the metabolic demands [20]. The second study demonstrated with a proline-deficient diet that intravenously infused L-arginine could act as a significant precursor for proline [9].

That L-arginine is indispensible for neonatal piglets is clear by the observation that life-threatening hyperammonemia develops when they are fed an L-arginine-deficient diet [21]. This is rapid when

parenterally fed but also occurs, although much slower, when the L-arginine-free diet is given enterally. Using hyperammonemia as an outcome measure, our group next assessed the ability of proline to ameliorate L-arginine deficiency. In piglets fed intravenously, an L-arginine- and proline-free diet rapidly led to hyperammonemia. This rapid rise was not affected by the addition of proline to the intravenous diet, indicating the importance of the splanchnic organs and first-pass metabolism. When the same diets were fed intragastrically, the L-arginine- and proline-free diet also led to hyperammonemia although the rise was not as rapid as when given intravenously. In this case, when an L-arginine-free diet was provided but with proline, the rate of rise in ammonia was significantly reduced indicating proline could act as a precursor for L-arginine when given enterally [21]. The group went further in the next series of experiments to assess differences in intragastric and intraportal diets [22]. The same results were found with intraportal diets being indistinguishable from the intravenous diets in the previous study. These results taken together indicate that only proline can act as a significant precursor for L-arginine and that this effect is dependent on the small intestine and only if feeds are given enterally.

This importance of an intact intestine for L-arginine synthesis was further confirmed by Urschel [23] who in parenterally fed piglets demonstrated that, by using glucagon-like peptide (GLP)-2 to inhibit intestinal atrophy, it was possible to maintain the ability of the bowel to use circulating proline to synthesize L-arginine, albeit still at a reduced rate from control enterally fed animals.

Using multitracer stable isotope methodology in 8–10-day-old piglets, Urschel et al. investigated the effects of an L-arginine-deficient diet on L-arginine synthesis from its dietary precursors [10]. The authors showed that proline contributes ~60 % of L-arginine synthesis, with the source of the remaining 40 % yet to be determined, and that citrulline formation is the limiting factor in L-arginine synthesis, demonstrating that all citrulline formed from proline is converted to L-arginine [10]. In the presence of an L-arginine-deficient diet, the piglets were able to upregulate L-arginine synthesis from proline.

In Vivo Studies: Human Neonate

Using stable isotopes we have been able to investigate L-arginine synthesis from proline in human neonates [18]. Fifteen stable growing preterm infants, between 30 and 34 weeks gestational age and <21 days of age, were studied. A primed, infusion of labeled L-arginine, proline, and glutamate was given to the infants over 12 h via the nasogastric tube. Enrichment of the isotopomers and their metabolites was measured in urine by tandem mass spectrometry.

The results confirmed that the findings from piglets, both in vivo and in vitro, are also valid in humans. There was no measurable enrichment of glutamate in plasma, indicating that all glutamate is metabolized on first pass through the splanchnic bed. However, none of the carbon skeleton of glutamate appeared in L-arginine, indicating that glutamate (and by extrapolation glutamine) is not a significant precursor for L-arginine in neonates. In keeping with the piglet data, there was only significant synthesis of L-arginine from proline. The rate of synthesis from proline was approximately four times the amount of L-arginine supplied by breast milk, indicating a significant rate of synthesis and metabolic demand on proline.

These results, taken together, indicate a co-dispensability of L-arginine and proline in human infants and also in piglets. The intake of L-arginine alone from breast milk is insufficient to meet the metabolic demands of this amino acid; however, when the total of proline and L-arginine is considered, there is more than enough given standard intakes of breast milk in term infants. It would appear though, using factorial methods, that these amino acids may become limiting in preterm infants given lower intakes and the higher metabolic demands of increased growth [18]. Whether this is clinically relevant needs further investigation.

In Vivo Studies: Adults

There have been few studies using isotopically labeled proline in older humans and none in adult larger mammal models. The most in-depth investigations were those of Jaksic et al. who performed a series of studies in both healthy men and patients after burn injury [24–26]. However, these studies primarily assessed proline synthesis and flux rates and did not determine synthesis of other amino acids from proline. Furthermore, all these studies used intravenous tracers; there have been no studies using labeled proline enterally to assess first-pass metabolism by the splanchnic organs. Certainly though, proline is at least conditionally indispensable in adult humans, as Jaksic et al. [26] showed no ill effects from a diet devoid of proline for 1 week and Tharakaran et al. [1] demonstrated the safety of 4 weeks of a diet lacking in L-arginine, proline, glutamate, and aspartate.

In order to elucidate proline metabolism in human adults, we performed a study using isotopically labeled L-arginine and proline given enterally to healthy men to determine the enteral flux of proline and if there was significant synthesis of L-arginine from dietary proline.

Five healthy men drinking a complete milkshake diet were fed, in addition, a primed intermittent "infusion" of enteral guanidino—$^{15}N_2$ L-arginine and ^{15}N Proline [17]. Enrichment of these tracers and labeled intermediaries ornithine and citrulline was measured in plasma by tandem mass spectrometry. The result indicated that there was small but significant synthesis of L-arginine from dietary proline, indicating that this synthetic pathway continues to exist in the adult enterocyte.

A further study indicated that glutamine could also act separately as both a nitrogen and carbon donor for L-arginine [27]. Taken together, these results indicate that there is considerable plasticity in the source of both the carbon skeleton and the nitrogen atoms of synthesized L-arginine.

These represent only preliminary studies however, and the importance of the synthesis of L-arginine and how it is regulated, especially in patient groups in whom gut function may be disrupted, remains to be elucidated.

Conclusion

In conclusion, it is clear that proline has a significant role as an L-arginine precursor in humans. Indeed the published work would support that in newborns preweaning, proline is the sole dietary precursor for L-arginine and is dependent on a functioning GI tract. In adults, there is considerable plasticity in L-arginine synthesis with both glutamine and proline able to act as precursor; however, our work demonstrates that in the physiological fed state a considerable proportion of dietary proline is used for L-arginine synthesis.

References

1. Tharakan JF, Yu YM, Zurakowski D, Roth RM, Young VR, Castillo L. Adaptation to a long term (4 weeks) L-arginine- and precursor (glutamate, proline and aspartate)-free diet. Clin Nutr. 2008;27(4):513–22.
2. Carcillo JA. Does L-arginine become a "near" essential amino acid during sepsis? Crit Care Med. 2003;31(2): 657–9.
3. Zello GA, Menendez CE, Rafii M, et al. Minimum protein intake for the preterm neonate determined by protein and amino acid kinetics. Pediatr Res. 2003;53(2):338–44.
4. Batshaw ML, Wachtel RC, Thomas GH, Starrett A, Brusilow SW. L-Arginine-responsive asymptomatic hyperammonemia in the premature infant. J Pediatr. 1984;105(1):86–91.
5. Heird WC, Nicholson JF, Driscoll Jr JM, Schullinger JN, Winters RW. Hyperammonemia resulting from intravenous alimentation using a mixture of synthetic L-amino acids: a preliminary report. J Pediatr. 1972;81(1):162–5.

6. Morris Jr SM. Enzymes of L-arginine metabolism. J Nutr. 2004;134(10 Suppl):2743S–7.
7. Jacquet H, Demily C, Houy E, et al. Hyperprolinemia is a risk factor for schizoaffective disorder. Mol Psychiatry. 2005;10(5):479–85.
8. Phang JM. Introduction to second proline symposium. Amino Acids. 2008;35(4):653–4.
9. Murphy SJ, Murphy JM, Ball RO. Proline is synthesized from intravenously infused L-arginine by piglets consuming low protein diets. Can J Anim Sci. 1996;76:435–41.
10. Urschel KL, Rafii M, Pencharz PB, Ball RO. A multitracer stable isotope quantification of the effects of L-arginine intake on whole body L-arginine metabolism in neonatal piglets. Am J Physiol Endocrinol Metab. 2007;293(3):E811–8.
11. Wu G. An important role for pentose cycle in the synthesis of citrulline and proline from glutamine in porcine enterocytes. Arch Biochem Biophys. 1996;336(2):224–30.
12. Wu G. Synthesis of citrulline and L-arginine from proline in enterocytes of postnatal pigs. Am J Physiol. 1997; 272(6 Pt 1):G1382–90.
13. Wu G, Borbolla AG, Knabe DA. The uptake of glutamine and release of L-arginine, citrulline and proline by the small intestine of developing pigs. J Nutr. 1994;124(12):2437–44.
14. Wu G, Flynn NE, Knabe DA. Enhanced intestinal synthesis of polyamines from proline in cortisoL-treated piglets. Am J Physiol Endocrinol Metab. 2000;279(2):E395–402.
15. Wu G, Knabe DA. L-Arginine synthesis in enterocytes of neonatal pigs. Am J Physiol. 1995;269(3 Pt 2):R621–9.
16. Wu G, Knabe DA, Flynn NE. Synthesis of citrulline from glutamine in pig enterocytes. Biochem J. 1994; 299(Pt 1):115–21.
17. Tomlinson C, Rafii M, Ball RO, Pencharz PB. L-Arginine can be synthesized from enteral proline in healthy adult humans. J Nutr. 2011;141(8):1432–6.
18. Tomlinson C, Rafii M, Sgro M, Ball RO, Pencharz P. L-Arginine is synthesized from proline, not glutamate, in enterally fed human preterm neonates. Pediatr Res. 2011;69(1):46–50.
19. Wu G, Knabe DA, Yan W, Flynn NE. Glutamine and glucose metabolism in enterocytes of the neonatal pig. Am J Physiol. 1995;268(2 Pt 2):R334–42.
20. Murphy JM, Murch SJ, Ball RO. Proline is synthesized from glutamate during intragastric infusion but not during intravenous infusion in neonatal piglets. J Nutr. 1996;126(4):878–86.
21. Brunton JA, Bertolo RF, Pencharz PB, Ball RO. Proline ameliorates L-arginine deficiency during enteral but not parenteral feeding in neonatal piglets. Am J Physiol. 1999;277(2 Pt 1):E223–31.
22. Bertolo RF, Brunton JA, Pencharz PB, Ball RO. L-Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. Am J Physiol Endocrinol Metab. 2003;284(5):E915–22.
23. Urschel KL, Evans AR, Wilkinson CW, Pencharz PB, Ball RO. Parenterally fed neonatal piglets have a low rate of endogenous L-arginine synthesis from circulating proline. J Nutr. 2007;137(3):601–6.
24. Jaksic T, Wagner DA, Burke JF, Young VR. Plasma proline kinetics and the regulation of proline synthesis in man. Metabolism. 1987;36(11):1040–6.
25. Jaksic T, Wagner DA, Burke JF, Young VR. Proline metabolism in adult male burned patients and healthy control subjects. Am J Clin Nutr. 1991;54(2):408–13.
26. Jaksic T, Wagner DA, Young VR. Plasma proline kinetics and concentrations in young men in response to dietary proline deprivation. Am J Clin Nutr. 1990;52(2):307–12.
27. Tomlinson C, Rafii M, Ball RO, Pencharz P. L-Arginine synthesis from enteral glutamine in healthy adults in the fed state. Am J Physiol Endocrinol Metab. 2011;301(2):E267–73.

Chapter 10
L-Arginine and Macrophages: Role in Classical and Alternative Activation

Jorge Lloberas, Manuel Modolell, and Antonio Celada

Key Points

- Macrophages play a key role in inflammation.
- At the inflammatory loci, macrophages first exert pro-inflammatory activity (tissue destruction), followed by an anti-inflammatory activity (tissue repair).
- L-Arginine is catabolized by nitric oxide synthase (NOS2) to form nitric oxide (pro-inflammatory activity) or by arginase 1 to form proline and polyamines (anti-inflammatory activity).
- A specific L-arginine transport system (Slc7a2, also called CAT2) is induced during the pro- and anti-inflammatory activation of macrophages.
- The expression of Slc7a2 is the limiting factor for the pro- and anti-inflammatory activation of macrophages.
- Arginase produced by macrophages converts L-arginine into polyamines, which are used for the proliferation of parasites such as *Leishmania major*. Also, the depletion of L-arginine by activated macrophages blocks specific T cell responses.
- Macrophages associated with tumors exert suppressive activity on T cells. This action can be mediated through macrophage-induced L-arginine depletion in the microenvironment.

Keywords Macrophage • Classical activation • Alternative activation • L-Arginine catabolism • L-Arginine transport • Nitric oxide synthase • Arginase

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Abbreviations

CATs	Cationic amino acid transporters
CCL2	CC-chemokine ligand 2
CD	Cluster of differentiation
CSF1r	Colony-stimulating factor 1 receptor
CTLs	Cytotoxic T lymphocytes
EGF-like	Epidermal growth factor-like
GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN	Interferon
IL	Interleukin
KO	Knockout
L	*Leishmania*
LPS	Lipopolysaccharide
Ly6	Lymphocyte antigen 6
M-CSF	Macrophage colony-stimulating factor
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NK	Natural killer
NO	Nitric oxide
NOS2	Nitric oxide synthese 2
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1
PDL1	PD1 ligand 1
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
TAM	Tumor-associated macrophages
Th	T helper
TNF	Tumor necrosis factor
TReg	Regulatory T cell
TSA	Trichostatin A

Introduction

Monocytes and macrophages are crucial effectors and regulators of the immune system. It has recently been shown that most tissue macrophages are produced locally, including microglia and Langerhans cells, among others [1]. Monocytes that circulate in the bloodstream are produced in the bone marrow and can be classified into two types. The first type has a surveillance function and is recognized by the cluster of differentiation (CD) surface markers, namely CD**14**$^{+}$CD**16**$^{++}$ in humans and Ly**6** (lymphocyte antigen **6**) C^{low} in mice, while the second type comprises effector cells that are involved in the inflammatory process and are recognized by CD**14**$^{++}$CD**16**$^{-}$ in humans and Ly**6**C^{high} in mice [1]. Monocytes perform immune surveillance by continuously checking the surrounding environment for signs of damage or infection. Once inside tissues, monocytes differentiate and become macrophages. In addition to eliminating pathogens, macrophages also perform other functions. They contribute to the clearance of dust and allergens in lungs (alveolar macrophages) and clear toxins from the liver (Kupffer cells) and senescent red blood cells from the spleen (splenic macrophage), and they induce immune tolerance in intestine (intestinal macrophage) [2]. Belonging to the myeloid lineage, macrophages have

diverse functions, including the initiation and resolution of inflammation, waste disposal, angiogenesis, bone remodeling, and the regulation of lipid metabolism, iron metabolism, and wound healing. Impairment of proper macrophage function leads to an imbalance in the immune response and, in extreme cases, to disease. These immune cells are characterized by the expression of specific surface markers, among these, CD**11**b, EGF-like (epidermal growth factor-like) module (Emr**1** or F**4**/**80**), CD**68**, colony-stimulating factor **1** receptor (CSF**1**r), lymphocyte antigen **6** (Ly**6**)C, and Ly**6**G [2].

Inflammation and Macrophages

Inflammation is the response of the body to injury. The word inflammation derives from the Latin *inflamatio*, meaning to set on fire, and since the times of Celsus more than 2000 years ago the cardinal signs of this condition are heat, redness, swelling, pain, and loss of function. Around three centuries ago, John Hunter, a Scottish surgeon, proposed that inflammation itself not be considered a disease but a salutary operation resulting from some form of aggression or disease. This early consideration highlights the relevance of not only inflammation but also the resolution of this condition [3]. Resolution includes the elimination of all inflammatory immune cells, such as granulocytes and pro-inflammatory macrophages, and cell debris. In addition, it allows the clearance of dead pathogens and the repair of damaged tissue, a process that, when correctly performed, prevents tissue scarring and loss of organ function.

A healthy immune system has a variety of tightly regulated mechanisms that can induce or downregulate inflammation according to requirements. When exacerbated and not properly downregulated, inflammation is damaging and causes disease. Atherosclerosis, autoimmunity, cancer, and chronic neurodegenerative diseases, to name a few, are among the list of more than a hundred inflammation-associated conditions [4].

Inflammation is a complex biological response involving several molecules and cells. This biological process is programmed first to remove the injurious stimuli and after to induce the healing process that leads to tissue repair. Inflammation is the most common response of the immune system to injury, infection, traumatism, and physical or chemical agents. In early stages of inflammation, vascularity and permeability increase, thus allowing fluids to move from the arterioles or venules to the extravascular space. Next, cells also move to the extravascular space in a precise order, starting with neutrophils, followed by monocytes, and ending with T lymphocytes (Fig. 10.1). Initially, there is a destructive phase produced mostly by neutrophils. After, macrophages reach the inflammatory site and are required to kill the microorganisms that have resisted the action of neutrophils, thus completing the destructive phase of inflammation. Next, macrophages remove the apoptotic bodies produced during this phase. This is then followed by tissue reconstruction, ending with the production of scar tissue. During the two phases of inflammation, macrophages exert two opposing roles that contribute to either tissue destruction or tissue repair. On the one hand, they show pro-inflammatory activity (M1 type) that occurs as a result of classical activation. On the other hand, to exert tissue repair functions, they carry out anti-inflammatory activity (M2 type) induced by alternative activation.

Macrophage Activation and l-Arginine

To perform their function, macrophages need to be properly activated through interaction with several molecules. This process implies that these cells undergo a series of functional, morphological, and biochemical modifications produced by the up- or downregulation of a large number of genes. Macrophages are multifunctional immune cells, and when activated, in a pro-inflammatory or anti-inflammatory fashion, the expression of other macrophage markers is up- or downregulated. For example, the major

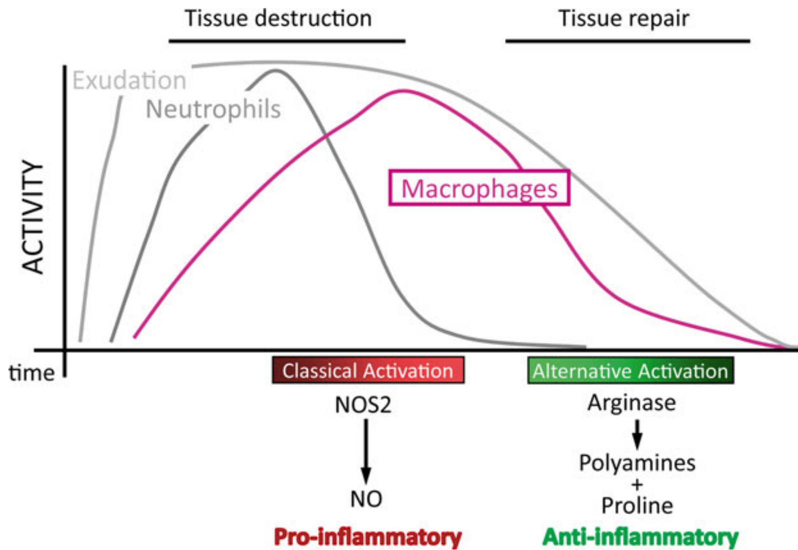

Fig. 10.1 Dual activity of macrophages at the inflammatory loci. Initially macrophages participate in the destruction of infectious agents, elimination of apoptotic bodies, and tissue damage (pro-inflammatory activity). After this phase, these cells undergo a series of modifications and become involved in tissue repair (anti-inflammatory activity)

histocompatibility complex (MHC) II and the mannose receptor are specific markers of these cells and represent examples of pro-inflammatory and anti-inflammatory activation, respectively [5]. In vitro, macrophages can be activated by T helper (Th) 1-type cytokines, such as interferon (IFN)-γ, and bacterial products, such as lipopolysaccharide (LPS), to become pro-inflammatory. These cells subsequently express highly destructive molecules, such as reactive oxygen species (ROS), nitric oxide (NO), oxygen-free radicals, and proteolytic enzymes [5]. While all these products serve to destroy microorganisms, they are also toxic for macrophages and may induce apoptosis. In this regard, macrophages have several mechanisms through which to deactivate pro-inflammatory activity [6]. Once activated by Th2 cytokines, such as interleukin (IL)-4 or IL-10, macrophages exert anti-inflammatory activity, which results in tissue repair [5] (Fig. 10.1).

Interestingly, although the pro- and anti-inflammatory phenotypes differ, they both involve the metabolism of L-arginine. This amino acid is processed by various biochemical pathways to yield the ultimate characteristics of the two macrophage phenotypes. IFN-γ and/or LPS produce the inducible NOS2, which catabolizes L-arginine into OH-arginine and then into NO (Fig. 10.2). In contrast, when macrophages are activated by IL-4, IL-10, or IL-13 to become anti-inflammatory, arginase 1 is produced [7]. This enzyme catabolizes L-arginine into urea and ornithine, which are then subsequently metabolized into proline and polyamines (putrescine, spermidine, and spermine). Polyamines induce cell proliferation, and proline is required for collagen production (Fig. 10.2). Also, it has been reported that polyamines stimulate several markers of IL-4-induced alternative activation in macrophages, producing an amplification loop of anti-inflammatory responses [8].

Therefore, during the anti-inflammatory phase, macrophages catalyze the reconstruction of damaged tissues by triggering cell proliferation and extracellular matrix reconstruction, the latter occurring during the final phases of inflammation. Taken together, these observations evidence that, during their pro-inflammatory phase, macrophages exert a destructive role while during the anti-inflammatory phase they repair the tissues damaged during the first phase.

The production of NOS2 or arginase is exclusive; macrophages exert either pro-inflammatory activity or an anti-inflammatory activity [7]. In fact, the induction of arginase 1 eliminates all the L-arginine available in the cell and then, despite unaltered mRNA, NO production is blocked. This inhibition occurs via inhibition of NOS2 mRNA translation [9].

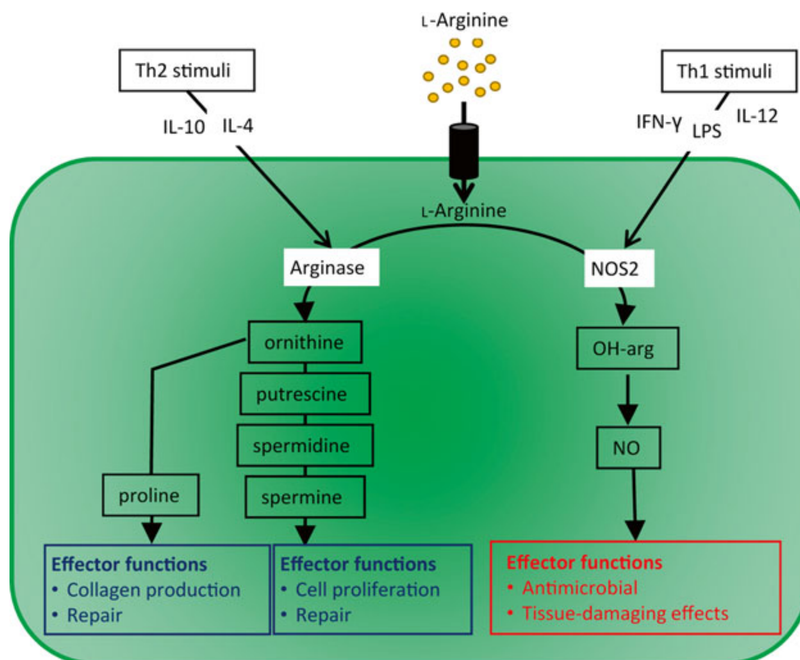

Fig. 10.2 Catabolism of L-arginine by macrophages activated by pro-inflammatory (Th1-type) cytokines (pro-inflammatory) or by anti-inflammatory (Th2-type) cytokines (anti-inflammatory). Th1 cytokines or bacterial products such as LPS trigger the production of NOS2, which then converts L-arginine into NO and citrulline, both of which exert tissue damage. Th2 cytokines induce arginase 1, which catabolizes L-arginine into proline and polyamines. The induction of arginase blocks the expression of NOS2

It is relevant that trichostatin A (TSA), which inhibits deacetylase activity, blocks LPS-dependent *nos2* expression at the transcriptional level [10] and also IL-4-induced arginase 1 [11]. LPS stimulates the expression of several genes that require new protein synthesis (secondary response genes) and others that do not (primary response genes). TSA inhibits secondary response genes by acting at the transcriptional level. We observed that this effect was due to recruitment at the gene promoter of CDK8 that associates with Med 12, Med13, and cyclin C to form a submodule that is a transcriptional negative regulator [10]. Also, TSA reduces C/EBPβ phosphorylation without affecting its binding to the *nos2* promoter [10]. In contrast, in IL-4-treated macrophages, TSA inhibited C/EBPβ binding to the *arginase 1* promoter [11]. This inhibitory effect is due to the acetylation on lysine residues 215–216, which are critical for DNA binding. These results demonstrate that the acetylation/deacetylation balance strongly influences the expression of genes involved in macrophage activation.

The pro- and anti-inflammatory activity of macrophages is tightly regulated. If excess pro-inflammatory activity occurs, then inflammation may become chronic. In contrast, under some pathological conditions, excess anti-inflammatory activation may lead to the development of fibrosis [12] (Fig. 10.3). Numerous cytokines derived from macrophages are key drivers of myofibroblast differentiation. These cells produce extracellular matrix (ECM) proteins, including collagen I. On the other hand, macrophages are crucial regulators of the processes driving fibrogenesis and can serve as a suppressor, degrading the fibrosis already established [13].

L-Arginine Transport During Macrophage Activation

In macrophages, L-arginine is a semi-essential amino acid required for the synthesis of proteins and the production of NO (classical activation) or polyamines and proline (alternative activation). Macrophages cannot synthesize L-arginine and therefore transporters are required to facilitate its uptake from the

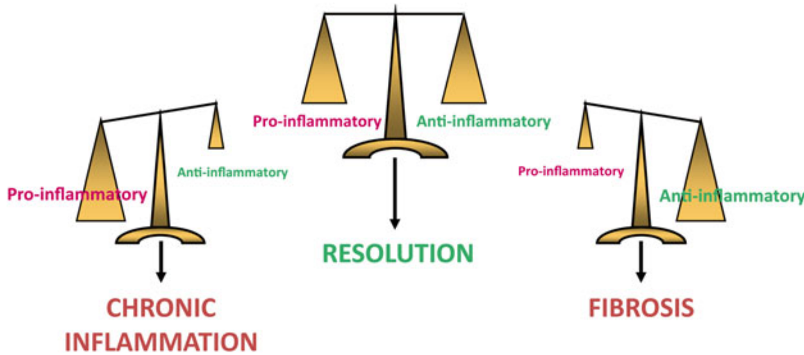

Fig. 10.3 Inflammation is a question of balances. Under physiological conditions, both pro- and anti-inflammatory activities follow one another in the inflammatory loci. If pro-inflammatory activity is enhanced, this may contribute to the chronicity of inflammation. In contrast, an excess of ant-inflammatory activity can cause increased fibrosis

extracellular milieu. In mammalian cells, the L-arginine flux through the plasma membrane is performed by four distinct mechanisms [14]: (1) system $B^{o,+}$, which is Na^+ and Cl^- dependent and used for neutral and cationic amino acids; (2) system $b^{o,+}$, which is Na^+ independent and handles both neutral and cationic amino acids; (3) system y^+, which interacts, either in the absence or in the presence of Na^+, with cationic amino acids and only very weakly (Km > 10 mM) with neutral amino acids; and (4) system y^+L, which handles cationic amino acids in a Na^+-independent fashion and in the presence of Na^+ also handles neutral amino acids. Various proteins account for the distinct transport activities. System $B^{o,+}$ is related to the $ATB^{o,+}$ transporter (gene *SLC6A14*); system $b^{o,+}$ is associated with the heteromeric amino acid transporter $b^{o,+}$-AT/rBAT (*SLC7A9* and *SLC3A1*, respectively); system y^+L is the product of the activity of the heteromeric amino acid transporters y^+LAT1/4F2hc and y^+LAT2/4F2hc, where the LAT1 and LAT2 systems refer to L-arginine transporters-1 (*SLC7A7*) and -2 (*SLC7A8*) while 4F2hc refers to *SLC3A2*; and system y^+ arises from the activity of cationic amino acid transporters (CAT)1–3 (*SLC7A1, SLC7A2*, *and SLC7A3*).

The activation of macrophages by pro- or anti-inflammatory stimuli leads to the induction of more than 400 genes. Consequently, to meet their metabolic demands, these cells take up exogenous L-arginine. This process is a key regulatory step for the normal behavior of macrophages during activation. To determine the L-arginine transport mechanism in these cells when they become activated, we used bone marrow-derived macrophages, which are non-transformed cells that respond to both pro- and anti-inflammatory stimuli. Incubation with IFN-γ and/or LPS led to the production of NO; however, no arginase activity was detected. In contrast, after incubation with IL-4, arginase activity was detected, but not NO production [15]. After testing the model, we sought to examine the L-arginine transport systems (y^+, B^{o+}, b^{o+}, and y^+L) used when macrophages become activated. Under basal conditions, little L-arginine uptake was detected in the cells, and y^+ activity was virtually absent. In contrast, the 4–5-fold increase in transport of this amino acid in the presence of Th1- or Th2-type cytokines was mediated by system y^+. Using quantitative polymerase chain reaction (PCR), we determined the expression of the genes involved in system y^+. *slc7A1* expression was low in untreated cells, and the expression was not modified by the treatments. In quiescent cells *slc7A2* was not detected but was induced under the treatment with both pro- and anti-inflammatory cytokines. Finally, *slc7A3* was not detected. Gene induction was not modified regardless of the presence or absence of L-arginine in the medium of the macrophage culture. All these data were confirmed by using macrophages from mice with disrupted *slc7A2*. In basal conditions, the amount of L-arginine uptake was similar in macrophages from control and *slc7A2* knockout (KO) mice; however, after activation, no increase in L-arginine transport in cells from the latter was observed. Although *nos2* was induced by pro-inflammatory stimuli in macrophages from *Slc7A2* KO mice, NO was not produced. Similarly, arginase 1 was induced in these cells through the effect of IL-4, but L-arginine was not catabolized. These results confirm that under both pro- and anti-inflammatory activation macrophages induce *slc7A2* expression, which is responsible for increased L-arginine transport (Fig. 10.4).

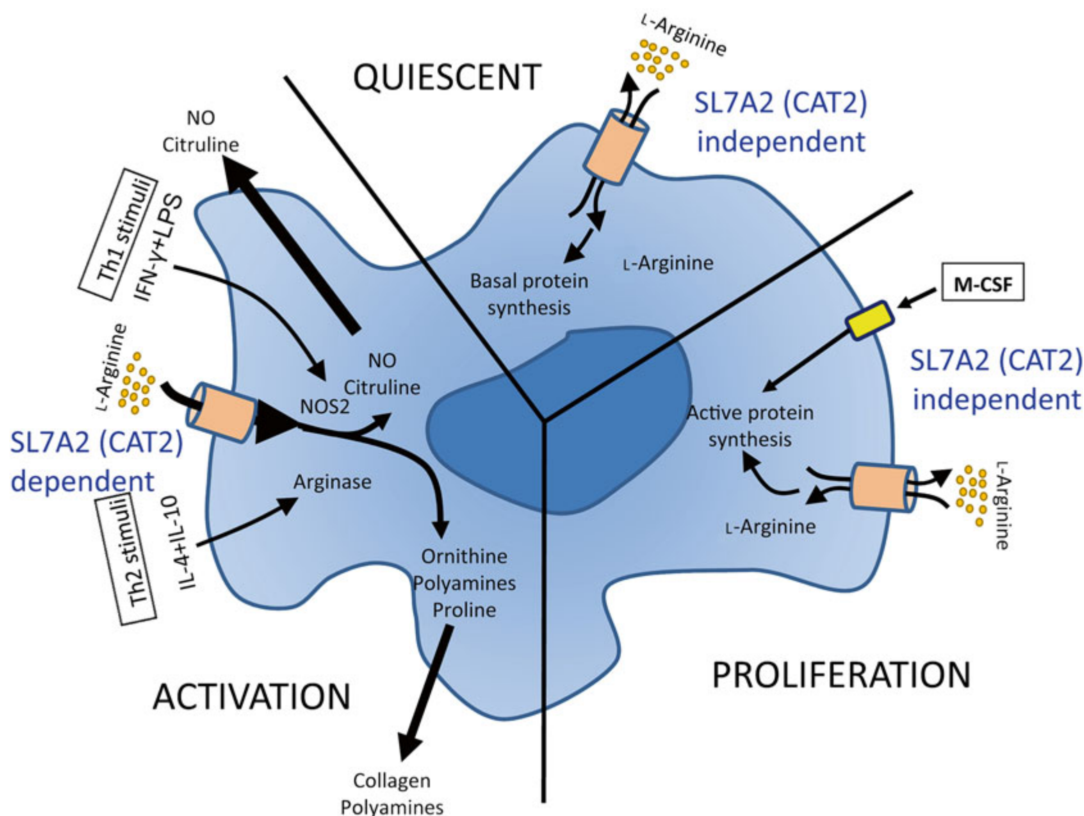

Fig. 10.4 L-Arginine transport in macrophages. During pro- or anti-inflammatory activation of these cells, the cationic amino acid transport SL7A2 (CAT2) is induced, allowing massive entry of L-arginine into the cell. During proliferation or when macrophages are quiescent, small amounts of L-arginine enter the cells by a SL7A2-independent system

Under the effect of several growth factors, macrophages proliferate. Among these factors, macrophage colony-stimulating factor (M-CSF) is the most powerful and the only one specific factor for this cell lineage. To meet their metabolic demands, macrophages require external L-arginine for proliferation and protein synthesis [16]. Interestingly, when macrophages become activated, proliferation is suppressed [17], thereby suggesting that these cells are able to exert only one activity or the other. Given these considerations, we addressed whether the behavior of L-arginine was the same under macrophage activation and proliferation. Treatment of the cells with M-CSF did not induce genes related to pro- (*nos2*) or anti-inflammatory (*arginase 1*) activation. We also found that when used during activation or proliferation, L-arginine was metabolized by distinct pathways. After incubation of activated macrophages with radiolabel L-arginine, most of the radioactivity was extracellular (in 6 h between 77 and 89 %) and was not bound to proteins. In contrast, in proliferating macrophages, around half of the radiolabeled material was extracellular (52 %) and half intracellular (47 %). Also, while the radioactivity outside the cells was not protein bound, that inside was. These observations suggest that during macrophage activation all the L-arginine in the media is depleted and catabolized, while during proliferation there is more L-arginine available than that required for protein synthesis and L-arginine not bound to protein is exchanged with that present in the media. Having identified these differences in L-arginine processing, we next tested the systems of L-arginine uptake during proliferation and activation. The latter process is associated with a drastic increase (4–5-fold) via y+ activity mediated by *slc7a2/cat2*. However, M-CSF induced only a modest increase in uptake (0.5-fold), independent of *slc7a2/cat2* and probably through *slc7a1/cat1*, which is constitutively expressed,

and this control is independent of proliferating or activating agents [16]. In fact, M-CSF-dependent proliferation was not modified when we used macrophages of *slc7a2* KO mice, again demonstrating differential uptake and metabolism of L-arginine when macrophages are treated with activating or proliferating agents (Fig. 10.5).

The granulocyte macrophage colony-stimulating factor (GM-CSF) induces modest macrophage proliferation but is a critical factor for differentiation to dendritic cells. GM-CSF also induces the expression of arginase 1, but to a lesser extent than anti-inflammatory cytokines such as IL-4. When macrophages are treated with GM-CSF, the transport activity increases through system y (>10-fold) [18]. The increase in L-arginine transport correlates with a rise in cationic amino acid transporter SL7A2, which was confirmed by using macrophages from the corresponding KO mice. Similar conclusions were obtained in *sl7a2* KO mice infected with *Leishmania amazonensis* [19].

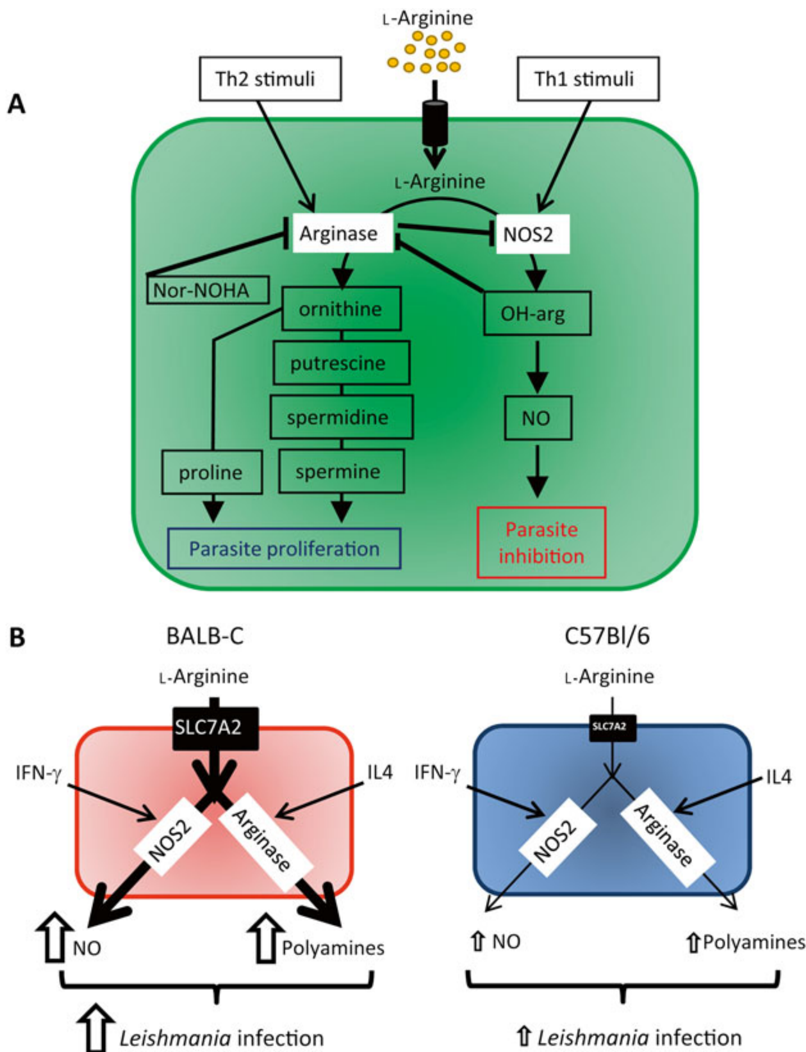

Fig. 10.5 L-Arginine metabolism in parasitic diseases. (**a**) L-Arginine is the substrate for both arginase and NOS2. Arginase is induced by Th2 cytokines and hydrolyzes L-arginine into ornithine and finally into polyamines, required for parasite growth. Th1 cytokines induce NOS2, which metabolizes L-arginine into NO, the metabolite responsible for killing the parasite. (**b**) In macrophages from BALB-C mice, the cationic amino acid transporter SLC7A2 (CAT2) allows the uptake of large amounts of L-arginine. In contrast, the reduced expression of this transporter in C57Bl/6 mice leads to lower amounts of L-arginine uptake by macrophages

L-Arginine, Macrophages, and Parasitic Diseases

Parasitic diseases affect millions of people. Host defense against many parasites requires Th**2** immunity (for example, the production of IL-**4** and IL-**10**). This immune response is necessary to kill or contain pathogens, as well as to repair the injuries caused during the cure of infection. However, an uncontrolled Th**2** response can cause tissue remodeling and fibrosis, particularly in organs like the lungs [20]. Many of the parasitic diseases associated with type **2** cytokines are characterized by the development of granulomas and fibrotic pathology, which are at the root of the disease. However, under experimental switching to a type **1** reaction, the pathologic response to parasites is reduced, thus demonstrating that differential activation of pro- or anti-inflammatory pathways are a critical determinant in the resolution of granulomas [21].

In humans, *Leishmania* produce lesions ranging from cutaneous to mucocutaneous and also cause visceral disease. The susceptibility of the murine model to *Leishmania* (*L.*) *major* differs depending on the mouse strain. Most strains develop small lesions that heal spontaneously and have been associated with the production of Th1 cytokines (particularly IFN-γ). In contrast, in some strains (BALB/c) with an increased production of IL-4, IL-10, and IL-13, the lesions increase progressively. However, recent data question this simplistic model [22]. *Leishmania* are intracellular parasites that live mainly in macrophages. In these cells, *Leishmania* are either killed or reproduced through their expansion in the host. In this regard, the balance between NOS2 and arginase 1 may be determinant for the fate of the parasite. In a series of experiments, we found that the amount of arginase in the lesions of mice correlates with the number of parasites load and the severity of the disease [23]. The inhibition of arginase by nor-NOHA in vivo reduces the activity of the enzyme, the number of parasites in the lesion, and the size of the lesion. To understand the role of macrophage-derived arginase, we infected macrophages in vitro with *L. major*. As expected, when the cells were treated with IL-4, the number of viable parasites increased. But under the inhibition of arginase activity, the number decreased drastically. The growth of parasites recovered when we added ornithine. These experiments demonstrate that polyamine synthesis induced by macrophage-derived arginase is required for the growth of the parasite.

Locally, at the level of the lesions of experimental leishmaniasis, the depletion of L-arginine by macrophages induces the suppression of specific T cell responses [22]. This decrease in L-arginine in the extracellular milieu affects *L. major*-specific T cells, which become hyporesponsive and produce lower levels of IL-4 and IL-10 [24]. Finally, the role of arginase in leishmaniasis is highlighted because arginase concentration in blood is a marker of disease severity with visceral leishmaniasis in humans [25].

It has been postulated that parasite-encoded arginase plays an important role in macrophage control of intracellular *Leishmania*. In fact, parasite-derived arginase may compete for intracellular L-arginine, thus decreasing the amount available to be converted through NOS2 into NO, thus reducing the response of the immune system to the parasites [26]. Using arginase-deficient *L. major*, it has been shown that the number of parasites during infection depends on parasite-derived arginase and that pathogenesis of the infection is cytokine independent [27]. Interestingly, the resistant C57BL/6 mice infected with arginase-deficient *L. major* take longer to resolve the disease. This delay is associated with an increase in programmed cell death-1 (PD-1) expression on $CD4^+$ T cells, which impairs proliferation and IFN-γ production. These observations suggest that parasite-derived arginase contributes to the immune response against the parasite [28].

In another group of experiments, we postulated that, if the differences in susceptibility to *L. major* between C57Bl/6 (non-healer strain) and BALB-C (healer strain) mice were due only to the production of Th1 or Th2 cytokines, then bone marrow-derived macrophages from both strains would be equally infected in the presence of IL-4. However, this was not the case, and macrophages from BALB-C were more infected than those from C57Bl/6 mice [29]. However, the macrophages of both

mouse strains showed comparable amounts of *nos2* and *arginase 1* after treatment with IFN-γ or IL-4. However, the production of NO or proline or polyamines was increased in macrophages from BALB-C animals. The observation that the levels of enzymes activity in the two types of macrophage were the same but the products of the reaction different prompted us to consider that the amount of L-arginine available inside the cells differed. Macrophages from BALB-C animals showed a marked increase in L-arginine uptake in relation to those from C57Bl/6A mice. This finding was attributed to a decreased induction of the L-arginine transporter *slc7a2* by pro- and anti-inflammatory cytokines. The decreased induction occurred at the transcriptional level and was caused by a deletion in the promoter of one of the 4 AGGG repeats of C57Bl/6 mice [29]. These results indicate not only the contribution of L-arginine to susceptibility to *Leishmania* infection but also the critical role of the L-arginine transport system. The latter may offer a therapeutic target through which to control macrophage activation.

The effect of L-arginine on parasites is not limited to *Leishmania*. Alternative activation of macrophages plays a critical role in helminth infection. The nematode *Brugia malayi* implanted in the peritoneal cavity of mice recruits macrophages expressing an anti-inflammatory phenotype, suppressing the proliferation of T cells upon antigen-specific stimulation [30]. Helminth infection leads to the mobilization of alternatively activated macrophages [31]. In summary, L-arginine uptake by activated anti-inflammatory macrophages facilitates the growth of parasites by providing polyamines, by inhibiting NO production, or by blocking T cell responses.

Arginine, Macrophages, and Cancer

Tumors are associated with macrophages that have been termed tumor-associated macrophages (TAMs). The presence of these cells is linked to poor prognosis, and they are involved in the development of metastasis [32]. In this regard, macrophages may contribute to tissue remodeling, allowing tumor growth through participation in wound healing and promotion of angiogenesis, as well as the induction of metastasis [33]. In recent years, TAMs have been claimed to exert suppressive activity, and the term "myeloid-derived suppressor cells" (MDSCs), which also includes neutrophils, has been widely used. Suppressor cells constitute a heterogeneous population that has not yet been clearly defined. The anarchic growth of tumor cells produces changes in the microenvironment, inducing hypoxia and inflammation, the latter attracting monocytes from the circulation through chemokines, such as CC-chemokine ligand 2 (CCL2) and CCL5. TAMs can be converted into MDSCs through several products released by tumor cells, including cytokines, chemokines, and other molecules such as tumor necrosis factor (TNF) and prostaglandin E2 (PGE2) [34]. In fact, TAMs develop the phenotype of activated anti-inflammatory macrophages upon the suppression of MHC class II molecules and IL-12 and also upon the expression of the anti-inflammatory cytokine IL-10. The lack of IL-12 production blocks the activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) and the induction of Th1 cells, thus producing an anti-inflammatory environment. Also, the production of CCL22 by TAMs attracts regulatory T (TReg) cells, which block T cell activation. TAMs may also affect activated T cells through the expression of PD1 ligand 1 (PDL1), which may induce apoptosis of T cells by the interaction with its receptor-programmed cell death protein 1 (PD1).

Most studies report that MDSCs have the capacity to inhibit antigen-specific T cell responses [35]. MDSCs extracted from tumors are able to block T cells activated by several systems. This finding has been attributed to the production of ROS and reactive nitrogen species by MDSCs [36]. One of these products, peroxynitrite ($ONOO^-$) oxidizes several molecules, producing cellular damage. Against this theory is the observation that MDSCs have an anti-inflammatory phenotype. Consequently, they do not express NOS2 and thus ROS cannot be induced.

However, another possible explanation for the inhibition of T cell responses may be related to the lack of L-arginine. In the microenvironment of the tumor, L-arginine is depleted as a result of arginase activity in macrophages [37]. Activated macrophages express the cationic amino acid transporter

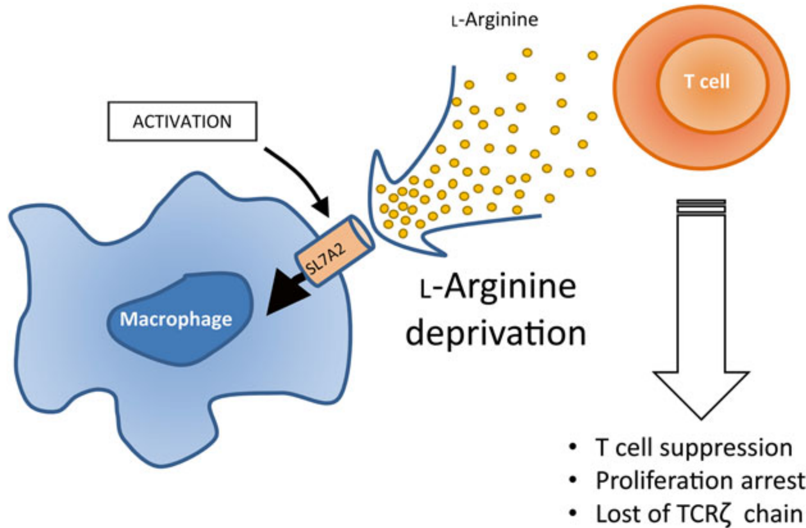

Fig. 10.6 Mechanisms of MDSC-dependent inhibition of T cell activation and proliferation. Activated macrophages induce a massive uptake of L-arginine, which in turn can lead to depletion of this amino acid in the microenvironment, thus affecting the proliferation and function of T cells

SL7A2 and thus the uptake of L-arginine increases enormously, depleting the microenvironment of this amino acid (Fig. 10.6). L-Arginine is required for the growth of activated T cells. In the absence of this molecule, T cells are arrested in the G0–G1 phase of the cell cycle and are unable to upregulate cyclin D3 and cdk4 expression. By contrast, T cells in the presence of L-arginine progress to S and G2-M phases [38]. These data are supported by experiments where arginase was inhibited using Nor-NOHA. Under these conditions, the loss of T cell function is prevented, resulting in an immune-mediated antitumor response, which inhibits tumor growth in a dose-dependent manner of the amount of inhibitor used [39]. Interestingly, STAT6 ($^{-/-}$) mice, which do not express arginase after IL-4 treatment, immunologically reject spontaneous metastatic mammary carcinoma, while STAT6-competent BALB/c mice succumb to metastatic disease [40]. In humans, CD8+ T cell antigen-specific cytotoxicity is not modified when arginase is absent, but antigen-specific proliferation is severely compromised [41].

Conclusions

Macrophages play a critical role during inflammation. The mechanism by which L-arginine is processed during the pro- or anti-inflammatory phases are hallmarks of macrophage activation. While tissue destruction is associated with NO production, tissue repair is characterized by the production of polyamines and proline. Macrophages cannot synthesize L-arginine and thus take it up by a specific system of transport. During both pro- and anti-inflammatory activation, macrophages take up huge amounts of L-arginine. This is attributed to expression of the cationic amino acid transporter SL7A2. Given that this transport system is the limiting factor for macrophage activation via pro- and anti-inflammatory pathway, it may offer a therapeutic target for the control of macrophage activation.

There is a strong correlation between L-arginine, macrophages, and two pathological situations, namely parasitic diseases and cancer. Depending how L-arginine is catabolized by macrophages, the products of these cells can destroy parasites (NO) or alternatively induce their growth (polyamines). In cancer, as in the case of parasites, the massive uptake of L-arginine by macrophages produces a

depletion of L-arginine in the microenvironment, thus affecting the proliferation and function of T cells.

References

1. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages and dendritic cells. Science. 2010;327:656–61.
2. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol. 2011;11:723–37.
3. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol. 2005;6:1191–7.
4. Couzin-Frankel J. Inflammation bares a dark side. Science. 2010;330:1621.
5. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol. 2011;11:750–61.
6. Valledor AF, Comalada M, Santamaría-Babi LF, Lloberas J, Celada A. Macrophage proinflammatory activation and deactivation: a question of balance. Adv Immunol. 2010;108:1–20.
7. Van den Bossche J, Lamers WH, Koehler ES, et al. Pivotal advance: arginase-1-independent polyamine production stimulates the expression of IL-4-induced alternatively activated macrophage markers while inhibiting LPS-induced expression of inflammatory genes. J Leukoc Biol. 2012;91:685–99.
8. Modolell M, Corraliza IM, Link F, Soler G, Eichmann K. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur J Immunol. 1995;25:1101–4.
9. Lee J, Ryu H, Ferrante RJ, Morris Jr SM, Ratan RR. Translational control of inducible nitric oxide synthase expression by L-arginine can explain the L-arginine paradox. Proc Natl Acad Sci U S A. 2003;100:4843–8.
10. Serrat N, Sebastian C, Pereira-Lopes S, Valverde-Estrella L, Lloberas J, Celada A. The response of secondary genes to lipopolysaccharides in macrophages depends on histone deacetylase and phosphorylation of C/EBPβ. J Immunol. 2014;192:418–26.
11. Serrat N, Pereira-Lopes S, Comalada M, Lloberas J, Celada A. Deacetylation of C/EBPβ is required for IL-4-induced arginase-1 expression in murine macrophages. Eur J Immunol. 2012;42:3028–37.
12. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009;158:638–51.
13. Duffield JS, Lupher M, Thannickal VJ, Wynn TA. Host responses in tissue repair and fibrosis. Annu Rev Pathol. 2013;8:241–76.
14. Fotiadis D, Kanai Y, Palacín M. The SLC3 and SLC7 families of amino acid transporters. Mol Aspects Med. 2013;34:139–58.
15. Yeramian A, Martin L, Serrat N, et al. L-Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. J Immunol. 2006;176:5918–24.
16. Yeramian A, Martin L, Arpa L, et al. Macrophages require distinct L-arginine catabolism and transport systems for proliferation and for activation. Eur J Immunol. 2006;36:1516–26.
17. Xaus J, Cardó M, Valledor AF, Soler C, Lloberas J, Celada A. Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. Immunity. 1999;11:103–13.
18. Martin L, Comalada M, Marti L, et al. Granulocyte-macrophage colony-stimulating factor increases l -arginine transport through the induction of CAT2 in bone marrow-derived macrophages. Am J Physiol Cell Physiol. 2006;290:C1364–72.
19. Wanasen N, MacLeod CL, Ellies LG, Soong L. L-arginine and cationic amino acid transporter 2B regulate growth and survival of Leishmania amazonensis amastigotes in macrophages. Infect Immun. 2007;75:2802–10.
20. Wilson MS, Wynn TA. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol. 2009;2:103–21.
21. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol. 2001;167:6533–44.
22. Modolell M, Choi BS, Ryan RO, et al. Local suppression of T cell responses by arginase-induced L-arginine depletion in nonhealing leishmaniasis. PLoS Negl Trop Dis. 2009;3:e480.
23. Kropf P, Fuentes JM, Fähnrich E, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. FASEB J. 2005;19:1000–2.

24. Munder M, Choi BS, Rogers M, Kropf P. L-arginine deprivation impairs Leishmania major-specific T-cell responses. Eur J Immunol. 2009;39:2161–72.
25. Abebe T, Takele Y, Weldegebreal T, et al. Arginase activity—a marker of disease status in patients with visceral leishmaniasis in Ethiopia. PLoS Negl Trop Dis. 2013;7:e2134.
26. Gaur U, Roberts SC, Dalvi RP, Corraliza I, Ullman B, Wilson ME. An effect of parasite-encoded arginase on the outcome of murine cutaneous leishmaniasis. J Immunol. 2007;179:8446–53.
27. Muleme HM, Reguera RM, Berard A, et al. Infection with arginase-deficient Leishmania major reveals a parasite number-dependent and cytokine-independent regulation of host cellular arginase activity and disease pathogenesis. J Immunol. 2009;183:8068–76.
28. Mou Z, Muleme HM, Liu D, et al. Parasite-derived arginase influences secondary anti-leishmania immunity by regulating programmed cell death-1-mediated $CD4^+$ T cell exhaustion. J Immunol. 2013;190:3380–9.
29. Sans-Fons MG, Yeramian A, Pereira-Lopes S, et al. L-Arginine transport is impaired in C57Bl/6 mouse macrophages as a result of a deletion in the promoter of Slc7a2 (CAT2), and susceptibility to Leishmania infection is reduced. J Infect Dis. 2013;207:1684–93.
30. Loke P, MacDonald AS, Robb A, Maizels RM, Allen JE. Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. Eur J Immunol. 2000;30:2669–78.
31. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminthes. Nat Rev Immunol. 2011;11:375–88.
32. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141:39–51.
33. Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol. 2009;9:259–70.
34. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12:253–68.
35. Movahedi K, Guilliams M, Van den Bossche J, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood. 2008;111:4233–44.
36. Lu T, Gabrilovich DI. Molecular pathways: tumor-infiltrating myeloid cells and reactive oxygen species in regulation of tumor microenvironment. Clin Cancer Res. 2012;18:4877–82.
37. Currie GA, Gyure L, Cifuentes L. Microenvironmental L-arginine depletion by macrophages in vivo. Br J Cancer. 1979;39:613–20.
38. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood. 2007;109:1568–73.
39. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64:5839–49.
40. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. J Immunol. 2005;174:636–45.
41. Munder M, Engelhardt M, Knies D, et al. Cytotoxicity of tumor antigen specific human T cells is unimpaired by L-arginine depletion. PLoS One. 2013;8:e63521.

Chapter 11
L-Arginine and TNFα Production in Macrophages: A Focus on Metabolism, Aging, Metabolic Syndrome, and Type 2 Diabetes

Charlotte Breuillard, Christophe Moinard, and Marie-Chantal Farges

Key Points

- In macrophages, L-arginine (Arg) may be synthesized from citrulline (Cit) via argininosuccinate synthase (ASS) and lyase (ASL). It is catabolized mainly via nitric oxide synthase (NOS) and arginase pathways.
- Macrophage polarization is associated with an orientation of Arg catabolism: macrophages with an M1 phenotype, characterized by a pro-inflammatory activity, present an overexpression of inducible NOS (iNOS) when macrophages with an M2 phenotype, which overexpress arginase, have anti-inflammatory properties.
- L-Arg is critical for macrophage cytokine response: it facilitates the activation of mitogen-activated protein kinase (MAPK) and consequently the production of tumor necrosis factor α (TNFα).
- Aging is associated with a depressed macrophage-mediated immune response.
- The "inflammaging state" associated with the age may be responsible for the macrophage "anergy."
- Arg downregulates TNFα production in peritoneal macrophages (PM) from type 2 diabetes (T2D).

Keywords Nitric oxide • Nitric oxide synthase • Arginases • Aging • Metabolic syndrome • Type 2 diabetes

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Abbreviations

ADC	L-Arginine decarboxylase
AP-1	Activator protein 1
Arg	L-Arginine
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
C/EBPβ	CAAT box enhancer binding protein β
CAT	Cationic amino acid transporter
Cit	Citrulline
DFMO	Difluoromethylornithine
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	Janus kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK-ERK	Mitogen-activated extracellular signal-regulated kinases—extracellular signal-regulated kinases
MHC	Major histocompatibility complex
MS	Metabolic syndrome
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet -}$	Superoxide anion
ODC	Ornithine decarboxylase
$ONOO^-$	Peroxynitrite
Orn	Ornithine
PM	Peritoneal macrophages
PMN	Polymorphonuclear neutrophil
PP2A	Protein phosphatase 2A
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes
TGFβ	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TPL-2	Tumor-promoting locus 2
ZDF	Zucker diabetic fatty

Introduction

L-Arginine (Arg) has long been known to be a major regulator of immunity via its metabolic and physiological functions. In the 1950s, it was classified as a non-essential amino acid by [1] Rose since L-Arg could be synthesized at the whole body level, mainly in the kidneys, after the conversion of intestinal citrulline (Cit) via argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL).

But things changed when Barbul et al. [2] observed, surprisingly, that Arg was an immunomodulator via a thymic effect. At the same time, they observed that Arg could be essential in several situations, like growth or sepsis. Finally in the 1990s, Albina et al. [3] demonstrated that macrophages were able to produce nitric oxide (NO). These pioneering observations opened a new field of research focused on the regulation of macrophage functions by Arg.

L-Arginine Metabolism in Macrophages

Macrophages

The monocyte/macrophage system is an essential cellular part in the innate immune response. Circulating monocytes are precursors of tissue macrophages. Whatever their tissue residence, macrophages are phagocytic cells and in this respect they clear invading pathogens such as bacteria, viruses, and transformed cells through oxygen-dependent and independent mechanisms. In addition, macrophages are potent cytokine producers and play a crucial role in a variety of processes ranging from antigen presentation to wound healing. Each of these biological properties is enhanced when macrophages are activated in response to various stimuli exposure, i.e., cytokines and/or pathogens.

In response to exogenous (i.e., lipopolysaccharide (LPS), pathogenic nucleic acids) or endogenous (e.g., oxidized lipids and proteins, heat shock proteins, and tumor necrosis factor alpha (TNFα) stimulation, toll-like receptors (TLR) are activated, leading to the immediate stimulation of the MyD88 (myeloid differentiation factor 88)-dependent pathway. This pathway is responsible for the stimulation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, resulting in activation of the two most important pro-inflammatory pathway transcription factors NF-κB and AP-1 (Activator protein 1). This leads to an enhanced expression of pro-inflammatory genes including cytokines (TNFα, interleukins (IL-1, IL-6) …) and inducible nitric oxyde synthase (iNOS) [4].

Activation of pro-inflammatory cytokine production, including TNFα, by TLR also occurs via activation of tumor-promoting locus-2 (TPL-2), a serine/threonine kinase which activates mitogen-activated extracellular signal-regulated protein kinases-extracellular signal-regulated kinase (MEK-ERK) pathway [5].

TNFα is a glycoprotein involved in systemic inflammation. On the site of inflammation, it acts on macrophages, inducing phagocytosis and cytokine, oxidant, and pro-inflammatory lipid production. TNFα is a powerfull proinflammatory agent and plays a central role in the initiation, perpertuation and also resolution of inflammation. [6].

L-Arginine Transporters in Macrophages

In peritoneal macrophages (PM), it seems that Arg transport is mediated mainly by cationic amino acid transporter (CAT)-2 (Fig. 11.1). Because Arg requirements increase in case of macrophage activation to induce the production of NO and polyamines, Arg transport is induced in stimulated macrophages. For more information regarding Arg transport, see Chap. 10 by J. Lloberas et al.

Moreover, it is important to note that macrophages are able to take up Cit, the direct Arg precursor. For Cit transport, two transport systems seem to coexist. The first one is a saturable system for neutral amino acid transport, and the second one is a competitive transport with Arg [7].

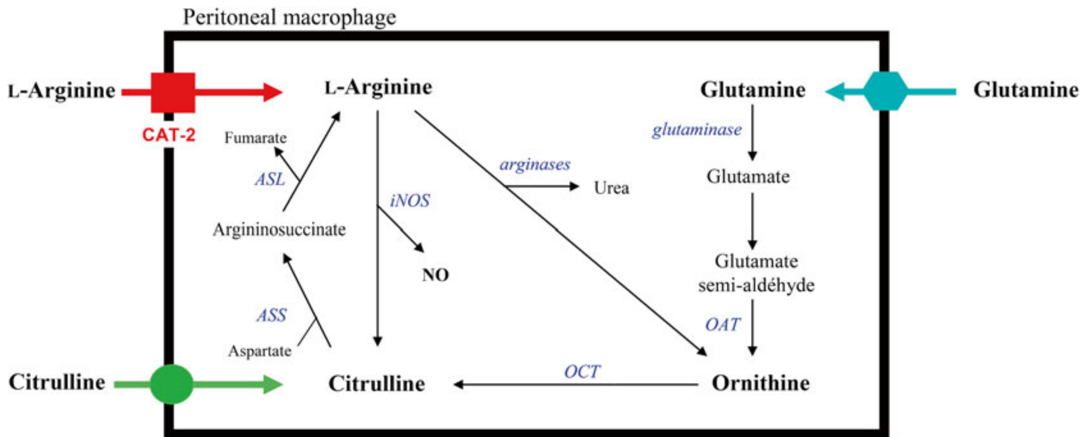

Fig. 11.1 L-Arginine metabolism in peritoneal macrophages. *CAT-2* cationic amino acid transporter -2, *iNOS* inducible nitric oxide synthase, *NO* nitric oxide, *ASS* argininosuccinate synthase, *ASL* argininosuccinate lyase, *OAT* ornithine aminotransferase, *OCT* ornithine carbamoyltransferase

L-Arginine Metabolism in Macrophages

L-Arginine Synthesis

Macrophages are able to produce Arg themselves by two distinct pathways (Fig. 11.1). As mentioned before, these cells are able to take up Cit and metabolize the latter into Arg via ASS and ASL. The importance of this metabolic pathway was underlined by Murphy and Newsholme [8] since these authors observed that 20 % of the Arg used for NO synthesis derived from Cit.

Moreover, they [8] have demonstrated the existence of Arg synthesis from glutamine in macrophages. Hence, glutaminase activity is increased in macrophage activation. This hypothesis is supported by results obtained using 6-diazo-5-oxo-norleucine, a glutaminase inhibitor, which downregulates NO production.

L-Arginine Catabolism

In macrophages, Arg is mainly metabolized by two enzymatic systems: NOS and arginases (Fig. 11.1). The first one, NOS (EC.1.14.13.39), converts Arg into NO and Cit. NOS activity is regulated by Arg availability and therefore by the other enzymes metabolizing Arg and Arg transport [9]. Among the three NOS isoforms, only iNOS, the inducible form, also called NOS-2, is present in PM, and its gene is not expressed at the basal state. As classically described, iNOS is regulated at its expression level by transcription and post-transcription mechanisms. Its expression is induced by all the factors that can activate transcription factor NF-κB and is associated with an increased Arg transport [10]. iNOS activity begins from 20 to 40 min after the stimulus, increases to reach a plateau between 4 and 8 h, and decreases within 24 h with enzyme degradation [11]. This isoform produces high NO quantities, continuously, up to several millimoles per hour for several days to fight against pathogen agents.

NOS metabolize Arg into Cit and NO. Cit is, as previously stated, an Arg precursor and accounts for 20 % of NO production. NO is a reactive nitrogen species which plays an important role in immunity. Produced in large quantities, it binds to superoxide anion ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$)

which gives it its cytotoxic properties [12]. NO is an essential factor to fight against viruses, bacteria, fungi, and parasites [13]. It acts in two different ways: directly, by itself or in association with $O_2^{\bullet-}$ to form $ONOO^-$, or indirectly to activate immune cells like polymorphonuclear neutrophils (PMNs), lymphocytes, and macrophages. Indeed, NO plays an important role in macrophage metabolism: $ONOO^-$ induces pro-inflammatory cytokine production, notably TNFα and IL-6, by a NF-κB pathway-dependent mechanism [14].

Arginases (EC 3.5.3.1) lead to Arg hydrolysis into ornithine (Orn) and urea. Arginases are negative regulators of NO production, because in metabolizing Arg, they reduce intracellular Arg availability and therefore its availability for iNOS [9]. There are two isoforms: arginase I and arginase II. The first one is a cytosolic enzyme mainly present in periportal hepatocytes, but also in immune cells such as macrophages. Arginase I overexpression occurs in the late phase of inflammation. It leads to a decrease of iNOS activity, which depends on Arg availability in order to avoid NO overproduction [15]. Its activity is inhibited by *N*-hydroxy-L-arginine, an intermediate of NO synthesis [16].

Arginase I also leads to the formation of polyamines from Arg, via Orn synthesis. Polyamines are biogenic amines that are involved in the immune response (both in the differentiation of immune cells and in the regulation of the inflammatory reaction). Their role in macrophages is confirmed since the presence of di-fluoromethylornithine (DFMO) in the medium culture, an inhibitor of the Orn decarboxylase (ODC), the limiting enzyme in polyamines synthesis, reduces the macrophage phagocytic capacity [17]. Moreover, the activation of macrophages by LPS results in the activation of ODC. This increase is maximum 4 h after stimulation and ODC activity returns to baseline after 8 h.

Arginase II is a mitochondrial enzyme whose expression could be induced by inflammatory stimuli in a very short time, in order to activate wound healing rapidly [18].

Arg is also metabolized in macrophages, to a lesser extent, by Arg decarboxylase (ADC), a mitochondrial enzyme which metabolizes Arg into agmatine. The latter is a competitive inhibitor of NOS and could be an endogenous regulator of NO synthesis [19]. However, it seems unlikely that the concentrations reached in vivo should be important enough to inhibit NO production, since agmatine is also an inhibitor of ADC [20]. Moreover, LPS causes a reduction in agmatine concentration through a decrease of ADC activity and an increase of agmatinase which hydrolyzes agmatine into urea and putrescine [21]. Agmatinase represents an alternate pathway for polyamines synthesis.

iNOS/Arginase Balance in Macrophages

Arg metabolism has a pivotal role in the activity of macrophages (Fig. 11.2). Indeed, macrophage activation by cytokines or pathogens induces a series of functional and structural modifications, called polarization, which leads to two populations of macrophages: M1 and M2. In response to T helper (Th) 1 cytokine activation, i.e., interferon (IFN)-γ or LPS, macrophages become M1, while in response to Th2 cytokine stimulation, i.e., IL-4, IL-10, or IL-13, they acquire the M2 phenotype. The M1/M2 phenotypes represent the two major and opposing activities of macrophages. One of the mechanisms involved in macrophage polarization occurs via the metabolic pathways of Arg, through iNOS (M1) or arginase I (M2). Classical activated M1 macrophages act during the early stages of inflammation, and, via the production of NO and other oxygen and nitrogen species, they kill pathogens or nearby host cells and produce pro-inflammatory cytokines (TNF-α, IL-6, and IL-12). M2 macrophages act in the late resolution phase of inflammation, regulate inflammatory response and adaptive Th1 immunity, clean debris with scavenger receptors, and repair tissues through the control of wound healing. These events are associated with an overexpression of arginase I [23].

Products of Th1 and Th2 responses also downregulate M2 and M1 activity, respectively. Macrophages that shift from an M1 to an M2 phenotype experience an increase in arginase expression that is concomitant with a reduction in iNOS expression [24]. The downregulation of iNOS

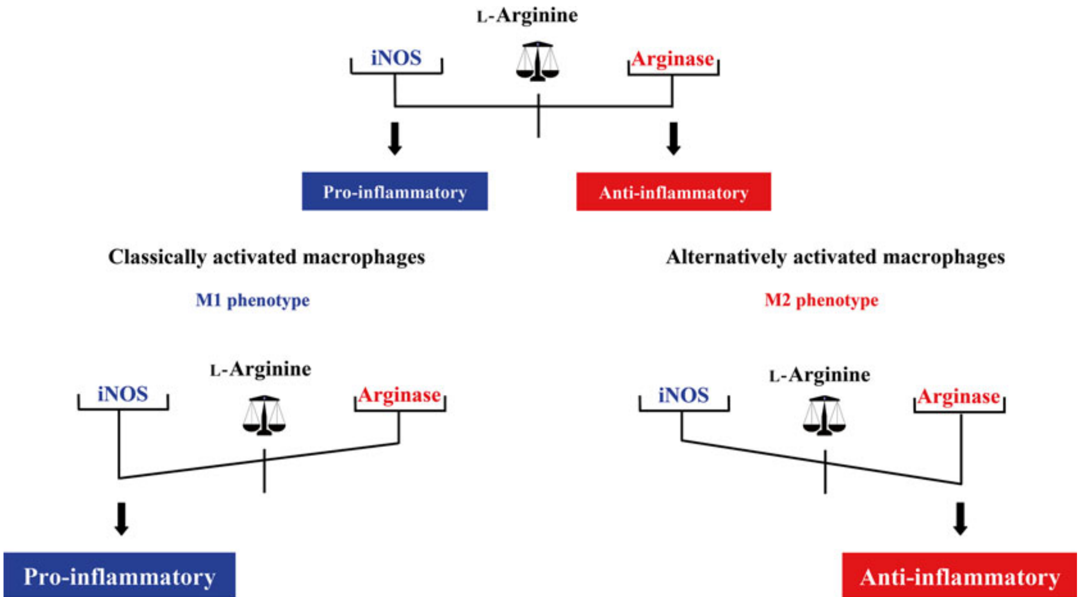

Fig. 11.2 iNOS/arginase imbalance. *iNOS* inducible nitric oxide synthase. Adapted from [22]

expression and the upregulation of arginase expression reflect a major shift in the metabolism of Arg. In wound healing, iNOS and arginase are induced in a time-coordinated manner [15]. While a high iNOS expression is associated with the initiation of the inflammatory phase, arginase expression comes later and is of prime importance in wound healing. The conversion of Arg into urea and Orn, and then into polyamines, initiates the repair phase of the inflammatory response. Such a shift is observed in dystrophin-deficient muscles, at the site of inflammation, while M1 macrophages promote muscle injury, M2 macrophages are involved in the repair of muscles [25].

Influence of L-Arginine and Its Metabolites on TNFα Production

Arg is an essential substrate for macrophage cytotoxicity and in particular for NO and cytokine production. Indeed, the activation of macrophages by LPS or IFN-γ results in an increase in the Arg influx in macrophages and a raised production of NO. In an in vitro study, an increase of Arg concentration (80–1000 μM) in the incubation medium is associated with an enhanced production of NO and cytotoxicity of murine PM, in a dose-dependent manner [26]. The capacity of Arg to modulate TNFα production has been observed in vivo. For example, in a model of burned rat, an Arg-enriched diet (1.85 g/kg/day) induces a decrease in the mRNA expression of pro-inflammatory cytokines (TNFα, IL-1, and IL-6) in different organs (spleen, thymus, lungs, and liver) and increased survival after stress [27]. In the same line, in endotoxemic rats, Arg supplementation (300 mg/kg administered intraperitoneally) reduces the production of cytokines from macrophages [28]. Finally, such an effect could also be observed in humans. For instance, Mendez et al. administered an Arg-enriched diet (25 g/day) to trauma patients. They evaluated the immune function after 1, 6, and 10 days of supplementation. It appears that Arg supplementation modulated TNFα production and prostaglandin E2 [29].

The effects of Arg on TNFα production could be due to direct effects of this amino acid on pro-inflammatory pathways, but also via its metabolite, NO.

Direct Effects

Arg seems to have a direct effect on immunity via the inhibition of activation of inflammatory factors' production inhibitors (Fig. 11.3). As previously described, the presence of microorganisms in the body is detected by TLR of innate immunity cells that activate NF-κB and MAPK pathways and, therefore, pro-inflammatory factors' expression, particularly via TPL-2. Mieulet et al. [30] showed that Arg is required to inhibit protein phosphatase 2 A (PP2A), an enzyme which itself inhibits the inactivating phosphorylation of TPL-2. This enables the activation of the MEK-ERK pathway and subsequent cytokine production, including TNFα.

Indirect Effects via Nitric Oxide

Arg seems also to regulate TNFα production via its metabolite, NO. Indeed, in the study on endotoxemic rats described previously, the authors suggest that the effects of Arg supplementation on cytokine production from macrophages are NO dependent [28]. Indeed, Arg would increase NO production by macrophages, which, by negative feedback, would decrease cytokine production. This effect could be related to a pretranscriptional effect by NF-κB modulation.

Aging

The age-related impairment of the immune function, i.e., immunosenescence, primarily affects the adaptive immunity, but it also impacts innate immunity [31].

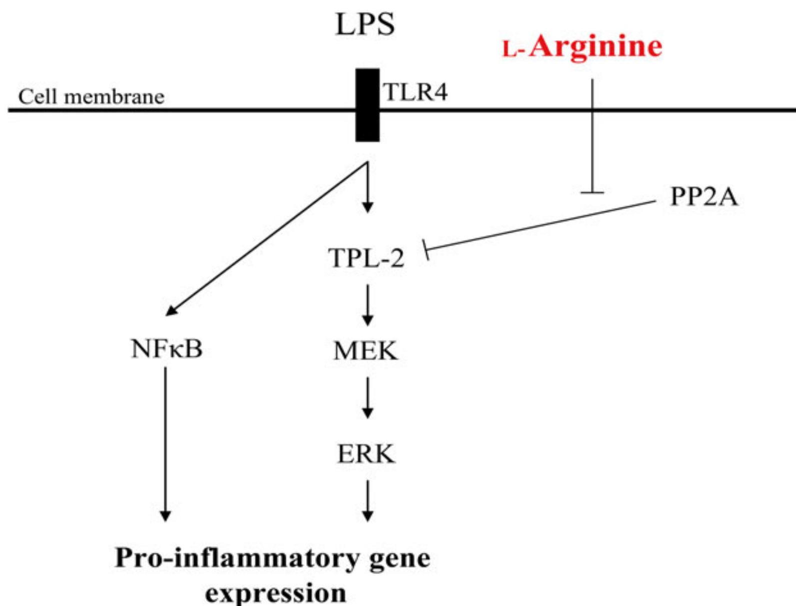

Fig. 11.3 Direct effects of L-arginine on pro-inflammatory gene expression. *LPS* lipopolysaccharide, *TLR4* toll-like receptor 4, *PP2A* protein phosphatase 2 A, *TPL-2* tumor-promoting locus 2, *MEK* mitogen-activated extracellular signal-regulated kinases, *ERK* extracellular signal-regulated kinases, *NF-κB* nuclear factor-κB. Adapted from [30]

Numerous studies have explored the effects of age on macrophage number and function; however, these have yielded conflicting sometimes opposing results. While a majority of the investigative, sometimes opposing, teams have reported declines in macrophage function with age, a substantial number of studies have demonstrated quite the opposite [32].

There is no clear evidence of an age-dependent effect on the generation of macrophages from their monocyte precursors [33]. Aging is associated with an increase in PM [34] and a decrease in Kupffer cells [35]. Quantitative and qualitative changes in adipose tissue macrophages with aging are illustrated by a decline in resident M2 macrophages and an increase in M1-infiltrated macrophages [36].

All these changes are accompanied by functional changes (Table 11.1). In healthy elderly subjects phagocytosis, free radical production, antigen presentation via major histocompatibility (MHC) class II molecules, prostaglandin E2, and pro-inflammatory cytokine production are altered [32]. In rodents, age effects on macrophage functions vary depending on the tissue site (alveolus, peritoneum, spleen, bone marrow) and maturity of the cell donors (naive or elicited) as well as on the nature of stimulant (IL-4, IFN-γ, or LPS) [37, 38]. In humans, if circulating levels of pro-inflammatory cytokines are elevated with age, the ex vivo macrophage cytokine production increases or decreases depending on the stimuli and the macrophage subpopulation studied. For example, upon TLR1/2 stimulation, both IL-6 and TNFα intracellular levels are reduced [39, 40]. In spite of this, pro-inflammatory cytokine production following stimulation of TLR2/6, TLR4, and TLR5 is preserved at an advanced age [40]. In contrast, basal and LPS-induced TNFα production through TLR4 by human monocytes increases with age [41].

Among the mechanisms involved in the age-dependent macrophage activation, a discrepancy regarding the level of TLR expression appears. In rodents, mRNA levels of TLR4 decrease with age [42], while TLR4 cell-surface expression is unaltered [43]. In humans, as compared to those of young adults, TLR1, TLR2, and TLR4 surface expressions on monocytes of older subjects are lower, unchanged, or slightly decreased, respectively [40]. Other defects particularly in TLR-mediated signal transduction, such as p38 and janus kinase (JNK) and MAPK, are involved in the differences between levels of macrophage cytokine production [43].

However, during the aging process, the apparent paradox of low cytokine production by macrophages and high plasma cytokine levels may be explained by an increased life span of macrophages and their constant activation by various stimuli and/or by an enhanced production of inflammatory mediators by other cells. Indeed, in response to TLR ligands, lymphocytes, and dendritic cells, macrophage-derived cells in peripheral tissues and the central nervous system, adipocytes, and myeloid-derived suppressor cells produce pro-inflammatory mediators [44, 45]. So, this constant stimulation could affect the phenotype, the functionality, and the activation properties of monocytes/macrophages and may be responsible for their "anergy." So the aging microenvironment, not really the monocytes/macrophages per se, plays a key role in defining the functionality and activation properties of macrophages in response to stimulation. This chronic low-grade inflammation, named "inflammaging" [46], plays a pivotal role in sarcopenia, frailty, and disability as it can be observed in a majority of age-related diseases, thus contributing to the elderly morbidity and mortality.

Table 11.1 Alterations of TNFα production by macrophages in aging

Tissue site	Ex vivo activation	Organism	Effect of aging	References
Alveolus	LPS	Mouse	↑	[37]
	LPS + IFN-γ	Mouse	↑	
Peritoneum	LPS	Mouse	↓	
	LPS + IFN-γ	Mouse	↓	
Spleen	LPS + IFN-γ	Mouse	↑	
Monocytes	TLR1/TLR2 ligand pam3cys + Brefeldin A	Human	↓	[39]

The symbols ↑ and ↓ indicate a statistically significant increase or decrease, respectively, with aging.

The literature comparing the effects of age on iNOS and arginase activity also leads to some discrepancies related to the experimental model, i.e., mouse lineage, age group studied, macrophage tissue site, and the nature of the stimulant. The stimulation of PM with LPS decreases NO production or increases the iNOS activity in old mice (72 weeks of age) compared to younger ones (8 weeks of age) [38, 47]. Bone marrow-derived macrophages obtained from both young and old mice were equally able to produce NO in response to IFN-γ, but old mice were more responsive to LPS [38]. Ex vivo macrophages from old mice (21 months) isolated from the alveoli and spleen produce more NO than their younger counterparts (12 and 2 months). Conversely, PM from old mice show the lowest NO production responses to stimuli than those of younger mice [37]. In old–compared to young–mice (72 *vs* 8 weeks) arginase I activity is lower in resident and elicited PM stimulated with LPS and in resident PM stimulated with IL-4 [38]. In an experimental model of Leishmaniasis, while the potential of bone marrow-derived classically activated macrophage to generate NO is preserved with age, old mice expressed a lower level of arginase I and a reduced arginase activity [48]. Since arginase and NOS use Arg as a substrate, the age-related low expression and activity of arginase I limit Orn and polyamines production and enhance NO synthesis, thereby reducing collagen formation and the proliferation of cells. Such a context might then result in delayed or ineffective wound healing and tissue regeneration but also in a protection against tumor formation in cells where mutations have already occurred or against parasite growth [48].

In the context of the decline of immune response with aging, some responses can be improved by targeting Arg metabolism. Arg supply at pharmacological doses is beneficial in conditions such as trauma, stress, burn, or injury; it improves immune functions and facilitates wound healing [49]. There is little evidence, however, that Arg is responsible for these positive effects since Arg is only one of the semi-essential amino acids and immune-enhancing diets contain other pharmacologically active components (e.g., omega 3 free fatty acids RNAs, antioxidant vitamins). Moreover, the experiments testing Arg supplementation alone failed to show clear immunologic effects [49]. Animal studies of splanchnic sequestration of amino acids in old rats show that age is associated with a reduced Arg hepatic uptake and utilization in the absence of an age-related effect on Arg portal flux [50].

Cit, which totally escapes the splanchnic sequestration, may be a potential candidate to supply Arg, as it could influence Arg availability and thus macrophage functions. In this way, we have recently evaluated the ability of a Cit-enriched diet to modulate macrophage functions in healthy aged rats. We have observed that Cit regulates macrophage behavior, with a lower NO production as compared to an isonitrogenous diet, but has no effects on TNFα production [51].

Metabolic Syndrome and Type 2 Diabetes

It is well known that metabolic syndrome (MS) and to a greater extent type 2 diabetes (T2D) are characterized by an immune dysfunction that leads to an increased susceptibility to infection and, in septic patients, high morbidity and mortality [52]. Various alterations in the immune defense system have been observed in animal models of insulin resistance, notably including disturbances of macrophage function, related to modifications of macrophage phenotype [53], hypoargininemia [54], or hyperglycemia [55].

Arg metabolism seems to be at the heart of macrophage dysfunction in MS and T2D. Indeed, the modifications of macrophage phenotype observed in case of T2D are characterized by Arg metabolism modifications. These macrophage modifications in T2D are first observed in macrophages of adipose tissue, with a switch from the M2 phenotype–macrophages with anti-inflammatory properties and an overexpression of arginase–to the M1 phenotype with a pro-inflammatory activity and an overexpression of iNOS [24]. Later, Lefèvre et al. [53] also showed modifications of the PM phenotype,

with alterations of Arg metabolism. Interestingly, these impairments lead to an increased susceptibility to some pathogens. Moreover, hypoargininemia that develops with MS and T2D could contribute, at least in part, to an increased susceptibility to infections in T2D patients, due to the very specific role played by Arg in macrophage functions [56].

Disturbances of macrophage function observed in MS and T2D come with an impaired cytokine production, but very few data are available on the topic and results differ from study to study (Table 11.2). In an insulin-resistant and obese animal model–ob/ob mice–an in vivo study shows that plasma TNFα concentration, after LPS injection, was lower in these rodents compared to their lean littermates [57]. However, Blanc et al. did not find the same result in Zucker rats. Indeed, there was no difference in TNFα production by PM in vitro between insulin-resistant and control rats [58]. Unfortunately, in both studies, no exploration of Arg metabolism was performed.

Concerning T2D, data are also contradictory. An in vivo study has shown that, in Zucker diabetic fatty (ZDF) *fa/fa* rats, TNFα plasma concentration after LPS injection was lower than in control rats [57]. This was confirmed by an in vitro study that showed that TNFα production by PM was lower in db/db mice [59]. On the contrary, Sherry et al. [55] demonstrated that *db/db* mice injected with LPS presented a higher TNFα concentration in serum and in peritoneal fluid than their lean littermates. This was accompanied by an increase of TNFα production by isolated PM, which seemed to be due to an enhanced p38 MAPK activity. Interestingly, incubating PM of control mice with high glucose concentration leads to an increased TNFα production [55]. A raised TNFα production in vitro by PM of T2D and obese rodents compared to their lean littermates was confirmed in *fa/fa* rats [54, 60]. In the first study, a higher TNFα production by macrophages of T2D rats was accompanied by a lower protein expression of arginase I, which is in accordance with the switch of polarization of PM in T2D animals.

As we have seen, hypoargininemia appears to be partly the cause of TNFα production impairment in T2D. It could be hypothesized that an increased Arg bioavailability could improve PM function and decrease TNFα production in T2D animal models (Table 11.3). In order to verify this hypothesis, PM from obese insulin-resistant Zucker rats and from T2D ZDF *fa/fa* rats were incubated in increased Arg concentration medium. In both cases, TNFα production by macrophages decreased with increasing Arg levels [54, 58]. In the second study, reduced TNFα production was accompanied by increased Arg uptake, Cit and Orn production, and arginase I protein expression.

In the same way as in vitro, Arg supply (5 g/kg/day) to T2D endotoxemic rats leads to a decrease of TNFα production by PM which were removed and cultured 4 days after LPS injection [61]. Interestingly, this modification of TNFα production was not accompanied by a modification of Arg uptake or Cit and Orn production but was well accompanied by an increase of arginase I protein expression. In a second study, graded Arg supply, in addition to diabetes-specific enteral formula

Table 11.2 Alterations in peritoneal macrophage TNFα production in metabolic syndrome and type 2 diabetes

	Strain of rodents	Activation	TNFα production	References
Metabolic syndrome	ob/ob *vs* lean	In vivo	Lower	[57]
	Zucker *vs* lean	In vitro	Equal	[58]
Type 2 diabetes	fa/fa *vs* lean	In vivo	Lower	[57]
	db/db *vs* db/+	In vitro	Lower	[59]
	db/db *vs* db/+	In vivo	Higher	[55]
	fa/fa *vs* lean	In vitro	Higher	[54, 60]

Ob/ob and db/db mice are invalidated for the leptin and its receptor, respectively. Fa/fa rat are knock out for the leptin receptor.

Table 11.3 Effects of Arg supply on TNFα production by isolated peritoneal macrophages (PM) in metabolic syndrome and type 2 diabetes

	Rodents	L-Arginine supply	TNFα production by isolated PM	References
Metabolic syndrome	Zucker rats	In vitro—2 mM—3 h	↓	[58]
Type 2 diabetes	fa/fa rats	In vitro—from 0.25 to 2 mM—3 h	↓	[54]
	fa/fa rats (endotoxemic)	In vivo—from 1 to 5 g/kg/day	–	[62]
	fa/fa rats (endotoxemic)	In vivo—5 g/kg/day—4 days	↓	[61]

The symbol ↓ indicates a statistically significant decrease, in the presence of metabolic syndrome or type 2 diabetes. Ob/ob and db/db mice are invalidated for the leptin and its receptor, respectively. fa/fa rat are knock out for the leptin receptor

given to T2D endotoxemic rats, had no effect on TNFα production, with no modification of arginase I protein expression [62]. However, it is difficult to come to the conclusion of an absence of Arg effects since infection is a complex interaction between host, immune cells, and intestinal integrity. In an in vivo bioluminescent imaging to follow bacterial invasion in rats with head injury, Moinard et al. [63] clearly demonstrated that Arg supply was able to reduce bacterial invasion, whereas classical parameters were not modified.

Due to the high Arg uptake by the liver and increased ureogenesis by Arg, Cit can also be a good candidate to supply Arg in case of MS or T2D. For this purpose, we evaluated the effects of Cit on macrophage cytokine production in vitro and observed that Cit decreased TNFα production and increased IL-6 production [60]. This latter, in the context of metabolic syndrome, is known to be beneficial in terms of insulin sensitivity and glucose homeostasis [64].

Conclusions and Perspectives

Macrophage Arg metabolism is complex. Arg availability in macrophages depends on its exogenous supply (dietary intake), (cellular protein breakdown, and endogenous de novo synthesis from amino acid (Gln, Orn, and Cit)) and its catabolism. Arg catabolism via iNOS or arginase is at the center of the macrophage polarization that occurs during inflammation from the induction to the resolution. While M1 macrophages produce NO species with antiproliferative and killing effects against pathogens and aberrant cells, M2 macrophages generate polyamines and proline with pro-proliferative effects and repair. Arg decreases TNFα production via a negative feedback exerted by NO. Advanced age impairs macrophage polarization and dysregulates the host response. In the same way, T2D leads, in most cases, to an increased TNFα production by PM which is associated with modifications of Arg metabolism, and increased Arg bioavailability leads to a decrease of TNFα production in these cells.

In the context of Arg deficiency, Arg provides an effective nutritional or pharmacotherapeutic treatment for many human diseases. Since Arg supply could be inefficient, or even harmful in some situations, and Cit is the only direct Arg precursor, exciting new roles for Cit in regulating cell and tissue functions in people in health and illness are promising.

References

1. Rose WC, Haines WJ, Warner DT. The amino acid requirements of man. V. The rôle of lysine, L-arginine, and tryptophan. J Biol Chem. 1954;206(1):421–30.
2. Barbul A, Rettura G, Levenson SM, Seifter E. L-Arginine: a thymotropic and wound-healing promoting agent. Surg Forum. 1977;28:101–3.
3. Albina JE, Abate JA, Henry WL. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. J Immunol. 1991;147:144–8.
4. West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol. 2006;22:409–37.
5. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, et al. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell. 2000;103:1071–83.
6. Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot Gene Expr. 2010;20:87–103.
7. Curis E, Nicolis I, Moinard C, Osowska S, Zerrouk N, Bénazeth S, et al. Almost all about citrulline in mammals. Amino Acids. 2005;29:177–205.
8. Murphy C, Newsholme P. Importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production. Clin Sci. 1998;95:397–407.
9. Mori M. Regulation of nitric oxide synthesis and apoptosis by arginase and L-arginine recycling. J Nutr. 2007;137:1616S–20.
10. Hammermann R, Dreissig MD, Mössner J, Fuhrmann M, Berrino L, Göthert M, et al. Nuclear factor-kappa B mediates simultaneous induction of inducible nitric-oxide synthase and up-regulation of the cationic amino acid transporter CAT-2B in rat alveolar macrophages. Mol Pharmacol. 2000;58:1294–302.
11. Liu SF, Barnes PJ, Evans TW. Time course and cellular localization of lipopolysaccharide-induced inducible nitric oxide synthase messenger RNA expression in the rat in vivo. Crit Care Med. 1997;25:512–8.
12. Xia Y, Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A. 1997;94:6954–8.
13. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005;5:641–54.
14. Matata BM, Galiñanes M. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. J Biol Chem. 2002;277:2330–5.
15. Albina JE, Mills CD, Henry WL, Caldwell MD. Temporal expression of different pathways of L-arginine metabolism in healing wounds. J Immunol. 1990;144:3877–80.
16. Hecker M, Nematollahi H, Hey C, Busse R, Racké K. Inhibition of arginase by NG-hydroxy-L-arginine in alveolar macrophages: implications for the utilization of L-arginine for nitric oxide synthesis. FEBS Lett. 1995;359:251–4.
17. Moinard C, Cynober L, de Bandt J-P. Polyamines: metabolism and implications in human diseases. Clin Nutr. 2005;24:184–97.
18. Morris SM, Kepka-Lenhart D, Chen LC. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol. 1998;275:E740–7.
19. Galea E, Regunathan S, Eliopoulos V, Feinstein DL, Reis DJ. Inhibition of mammalian nitric oxide synthases by agmatine, an endogenous polyamine formed by decarboxylation of L-arginine. Biochem J. 1996;316:247–9.
20. Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ. Agmatine: an endogenous clonidine-displacing substance in the brain. Science. 1994;263:966–9.
21. Sastre M, Galea E, Feinstein D, Reis DJ, Regunathan S. Metabolism of agmatine in macrophages: modulation by lipopolysaccharide and inhibitory cytokines. Biochem J. 1998;330:1405–9.
22. Cynober L, Moinard C, De Bandt J-P. The 2009 ESPEN Sir David Cuthbertson. Citrulline: a new major signaling molecule or just another player in the pharmaconutrition game? Clin Nutr. 2010;29:545–51.
23. Zhou D, Huang C, Lin Z, Zhan S, Kong L, Fang C, et al. Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. Cell Signal. 2014;26:192–7.
24. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest. 2007;117:175–84.
25. Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG. Shifts in macrophage phenotypes and macrophage competition for L-arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Hum Mol Genet. 2009;18:482–96.
26. Norris KA, Schrimpf JE, Flynn JL, Morris Jr SM. Enhancement of macrophage microbicidal activity: supplemental L-arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of Trypanosoma cruzi. Infect Immun. 1995;63:2793–6.

27. Saito H, Trocki O, Wang SL, Gonce SJ, Joffe SN, Alexander JW. Metabolic and immune effects of dietary L-arginine supplementation after burn. Arch Surg. 1987;122:784–9.
28. Madden HP, Breslin RJ, Wasserkrug HL, Efron G, Barbul A. Stimulation of T cell immunity by L-arginine enhances survival in peritonitis. J Surg Res. 1988;44:658–63.
29. Mendez C, Jurkovich GJ, Wener MH, Garcia I, Mays M, Maier RV. Effects of supplemental dietary L-arginine, canola oil, and trace elements on cellular immune function in critically injured patients. Shock. 1996;6:7–12.
30. Mieulet V, Yan L, Choisy C, Sully K, Procter J, Kouroumalis A, et al. TPL-2-mediated activation of MAPK downstream of TLR4 signaling is coupled to L-arginine availability. Sci Signal. 2010;3:ra61.
31. Shaw AC, Goldstein DR, Montgomery RR. Age-dependent dysregulation of innate immunity. Nat Rev Immunol. 2013;13:875–87.
32. Lloberas J, Celada A. Effect of aging on macrophage function. Exp Gerontol. 2002;37:1325–31.
33. Sebastián C, Espia M, Serra M, Celada A, Lloberas J. MacrophAging: a cellular and molecular review. Immunobiology. 2005;210:121–6.
34. Linehan E, Dombrowski Y, Snoddy R, Fallon PG, Kissenpfennig A, Fitzgerald DC. Aging impairs peritoneal but not bone marrow-derived macrophage phagocytosis. Aging Cell. 2014;13:699–708.
35. Hilmer SN, Cogger VC, Le Couteur DG. Basal activity of Kupffer cells increases with old age. J Gerontol A Biol Sci Med Sci. 2007;62:973–8.
36. Lumeng CN, Liu J, Geletka L, Delaney C, Delproposto J, Desai A, et al. Aging is associated with an increase in T cells and inflammatory macrophages in visceral adipose tissue. J Immunol. 2011;187:6208–16.
37. Kohut ML, Senchina DS, Madden KS, Martin AE, Felten DL, Moynihan JA. Age effects on macrophage function vary by tissue site, nature of stimulant, and exercise behavior. Exp Gerontol. 2004;39:1347–60.
38. Cecílio CA, Costa EH, Simioni PU, Gabriel DL, Tamashiro WMSC. Aging alters the production of iNOS, arginase and cytokines in murine macrophages. Braz J Med Biol Res. 2011;44:671–81.
39. Nyugen J, Agrawal S, Gollapudi S, Gupta S. Impaired functions of peripheral blood monocyte subpopulations in aged humans. J Clin Immunol. 2010;30:806–13.
40. Van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, et al. Age-associated defect in human TLR-1/2 function. J Immunol. 2007;178:970–5.
41. Hearps AC, Martin GE, Angelovich TA, Cheng W-J, Maisa A, Landay AL, et al. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. Aging Cell. 2012;11:867–75.
42. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. J Immunol. 2002;169:4697–701.
43. Boehmer ED, Goral J, Faunce DE, Kovacs EJ. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. J Leukoc Biol. 2004;75:342–9.
44. Agrawal A, Agrawal S, Cao J-N, Su H, Osann K, Gupta S. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. J Immunol. 2007;178:6912–22.
45. Enioutina EY, Bareyan D, Daynes RA. A role for immature myeloid cells in immune senescence. J Immunol. 2011;186:697–707.
46. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69 Suppl 1:S4–9.
47. Chen LC, Pace JL, Russell SW, Morrison DC. Altered regulation of inducible nitric oxide synthase expression in macrophages from senescent mice. Infect Immun. 1996;64:4288–98.
48. Müller I, Hailu A, Choi B-S, Abebe T, Fuentes JM, Munder M, et al. Age-related alteration of arginase activity impacts on severity of leishmaniasis. PLoS Negl Trop Dis. 2008;2:e235.
49. Nieves C, Langkamp-Henken B. L-Arginine and immunity: a unique perspective. Biomed Pharmacother. 2002;56:471–82.
50. Jourdan M, Deutz NEP, Cynober L, Aussel C. Features, causes and consequences of splanchnic sequestration of amino acid in old rats. PLoS One. 2011;6:e27002.
51. Breuillard C, Curis E, Le Plenier S, Cynober L, Moinard C. [Effects of citrulline on aged rats' macrophages functionnality: in vivo and in vitro study. Nutr Clin Métab. 2014;28 Suppl 1:S221.
52. Carton JA, Maradona JA, Nuño FJ, Fernandez-Alvarez R, Pérez-Gonzalez F, Asensi V. Diabetes mellitus and bacteraemia: a comparative study between diabetic and non-diabetic patients. Eur J Med. 1992;1:281–7.
53. Lefèvre L, Galès A, Olagnier D, Bernad J, Perez L, Burcelin R, et al. PPARγ ligands switched high fat diet-induced macrophage M2b polarization toward M2a thereby improving intestinal Candida elimination. PLoS One. 2010;5:e12828.
54. Breuillard C, Belabed L, Bonhomme S, Blanc-Quintin M-C, Neveux N, Couderc R, et al. L-Arginine availability modulates L-arginine metabolism and TNFα production in peritoneal macrophages from Zucker diabetic fatty rats. Clin Nutr. 2012;31:415–21.
55. Sherry CL, O'Connor JC, Kramer JM, Freund GG. Augmented lipopolysaccharide-induced TNF-alpha production by peritoneal macrophages in type 2 diabetic mice is dependent on elevated glucose and requires p38 MAPK. J Immunol. 2007;178:663–70.

56. Belabed L, Senon G, Blanc M-C, Paillard A, Cynober L, Darquy S. The equivocal metabolic response to endotoxaemia in type 2 diabetic and obese ZDF rats. Diabetologia. 2006;49:1349–59.
57. Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, et al. Leptin regulates proinflammatory immune responses. FASEB J. 1998;12:57–65.
58. Blanc M-C, Moinard C, Béziel A, Darquy S, Cynober L, De Bandt J-P. L-Arginine and glutamine availability and macrophage functions in the obese insulin-resistant Zucker rat. J Cell Physiol. 2005;202:153–9.
59. Zykova SN, Jenssen TG, Berdal M, Olsen R, Myklebust R, Seljelid R. Altered cytokine and nitric oxide secretion in vitro by macrophages from diabetic type II-like db/db mice. Diabetes. 2000;49:1451–8.
60. Breuillard C, Bonhomme S, Couderc R, Cynober L, De Bandt J-P. In vitro anti-inflammatory effects of citrulline on peritoneal macrophages in Zucker diabetic fatty rats. Br J Nutr. 2014;13:1–5.
61. Bonhomme S, Belabed L, Blanc M-C, Neveux N, Cynober L, Darquy S. L-Arginine-supplemented enteral nutrition in critically ill diabetic and obese rats: a dose-ranging study evaluating nutritional status and macrophage function. Nutrition. 2013;29:305–12.
62. Breuillard C, Darquy S, Curis E, Neveux N, Garnier J-P, Cynober L, et al. Effects of a diabetes-specific enteral nutrition on nutritional and immune status of diabetic, obese, and endotoxemic rats: interest of a graded L-arginine supply. Crit Care Med. 2012;40:2423–30.
63. Moinard C, Barbar S, Choisy C, Butel M-J, Francis Bureau M, Hasselmann M, et al. L-Arginine reduces bacterial invasion in rats with head injury: an in vivo evaluation by bioluminescence. Crit Care Med. 2012;40:278–80.
64. Pedersen BK, Febbraio MA. Point: interleukin-6 does have a beneficial role in insulin sensitivity and glucose homeostasis. J Appl Physiol. 2007;102:814–6.

Chapter 12
L-Arginine Metabolism Impairment in Sepsis and Diseases: Causes and Consequences

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Key Points

- Many diseases (such as sepsis, cystic fibrosis, inherited metabolic diseases) are characterized by an impairment of L-arginine homeostasis.
- Most of these pathologies present common features of L-arginine deficiency except for inherited arginase deficiency which results in L-arginine increase.
- L-Arginine deficiency is usually associated with a decrease in nitric oxide (NO) availability.
- In sepsis, a decrease in plasma L-arginine concentration is observed, resulting in a worse survival rate.
- In Chronic obstructive pneumopathy disease (COPD) patients, de novo L-arginine synthesis/formation is largely depressed and leads to a downregulation of NO synthesis.

Keywords Nitric oxide • Cystic fibrosis • Type 2 diabetes • Endothelial dysfunction • Chronic obstructive pneumopathy disease • Inherited disease syndromes • Arginase I • Sepsis

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Abbreviations

AA	Amino acids
ADMA	Asymmetric dimethylarginine
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthetase
ATP	Adenosine triphosphate
BH4	Tetrahydrobiopterin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pneumopathy disease
CPS	Carbamoylphosphate synthetase
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
ICU	Intensive care units
iNOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
L-NMMA	NG-methyl-L-arginine
LPI	Lysinuric protein intolerance
LPS	Lipopolysaccharide
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
NAGS	*N*-acetylglutamate synthetase
NO	Nitric oxide
NOS	Nitric oxide synthase
OTC	Ornithine transcarbamylase
T2D	Type 2 diabetes
ZDF	Zucker diabetic fatty

Introduction

Sepsis is recognized as a common cause for admission in intensive care unit (ICU). In general, sepsis and many diseases lead to alterations of the metabolism of amino acids (AA), among them L-arginine [1] more especially. Indeed, in humans, sepsis is characterized by a substantial decrease in L-arginine pools. This decrease of L-arginine concentration is usually due to a major increase of its consumption and a decrease of its endogenous production leading to the concept of "L-arginine deficiency" [1]. Hence, in sepsis, it is generally admitted that endogenous synthesis (i.e., L-arginine is a non-essential amino acid) cannot meet the needs and L-arginine becomes a conditionally essential AA [2]. This may have important consequences. As a matter of fact, L-arginine is not only a component of proteins but also a molecule that can generate a number of active metabolites (Fig. 12.1): L-arginine may be the precursor of nitric oxide (NO, which is essential for the immune system), of ornithine (which is recognized as a polyamine precursor), or of agmatine (which is a major regulator of cell functions). Moreover, L-arginine is an important element in muscle energy: after reacting with glycine and methionine, it allows the formation of creatine. Finally, L-arginine acts as a secretagogue (such as insulin, glucagon, growth hormones, prolactin, and catecholamines) [3]. This could explain why the impairment of L-arginine homeostasis in sepsis and several diseases can contribute to pathophysiological alterations.

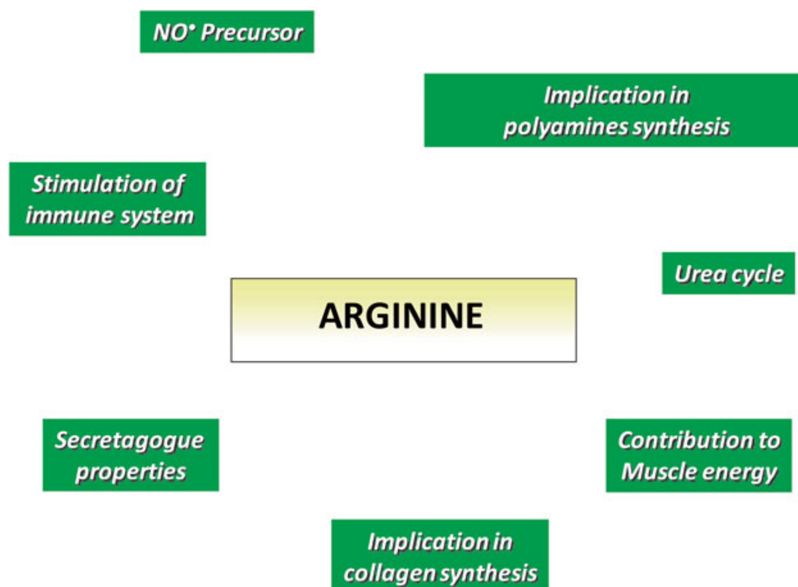

Fig. 12.1 General overview of ARG L-arginine properties

Impairment of L-Arginine Homeostasis in Sepsis

Causes

In sepsis, nitrogen homeostasis is largely sustained, while AA metabolism undergoes major modifications in order to adapt to increasing nitrogen requirements. In healthy conditions, the L-arginine flux at the whole-body level is estimated to be 70–90 μmol/kg/h, which represents around 15–20 g/day (with 10–15 % coming from daily dietary intake and 85–90 % from de novo synthesis) and the physiological plasma L-arginine concentration in a fasted state is around 100 μmol/l [4]. In sepsis, a decrease in plasma L-arginine concentration was observed, resulting in a worse survival rate. The reason is a profound modification of L-arginine turnover; using stable isotopes in septic patients, it was demonstrated that endogenous L-arginine production was dramatically reduced, this being caused by a decrease of citrulline intestinal production (citrulline is synthesized by ornithine transcarbamylase (OTC) in enterocytes) [5, 6]. In fact, citrulline is almost exclusively synthesized by the intestine from glutamine [7, 8]. Its depletion, consecutively observed in sepsis, leads to a reduced plasma citrulline and results in reduced de novo L-arginine production in adult sepsis (yet this phenomenon was never observed in septic children who have an increase in their whole-body citrulline production suggesting an age-related phenomenon [9]). The importance of the gut is supported by the fact that decreased renal function does not affect de novo L-arginine production, even if kidneys are the main site of L-arginine production from citrulline [6]. This phenomenon could be partially compensated for by the major increase of proteolysis which may preserve L-arginine availability. In a mouse model of OTC deficiency (a mouse *(spf(ash)* that has low OTC activity and characterized by a low citrulline production), endotoxemia enhances protein breakdown with maintenance of NO production [10]. The major consequence of L-arginine depletion is a decrease of NO production and an activation of arginase activity (due to an overactivation of the urea cycle). However, L-arginine is the precursor of NO, which is

essential for immune cells (due to its antimicrobial activities) in this situation. It is classically admitted that any impairment of its homeostasis could be deleterious in these patients: an increase of NO production is responsible for nitrosative stress whereas NO deficiency is associated with dysimmunity [8]. It has been proposed that the impairment of NO production could explain the diminished microcirculation and organ perfusion, which are of major consequences in septic shocks (and could lead to multiple organ failure) [6, 8]. Interestingly, it was demonstrated, in a pig model of sepsis, that the nutritional status could affect metabolic response to endotoxemia: a caloric restriction is associated with a decrease in L-arginine appearance and a lower NO production after endotoxin challenge [11]. This work suggests that malnutrition (which is classically observed in septic patients) could exacerbate L-arginine depletion.

Consequences

In sepsis, a reduced plasma L-arginine concentration is associated with a poor outcome. It has been reported that increased mortality in septic patients would be related to elevated asymmetric dimethylarginine (ADMA) levels [12]. Methylarginines, such as ADMA and NG-methyl-L-arginine (L-NMMA), are the most powerful endogenous and competitive nonspecific NOS inhibitors that compete with L-arginine for nitric oxide synthase (NOS) (Fig. 12.2) and for its intracellular transport [12]. These molecules are issued from proteolysis (posttranslational modified proteins that contain methylated L-arginine residues). In sepsis, hypercatabolism associated with an impaired renal function can thus contribute to elevated levels of methylarginines while L-arginine concentration is depressed. The consequence is a decrease of the L-arginine-to-dimethylarginine ratio which is an independent factor of a poor outcome [13].

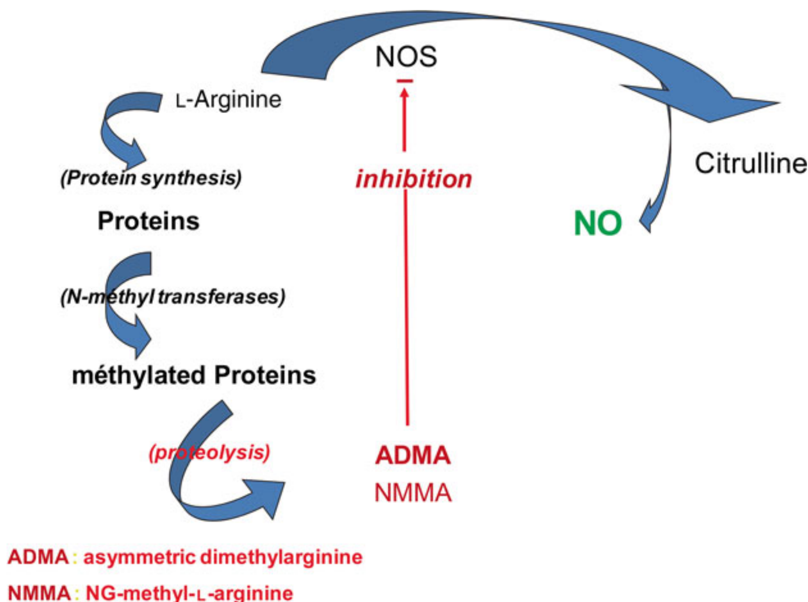

Fig. 12.2 Effect of asymmetric dimethylarginine on NO (nitric oxide) synthase activity. ADMA and NG-methyl-L-arginine (L-NMMA) are endogenous and competitive nonspecific NOS (nitric oxide synthase) inhibitors (derived from proteolysis) that compete with L-arginine for NOS. *ADMA* asymmetric dimethylarginine, *NMMA* NG-methyl-L-arginine

Moreover, in sepsis, L-arginine is essential to fight pathogens; it can act via NO (which is essential for immune cell activity and preventing intestinal hypoperfusion) [14]. Indeed, NO is a cytotoxic agent that can inhibit the respiratory chain (inhibition of complex I and complex II) and the Krebs cycle (aconitase inhibition) [15, 16]. It also has the ability to inhibit ribonucleotide reductase, an enzyme involved in the synthesis and repair of DNA [17].

L-Arginine is also an important polyamine precursor (via ornithine synthesis): polyamines are biogenic amines that are involved in many physiological functions, such as immunity (both the differentiation of immune cells and the regulation of the inflammatory reaction) and intestine trophicity (polyamines are essential to all intestinal repair processes) [18].

Furthermore, L-arginine is a direct precursor of agmatine, a polyamine that is synthesized following decarboxylation of L-arginine by L-arginine decarboxylase [18]. This molecule was first discovered in plants, bacteria, and invertebrates. In 1994, agmatine was isolated from mammalian brains [18]. Since then, many of its functions have been discovered. Agmatine could in particular play a role during sepsis. Indeed, it is able to inhibit inducible NOS (iNOS) activity [19]. Agmatine administration to endotoxemic rats prevents the decrease in their blood pressure and renal impairment classically associated with sepsis. Moreover, agmatine treatment increases the survival of LPS-treated mice [20].

L-Arginine is also known to be essential in sepsis to favor wound healing (as L-arginine deficiency is to delay wound healing). L-Arginine, via the synthesis of proline and hydroxyproline, promotes the deposition of collagen and thus provides better wound healing [21]. However, the effect of L-arginine in wound healing processes is not limited to its ability to generate proline and hydroxyproline. It is also related to NO production since the inhibition of iNOS (by competitive inhibitors or in iNOS knockout animals) decreases collagen deposition and breaking strength of incisional wounds and impairs healing in various wound models [22]. Finally, a recent paper by Fujiwara et al. proposes novel mechanistic insights into the positive effects of L-arginine on wound healing (via an activation the GPRC6A-ERK1/2 and PI3K/Akt signaling pathway) [23].

Considering the importance of all the metabolic pathways described above in sepsis, this can explain that the impairment of L-arginine homeostasis in sepsis leads to a poor prognosis.

Sepsis in Diabetes

It is well known that type 2 diabetes (T2D) is characterized by an impairment of L-arginine metabolism [24] as in sepsis, yet, surprisingly, L-arginine metabolism in sepsis in a situation of T2D has seldom been studied. To the best of our knowledge, there are no studies in humans, and only one in animal models [25]: The authors explored the metabolic response to endotoxin challenge in Zucker diabetic fatty (ZDF) rats. Contrary to control rats, endotoxemia induced neither L-arginine plasma concentration modifications nor a citrulline plasma concentration decrease in ZDF rats. It could be due to a lack of increased citrulline to L-arginine conversion in kidneys, which is consistent with renal complications in case of T2D [26]. On the other hand, endotoxemia induced no modification of muscular and intestinal AA concentrations. So, L-arginine metabolism is well impaired in endotoxemic and T2D rats (with modifications of L-arginine and citrulline plasma concentrations), but it does not seem to be deleterious. Surprisingly as a matter of fact, these rats were less sick than control ones, with a better cumulative nitrogen balance and a lower urinary 3-methylhistidine:creatinine ratio (the urinary marker of muscle proteolysis). The importance of L-arginine supplementation in this situation was investigated in two studies which evaluated the importance to sustain L-arginine intake. In the first one [27], the authors proposed that increasing L-arginine supplementation in endotoxemic ZDF rats would make it possible to improve glucose and lipid metabolism, but L-arginine supplementation had no effect. In the second study [28], L-arginine addition in endotoxemic ZDF rats had no more effects on glucose and lipid metabolism than diabetes-specific enteral formula alone. In conclusion, modifications of L-arginine metabolism in case of sepsis and T2D are well established, but their

consequences are not totally understood and it is difficult to discriminate the part of T2D, the part of sepsis, and their interaction. Many more researches are required to understand the metabolic specificity of L-arginine impairment in septic T2D patients.

Impairment of L-Arginine Homeostasis in Chronic Obstructive Pneumopathy Disease

Definition

Chronic obstructive pneumopathy disease (COPD) is a major health problem worldwide since it was the fourth cause of death in 2013. COPD affects 11 % of the population over 40 years of age—rising to 50 % in heavy smokers—and is responsible for 5.2 million disability-adjusted life years lost annually in the EU at a minimum cost of almost 300 billion € [29]. COPD is characterized by lung and systemic disorders that have a major impact on patient outcomes. Among them, endothelial dysfunction and muscle weakness—or sarcopenia—are some of the prime causes of morbidity and mortality. Until recently, this systemic dimension was considered an irreversible consequence of the disease progression and was not adequately addressed apart from pulmonary rehabilitation [30]. However, muscle weakness is of major importance in patient outcomes due to the fact that muscle mass is a determinant of the respiratory function. It directly impacts the life quality, morbidity, and mortality of COPD patients, with around 40 % of them showing a weight loss, i.e., >5 % of their body weight over the previous 3 months or 10 % over the previous 6 months. Even in normal weight—or overweight—COPD patients, a decrease in their lean body mass is highly prevalent. At the same time, 25 % of COPD die from cardiovascular diseases due to endothelial dysfunction which is usually considered as a consequence of the systemic inflammation observed in this population [30].

L-Arginine Metabolism Impairment in COPD

It has been proposed that this metabolic disturbance could be related to an impairment of L-arginine metabolism. In fact, it has very recently been observed that COPD patients exhibit a decrease in L-arginine synthesis, which, in turn, would decrease NO production (a vasodilator recognized for its antiatherogenic properties) and thus impair endothelial function.

The results clearly indicated, as observed in sepsis, that COPD was associated with a huge decrease in intestinal citrulline production. Jonker et al. [31] evaluated, in moderate to severe stable COPD, whole-body L-arginine and citrulline production, de novo L-arginine synthesis, and NO production. They observed that de novo L-arginine was largely depressed (around −30 % compared to healthy controls), which led to a downregulation of NO synthesis (−60 %). This phenomenon could be related to inflammation that is known to decrease intestinal citrulline production [32]. But at the same time, the role of hypoxia could be implicated; exposition of healthy rats to 45 % air in N_2 for 5 h induced a large decrease in citrulline and L-arginine concentration in plasma (−30 %) [33]. Moreover, it has been suggested that cigarette smoke and inflammation may increase arginase expression. The consequence would be a decrease in L-arginine availability for NO synthesis. Besides, arginase activity may increase L-ornithine downstream products like aliphatic polyamines and L-proline that promote cell proliferation (polyamines) and collagen synthesis (proline), thus favoring tissue remodeling.

This hypothesis was supported by the fact that plasma ornithine concentration was higher in COPD patients compared to healthy controls [34]. Moreover, in a guinea pig model of COPD, an upregulation of arginase was observed and it was well correlated to pulmonary inflammation. However, when animals were pretreated by 2(*S*)-amino-6-boronohexanoic acid (an arginase inhibitor), these alterations were totally prevented [35].

Impairment of L-Arginine Homeostasis in Cystic Fibrosis

Definition

Cystic fibrosis (CF), also called mucoviscidosis, is a genetic disorder affecting mucin-producing organs, like the pancreas, liver, intestine, and mainly the lungs. The disease results from the mutation of the CFTR (cystic fibrosis transmembrane conductance regulator) gene protein which regulates the formation of mucus [36]. It leads to airway dysfunction characterized by chronic inflammation, chronic bacterial infection, and recurrent infection-associated pulmonary exacerbations that significantly deteriorate pulmonary function [37] (Fig. 12.3).

L-Arginine Metabolism Impairment in CF

Abnormalities in L-arginine/NO metabolism in patients with CF have been evidenced mostly in the lungs and airways, but they have also been reported at the whole-body level.

On the pulmonary level, NO is a key messenger molecule which plays a role in neurotransmission, smooth muscle relaxation, and bronchodilation [38]. While airway NO production is usually increased in pulmonary inflammation, fractional exhaled NO is decreased in airway fluids of patients with CF

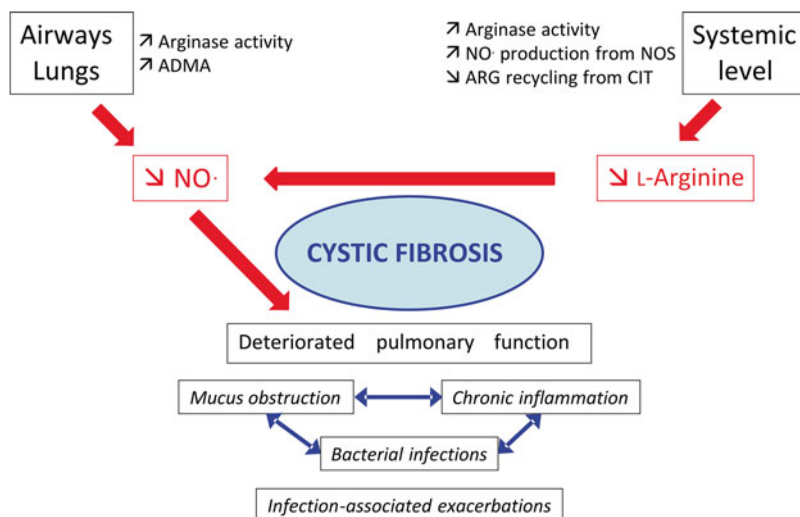

Fig. 12.3 L-Arginine/NO metabolism impairment in patients with cystic fibrosis. Cystic fibrosis is characterized by abnormalities in L-arginine/NO metabolism. In patients cystic fibrosis has been evidenced mostly in the lungs and airways, but it has also been reported at the whole-body level

in spite of the inflammatory nature of the disease [39]. Two phenomena contribute to the local NO deficiency: one is the significant increase in arginase activity in CF airways [40], and the second is the increase of the endogenous competitive NO synthase inhibitor ADMA [41].

In addition, systemic bioavailability of L-arginine and NO is probably involved. Pulmonary exacerbations in patients with CF induce elevated systemic arginase levels and proportionally reduce plasma L-arginine concentrations. This is confirmed as those parameters are normalized after treatment with antibiotics [42]. Engelen et al. [43] have observed that NO production rate and plasma L-arginine levels were similar between healthy controls and patients with CF. Interestingly, patients with CF and nutritional failure presented lowered systemic L-arginine concentrations [43]. Among several possible causes, decreased L-arginine intake and lowered intestinal absorption may be ruled out since caloric intake and weight gain were comparable in patients with or without nutritional failure. The involvement of an increased consumption of L-arginine through an increased whole-body NO production from NOS, but also insufficient L-arginine recycling from citrulline, has been evidenced. In addition, a decreased de novo synthesis of L-arginine from other amino acids remains to be studied as a possible cause [43].

Finally, interaction between systemic and pulmonary abnormalities should be addressed in future studies combining whole-body and organ-specific assessments of L-arginine metabolism in CF patients [44].

Therapeutic Interventions

Several possibilities are envisaged to treat L-arginine/NO imbalance in CF. One direct therapeutic approach consists in inhaling L-arginine, which acutely and transiently improves pulmonary function by increasing airway NO formation [45]. However, a sustained local delivery of L-arginine may be deleterious through the promotion of lung fibrosis via downstream products of arginase activity such as polyamines [41]. Molecules like selective arginase inhibitors [46, 47] and statins [48, 49]—envisaged for the treatment of asthma—may be of interest in CF. Another possibility relies on ADMA competitors like melatonin that could potentially improve pulmonary function through local NO production [41].

Impairment of L-Arginine Homeostasis in Other Inherited Disease Syndromes

Definition

Inherited or genetic diseases result from abnormalities in the genome. Among the tremendous variety of inherited disorders, some induce L-arginine/NO metabolism impairment. This is the case for most inherited disorders of the urea cycle, but also for lysinuric protein intolerance, and mitochondrial disorders.

Inherited Disorders of the Urea Cycle

The urea cycle has two roles. It is the final common pathway in mammals to detoxify ammonia and to remove surplus amino group nitrogen. It also produces L-arginine by de novo synthesis. The urea

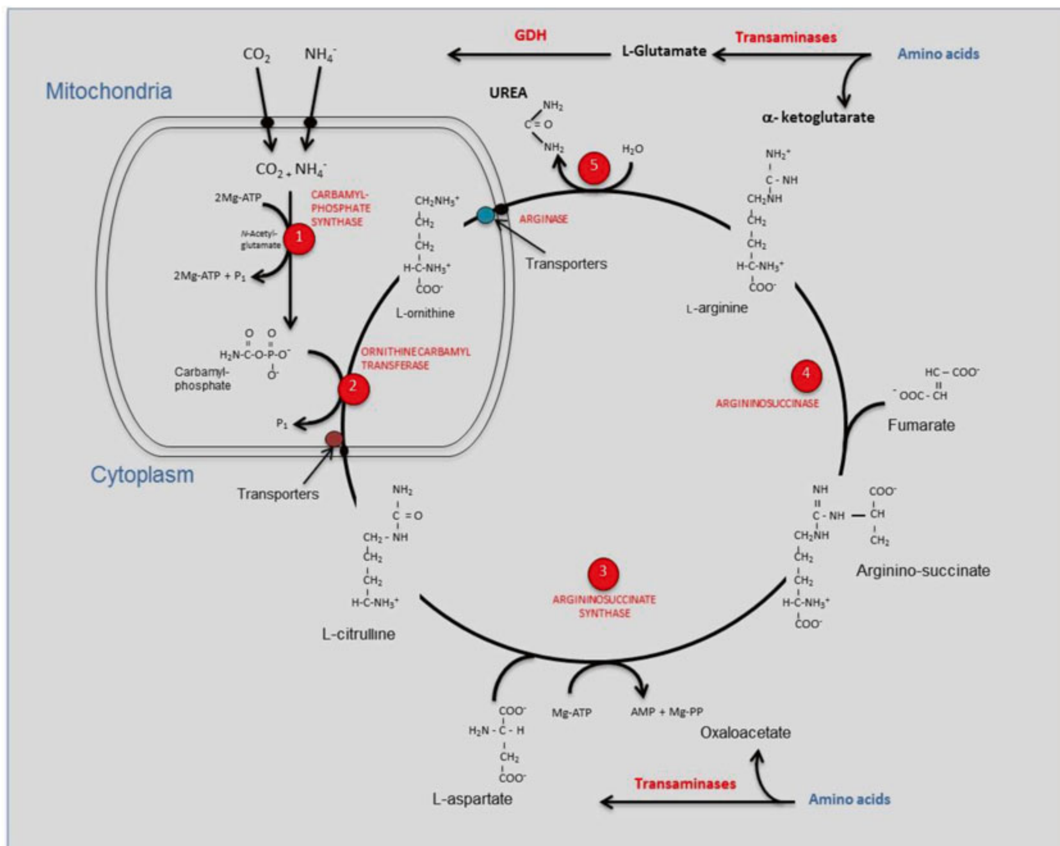

Fig. 12.4 Schematic representation of the urea cycle. L-Arginine metabolism in urea cycle (which mainly occurred in hepatocytes). The urea cycle comprises six enzyme reactions, the first three being intramitochondrial and the others being cytosolic

cycle comprises six enzyme reactions, the first three being intramitochondrial and the others being cytosolic (Fig. 12.4).

Inherited disorders of the urea cycle are inborn metabolic diseases caused by the deficiency of enzymes including carbamoylphosphate synthetase (CPS), OTC, argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), arginase, and *N*-acetylglutamate synthetase (NAGS). Out of the six disorders, OTC deficiency has the highest incidence, while arginase and NAGS deficiencies have the lowest [50]. While the first four deficiencies are responsible for reduced plasma L-arginine concentrations, the arginase defect leads to hyperargininemia. NAGS deficiency does not affect L-arginine metabolism [51]. All these diseases lead to hyperammonemia with subsequent neurological damage (for complete review, see [51]). Their treatment aims at reducing plasma ammonemia quickly, decreasing production of waste nitrogen, disposing of waste nitrogen by using alternative pathways to the urea cycle, and meeting all the nutritional needs, particularly by replacing the deficient amino acid by L-arginine or citrulline (except in patients suffering from arginase deficiency) and by providing essential amino acids [52].

Lysinuric Protein Intolerance

Lysinuric protein intolerance (LPI) is an inborn defect of the transport of cationic amino acids, lysine, L-arginine, and ornithine, affecting their intestinal absorption and renal reabsorption. It is caused by mutations in SLC7A7 encoding the y+LAT1 protein [53]. The disease is a severe multisystem one, far more complex than a classic urea cycle disorder. Since the characteristics of acute episodes are hyperammonemia, low levels of plasma L-arginine, lysine, and ornithine, as well as an increased urinary excretion of these amino acids, a low protein diet and citrulline supplementation have first been considered as an appropriate treatment. However, intracellular accumulation of L-arginine due to defective efflux from the cell leads to NO overproduction that may be responsible for immune dysfunction and serious complications in severe cases. In such situations, excessive citrulline supply may be detrimental. Current therapeutic recommendations consist in lower oral citrulline supplementation and strict protein restriction to treat urea cycle dysfunction; this is associated with the management of growth failure, osteopenia, and immune dysfunction [53].

Mitochondrial Disorders

Mitochondrial disorders are inherited diseases characterized by the dysfunction of the mitochondrial respiratory chain. They result in a defective production of ATP that fails to meet the energy needs of various organs, particularly those with a high-energy demand like the central nervous system, skeletal and cardiac muscles, kidneys, liver, and endocrine systems. NO deficiency is another aspect of these diseases that may play a key role in the pathogenesis of various complications such as stroke-like episodes, myopathy, diabetes, and lactic acidosis [54]. MELAS syndrome—mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes—is one of the most frequent inherited mitochondrial disorders [55]. The origins of the mechanisms responsible for NO deficiency may be multifactorial and may involve a defective NO production due to endothelial dysfunction, NO scavenging by cyclooxygenases in cyclooxygenase-positive sites, NO shunting into reactive nitrogen species formation, decreased availability of L-arginine and citrulline, and increased ADMA concentrations [54]. Since L-arginine and citrulline act as NO precursors, their administration has been proposed to treat MELAS syndrome. While both amino acids are able to improve NO production, citrulline raises NO availability to a greater extent than L-arginine thanks to a substantial increase in de novo L-arginine synthesis. Such supplementations improve the clinical symptoms associated with stroke-like episodes. Their therapeutic utility needs to be evaluated in other mitochondrial disorders [55].

Impairment of L-Arginine Homeostasis in Endothelial Dysfunction

Endothelial dysfunction identified early in the development of atherosclerosis is exacerbated by hyperglycemia and insulin resistance of T2D and aggravated by obesity. The endothelium is a biologically active barrier between blood and the arterial wall. It plays a key role in arterial vasoreactivity and in inhibiting the adhesion of leukocytes and platelets as well as the proliferation of smooth muscle cells (Fig. 12.5). It is also involved in the protection of the vascular wall against oxidative stress. Endothelial dysfunction is partly due to an impairment of the endothelial nitric oxide synthase (eNOS). The consequence is a decrease of the NO production and smooth muscle cell relaxation [56].

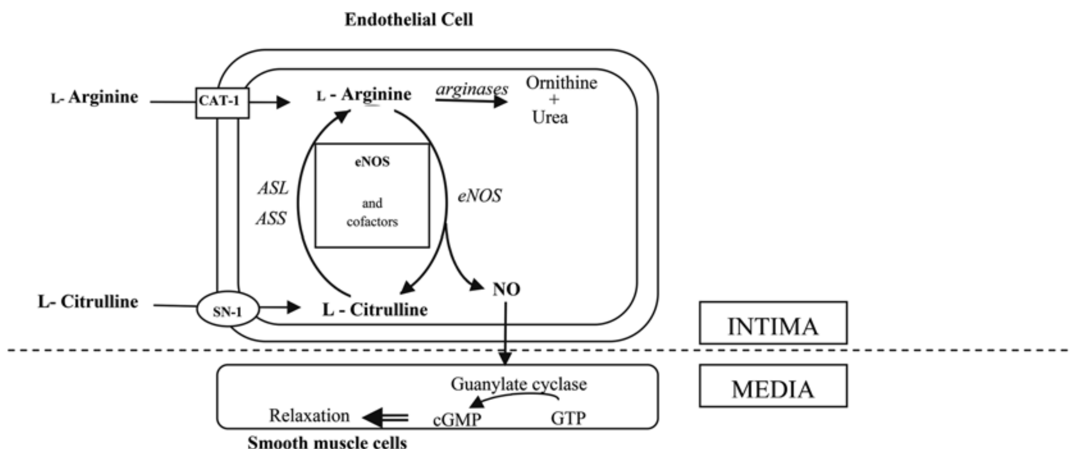

Fig. 12.5 L-Arginine crossroad in endothelial cell. Intracellular citrulline–L-arginine cycle and its relationship with NO metabolism. ASL (argininosuccinate lyase), ASS (argininosuccinate synthase), eNOS (endothelial Nitric Oxide Synthase)

L-Arginine Metabolism Impairment in Endothelial Dysfunction

The frequency of cardiovascular complications in diabetes can be explained by abnormalities in the metabolism of L-arginine. In this regard, plasma L-arginine concentrations are decreased in experimental models of T2D [25] as well as in diabetic patients [57], leading to a reduced bioavailability of L-arginine, which in turn blunts the production of NO by endothelial cells. Cell production of NO depends on the L-arginine availability and on the balance between NOS and arginases. This hypothesis was confirmed by Beleznai et al. [58], who removed the coronary artery in surgery patients (diabetic or not). The authors observed a sharp increase in the expression of arginase I at the vascular level in diabetic patients. Moreover, the data obtained by immunohistochemistry demonstrated the co-localization of arginase I and eNOS. The implication of this L-arginine unbalance was confirmed in diabetic rats using an arginase inhibitor which allowed the restoration of coronary circulation [59]. In addition, ADMA, which is increased in diabetics, competes with L-arginine for its intracellular transport by CAT-1, as well as for NO synthesis by eNOS [60]. Moreover, eNOS is impaired because insulin resistance induces a decrease in eNOS activation due to an altered Akt (PDK/PKB) pathway. Other factors are also involved in the decrease in NO production in diabetes: there is a decrease in tetrahydrobiopterin (BH4) availability which promotes eNOS decoupling and peroxynitrite (eNOS inhibitor) production [60]. Finally, NO availability is further decreased by NADPHox endothelial activation which is stimulated by oxidized LDL (low-density lipoprotein) (via PKC), hyperglycemia, and/or an increase of free fatty acids [60].

Conclusions and Prospects

In conclusion, it appears that an impairment of L-arginine homeostasis is observed in many diseases. They may be caused by various factors, but, finally, the consequences are very similar: a decrease of L-arginine availability leading, in many cases, to a decrease in NO production. The causes may be a

decrease in NO production (inhibition of NOS) or a decrease of L-arginine availability due to high consumption (due to an increase of arginase activity). Several approaches have been suggested to increase L-arginine availability in these pathologies: increasing L-arginine intake (or citrulline, its direct precursor), stimulating NOS activity, or inhibiting arginase I. In spite of the fact that L-arginine was reduced in patients with sepsis and that it had a deleterious impact on mortality, many studies investigated the interest of an L-arginine supplementation in case of sepsis, but the results are largely controversial [14]. Other strategies are proposed (the use of citrulline instead of L-arginine, the use or arginase inhibitors,…), yet, despite some positive results, no definitive strategy has been presented and many researches are still required to optimize these fields.

References

1. Ventura G, Moinard C, Segaud F, Le Plenier S, Cynober L, De Bandt JP. Adaptative response of nitrogen metabolism in early endotoxemia: role of ornithine aminotransferase. Amino Acids. 2010;39:1417–26.
2. Pribis JP, Zhu X, Vodovotz Y, Ochoa JB. Systemic L-arginine depletion after a murine model of surgery or trauma. JPEN J Parenter Enteral Nutr. 2012;36:53–9.
3. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37:1–17.
4. Castillo L, Chapman TE, Sanchez M, Yu YM, Burke JF, Ajami AM, Vogt J, Young VR. Plasma L-arginine and citrulline kinetics in adults given adequate and L-arginine-free diets. Proc Natl Acad Sci U S A. 1993;90:7749–53.
5. Kao CC, Bandi V, Guntupalli KK, Wu M, Castillo L, Jahoor F. L-Arginine, citrulline and nitric oxide metabolism in sepsis. Clin Sci. 2009;117:23–30.
6. Luiking YC, Poeze M, Ramsay G, Deutz NE. Reduced citrulline production in sepsis is related to diminished de novo L-arginine and nitric oxide production. Am J Clin Nutr. 2009;89:142–52.
7. Bahri S, Zerrouk N, Aussel C, Moinard C, Crenn P, Curis E, Chaumeil JC, Cynober L, Sfar S. Citrulline: from metabolism to therapeutic use. Nutrition. 2013;29:479–84.
8. de Betue CT, Deutz NE. Changes in L-arginine metabolism during sepsis and critical illness in children. Nestle Nutr Inst Workshop Ser. 2013;77:17–28.
9. Argaman Z, Young VR, Noviski N, Castillo-Rosas L, Lu XM, Zurakowski D, Cooper M, Davison C, Tharakan JF, Ajami A, Castillo L. L-Arginine and nitric oxide metabolism in critically ill septic pediatric patients. Crit Care Med. 2003;31:591–7.
10. Luiking YC, Hallemeesch MM, van de Poll MC, Dejong CH, de Jonge WJ, Lamers WH, Deutz NE. Reduced citrulline availability by OTC deficiency in mice is related to reduced nitric oxide production. Am J Physiol Endocrinol Metab. 2008;295:E1315–22.
11. Poeze M, Bruins MJ, Luiking YC, Deutz NE. Reduced caloric intake during endotoxemia reduces L-arginine availability and metabolism. Am J Clin Nutr. 2010;91:992–1001.
12. Leiper J, Vallance P. Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. Cardiovasc Res. 1999;43:542–8.
13. Gough MS, Morgan MAM, Mack CM, Darling DC, Frasier LM, Doolin KP, Apostolakos MJ, Stewart JC, Graves BT, Arning E, Bottiglieri T, Mooney RA, Frampton MW, Pietropaoli AP. The ratio of L-arginine to dimethylarginines is reduced and predicts outcomes in patients with severe sepsis. Crit Care Med. 2011;39:1351–8.
14. Moinard C, Barbar S, Choisy C, Butel MJ, Bureau MF, Hasselmann M, Cynober L, Charrueau C. L-Arginine reduces bacterial invasion in head-injury rats: an in vivo evaluation by bioluminescence. Crit Care Med. 2012;40:278–80.
15. Clementi E, Brown GC, Feelisch M, Moncada S. Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc Natl Acad Sci U S A. 1998;95:7631–6.
16. Kelly E, Morris SM, Billiar TR. Nitric oxide, sepsis, and L-arginine metabolism. JPEN J Parenter Enteral Nutr. 1995;19:234–8.
17. Kröncke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. Biol Chem Hoppe Seyler. 1995;376:327–43.
18. Moinard C, Cynober L, De Bandt J-P. Polyamines: metabolism and implications in human diseases. Clin Nutr. 2005;24:184–97.
19. Raghavan SA, Dikshit M. Vascular regulation by the L-arginine metabolites, nitric oxide and agmatine. Pharmacol Res. 2004;49:397–414.

20. Satriano J, Schwartz D, Ishizuka S, et al. Suppression of inducible nitric oxide generation by agmatine aldehyde: beneficial effects in sepsis. J Cell Physiol. 2001;188:313–20.
21. Barbul A. L-Arginine: biochemistry, physiology, and therapeutic implications. J Parenter Enteral Nutr. 1986;10: 227–38.
22. Witte MB, Barbul A. Role of nitric oxide in wound repair. Am J Surg. 2002;183:406–12.
23. Fujiwara T, Kanazawa S, Ichibori R, Tanigawa T, Magome T, Shingaki K, Miyata S, Tohyama M, Hosokawa K. L-Arginine stimulates fibroblast proliferation through the GPRC6A-ERK1/2 and PI3K/Akt pathway. PLoS One. 2014;9:e92168.
24. Tessari P, Cecchet D, Cosma A, Puricelli L, Millioni R, Vedovato M, Tiengo A. Insulin resistance of amino acid and protein metabolism in type 2 diabetes. Clin Nutr. 2011;30:267–72.
25. Belabed L, Senon G, Blanc M-C, Paillard A, Cynober L, Darquy S. The equivocal metabolic response to endotoxaemia in type 2 diabetic and obese ZDF rats. Diabetologia. 2006;49:1349–59.
26. Afkarian M, Sachs MC, Kestenbaum B, Hirsch IB, Tuttle KR, Himmelfarb J, et al. Kidney disease and increased mortality risk in type 2 diabetes. J Am Soc Nephrol. 2013;24:302–8.
27. Bonhomme S, Belabed L, Blanc M-C, Neveux N, Cynober L, Darquy S. L-Arginine-supplemented enteral nutrition in critically ill diabetic and obese rats: a dose-ranging study evaluating nutritional status and macrophage function. Nutrition. 2013;29:305–12.
28. Breuillard C, Darquy S, Curis E, Neveux N, Garnier J-P, Cynober L, de Bandt J.P. Effects of a diabetes-specific enteral nutrition on nutritional and immune status of diabetic, obese, and endotoxemic rats: interest of a graded L-arginine supply. Crit Care Med. 2012;40:2423–30.
29. Patel JG, Nagar SP, Dalal AA. Indirect costs in chronic obstructive pulmonary disease: a review of the economic burden on employers and individuals in the United States. Int J Chron Obstruct Pulmon Dis. 2014;9:289–300.
30. Antonione R. Nutrition in cardiac and pulmonary disease. In: Sobotka L, editor. Basic in clinical nutrition. 4th ed. Prague: Galen; 2011. p. 485–93.
31. Jonker R, Deutz N, Erbland M, Anderson P, Engelen M. Whole body de novo L-arginine production and NO synthesis are reduced in COPD patients. Clin Nutr. 2013;32:S5–6.
32. Boutry C, Matsumoto H, Bos C, Moinard C, Cynober L, Yin Y, Tomé D, Blachier F. Decreased glutamate, glutamine and citrulline concentrations in plasma and muscle in endotoxemia cannot be reversed by glutamate or glutamine supplementation: a primary intestinal defect? Amino Acids. 2012;43:1485–98.
33. Muratsubaki H, Yamaki A. Profile of plasma amino acid levels in rats exposed to acute hypoxic hypoxia. Indian J Clin Biochem. 2011;26:416–9.
34. Pera T, Zuidhof AB, Smit M, Menzen MH, Klein T, Flik G, Zaagsma J, Meurs H, Maarsingh H. Arginase inhibition prevents inflammation and remodeling in a Guinea pig model of chronic obstructive pulmonary disease. J Pharmacol Exp Ther. 2014;349:229–38.
35. Rutten EP, Engelen MP, Wouters EF, Schols AM, Deutz NE. Metabolic effects of glutamine and glutamate ingestion in healthy subjects and in persons with chronic obstructive pulmonary disease. Am J Clin Nutr. 2006;83:115–23.
36. Ehre C, Ridley C, Thornton DJ. Cystic fibrosis: an inherited disease affecting mucin-producing organs. Int J Biochem Cell Biol. 2014;52:136–45.
37. Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med. 2003;168:918–51.
38. Ricciardolo FL, Sterk PJ, Gaston B, Folkerts G. Nitric oxide in health and disease of the respiratory system. Physiol Rev. 2004;84:731–65.
39. Elphick HE, Demoncheaux EA, Ritson S, Higenbottam TW, Everard ML. Exhaled nitric oxide is reduced in infants with cystic fibrosis. Thorax. 2001;56:151–2.
40. Grasemann H, Schwiertz R, Matthiesen S, Racké K, Ratjen F. Increased arginase activity in cystic fibrosis airways. Am J Respir Crit Care Med. 2005;172:1523–8.
41. Grasemann H, Al-Saleh S, Scott JA, Shehnaz D, Mehl A, Amin R, Rafii M, Pencharz P, Belik J, Ratjen F. Asymmetric dimethylarginine contributes to airway nitric oxide deficiency in patients with cystic fibrosis. Am J Respir Crit Care Med. 2011;183:1363–8.
42. Grasemann H, Schwiertz R, Grasemann C, Vester U, Racké K, Ratjen F. Decreased systemic bioavailability of L-arginine in patients with cystic fibrosis. Respir Res. 2006;7:87.
43. Engelen MPKJ, Com G, Luiking YC, Deutz NE. Stimulated nitric oxide production and L-arginine deficiency in children with cystic fibrosis with nutritional failure. J Pediatr. 2013;163:369–75.
44. Grasemann H, Pencharz B. L-Arginine metabolism in patients with cystic fibrosis. J Pediatr. 2013;163:317–9.
45. Grasemann H, Kurtz F, Ratjen F. Inhaled L-arginine improves exhaled nitric oxide and pulmonary function in patients with cystic fibrosis. Am J Respir Crit Care Med. 2006;174:208–12.
46. Ilies M, Di Costanzo L, North ML, Scott JA, Christianson DW. 2-aminoimidazole amino acids as inhibitors of the binuclear manganese metalloenzyme human arginase I. J Med Chem. 2010;53:4266–76.

47. North MLN, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. Am J Physiol Lung Cell Mol Physiol. 2009;296:L911–20.
48. Zeki AA, Bratt JM, Rabowsky M, Last JA, Kenyon NJ. Simvastatin inhibits goblet cell hyperplasia and lung arginase in a mouse model of allergic asthma: a novel treatment for airway remodeling? Transl Res. 2010;156:335–49.
49. Ahmad T, Mabalirajan U, Sharma A, Aich J, Makhija L, Ghosh B, Agrawal A. Simvastatin improves epithelial dysfunction and airway hyperresponsiveness: from asymmetric dimethyl-arginine to asthma. Am J Respir Cell Mol Biol. 2011;44:531–9.
50. Crombez EA, Cederbaum SD. Hyperargininemia due to liver arginase deficiency. Mol Genet Metab. 2005;84:243–51.
51. Leonard JV. disorders of the urea cycle and related enzymes. In: Fernandes J, Saudubray JM, Van Den Berghe G, Walters JH, editors. Inborn metabolic diseases—diagnosis and treatment. 4th ed. Berlin: Springer; 2006. p. 263–72.
52. Walker V. Ammonia toxicity and its prevention in inherited defects of the urea cycle. Diabetes Obes Metab. 2009;11:823–35.
53. Ogier de Baulny H, Schiff M, Dionisi-Vici C. Lysinuric protein intolerance (LPI): a multi organ disease by far more complex than a classic urea cycle disorder. Mol Genet Metab. 2012;106:12–7.
54. El-Hattab AW, Emrick LT, Craigen WJ, Scaglia F. Citrulline and L-arginine utility in treating nitric oxide deficiency in mitochondrial disorders. Mol Genet Metab. 2012;107:247–52.
55. El-Hattab AW, Emrick LT, Chanprasert S, Craigen WJ, Scaglia F. Mitochondria: role of citrulline and L-arginine supplementation in MELAS syndrome. Int J Biochem Cell Biol. 2014;48:85–91.
56. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004;114:1752–61.
57. Lucotti PC, Setola E, Monti LD, Galluccio E, Costa S, Sandoli EP, Fermo I, Rabaiotti G, Gatti R, Piatti P. Beneficial effects of oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab. 2006;291:E906–12.
58. Beleznai T, Feher A, Spielvogel D, Lansman SL, Bagi Z. Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes. Am J Physiol Heart Circ Physiol. 2011;300:H777–83.
59. Grönros J, Jung C, Lundberg JO, Cerrato R, Ostenson CG, Pernow J. Arginase inhibition restores in vivo coronary microvascular function in type 2 diabetic rats. Am J Physiol Heart Circ Physiol. 2011;300:H1174–81.
60. Okon EB, Chung AWY, Rauniyar P, Padilla E, Tejerina T, McManus BM, Luo H, van Breemen C. Compromised arterial function in human type 2 diabetic patients. Diabetes. 2005;54:2415–23.

Chapter 13
Use of L-Arginine with Growth Hormone-Releasing Hormone (GHRH) and the Endocrine Response

Giulia Brigante and Vincenzo Rochira

Key Points

- Arginine elicits pituitary GH secretion by activating several pathways, the most important of which, in vivo, is the inhibition of SST at hypothalamic level.
- Arginine acts as a GH secretagogue in several species including humans.
- Intravenous L-arginine administration elicits GH secretion in a faster manner compared to oral administration.
- Intravenous L-arginine potentiates the pituitary responsiveness to GHRH resulting in a supramaximal GH release in terms of GH peak and area under the curve when administered together.
- Arginine test is based on intravenous infusion of L-arginine alone and leads to misclassification of GHD due to the high rate of false positivity and poor reproducibility especially in adults.
- GHRH+Arg test is useful for the clinical diagnosis of GHD in both childhood and adulthood, but its results should be interpreted according to other patient's clinical data.
- Several particular conditions (e.g., obesity, HIV infection, congenital estrogen deficiency) might impair the GH response to GHRH+Arg (i.e., biochemical GHD) even in the absence of a truly, clinical GHD.

Keywords L-Arginine • Growth hormone • Growth hormone-releasing hormone plus L-arginine test • GH-IGF-I axis • GH secretagogues • IGF-I • Amino acids

Abbreviations

cAMP	Cyclic AMP
FFA	Free fatty acids
GH	Growth hormone

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GHRH	Growth hormone-releasing hormone
GHRH+Arg	Growth hormone-releasing hormone plus L-arginine
GHRP	Growth hormone-releasing peptide
i.v.	Intravenous
IGF-I	Insulin-like growth factor I
ITT	Insulin tolerance test
SST	Somatostatin

Introduction

L-Arginine is one of the most common natural amino acids that takes part to the structure of the messenger ribonucleic acid (mRNA). In mammals L-arginine is a semiessential or an essential amino acid depending on age.

The endocrinological history of L-arginine started with the discovery of its secretagogue property on GH and its first use by the athletic population in efforts to enhance the exercise-induced growth hormone (GH) response [1].

The GHRH-GH-IGF-I Axis

GH is a peptide hormone synthesized, stored, and secreted by the somatotropic cells of the anterior pituitary gland [2, 3]. GH secretion production is directly stimulated by GH-releasing hormone (GHRH), which is secreted from neurosecretory nerve terminals of the hypothalamic arcuate neurons. GHRH is carried by the hypothalamo-hypophyseal portal system to its target, the GHRH-receptor present on the surface of pituitary somatotropic cells (Fig. 13.1), where gene transcription is activated by cyclic adenosine monophosphate-dependent mechanisms [2, 3].

Besides this positive stimulus, the GH secretion is negatively regulated also by somatostatin (SST), which has a direct inhibitory effect on the GH secretion from the pituitary gland [2, 3]. Moreover, various synthetically produced GH-releasing compounds and the natural hormone ghrelin, secreted by the stomach, may have a role both in increasing the release of GHRH and inhibiting SST action, thereby obtaining a powerful stimulation of GH secretion (Fig. 13.1) [4]. It is known that stress, hypoglycemia, and ingestion of protein (high levels of circulating amino acids) stimulate GH secretion, while high levels of glucose and fat-free acids inhibit it [3]. Several natural (endogenous or exogenous) and synthetic compounds are able to regulate GH secretion [2, 3, 5, 6], and most of them are listed in Table 13.1. Balancing these stimuli, the pituitary somatotropic cells secrete GH in a pulsatile manner and in a circadian rhythm with a maximal release in the second half of the night [2].

GH acts both directly through its own receptor and indirectly through the induced production of insulin-like growth factor I (IGF-I). IGF-I is synthesized both in the liver and in the periphery and is an important mediator of GH actions (Fig. 13.1). It circulates bound to a number of different binding proteins, of which IGFBP-3 is the most important [2, 3]. Circulating IGF-I has been also shown to act as a negative feedback regulator of GH gene expression at the level of the promoter [3, 7].

GH exerts its effects by binding to a single-chain plasma transmembrane glycoprotein receptor. The highest concentration of receptors is in the liver, cartilage, adipose tissue, heart, kidneys, intestine, lungs, pancreas, and skeletal muscle [2, 3]. Considering these localizations, it derives that the actions of GH are to promote longitudinal growth in childhood and to modulate metabolism and body composition [2]. In particular, it promotes glucose production, insulin antagonism, lipolysis, and protein anabolism, acting directly or indirectly through IGF-I [2, 3].

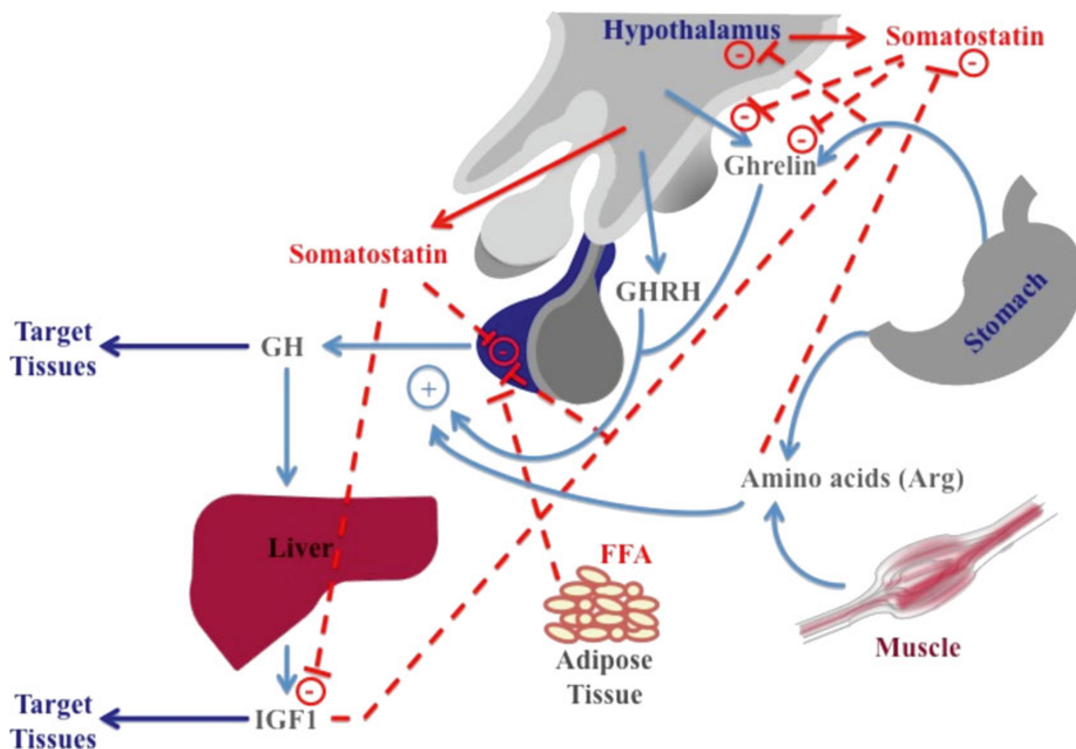

Fig. 13.1 Regulation of growth hormone secretion: the GH-IGF-I axis. *GH* growth hormone, *FFA* free fatty acids, *Arg* l-arginine, *min* minutes

L-Arginine and the GH-IGF-I Axis

Mechanisms of L-Arginine Action on GH-IGF-I Axis: Animal Studies

Amino acids have been pointed out as potent stimulators of GH secretion [5] (Table 13.1). Among them, L-arginine has been considered one of the most effective GH releasers in man [8], and this action is highly conservative since it is present in several species such as baboons [9], rats [10, 11], goats [12], rabbits [13], and pigs [14]. L-Arginine seems to stimulate pituitary GH release through the inhibition of SST secretion [5, 15]. SST is a peptide hormone produced by delta cells of the pancreas and by neurons of the ventromedial nucleus of the hypothalamus (Fig. 13.1). SST inhibits the GH-IGF-I axis both centrally and peripherally at the level of both the pituitary GH release and hepatocyte IGF-I transcription, respectively (Fig. 13.1) [16]. The mechanisms of action by which L-arginine induces GH release, however, have never been definitively clarified.

In Vitro Studies

Several in vitro studies suggest a direct action of L-arginine on somatotropic cells at pituitary level resulting in GH release into the blood (Fig. 13.1). Accordingly, several amino acids are able to increase GH release from primary caprine pituitary somatotropic cells; this direct effect is probably due to increased intracellular calcium concentrations since it can be prevented by adding the calcium

Table 13.1 Main endogenous and exogenous (natural or synthetic) GH secretagogues and inhibitors

	GH secretagogues	GH inhibitors
Endogenous	GHRH	Somatostatin
	Ghrelin	GH
	Endorphins	IGF-I
	Enkephalins	
	Hypoglycemia	
	Vigorous exercise	
Endogenous and exogenous (natural)	L-Dopa	Serum glucose
	L-Arginine	Free fatty acids
	Histidine	
	Lysine	
	Valine	
	Phenylalanine	
	Leucine	
	Methionine	
	Threonine	
	Ornithine	
	Androgens	
	Estrogens	
Exogenous GH	Clonidine	
	Hexarelin	
	GHRP-1	
	GHRP-2	
	GHRP-6	
	Pyridostigmine	
	GHRH analogs (tesamorelin, sermorelin)	

GHRP growth hormone-releasing peptide

antagonist nifedipine [12]. The direct effect of L-arginine on somatotropic cells has been well elucidated in rats [10]. An in vitro study of cultured rat anterior pituitary cells demonstrated that the L-arginine-induced rise of intracellular calcium was due to cell membrane depolarization and was associated to prolactin release [10]. This mechanism might be involved also for the release of other pituitary hormones such as GH and might represent an additional mechanism—other than SST inhibition—by which L-arginine exerts its secretagogue activity [10]. In addition, Adriao et al. demonstrated that the infusion of L-arginine leads to a 2.3-fold increase in pituitary GH mRNA levels within the pituitary of rats sacrificed after L-arginine exposure as well as in cultured anterior pituitary cells of rat in vitro [11]. Thus, direct L-arginine effect on somatotropic cells seems to involve both membrane depolarizations and probably results in the subsequent release of the intracellular GH reserve as well as in an increased GH synthesis due to direct effect on gene transcription [10, 11]. Both of these two mechanisms operate separately with respect to L-arginine-induced inhibition of SST; the latter, in fact, follows a different pathway resulting in an increase in cyclic AMP (cAMP) levels in somatotrophs and subsequent protein kinase A activation [2]. Several other mediators, such as nitric oxide, or changes in the expression of genes like PIT 1 might be recruited in these pathways and might also account for L-arginine-mediated elicitation of GH secretion. It is expected that phosphorylation of pituitary-specific transcriptional factors like PIT 1 should occur, which could result in marked alterations in GH gene transcription [17]. Thus, it is possible that the increase in GH mRNA levels observed after L-arginine administration might be the result of the activation of one of these pathways [11], the real nature of which remains to be determined. Of sure, slight hypoglycemia induced by L-arginine does not explain the rise in serum GH since the fall in serum glucose is too small to induce GH release [5].

In Vivo Studies

Independently of the mechanisms of action, all in vivo studies pointed out that L-arginine is able to stimulate GH secretion through the inhibition of the SST suppressive stimulus at hypothalamic level [15] (Fig. 13.1). Intravenous administration of L-arginine, in fact, is able to increase serum GH in several species in vivo [9–13]. In rats, GH rise after L-arginine infusion is prevented by the administration of antiserum against somatostatin [18]. The action of L-arginine on the SST pathway is further substantiated by the lack of GH increase after L-arginine administration in pigs with transected pituitary stalk [14] as well as in rats with ventromedial nuclei lesion [18]. Again, in both male and female wild-type mice, a bolus injection of L-arginine increases serum GH levels, while this effect was a loss in SST-knockout mice [19].

Apart from the main mechanism by which L-arginine stimulates GH release through reducing endogenous hypothalamic SST tone on the pituitary, other mechanisms involving or not SST have been clarified by in vivo studies in animals. L-Arginine stimulates even the peripheral production of SST and insulin by pancreatic delta and beta cells, respectively. This mechanism, even though less important, could be involved in GH release induced by L-arginine (Fig. 13.1) [16, 19]. Also the oral chronic administration of L-arginine increases, in rats, the pituitary GH mRNA content [20] in the same way as it is observed when the amino acid is acutely administered intravenously [11]. These in vitro and in vivo studies reinforce the role for L-arginine not only as a GH secretagogue but also as a stimulant of GH synthesis.

Role of L-Arginine on GH Secretion in Humans

In the 1960s, it became possible to measure changes of GH plasma levels in response to physiologic stimuli [21], thanks to the development of radioimmunoassay method for the precise determination of human GH [22]. In particular, several studies of the effect of intravenous amino acids on the GH secretion were carried out. It was already known that the intravenous infusion of L-arginine is followed by a notable rise in plasma insulin, but with a slight fall in blood glucose, despite high insulin serum levels. This suggested that, following the amino acid infusion, there might be a release not only of insulin but also of a substance counteracting insulin action on glucose [8, 21]. As GH was considered a possible candidate, the effects of L-arginine (30 g) infusion were investigated discovering that intravenous L-arginine brings to an 11-fold increase in plasma GH other than a rise in plasma insulin [8]. In the meanwhile, another study confirmed that the basic amino acids L-arginine, histidine, and lysine all elicit a clear increase in GH levels, an effect that is independent from blood glucose changes [23]. In particular, a dose of 30 g of L-arginine infused i.v. over a 30-min period resulted to provoke the greatest rise in plasma GH [23]. The subsequent step was to study possible differences in GH secretion induced by L-arginine among healthy subjects and subjects with dwarfism or known hypopituitarism, thus opening the way to the use of L-arginine infusion as a possible alternative to insulin tolerance test (ITT) for the diagnosis of GH deficiency (GHD) in humans [24, 25] (see the following paragraphs for details).

Several studies were also addressed to elucidate how L-arginine enhances GH secretion by investigating in vivo the effects on GH secretion of the combined administration of L-arginine and GHRH. Even using a supramaximal dose (100 mcg) of GHRH, the serum GH response to that GHRH dosage is significantly increased by the L-arginine infusion [15]. This observation suggested that L-arginine stimulates GH by inhibiting endogenous SST rather than by further stimulating endogenous GHRH release, the latter pathway being just overstimulated by a very high dose of GHRH [15]. Moreover, differently from levodopa, ornithine—the active form of L-arginine—was unable to modify

plasma GHRH levels in humans [26]. This is in agreement with the evidence that L-arginine administered after an earlier stimulation of GH secretion by GHRH induces a second pronounced GH rise [27]. Also in vitro data using rat anterior pituitary cells failed to demonstrate a direct influence of L-arginine on GHRH-stimulated GH release: L-Arginine at different doses was not able to stimulate GH release, and when cells were incubated with L-arginine and GHRH, the GH increase was not greater than that in response to GHRH alone. This observation should be due to the absence of SST system in such an in vitro model that prevents L-arginine to exert its effect [15]. All these findings together with evidence coming from the in vitro studies (see previous subheading for details) support the idea that L-arginine acts independently from the GHRH pathway and inhibits endogenous SST activity.

Another interesting aspect of the GH response to L-arginine is that it does not differ between elderly and young subjects differently from what happens for other stimuli [6]. It has been demonstrated that the responsiveness of somatotropic cells to GHRH is reduced in elderly humans but it is totally restored by L-arginine, suggesting an increased somatostatinergic activity in aging and reinforcing the concept that L-arginine acts on GH through SST inhibition [28].

Following studies on the consequences of intravenous L-arginine administration, other investigators focused their attention on the effects of oral L-arginine on GH release. Chronic administration of oral L-arginine for 30 days has been shown to increase resting GH levels in postmenopausal women [29]. Data on the effects of acute administration of oral L-arginine are more controversial. Marcell et al. did not find any GH change after oral L-arginine [30], while several other authors reported an increase of various degrees in serum GH [31, 32]. The data of Collier et al. indicated that the effect of oral L-arginine is not dose dependent. Accordingly, 5 and 9 g of oral L-arginine result in a significant GH response compared to placebo, while 13 g of L-arginine has a substantially lesser effect; the rise in GH occurs 30 min after ingestion, with a peak in GH concentration 60 min after ingestion [32]. The use of a combination of more than one amino acid seems to be more effective than a single amino acid in eliciting GH secretion [31], probably because it ensures at least one amino acid will be absorbed and able to be transported to the hypothalamus. However, oral L-arginine administration, even if effective, results in a less dramatic increase in GH concentrations compared to intravenous administration [32]. Moreover, oral L-arginine induces the GH rise a little later than intravenous L-arginine, with a delay in GH peak of about 10 min [32]. It makes sense considering that intravenous introduction of the bolus is a faster method of introducing L-arginine into the bloodstream.

Summarizing, both intravenous and oral administration of L-arginine in humans increases pituitary GH release by suppressing endogenous SST release at the hypothalamic level, while intravenous L-arginine potentiates the maximal pituitary somatotropic responsiveness to GHRH.

Clinical Use of L-Arginine in the Diagnosis of GH Deficiency in Humans

Considering its capacity to induce GH release, L-arginine was investigated as a diagnostic tool for the detection of GHD. GHD is a condition that has different consequences according to age of onset. When GHD occurs before puberty, the child has a slow or flat rate of growth [33]. In adults GHD leads to impairment in body composition and function, as well as to deranged lipoprotein and carbohydrate metabolism and increased cardiovascular morbidity [34]. GHD can be caused by congenital condition (genetic, associated with brain structural defects, associated with midline facial defects) or acquired (trauma, central nervous system infections, tumors of hypothalamus or pituitary, infiltrative/granulomatous diseases, cranial irradiation, neurosurgery, idiopathic) [35] and shares all the other common causes of hypopituitarism [36].

The diagnosis of GHD is a challenge. Since GH is secreted from anterior pituitary in a pulsatile manner and in a circadian rhythm, the concentration of GH in random samples of serum does not

provide a clear differentiation between the normal and the hyposomatotropic individual [34, 37]. Moreover, the assay of IGF-I per se does not establish the diagnosis of GHD. In fact, normal levels of IGF-I do not exclude GHD as a consequence of significant IGF-I value overlap between normal and GHD subjects [34].

Accordingly, the demonstration of a rise in serum GH after a secretagogue stimulus appears to be the only way to quantify the synthetic and secretory capacity of the somatotropic cells. At present, the ITT remains the diagnostic test of choice [34] and is based on the counterregulatory response carried out by a normal pituitary gland, which secretes GH in response to the severe hypoglycemia induced by insulin intravenous administration. However, ITT should be performed only in experienced endocrine units where the test is performed frequently due to the high risk of severe hypoglycemia, especially in children and older subjects. It is contraindicated in patients with electrocardiographic evidence or history of ischemic heart disease or in patients with seizure disorders; moreover, there is less experience in patients over the age of 60 years [34].

The L-arginine infusion can be considered an innocuous, reliable stimulus of GH secretion in the study of hyposomatotropism, which avoids the undesirable side effects of insulin-induced hypoglycemia [25]. The procedure is standardized [25, 38] and is briefly summarized in Table 13.2. The administration of L-arginine alone can be considered [21, 23, 24], but this test has less established diagnostic value compared to ITT, especially in adults [34]. In fact, when the sensitivity and specificity of L-arginine infusion test are considered in comparison with ITT and other four different tests, L-arginine alone should not be considered a reliable alternative [38]. In particular, L-arginine alone leads to a small increase in serum GH and a low GH peak even in healthy subjects (Fig. 13.2) with an overlap between GHD and normal subjects resulting in misclassification due to the high rate of false-positive [38]. Only in patients with GHD of hypothalamic origin, e.g., those having received irradiation of the hypothalamic-pituitary region, L-arginine alone may be useful [38].

In clinical practice, the diagnostic test based on the infusion of L-arginine alone (Table 13.2) has been abandoned in adults due to the very low diagnostic value [38, 40], but is still used for the diagnosis of GHD in children coupled with a second not supramaximal test (with only one secretagogue administered) and clinically integrates available data (auxological, clinical, radiological) [33, 41]. Another possibility for improving the diagnosis of true GHD is to use more stringent cutoffs or a supramaximal test [41–43].

Table 13.2 Procedure and interpretation of the diagnostic test for GHD based on L-arginine infusion

Provocative test	Dosage (i.v.)	Timing of infusion (after an overnight fast)	Timing of blood sampling (min)	GH peak cutoffs for the GHD diagnosis	
				Children (μg/L)	Adults (μg/L)
L-Arginine	0.5 g/kg of body weight in saline solution	Over 30 min (from −30 to 0 min)	0–30–60[a]–90–120	<10[b]	<10[c]
GHRH+Arg	*L-Arginine*, 0.5 g/kg of body weight in saline solution; *GHRH*, 1 μg/kg	*L-Arginine*, over 30 min (from −30 to 0 min); *GHRH*, after the end of L-arginine infusion	0–15–30–45[a]–60[a]–90–120	<20[d]	<9

[a]Expected timing for GH peak
[b]Several authors consider the cutoff of 7 μg/L as more appropriate; other authors consider GHD for values <5 μg/L and values between 5 and 10 μg/L as a gray area in which partial GHD is possible
[c]This cutoff has poor diagnostic value in adults due to the low sensitivity and specificity of L-arginine test for the diagnosis of GHD in adults [34, 38]
[d]Several authors consider the cutoff of 24 μg/L as more appropriate in order to avoid false negative [39]
GHD growth hormone deficiency, *Arg* L-arginine

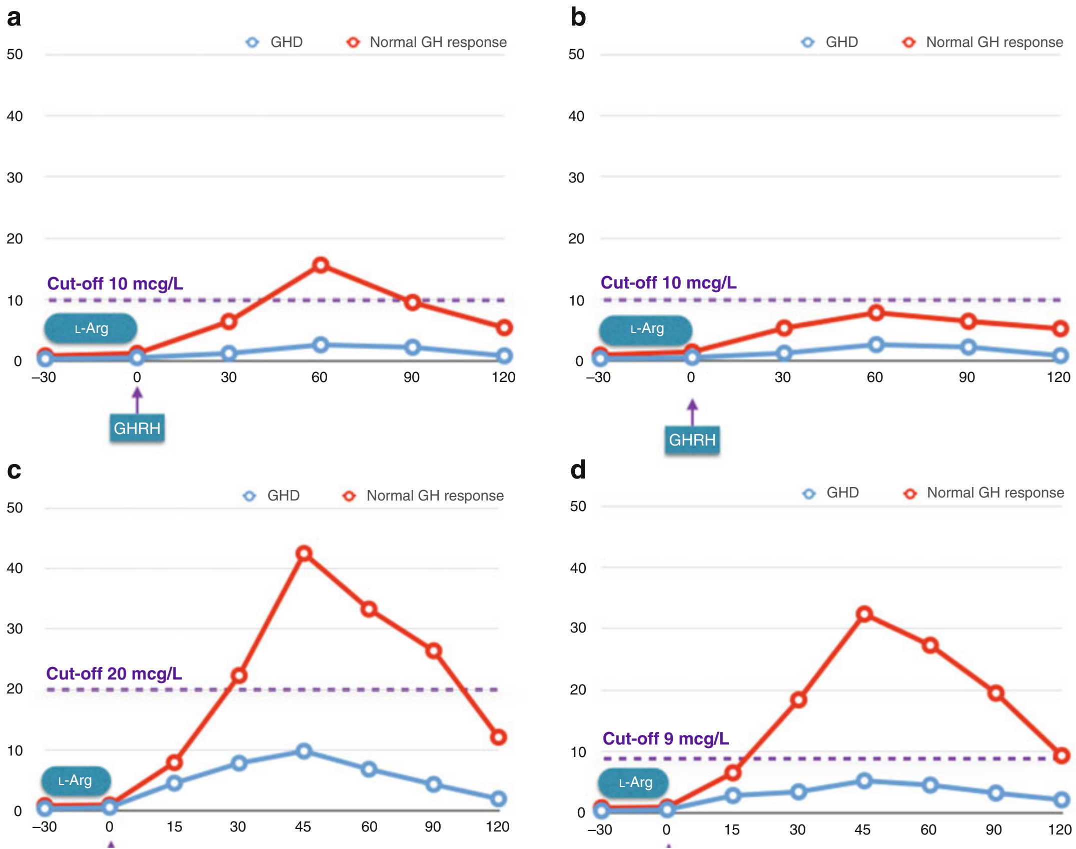

Fig. 13.2 Different patterns of GH response to l-arginine in children (**a**) and adults (**b**) and to GHRH+Arg in children (**c**) and adults (**d**). By increasing the GH peak and the area under the *curve* (through the supramaximal GHRH+Arg test) allows to better split up GHD patients from healthy subjects. The supramaximal stimulation allows also to avoid overlap between these two types of response. In (**b**) this overlap is evident and could lead to GHD misclassification

Clinical Use of Combined GHRH and L-Arginine Infusion in the Diagnosis of GH Deficiency in Humans

Physiological Significance

Considering the difficult management of the gold standard test used for diagnosing GHD in both children and adults, ITT, and the low reliability of L-arginine test, new and more potent stimuli of GH secretion have been progressively tested [42]. Among them, GHRH has been largely studied considering its physiological GH secretagogue action. Anyway, testing with GHRH alone has low value as a provocative test. In fact, the mean GH response to GHRH is not significantly higher than that elicited by ITT, and an absent GH response is possible in some normal adults [44] as well as in children [42]. A marked intraindividual variability of the somatotropic responsiveness to GHRH given alone was demonstrated, probably due to the spontaneous fluctuations in the hypothalamic SST release [45]. So, given alone, GHRH is unable to reliably explore the secretory capacity of somatotropic cells and has no diagnostic reliability [45], similarly to what happens for other GH secretagogues when used alone [38, 40, 42].

It is well recognized that the somatotropic responsiveness to GHRH is strongly potentiated by L-arginine and pyridostigmine, a cholinergic agonist, which both markedly potentiate its GH-releasing activity via concomitant inhibition of hypothalamic SST release [15, 44]. The importance of SST on the regulation of GHRH effect on GH has been clearly shown in animals, in which SST antiserum or hypothalamic deafferentation abolishes somatostatinergic activity on somatotropic cells [14, 18, 19] (see previous paragraph on animal studies for details). The pharmacological removal of SST inhibition allows also a more potent and reproducible stimulatory effect of GHRH on GH secretion in humans resulting in higher serum GH peak and a greater amplitude of the area under the curve of GH secretion after stimulation (Fig. 13.2) [45]. Thus, the combined administration of L-arginine and GHRH (GHRH+Arg) in humans should be considered a supramaximal provocative test for GH since it leads to GH levels higher than those observed after GHRH or L-arginine alone (Fig. 13.2) [6]. By rising the rise of serum GH after the provocative stimulus with a supramaximal testing (with two secretagogues) produces the sharpest separation between GHD patients and healthy subjects since the increased height of the secretive curve allows GH peak obtained from GHD patients to diverge from that of healthy subjects resulting in a better definition of diagnostic cutoffs (Fig. 13.2) [38, 46]. In addition, this test has also the advantage of reducing the intraindividual variability of somatotropic responsiveness to the neurohormone alone [47].

Use of GHRH+Arg in the Diagnosis of GHD

The combined administration of L-arginine and GHRH is safe and provides a strong stimulus to GH secretion and thus could be used as an alternative to the ITT as a test of pituitary GHD in adults [46] and children [6, 39, 48]. In fact, testing with GHRH+Arg has been shown to be one of the most potent stimuli of GH secretion in normal children [42], and later data confirmed this evidence in adults [44]. Because the GHRH+Arg test is generally well tolerated and free of the potentially serious side effect of hypoglycemia, it began to gain a wider use for patients with suspected GHD of pituitary origin, the majority of patients with suspect GHD. Especially in children, this test is more secure and has a better diagnostic value than L-arginine alone in terms of sensitivity and specificity [42] and could be a valid alternative to ITT also for the diagnosis of childhood-onset GHD [39].

Nowadays, even if ITT remains the gold standard, GHRH+Arg test is considered more than simply the most promising alternative. When the diagnostic reliability of ITT and GHRH+Arg test is compared in a large population of hypopituitary adults, the GHRH+Arg test results, at least, as sensitive as ITT, provided that appropriate cutoff limits are considered [46]. The GH response to both tests is positively correlated, but GHRH+Arg is a more potent stimulus of GH secretion than ITT, even in GHD patients [46]. Moreover, the GH response to GHRH+Arg, but not to ITT, is positively associated with IGF-I levels [46]. Also considering the ability of the test to discriminate healthy subjects from GHD, the GHRH+Arg test performs well, as shown by an area under the ROC curve of 0.968 in the study of Biller et al. [38]. All these considerations apply also to children insomuch as GHRH+Arg is now commonly used in the clinic for the diagnosis of childhood-onset GHD [39, 42, 48]. As far as the use of GHRH+Arg diagnosis of GHD is concerned, different cutoffs for serum GH peak should be considered in adult-onset and childhood-onset GHD A cutoff of serum GH peak 9 μg/L is currently considered for the diagnosis of GHD in adults [28, 35, 44, 46], while a cutoff of 20 μg/L is used for children [39, 42, 48] (Table 13.2). The procedure for performing GHRH+Arg test is standardized and is briefly summarized in Table 13.2 together with current interpretation of the results.

Another important point favoring GHRH+Arg as a good test for the diagnosis of GHD is that it has an extremely good safety profile and no contraindications. The most common minor side effect is transient facial flushing induced by GHRH, which occurs in approx 30 % of the subjects tested [45]. Administering 0.5 g/kg of L-arginine hydrochloride over 30 min is basically devoid of side effects;

increasing the speed of the infusion could induce nausea and vomiting [45]. Chronic renal failure is basically the unique contraindication to L-arginine load in humans [49].

Value of GHRH+Arg in Comparison with Other Tests for the Diagnosis of GHD

The biggest advantage of GHRH+Arg compared to the gold standard, ITT, is that it is safer and has less contraindication. Moreover, up to a sixfold difference in GH peak has been demonstrated on different days in healthy adults undergoing ITT, regardless of the degree of hypoglycemia [50]. On the contrary, GHRH+Arg seems to have a high intraindividual reproducibility [38, 47].

Considering other GH secretagogue stimuli, the diagnostic value of clonidine provocative testing has been shown to be very limited [45] and less useful in adults than in children [34]. On the other hand, L-arginine and glucagon alone could be useful but are less discriminatory than ITT [45]. However, the poor within-subject reproducibility of these classic provocative tests is well recognized [44, 50], and they have less established diagnostic value compared to ITT [45].

In a very interesting study, Biller et al. evaluated the relative performance of GHRH+Arg, ITT, L-arginine alone, clonidine, levodopa, and the combination of L-arginine plus levodopa in adults [38]. The overall performance of the GHRH+Arg test, with 95 % sensitivity and 91 % specificity at a GH cutoff of 4.1 μg/L, compares well to ITT, which has an optimal GH cutoff of 5.1 μg/L (96 % sensitivity and 92 % specificity), while the performance of the other tests is much poorer. The greatest diagnostic accuracy is obtained with the ITT and the GHRH+Arg test. There is more overlap between GHD patients and control subjects for L-arginine, levodopa, and L-arginine plus levodopa. Defining test-specific cut points to improve the sensitivity and specificity of these tests, the L-arginine plus levodopa test appears to be a reasonable third choice [38].

Compared to other tests, the GH response to GHRH+Arg also proves to be independent of age [6, 28]. On the contrary, the potentiating effect of pyridostigmine on the GH response to GHRH decreases with aging, and it has been shown that a GHRH+pyridostigmine test distinguishes GHD from normal subjects in young adults but not in elderly subjects [28]. Moreover, pyridostigmine often induces classic cholinergic side effects and is contraindicated in clinical situations such as in elderly subjects and in the presence of arrhythmic heart disease [28]. Also the responsiveness of somatotropic cells to GHRH is known to be reduced in elderly humans, but it is totally restored by L-arginine, suggesting that aging could be characterized by a higher SST tone [28]. Since ITT is contraindicated in elderly subjects because hypoglycemia and the adrenergic response could trigger ischemic attack, GHRH+Arg is the test of choice to distinguish GHD from normal elderly subjects [45]. Finally, compared to ITT, L-arginine alone, clonidine, levodopa, and the combination of L-arginine plus levodopa, the GHRH+Arg test is preferred by the patients, thanks to the lack of side effects [38].

Potential Limitations of GHRH+Arg Testing

Several limitations of the GHRH+Arg test pose several troubles concerning a correct diagnosis of GHD and do not allow to discriminate with certainty true GHD from other clinical or laboratoristic conditions resulting in biochemical GHD (i.e., an abnormally decreased response of GH to GHRH+Arg without certain clinical correlates of hypothalamic-pituitary disease). All these limits might induce the clinician to GHD misclassification. However, if the results of biochemical testing GH secretion are

coupled with clinical data and enclosed into the appropriate clinical context, the risk of misclassification could be reduced [33–35, 43]. The main limitations of the GHRH+Arg test are due to (1) cutoff definition, (2) testing variables, (3) sexual dimorphism and patient's hormonal status, and (4) special clinical situations.

Limitations Due to Cutoff Definition

The use of cutoffs or thresholds for normal GH response remains arbitrary since several differences have been found in different settings due to variance in hormonal assays used, population heterogeneity, lack of reproducibility in the same patient, and several other factors [38, 42]. When a GH response to GHRH+Arg is slightly above the diagnostic cutoff, the outcome remains doubtful and might be related to a condition of partial GHD [51]; often it needs to be confirmed by a subsequent test. Again, the results based on the cutoffs proposed for the GHD diagnosis should be ever interpreted according with all other patient's clinical data [33–35, 43].

Testing Variables

Several factors could impact on the results of the GHRH+Arg test. The setting of GHRH+Arg procedures may differ according to some patient's components (e.g., inadequate overnight fasting), laboratory variables (e.g., inaccurate GH assay), and testing problems (e.g., inappropriate timing for secretagogues infusion or for blood sampling). Furthermore, practical problems could compromise the possibility to run a test with GHRH+Arg, for example, GHRH is currently unavailable for clinical use in the USA, and, in this case, glucagon test could be considered as alternative to ITT [52].

Sexual Dimorphism and Patient's Hormonal Status

Another possible confounding factor in the evaluation of GH response after GHRH+Arg test is that a sex difference in the response to L-arginine alone has been reported [21]. As a consequence of differences in serum estrogens, in females a greater responsiveness to L-arginine occurs with respect to men and during midcycle compared to menstrual period [21]. Anyway, this gender difference seems lost when L-arginine is used in combination with GHRH [53, 54]. Furthermore, the response to GHRH+Arg test is slightly different in aging [28, 46]. In addition several other hormonal deficits or hormonal therapies might influence the GH response to GHRH+Arg. It is known that mainly sex steroids but also thyroid hormones and adrenal steroids alter the amount of the synthesized and stored GH [35].

Special Clinical Situations

Several clinical conditions are associated to an impaired pituitary GH reserve; some of them are very common and should be considered in clinical practice.

Obesity

The confounding effect of obesity on the evaluation of somatotropic function needs particular attention owing to its common occurrence. It is well known that somatotropic function is negatively associated with body mass, and, in fact, both spontaneous and stimulated GH secretion is clearly reduced in obese patients [45]. It was also demonstrated that the mean GH response to GHRH+Arg is negatively associated with BMI, being markedly reduced in obese patients [55]. In a considerable percentage of obese patients, the GH response to GHRH+Arg is as impaired as that in hypopituitaric patients with GHD [55]. Taking into account the fact that hypopituitaric patients are often overweight, it is strongly recommended that adult GHD is suspected in the appropriate clinical context and considering more restrictive GH cutoff values in obese and overweight subjects. Thus, in the overweight population, the highest specificity and sensibility of GHRH+Arg test were found using a peak GH cutoff point of 8.0 μg/L, while in the obese population, the cutoff point is settled to the lower value of 4.2 μg/L [56] (Table 13.3). Applying these specific BMI-related cutoffs, the GHRH+Arg test can be considered a reliable tool for the diagnosis of adult GH deficiency in lean, overweight, and obese patients.

Transition Period

Children with a diagnosis of childhood-onset GHD need to be retested for the confirmation of GHD even during adulthood [34, 57]. In the transition period, this retesting should be performed after withdrawal of GH treatment, and GHRH+Arg is a valid test useful to confirm the persistence or not of GHD [58]. The response to GHRH+Arg in late adolescents and young adults is very close to that of children [58], and the cutoffs of 19 mg/L for the diagnosis of GHD are similar [57, 58] (Table 13.3).

Hypothalamic Defects

Because GHRH acts directly on the pituitary, its administration both alone and in combination with L-arginine could induce clear GH response only in hypopituitaric patients with GHD owing to hypothalamic GHRH defect [59], leading to false-negative results. This possibility seems unlikely, however. In fact, there is a strong positive association between the GH peak after GHRH+Arg and ITT [45], and it has been demonstrated that the integrity of the hypothalamus-pituitary connection (evaluated by magnetic resonance imaging) is essential for GHRH+Arg as well as for ITT to express their GH-releasing activity [48]. It means that the combination of GHRH+Arg is able to test the functionality of both the hypothalamic and the pituitary level of somatotropic axis.

Table 13.3 Cutoffs for GH peak in some particular clinical conditions

Clinical condition	GH peak cutoff (μg/L)	Peculiar characteristics
Obesity	<4.2	Free fatty acids exhibit an inhibitory effect on GH secretion; visceral adiposity should be evaluated and/or taken into account
Overweight	<8.0	Visceral adiposity should be evaluated and/or taken into account
Transition period	<19	Response to GHRH+Arg is quite similar to that of children; transition involves a period until 6–7 years after achievement of final height
HIV infection	<7.5	Consider BMI and visceral adiposity; usually GHD is only biochemical and does not need treatment; probably a subset of these patients has true GHD (more researches needed)

HIV Infection

The secretory response of GH is impaired in HIV-infected patients [60]. Biochemical GHD after GHRH+Arg is more frequent in HIV-infected males than in HIV-infected females and is present in about 20–30 % of all patients [61]. The real nature of this defective response to GH provocative stimuli is still not defined, but several factors including visceral obesity due to HIV-related lipodystrophy, the effects of antiretroviral drugs, and the HIV infection, per se, all contribute to biochemical GHD [60, 61]. In this particular setting, the cutoffs established for the adult population should not be used, and more appropriate cutoffs (7.5 μg/L), accounting also for visceral fat accumulation and the HIV condition, have been proposed [60, 61] (Table 13.3).

Other Rare Clinical Conditions

The GH response to GHRH+Arg is quite abolished in patients with congenital aromatase deficiency and is not restored by estrogen replacement treatment [62]. Notwithstanding the presence of severe GHD at the provocative test, these patients are tall [62], similarly to what happens in children with hypopituitarism due to the interruption of the pituitary stalk [63] and in some children operated for craniopharyngioma [63, 64].

Who to Test?

By considering all the issues previously reported, GHRH+Arg should not be performed on a large scale or as a screening, but it should be offered to patients according with the appropriate clinical context [28, 35]. In childhood, the usual circumstance in which GH status needs to be assessed is the child with standing height SD score below −2 and a growth velocity below the 10–25th centile in whom other causes of poor growth have been excluded [33, 43, 59]. In adults, an evaluation for GHD should be considered only in patients with evidence of hypothalamic-pituitary disease or with childhood onset of GHD and in subjects who have received cranial irradiation or a brain injury [28, 35].

In some particular conditions, such as HIV infection, GHRH+Arg should not be performed outside research protocols since GH replacement treatment is still not indicated in these patients, even though a subgroup of them could be truly GH deficient and might probably benefit from GH therapy [60, 61]. Accordingly, the finding of isolated biochemical GHD is not sufficient for the diagnosis of true GHD because it needs to be coupled with a documented pituitary or hypothalamic disease [28, 35].

Other clinical conditions such as idiopathic adult GHD [65] and partial GHD [51] can be established through GHRH+Arg, but, even though the usefulness of GHRH+Arg for detecting these conditions is undoubted, its clinical utility remains uncertain since these conditions have not been classified as true clinical entities [51, 65].

Conclusions

Over the past 40 years, a vast amount of information has been accrued relating to the endocrinological action of L-arginine. All these information allowed to define the clinical use of L-arginine and to better know the pathophysiology of the GH-IGF-I axis. In particular, the studies on this issue allowed to

standardize a useful test for the investigation of GH secretion that is largely used in clinic. Considerable further study will be required to solve some unsolved issue that still remains. Among them, the real effect of oral L-arginine administration and the individual variation in the hormonal response to L-arginine need to be better defined.

References

1. Di Luigi L, Guidetti L, Pigozzi F, et al. Acute amino acids supplementation enhances pituitary responsiveness in athletes. Med Sci Sports Exerc. 1999;31:1748–54.
2. Casanueva FF. Physiology of growth hormone secretion and action. Endocrinol Metab Clin N Am. 1992;21:483–517.
3. Khoo B, Grossman A. Normal physiology of ACTH and GH release in the hypothalamus and anterior pituitary in man. In: De Groot LJ, editor. Section of pituitary disease and neuroendocrinology. Online Endotext. www.endotext.com. Updated February, 2011. Accessed 28 Mar 2014.
4. van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev. 2004;25:426–57.
5. Reichlin S. Regulation of somatotropic hormone secretion. In: Knobil E, Sawyer WH, editors. Handbook of physiology. Part II: The pituitary gland and its neuroendocrine control. Washington, DC: American Physiological Society; 1974. p. 405–8.
6. Ghigo E, Bellone J, Mazza E, et al. L-Arginine potentiates the GHRH but not the pyridostigmine-induced GH secretion in normal short children: further evidence for a somatostatin suppressing effect of L-arginine. Clin Endocrinol. 1990;32:763–7.
7. Romero CJ, Pine-Twaddell E, Sima DI, et al. Insulin-like growth factor 1 mediates negative feedback to somatotroph GH expression via POU1F1/CREB binding protein interactions. Mol Cell Biol. 2012;32:4258–69.
8. Merimee TJ, Lillicrap DA, Rabinowitz D. Effect of L-arginine on serum-levels of human growth-hormone. Lancet. 1965;2:668–70.
9. Stewart JK, Koerker DJ, Goodner CJ. Effects of branched chain amino acids on spontaneous growth hormone secretion in the baboon. Endocrinology. 1984;115:1897–900.
10. Villalobos C, Nunez L, Garcia-Sancho J. Mechanisms for stimulation of rat anterior pituitary cells by L-arginine and other amino acids. J Physiol. 1997;502:421–31.
11. Adrião M, Chrisman CJ, Bielavsky M, Olinto SC, Shiraishi EM, Nunes MT. L-Arginine increases growth hormone gene expression in rat pituitary and GH3 cells. Neuroendocrinology. 2004;79:26–33.
12. Ohata Y, Maruyama Y, Katoh K, Sasaki Y. Growth hormone release induced by an amino acid mixture from primary cultured anterior pituitary cells of goat. Domest Anim Endocrinol. 1997;14:99–107.
13. Mclntyre HB, Odell WD. Physiological control of growth hormone in the rabbit. Neuroendocrinology. 1974;16:8–21.
14. Anderson LL, Ford JJ, Klindt J, Molina JR, Vale WW, Rivier J. Growth hormone and prolactin secretion in hypophysial stalk-transected pigs as affected by growth hormone and prolactin-releasing and inhibiting factors. Proc Soc Exp Biol Med. 1991;196:194–202.
15. Alba-Roth J, Müller OA, Schopohl J, von Werder K. L-Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. J Clin Endocrinol Metab. 1988;67:1186–9.
16. Murray RD, Kim K, Ren SG, Chelly M, Umehara Y, Melmed S. Central and peripheral actions of somatostatin on the growth hormone-IGF-I axis. J Clin Invest. 2004;114:349–56.
17. Lin C, Lin SC, Chang CP, Rosenfeld MG. Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. Nature. 1992;360:765–8.
18. Mori T, Inoue S, Egawa M, Takamura Y, Minami S, Wakabayashi I. Impaired growth hormone secretion in VMH lesioned rats. Int J Obes Relat Metab Disord. 1993;17:349–53.
19. Córdoba-Chacón J, Gahete MD, Pozo-Salas AI, Castaño JP, Kineman RD, Luque RM. Endogenous somatostatin is critical in regulating the acute effects of L-arginine on growth hormone and insulin release in mice. Endocrinology. 2013;154:2393–8.
20. de Castro Barbosa T, Lourenço Poyares L, Fabres Machado U, Nunes MT. Chronic oral administration of L-arginine induces GH gene expression and insulin resistance. Life Sci. 2006;79:1444–9.
21. Merimee TJ, Rabinowtitz D, Fineberg SE. L-Arginine-initiated release of human growth hormone. Factors modifying the response in normal man. N Engl J Med. 1969;280:1434–8.
22. Glick SM, Roth J, Yalow RS, Berson SA. Immunoassay of human growth hormone in plasma. Nature. 1963;199:784–7.

23. Knopf R, Conn J, Fajans S, Floyd J, Guntsche E, Rull J. Plasma growth hormone response to intravenous administration of amino acids. J Clin Endocrinol Metab. 1965;25:1140–4.
24. Merimee TJ, Rabinowitz D, Riggs L, Burgess JA, Rimoin DL, McKusick VA. Plasma growth hormone after L-arginine infusion. Clinical experiences. N Engl J Med. 1967;276:434–9.
25. Parker ML, Hammond JM, Daughaday WH. The L-arginine provocative test: an aid in the diagnosis of hyposomatotropism. J Clin Endocrinol Metab. 1967;27:1129–36.
26. Brion DE, Donnadieu M, Liapi C, et al. Plasma growth hormone releasing factor levels in children: physiological and pharmacologically induced variations. Horm Res. 1986;24:116–20.
27. Page MD, Dieguez C, Valcavi R, Edwards C, Hall R, Scanlon MF. Growth hormone (GH) responses to L-arginine and L-dopa alone and after GHRH pretreatment. Clin Endocrinol (Oxf). 1988;28:551–8.
28. Ghigo E, Arvat E, Gianotti L, et al. Human aging and the GH-IGF-I axis. J Pediatr Endocrinol Metab. 1996;3:271–8.
29. Blum A, Cannon 3rd RO, Costello R, Schenke WH, Csako G. Endocrine and lipid effects of oral L-arginine treatment in healthy postmenopausal women. J Lab Clin Med. 2000;135:231–7.
30. Marcell TJ, Taaffe DR, Hawkins SA, et al. Oral L-arginine does not stimulate basal or augment exercise-induced GH secretion in either young or old adults. J Gerontol A Biol Sci Med Sci. 1999;54:M395–9.
31. Isidori A, Lo Monaco A, Cappa M. A study of growth hormone release in man after oral administration of amino acids. Curr Med Res Opin. 1981;7:475–81.
32. Collier SR, Casey DP, Kanaley JA. Growth hormone responses to varying doses of oral L-arginine. Growth Horm IGF Res. 2005;15:136–9.
33. Growth Hormone Research Society. Consensus guidelines for the diagnosis and treatment of growth hormone (GH) deficiency in childhood and adolescence: summary statement of the GH Research Society. GH Research Society. J Clin Endocrinol Metab. 2000;85:3990–3.
34. Ghigo E, Aimaretti G, Corneli G. Diagnosis of adult GH deficiency. Growth Horm IGF Res. 2008;18:1–16.
35. Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Shalet SM, Vance ML, Endocrine Society's Clinical Guidelines Subcommittee, Stephens PA. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2006;91:1621–34.
36. Schneider HJ, Aimaretti G, Kreitschmann-Andermahr I, Stalla GK, Ghigo E. Hypopituitarism. Lancet. 2007;369:1461–70.
37. Utiger RD, Parker ML, Daughaday WH. Studies on human growth hormone. I. A radio-immunoassay for human growth hormone. J Clin Invest. 1962;41:254–61.
38. Biller BM, Samuels MH, Zagar A, et al. Sensitivity and specificity of six tests for the diagnosis of adult GH deficiency. J Clin Endocrinol Metab. 2002;87:2067–79.
39. Maghnie M, Cavigioli F, Tinelli C, et al. GHRH plus L-arginine in the diagnosis of acquired GH deficiency of childhood-onset. J Clin Endocrinol Metab. 2002;87:2740–4.
40. Hazem A, Elamin MB, Malaga G, et al. The accuracy of diagnostic tests for GH deficiency in adults: a systematic review and meta-analysis. Eur J Endocrinol. 2011;165:841–9.
41. Murray PG, Clayton PE. Disorders of growth hormone in childhood. In: De Groot LJ, editor. Section of pediatric endocrinology. Online Endotext. www.endotext.com. Updated December, 2013. Accessed 28 Mar 2014.
42. Ghigo E, Bellone J, Aimaretti G, et al. Reliability of provocative tests to assess growth hormone secretory status. Study in 472 normally growing children. J Clin Endocrinol Metab. 1996;81:3323–7.
43. Stanley T. Diagnosis of growth hormone deficiency in childhood. Curr Opin Endocrinol Diabetes Obes. 2012;19:47–52.
44. Aimaretti G, Baffoni C, DiVito L, et al. Comparisons among old and new provocative tests of GH secretion in 178 normal adults. Eur J Endocrinol. 2000;142:347–52.
45. Ghigo E, Aimaretti G, Arvat E, Camanni F. Growth hormone-releasing hormone combined with L-arginine or growth hormone secretagogues for the diagnosis of growth hormone deficiency in adults. Endocrine. 2001;15:29–38.
46. Aimaretti G, Corneli G, Razzore P, et al. Comparison between insulin-induced hypoglycemia and growth hormone (GH)-releasing hormone + L-arginine as provocative tests for the diagnosis of GH deficiency in adults. J Clin Endocrinol Metab. 1998;83:1615–8.
47. Valetto MR, Bellone J, Baffoni C, et al. Reproducibility of the growth hormone response to stimulation with growth hormone-releasing hormone plus L-arginine during lifespan. Eur J Endocrinol. 1996;135:568–72.
48. Maghnie M, Salati B, Bianchi S, et al. Relationship between the morphological evaluation of the pituitary and the growth hormone (GH) response to GH-releasing hormone plus L-arginine in children and adults with congenital hypopituitarism. J Clin Endocrinol Metab. 2001;86:1574–9.
49. Underwood L, Van Wyk J. In: Wilson JD, Foster DW, editors. Williams textbook of endocrinology. Philadelphia: Saunders; 1992.
50. Vestergaard P, Hoeck HC, Jakobsen PE, Laurberg P. Reproducibility of growth hormone and cortisol responses to the insulin tolerance test and the short ACTH test in normal adults. Horm Metab Res. 1997;29:106–10.

51. Murray RD, Bidlingmaier M, Strasburger CJ, Shalet SM. The diagnosis of partial growth hormone deficiency in adults with a putative insult to the hypothalamo-pituitary axis. J Clin Endocrinol Metab. 2007;92:1705–9.
52. Kargi AY, Merriam GR. Testing for growth hormone deficiency in adults: doing without growth hormone-releasing hormone. Curr Opin Endocrinol Diabetes Obes. 2012;19:300–5.
53. Qu XD, Gaw Gonzalo IT, Al Sayed MY, et al. Influence of body mass index and gender on growth hormone (GH) responses to GH-releasing hormone plus L-arginine and insulin tolerance tests. J Clin Endocrinol Metab. 2005;90:1563–9.
54. Colao A, Di Somma C, Savastano S, et al. A reappraisal of diagnosing GH deficiency in adults: role of gender, age, waist circumference, and body mass index. J Clin Endocrinol Metab. 2009;94:4414–22.
55. Maccario M, Valetto MR, Savio P, et al. Maximal secretory capacity of somatotrope cells in obesity: comparison with GH deficiency. Int J Obes Relat Metab Disord. 1997;21:27–32.
56. Corneli G, Di Somma C, Baldelli R, et al. The cut-off limits of the GH response to GH-releasing hormone-arginine test related to body mass index. Eur J Endocrinol. 2005;153:257–64.
57. Gasco V, Corneli G, Beccuti G, et al. Retesting the childhood-onset GH-deficient patient. Eur J Endocrinol. 2008;159 Suppl 1:S45–52.
58. Corneli G, Di Somma C, Prodam F, et al. Cut-off limits of the GH response to GHRH plus L-arginine test and IGF-I levels for the diagnosis of GH deficiency in late adolescents and young adults. Eur J Endocrinol. 2007;157:701–8.
59. Shalet SM, Toogood A, Rahim A, Brennan BM. The diagnosis of growth hormone deficiency in children and adults. Endocr Rev. 1998;19:203–23.
60. Zirilli L, Orlando G, Carli F, et al. GH response to GHRH plus L-arginine is impaired in lipoatrophic women with human immunodeficiency virus compared with controls. Eur J Endocrinol. 2012;166:415–24.
61. Brigante G, Diazzi C, Ansaloni A, et al. Gender differences in GH response to GHRH+ARG in lipodystrophic patients with HIV: a key role for body fat distribution. Eur J Endocrinol. 2014;170:685–96.
62. Rochira V, Zirilli L, Maffei L, et al. Tall stature without growth hormone: four male patients with aromatase deficiency. J Clin Endocrinol Metab. 2010;95:1626–33.
63. Den Ouden DT, Kroon M, Hoogland PH, Geelhoed-Duijvestijn PH, Wit JM. A 43-year-old male with untreated panhypopituitarism due to absence of the pituitary stalk: from dwarf to giant. J Clin Endocrinol Metab. 2002;87:5430–4.
64. Faustini-Fustini M, Balestrieri A, Rochira V, Carani C. The apparent paradox of tall stature with hypopituitarism: new insights from an old story. J Clin Endocrinol Metab. 2003;88:4002–3.
65. Melmed S. Idiopathic adult growth hormone deficiency. J Clin Endocrinol Metab. 2013;98:2187–97.

Chapter 14
Serum L-Arginase in Healthy Subjects and Nitric Oxide

Keiki Ogino and Kei Takemoto

Key Points

- Concentration of L-arginase in serum increases in various diseases including carcinoma and liver damages. Therefore, measurement of L-arginase in healthy subject is important to diagnose and monitor diseases.
- Traditionally, enzymatic activity assay was used for evaluation of serum L-arginase. Recently, enzyme-linked immunosorbent assay specific for L-arginase enabled more accurate determination.
- The concentration of serum L-arginase in healthy humanity is ng/mL orvder.
- Hemolysis and storage condition may influence L-arginase measurement.
- L-Arginase activity or concentration in serum is increased in various diseases. There are many factors that correlate with L-arginase concentration but under discussion due to the lack of investigation.
- Increasing L-arginase may affect the function of vascular endothelium because L-arginase competes with nitric oxide synthase as consumer of L-arginine.

Keywords Enzyme-linked immunosorbent assay • Hemolysis • Oxidative stress • Carcinoma • Diabetes mellitus

Abbreviations

3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy-2′-deoxyguanosine
AFP	Alpha-fetoprotein
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
CDL	Choledocholithiasis

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EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FENO	Fractional exhaled nitric oxide
FVE1%	Percent predicted forced expiratory volume in 1 second
HbA1c	Hemoglobin A1c
HCC	Hepatocellular carcinoma
HDL-C	High-density lipoprotein cholesterol
hs-CRP	High-sensitivity C-reactive protein
IgE	Immunoglobulin E
LC	Liver cirrhosis
LDH	Lactate dehydrogenase
LDL-C	Low-density lipoprotein cholesterol
NO	Nitric oxide
NOS	Nitric oxide synthase
RBC	Red blood cell
SNP	Single nucleotide polymorphism
WBC	White blood cell

Introduction

Blood flow is essential for human to maintain the life and also a very important biosample for physicians and clinical researchers to observe the condition of the human body. The reasons why investigating human serum is important are as follows: (**1**) human serum is easy to collect, (**2**) human serum contains various chemicals or biomarkers suitable for diagnosis, and (**3**) huge quantities of clinical test protocols using serum are already established and even automated.

In the past 60 years, L-arginase elevation in serum has been in interest in the aspect of biomarker for diseases, especially carcinoma and liver injuries. Also, L-arginase was quantified in patients who had been undergone partial liver transplantation, and it was concluded that monitoring of L-arginase concentration is useful for evaluation whether the operation was successful or not [1]. L-Arginase is expected to be a better marker indicating hepatic injury, because traditional factors including ALT, AST, and LDH are expressed in various extrahepatic tissues [2].

L-Arginase catalyzes the reaction of L-arginine to urea and ornithine, and is the core enzyme in urea cycle [3]. Two isoforms, namely L-arginase I and L-arginase II, are known. L-Arginase I is expressed mainly in the liver, the so-called "liver-type L-arginase." L-Arginase II is mainly expressed in extrahepatic organs, particularly in the kidney and prostate. Relationship between L-arginase and NOS has been noted for two reasons. Firstly, L-arginase may compete against NOS to decrease the production of NO, because the substrate is common, namely L-arginine. Secondly, L-arginine is converted to citrulline and nitric oxide, and citrulline is the source of proline and polyamine synthesis, and causes airway remodeling and vascular dysfunction.

Therefore, for these reasons, it is very important to evaluate the state of L-arginase in serum from healthy subjects to compare with patients. However, currently, serum L-arginase concentration in healthy subjects is not widely investigated and also not employed as clinical test item. In this article, current status of L-arginase in serum will be summarized, including the methodology of measurement, retrieved value of L-arginase concentration, and correlation with other intrinsic factors. Also, mutual intervention between L-arginase and NOS via L-arginine, their common substrate, will be described.

Measurement Methods of Serum L-Arginase

Today, measurement of human serum L-arginase is classified into two following principles: (1) determination of enzymatic activity and (2) direct quantification of the concentration of L-arginase protein. In general, almost determination of serum L-arginase had been evaluated by its enzyme activity. Both of the two metabolites, namely, ornithine and urea, were used to determine L-arginase activity with photometrical methods [4, 5], and commercial colorimetric assay kit is available. Radioisotope has also been used to determine L-arginase activity. In this case, [^{14}C-guanidino] L-arginine was converted to [^{14}C] urea, further metabolized to $^{14}CO_2$ by urease, and finally detected as $Na_2{}^{14}CO_3$ [6].

However, determination of L-arginase activity has some difficulties. In most cases, the result is shown as arbitrary unit (e.g., micromole released ornithine or urea per minute). Removal of urea by dialysis or ammonium sulfate precipitation may be required, since serum contains intrinsic urea (normally 80–200 mg/L). Additionally, the prediction of L-arginase quantity may be difficult on the ground of enzymatic activity.

Today, ELISA kits for both L-arginases I and II are commercially available from a few manufacturers. By using these kits (according to the manufacturer's instruction), L-arginase I can be detectable at ng/mL order. Also, ELISA system can be established by researcher themselves, because antibodies against human L-arginase I and II are widely available. About 20 years ago, the earliest ELISA system was developed using recombinant or purified human L-arginase [2, 7]. Other research groups also established their ELISA/EIA systems independently [8–10]. Reliability of individual assays was sufficiently validated.

Our laboratory also developed a sandwich ELISA system [11]. Briefly, recombinant L-arginase I protein was retrieved from *Escherichia coli*, and purified L-arginase I was immunized to rabbits to raise polyclonal antibodies. New ELISA system was validated with intra- and inter-assay coefficient of variation, stability at room temperature at 4 °C, repeated freeze-thaw stability, and dilution linearity. Additionally, the potential of original ELISA kit was compared with existing commercial kit (Hycult Biotech). Minimum and maximum detection concentration was 0.085 and 500 ng/mL, respectively, superior to the commercial kit (0.4–100 ng/mL). However, the mean results were quite different between two ELISA systems (20.3 ± 4.7 by commercial and 4.7 ± 0.2 ng/mL by independent ELISA, respectively), provably due to difference of the nominal amount of L-arginase standard. Meanwhile, result of Spearman's correlation was almost overlapped between commercial and our original ELISA.

ELISA would become a mainstream method for evaluation of L-arginase in serum, because accurate concentration can be calculated and required serum volume is smaller than traditional L-arginase enzymatic activity assay.

Factors That Affect During Measurement for L-Arginase ELISA

The measured value of L-arginase concentration is strongly affected by hemolysis, because L-arginase is also expressed in human erythrocytes. The effect of hemolysis on L-arginase ELISA was investigated in the earliest report [2]. By "spike-and-recovery" test of hemoglobin, measured "serum L-arginase" value increased linearly, up to 300 mg/L hemoglobin contamination. The literature concluded that hemolysis should be avoided, while it is also proposed that measurement of hemoglobin concentration is the aid for correcting the effect of hemolysis. Meanwhile, the same report concluded that subvisible hemolysis (~100 ng/mL hemoglobin) would not be exceeded basal activity. In sickle cell anemia patients, serum L-arginase level was associated with total bilirubin, indirect bilirubin, and AST [12]. These factors are the biomarker of hemolysis.

However, even if hemolysis is unavoidable, the accurate concentration derived from serum itself would be measurable. It was revealed that the ratio between hemoglobin concentration and L-arginase concentration which originates in erythrocyte was almost constant, and hemoglobin concentration can be quantified easily by measuring peroxidase-like activity [10]. Moreover, an alternative spliced L-arginase I variant is discovered in erythrocyte [13]. A noticeable feature of the variant form is insertion of 24 nucleotides (8 amino acids) without elimination of enzymatic activity. The hemolysis problem could be resolved with an ELISA system that is capable of distinguishing "liver-type" L-arginase I and its erythrocyte variant more strictly.

Additionally, the temperature and transporting condition should be taken in consideration. Our previous report indicated that measurement of L-arginase concentration was not influenced by storage of serum at room temperature or 4 °C for 24 h [11]. Meanwhile, a previous report mentioned about instability of L-arginase in serum [7]. The L-arginase activity attenuated immediately (~3 days) when the serum was stored at 20 °C or 37 °C. Even at 4 °C, the report indicated that serum can be stored safely for only 1 or 2 days [7]. Additionally, half-life of liver-type L-arginase in blood was estimated to be 1 h [1] or 5–6 h [2]. In conclusion, concentration of L-arginase should be determined, or serum samples should be frozen, as quickly as possible. Otherwise, "cold-chain" transport system may be necessary to avoid loss of L-arginase.

Concentration of L-Arginase in Healthy Subjects

The measured concentrations of L-arginase by ELISA in healthy mankind are summarized in Table 14.1. The results somewhat vary among the investigations, but range is commonly ng/mL order. Although the reason for the inter-measurement difference is unclear, diversity among the experimental groups may affect measurement values (see next section). According to a previous data that was retrieved from 130 healthy Japanese workers, the minimum and maximum value was 0.94 and 108.1 ng/mL, respectively [20]. Taking the report into consideration, all of these values retrieved previously would be rational.

Table 14.1 L-Arginase concentration by enzyme-linked immunosorbent assay in healthy human serum or plasma

	L-Arginase concentration (ng/mL)						
Origin	Total	*n*	Male	*n*	Female	*n*	References
Plasma	51 ± 0.3	143					[7]
Serum	31.79 ± 14.66	58					[9]
Serum	20 ± 6	9					[14]
Serum	14.7	28					[12]
Serum	9.97 ± 4.91	24					[15]
Serum	32.6 ± 22.3	278	32.5 ± 22.4	142	32.6 ± 22.2	136	[16]
Serum	27.2 ± 12.9	30					[17]
Serum	58 ± 41	33					[18]
Serum	21.9 ± 9.2	19					[19]
Serum[a]	20.3 ± 0.7	721	23.6 ± 1.1	276	18.2 ± 1	445	[11]
Serum[b]	4.7 ± 0.2	721	4.8 ± 0.2	276	4.7 ± 0.3	445	[11]
Serum	14.6	130	11.6	57	14	73	[20]
Serum	24.6 ± 27.6	15					[21]

Concentration of L-arginase in healthy humanity was determined by enzyme-linked immunosorbent assay (ELISA). Some studies investigated the value in male and female separately. Data are represented as mean (±SD)

[a]Measured by commercially available ELISA kit

[b]Measured by original ELISA kit

Table 14.2 L-Arginase enzymatic activity in healthy human serum or plasma

Origin	L-Arginase activity	Unit	*n*	Reference
Serum	9.48 ± 2.13	U/L	50	[22]
Serum	5.2 ± 2.3	U/L	65	[23]
Serum	33.64 ± 16.19	U/L	25	[24]
Serum	80.1 ± 29.37	IU/L	30	[25]
Serum	5.6	U/L[a]	90	[26]
Serum	6.9	U/L[a]	29	[27]
Serum	5.7 ± 2.4	U/L	70	[28]
Serum	14 ± 1	mU/mL	19	[6]
Serum	3.4 ± 0.28	U/L	22	[29]
Serum	2.18	U/L	15	[30]
Plasma	18.7 ± 10.62	U/g protein	22	[31]

[a]Data are shown as median. Other data are shown by mean (±SD)
Enzymatic activity of L-arginase was measured by various methods. Note that data does not reflect the amount of enzyme and the unit (U or IU) is arbitrary (typically, amount of enzymatic metabolites/min)

However, at present, normal concentration range of serum L-arginase has not been established yet. Since ELISA is comparatively novel technique, there is a lack of cases, especially systematic and extensive surveys. Additionally, in most studies, the concentration of L-arginase in healthy human beings was calculated to recruit sorely as control toward patients. Therefore, the status (e.g., age, case number, the ratio of male/female) of control group is very diverse. Further inclusive investigation that is targeted for healthy human may be needed to determine normal range and to use as a clinical parameter. Also, information about L-arginase II concentration in human serum is not available.

Also, the values based on enzymatic activity are shown in Table 14.2. The same as serum concentration, enzymatic activity is also varied. The concentration range was investigated in women as the control of breast cancer patients [28], from 0.6 to 12.0 U/L (one unit was defined as 1 μmol product per minute at 37 °C). It should be noted that the values did not reflect accurate L-arginase concentration in the blood. Additionally, arbitral "enzyme unit" could cause some difficulties to compare the results among studies.

Factors Correlated with Serum L-Arginase

Some previous reports investigated the correlation between L-arginase concentration in serum and various clinical parameters in healthy subjects and patients. In our laboratory, L-arginase concentration data were retrieved from 30 to 721 healthy individuals by four cross-sectional studies [11, 16, 17, 20] and investigated to examine the relationship using Spearman's correlation. Characteristic data are summarized in Table 14.3. The results were similar among these studies. Concentration of serum L-arginase showed significant negative correlation with age and HDL-C concentration and positive correlations with BMI, WBC, RBC, hs-CRP, 8-OHdG, and ALT in plural studies. Meanwhile, there was no correlation with NOx or urea concentration in serum and lifestyle habits such as alcohol consumption, smoking, and exercise.

Multiple regression analyses for L-arginase I were also carried out [16, 20]. The results are summarized in Table 14.4. L-Arginase I concentration was associated with HbA1c, 8-OHdG, and 8-isoprostane. Therefore, it suggested that L-arginase I may be a new biomarker for prediction of oxidative stress and diabetes mellitus. In another study, association of 3-NT and L-arginine with L-arginase was observed.

Table 14.3 Correlation of serum L-arginase I with clinical factors

References	n	Correlation coefficient								
		General			Hematological			Oxidative stress		
		Age	Sex	BMI	WBC	RBC	IgE	8-OHdG	8-isoprostane	3-NT
[16]	278	–0.128*	–	0.187**	0.302**	0.218**	–	0.101	0.121*	–
[17]	30	–0.393*	–	–	0.307	0.359	0.076	–	–	–
[11][a]	721	–0.069	–0.201**	0.159**	0.301**	0.269**	–	0.094*	0.057	–
[11][b]	721	–0.078*	–0.070	0.109**	0.340**	0.209**	–	0.139*	0.010	–
[20]	130	0.039	–0.112	0.022	0.134	–	0.084	–0.095	–	0.334**
		Atherosclerosis			Diabetic			Respiratory		
		hs-CRP	LDL-C	HDL-C	HbA1c	Insulin	Glucose	FVE1%	FENO	
[16]	278	0.125*	0.004	–	–	–	–	–	–	
[17]	30	0.147	–	–	–	–	–	–	–	
[11][a]	721	0.187**	0.104**	–0.155**	–0.016	0.055	0.029	–	–	
[11][b]	721	0.154**	0.071	–0.085*	0.033	0.041	0.012	–	–	
[20]	130	0.096	–0.148	–	–	–	–	–0.219*	0.220*	
		L-Arginase related						Lifestyle		
		L-Arginine	L-Citrulline	L-Ornithine	ALT	NOx		Smoking	Exercise	Alcohol
[16]	278	–	–	–	0.153*	0.014		–	–	–
[17]	30	–	–	–	0.196	0.054		–	–	–
[11][a]	721	–	–	–	0.144**	0.0002		0.016	–0.036	0.065
[11][b]	721	–	–	–	0.075*	–0.013		0.029	–0.060	–0.024
[20]	130	–0.275**	–0.054	0.165	0.101	0.099		–0.033	–	0.008

The relationship between L-arginase I in serum and various clinical parameters is shown in this table. Interestingly, age and BMI are significantly correlated with serum L-arginase I concentration, while lifestyle is not. Oxidative stress caused by free radical and lipid peroxidation showed correlations with L-arginase concentration

–: Data not available

Significant correlation (*$P<0.05$; **$P<0.01$) between serum L-arginase I and each parameter

[a]Measured by commercially available ELISA kit

[b]Measured by original ELISA kit

Table 14.4 Multiple regression analysis for L-arginase I by stepwise method

References	Explanatory variable	β	p	Adjusted R^a
[16]	WBC	0.250	<0.001	0.164
(n = 274)[b]	RBC	0.147	0.012	
	8-OHdG	0.178	0.002	
	Age	–0.185	0.003	
	HbA1c	0.159	0.007	
	8-Isoprostane	0.126	0.025	
[20]	3-NT	0.281	0.001	0.205
(n = 130)[a]	FVE1 %	–0.199	0.013	
	FENO	0.171	0.033	
	L-Arginine	–0.163	0.049	

The result of previous multiple regression analyses was shown in this table. Biomarkers for oxidative stress (8-OHdG, 3-NT, and 8-isoprostane) were associated with L-arginase I, the same as correlation studies (Table 14.3). β indicates standardized partial regression coefficient

[a]Explanatory variables included were sex, age, systolic blood pressure, FVE1 %, ALT, NOx, ferritin, ceruloplasmin, L-arginine, L-citrulline, L-ornithine, LDL-C, BMI, FENO, 8-OHdG, WBC, hs-CRP, IgE, smoking, and alcohol drinking

[b]Explanatory variables included were sex, ALT, NOx, H_2O_2, LDL-C, uric acid, and BMI

However, the results often complicate among studies. For example, correlation between age and L-arginase was investigated in some previous reports. A positive correlation was indicated in healthy women, while no change was observed as a whole [7]. Moreover, as described above, negative correlation between age and L-arginase was found in healthy subjects. The exact reason for the discrepancy is unresolved yet, though would be caused by smaller scale and biases for the surveyed subjects in individual research, as same as described in former section. Conversely, some specific condition (e.g., in child or female) may reveal significant unknown correlation. Our previous report supports this hypothesis, because some significant correlations with L-arginase I were observed in detailed group (e.g., alcohol consumption, age, or sex) [16].

Also, information about the relationship between genetic condition of L-arginase and actual activity is lacking. Although some SNPs were identified in L-arginase I, the influence on enzymatic activity is not evaluated. Racial differences are also lacking for information unlike some drug metabolism enzymes (e.g., cytochrome P450) [32]. Correlation among L-arginase and other intrinsic parameters is still under discussion.

Serum L-Arginase Concentration and Diseases

The concentration or enzymatic activity of L-arginase can be affected by various diseases, as indicated in Table 14.5. In almost case, L-arginase in blood is increased, while some exceptions were observed. In childhood asthma, enzymatic activity was decreased than healthy control [31], while concentration of L-arginase was increased in adult asthmatic patient [17]. Lower L-arginase activity in childhood asthma patient could be explained with lower manganese (cofactor of L-arginase) concentration in plasma. Moreover, significant L-arginase elevation was observed only in nonatopic asthma group [17]. In β-thalassemia, serum L-arginase level is not changed significantly in "hypertransfusion" therapy (hemoglobin concentration is kept from 9.5 to 10.0 g/dL) compared to healthy subjects, although higher concentration was observed in patients without hypertransfusion [19]. In colorectal cancer, serum L-arginase activity was insignificant, while higher L-arginase activity was observed in tumor tissue samples from the same group [29].

Table 14.5 List of diseases and therapies with significant changes of L-arginase in blood

Disease or therapy	Method	References
Significant upregulation		
Cancer and benign growth		
Breast cancer	Activity	[22, 28]
Colorectal cancer	Activity	[23, 27]
Hepatocellular carcinoma	Activity	[26]
Benign prostatic hypertrophy	Activity	[24]
Others		
Major depressant	Activity	[25]
Choledocholithiasis	Activity	[26]
Liver cirrhosis	Activity	[26]
Pulmonary arterial hypertension	Activity	[6]
Hemodialysis	Activity	[30]
Sickle cell disease	ELISA	[12, 14]
β-Thalassemia, without hypertransfusion	ELISA	[19]
Obstructive sleep apnea	ELISA	[21]
Asthma	ELISA	[17]
Pulmonary embolism with tricuspid regurgitation	ELISA	[33]
Coronary heart disease with hemodialysis	ELISA	[15]
Myocardial infarction	ELISA	[18]
Autoimmune hepatitis	EIA	[8]
Hepatitis by HCV	EIA	[8]
Liver transplantation	ELISA	[1, 10]
Significant downregulation		
Prostate cancer	Activity	[24]
Asthma in childhood	Activity	[31]

EIA enzyme immunoassay, *ELISA* enzyme-linked immunosorbent assay
Previous investigations suggested that these diseases affect the concentration or enzymatic activity of L-arginase in blood. Especially, L-arginase is augmented by some kinds of cancer, liver complaints, and blood disorders

For the purpose of monitoring disease condition, L-arginase is also considered as a useful biomarker. In liver transplantation, L-arginase is available for judging hepatocellular damage as well as AST and ALT or even better, because of faster elevation and shorter elimination from blood [1]. Six days after surgery on colorectal cancer, significant decrease of L-arginase activity was observed [27]. However, in colorectal cancers, higher L-arginase activity was observed in death or liver metastasis [23]. Graft rejection after liver transplantation caused elevation of L-arginase activity in bile, while gradual reduction of L-arginase activity was observed in successful operation [26]. Therefore, reduced L-arginase activity may be helpful for the decision of recovery.

Correlations between L-arginase I and clinical parameters were also investigated in some studies. Generally, significant correlations are observed with the factors closely related to the disease (e.g., bilirubin and ferritin in β-thalassemia [19], hs-CRP in myocardial infarction [18], nitric oxide and manganese in childhood asthma [31]).

Cutoff values were explored in some studies to diagnose diseases. In the previous report, L-arginase enzymatic activity was measured to diagnose choledocholithiasis, hepatocellular carcinoma, and liver cirrhosis [26]. The sensitivity was very high in HCC (96 %) and LC (92 %) but low in CDL (33 %). In hepatocellular carcinoma determination, the use of L-arginase as a biomarker was better than AFP in the aspect of sensitivity, which had been considered as the most useful biomarker. In breast cancer, cutoff value 8 U/L in serum was determined based on Chinard's method [4]. Its sensitivity and specificity were 63 % and 60 %, respectively [28]. In another investigation, three cutoff values were compared in breast cancer patients. It was concluded that cutoff value 13.74 U/L (healthy 9.48 U/L) was the highest accuracy for diagnosis [22]. In colorectal cancer and its metastasis to liver, 12 U/L cutoff value was determined with 82 % of specificity [27].

L-Arginase II may be involved in the elevation of total L-arginase activity in some disorders. In HCC and LC, both L-arginase I and L-arginase II were increased in protein level [26]. Protein expression of L-arginase II was also detected in tissues from breast cancer patients, but was undetectable in the serum [22]. Therefore, the presence of L-arginase II in serum may be useful in the determination of these disorders, while serum concentration of L-arginase II and contribution of L-arginase II is scarcely investigated.

Overall, L-arginase in blood is not specific marker for a disease because enzymatic activity or concentration of L-arginase is elevated in various disorders. In other words, L-arginase and its change could be a universal biomarker for prevention, diagnosis, and monitoring, especially in carcinoma and liver damages.

Interactions with NOS via L-Arginine Consumption

L-Arginine is the substrate for not only L-arginase but also nitric oxide synthase, L-arginine/glycine amidinotransferase, and L-arginine decarboxylase [34]. The relationship between L-arginase and NOS has been widely investigated, because competition between these two enzymes can be influenced to various factors. Elevation of L-arginase activity may cause harmful effects. Ornithine is the material for proline and polyamines. NOS can produce superoxide under hypoarginic condition. Therefore, overexpression of L-arginase is deleterious according to two aspects: (1) enhanced synthesis of polyamine and (2) oxidative stress caused by reactive oxygen/nitrogen species from uncoupled NOS. In healthy subjects, negative association of L-arginine concentration and positive association of 3-NT with L-arginase I were observed simultaneously [20]. The result implied that insufficiency of L-arginine was caused by increasing L-arginase I and further leads to the generation of 3-NT by uncoupled NOS because of depletion of L-arginine.

NO, the product of NOS-catalyzed reaction, is unstable, because NO is rapidly converted to nitrite or nitrate. However, these ions are easy to measure using colorimetric Griess test or iodine-ozone-based chemiluminescent assay [35]. In healthy subjects, no significant association of serum L-arginase I concentration with NOx ($NO_2^-+NO_3^-$) was observed in plural analyses [11, 17, 20]. However, significant correlation between serum L-arginase I and FENO was detected. The reason(s) for the association is unknown.

Release of NO from endothelial cells contributes homeostasis of vascular function. Some supplementation therapy was attempted for amelioration of L-arginase overexpression. Functional status of the heart was improved by short-term oral administration of L-arginine (6 weeks) to the patients with heart failure [36]. Meanwhile, in myocardial infarction, long-term (6 months) consumption of L-arginine was not concluded to be recommended to improve acute myocardial infarction [37]. Therefore, to suppress overexpression of L-arginase would be more important than symptomatic therapy.

As mentioned above, increase of blood L-arginase level is observed in diseases. Elevating L-arginase is not only the marker of these disorders but also the cause of injurious effect by indirectly inhibition of NO. Therefore, in this aspect, monitoring of blood L-arginase is important.

Conclusion

To determine L-arginase concentration in serum from well-conditioned human subject is important, because elevation of serum L-arginase level (both of enzymatic activity and concentration) is observed in many disorders.

To date, L-arginase activity has been measured indirectly, by the amount of urea and ornithine and the metabolites of L-arginase as enzymatic activity. Now, exact amount of L-arginase itself can be determined by ELISA. This technique seems to be revolutionary, because the unit is identical, while the results of traditional L-arginase activity assay were indicated by nonunique "enzyme units." Therefore, ELISA also enabled inter-assay comparison. In healthy subjects, serum or plasma L-arginase concentration is estimated to be ng/mL order.

Measurement of L-arginase may be useful as universal biomarker for prevention, diagnosis, and monitoring of various diseases. At present, however, L-arginase in blood is not considered to be a clinical test parameter nor is available as automatic and systematic measurement. There are many unknown factors related to L-arginase concentration in the blood of healthy or unhealthy mankind. Comprehensive and extensive survey may be essential to define concentration range to evaluate at normal level. Investigation of relationship between intrinsic parameter and L-arginase would be helpful to determine normality of the concentration. Also, change of L-arginase concentration in disorders should be investigated and collected in the future.

References

1. Ikemoto M, Tsunekawa S, Tanaka K, et al. Liver-type L-arginase in serum during and after liver transplantation: a novel index in monitoring conditions of the liver graft and its clinical significance. Clin Chim Acta. 1998; 271:11–23.
2. Ikemoto M, Ishida A, Tsunekawa S, et al. Enzyme immunoassay of liver-type L-arginase and its potential clinical application. Clin Chem. 1993;39:794–9.
3. Durante W, Johnson FK, Johnson RA. L-Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin Exp Pharmacol Physiol. 2007;34:906–11.
4. Chinard FP. Photometric estimation of proline and ornithine. J Biol Chem. 1952;199:91–5.
5. Geyer JW, Dabich D. Rapid method for determination of L-arginase activity in tissue homogenates. Anal Biochem. 1971;39:412–7.
6. Xu W, Kaneko FT, Zheng S, et al. Increased L-arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. FASEB J. 2004;18:1746–8.
7. Wang SR, Chen ML, Huang MH, et al. Plasma L-arginase concentration measured by an enzyme-linked immunosorbent assay (ELISA) in normal adult population. Clin Biochem. 1993;26:455–60.
8. Kimura M, Tatsumi KI, Tada H, et al. Enzyme immunoassay for autoantibodies to human liver-type L-arginase and its clinical application. Clin Chem. 2000;46:112–7.
9. Huang LW, Liu HW, Chang KL. Development of a sandwich ELISA test for L-arginase measurement based on monoclonal antibodies. Hybridoma. 2001;20:53–7.
10. Ikemoto M, Tsunekawa S, Awane M, et al. A useful ELISA system for human liver-type L-arginase, and its utility in diagnosis of liver diseases. Clin Biochem. 2001;34:455–61.
11. Ogino K, Murakami I, Wang DH, et al. Evaluation of serum L-arginase I as an oxidative stress biomarker in a healthy Japanese population using a newly established ELISA. Clin Biochem. 2013;46:1717–22.
12. Vilas-Boas W, Cerqueira BA, Zanette AM, et al. L-Arginase levels and their association with Th17-related cytokines, soluble adhesion molecules (sICAM-1 and sVCAM-1) and hemolysis markers among steady-state sickle cell anemia patients. Ann Hematol. 2010;89:877–82.
13. Kim PS, Iyer RK, Lu KV, et al. Expression of the liver form of L-arginase in erythrocytes. Mol Genet Metab. 2002;76:100–10.
14. Morris CR, Suh JH, Hagar W, et al. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. Blood. 2008;111:402–10.
15. Eleftheriadis T, Liakopoulos V, Antoniadi G, et al. L-Arginase type I as a marker of coronary heart disease in hemodialysis patients. Int Urol Nephrol. 2011;43:1187–94.
16. Ogino K, Takahashi N, Takigawa T, et al. Association of serum L-arginase I with oxidative stress in a healthy population. Free Radic Res. 2011;45:147–55.
17. Ogino K, Obase Y, Takahashi N, et al. High serum L-arginase I levels in asthma: its correlation with high-sensitivity C-reactive protein. J Asthma. 2011;48:1–7.
18. Bekpinar S, Gurdol F, Unlucerci Y, et al. Serum levels of L-arginase I are associated with left ventricular function after myocardial infarction. Clin Biochem. 2011;44:1090–3.

19. El-Hady SB, Farahat MH, Atfy M, et al. Nitric oxide metabolites and L-arginase I levels in β-thalassemic patients: an Egyptian study. Ann Hematol. 2012;91:1193–200.
20. Ogino K, Wang DH, Kubo M, et al. Association of serum L-arginase I with L-arginine, 3-nitrotyrosine, and exhaled nitric oxide in healthy Japanese workers. Free Radic Res. 2014;48:137–45.
21. Choudhury B, Srivastava S, Choudhury HH, et al. L-Arginase and C-reactive protein as potential serum-based biomarker of head and neck squamous cell carcinoma patients of north east India. Tumour Biol. 2014;35:6739–48.
22. Polat MF, Taysi S, Polat S, et al. Elevated serum L-arginase activity levels in patients with breast cancer. Surg Today. 2003;33:655–61.
23. Porembska Z, Skwarek A, Mielczarek M, et al. Serum L-arginase activity in postsurgical monitoring of patients with colorectal carcinoma. Cancer. 2002;94:2930–4.
24. Elgün S, Keskineğe A, Yilmaz E, et al. Evaluation of serum L-arginase activity in benign prostatic hypertrophy and prostatic cancer. Int Urol Nephrol. 1999;31:95–9.
25. Elgün S, Kumbasar H. Increased serum L-arginase activity in depressed patients. Prog Neuropsychopharmacol Biol Psychiatry. 2000;24:227–32.
26. Chrzanowska A, Graboń W, Mielczarek-Puta M, et al. Significance of L-arginase determination in body fluids of patients with hepatocellular carcinoma and liver cirrhosis before and after surgical treatment. Clin Biochem. 2014;47:1056–9.
27. Graboń W, Mielczarek-Puta M, Chrzanowska A, Barańczyk-Kuźma A. L-arginine as a factor increasing L-arginase significance in diagnosis of primary and metastatic colorectal cancer. Clin Biochem. 2009;42:353–7.
28. Porembska Z, Luboiński G, Chrzanowska A, et al. L-Arginase in patients with breast cancer. Clin Chim Acta. 2003;328:105–11.
29. del Ara RM, González-Polo RA, Caro A, et al. Diagnostic performance of L-arginase activity in colorectal cancer. Clin Exp Med. 2002;2:53–7.
30. Tektaş AK, Uslu S, Yalçin AU, et al. Effects of lipoprotein-associated phospholipase A2 on L-arginase/nitric oxide pathway in hemodialysis patients. Ren Fail. 2012;34:738–43.
31. Kocyigit A, Zeyrek D, Keles H, et al. Relationship among manganese, L-arginase, and nitric oxide in childhood asthma. Biol Trace Elem Res. 2004;102:11–8.
32. The Human Cytochrome P450 (CYP) Allele Nomenclature Database. http://www.cypalleles.ki.se/. Accessed 26 Sept 2014.
33. Kline JA, Watts J, Courtney D, et al. Severe pulmonary embolism decreases plasma L-arginine. Eur Respir J. 2014;43:906–9.
34. Morris Jr SM. Recent advances in L-arginine metabolism: roles and regulation of the L-arginases. Br J Pharmacol. 2009;157:922–30.
35. Yang BK, Vivas EX, Reiter CD, et al. Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. Free Radic Res. 2003;37:1–10.
36. Taddei S, Virdis A, Mattei P, et al. Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. Circulation. 1996;94:1298–303.
37. Schulman SP, Becker LC, Kass DA, et al. L-arginine therapy in acute myocardial infarction: the vascular interaction with age in myocardial infarction (VINTAGE MI) randomized clinical trial. JAMA. 2006;295:58–64.

Part III
L-Arginine Status in Cells Related to Organ Damage and Disease

Chapter 15
Protein L-Arginine Methylation of RNA-Binding Proteins and Their Impact on Human Diseases

Michael C. Yu and Christopher A. Jackson

Key Points

- RNA-binding proteins are responsible for a plethora of important biological functions, and defects in how these proteins function have been shown to play a role in the etiology for a number of debilitating human diseases.
- Many RNA-binding proteins contain a type of posttranslational modification called protein L-arginine methylation. L-Arginine methylation has been shown to be critical in controlling how these RNA-binding proteins function in a cell.
- At the molecular level, protein L-arginine methylation of RNA-binding proteins can affect their protein–protein interactions, protein–RNA interactions, protein and RNA stability, and subcellular protein localization.

Keywords Protein L-arginine methylation • RNA-binding proteins • PRMT • Posttranslational modification • Protein–protein interaction

Abbreviations

AdoMet	*S*-adenosyl-L-methionine
AdOX	Adenosine dialdehyde
aDMA	Asymmetric dimethylarginine
ALS	Amyotrophic lateral sclerosis
ARE	AU-rich element
FMRP	Fragile X mental retardation protein
FUS	Fused in sarcoma
GAR	Glycine-arginine rich
hnRNP	Heterogeneous nuclear ribonucleoprotein

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MMA	Monomethylarginine
OPMD	Oculopharyngeal muscular dystrophy
PGM	Proline-glycine-methionine
PRMT	Protein L-arginine methyltransferase
RBDs	RNA-binding domains
RBP	RNA-binding protein
RRM	RNA recognition motif
sDMA	Symmetric dimethylarginine
SMA	Spinal muscular dystrophy
STAR	Signal transduction and activation of RNA
UTR	Untranslated regions

Introduction

RNA-binding proteins (RBPs) are important for regulating the transcriptional expression of genes, as well as controlling the production of multiple transcriptional isoforms from a single gene by modulating pre-mRNA splicing [1]. These proteins influence multiple aspects of RNA metabolism, including maturation, surveillance, subcellular localization, nucleocytoplasmic transport, and degradation [1]. RNA-binding proteins contain one or more RNA-binding domains (RBDs) that specify their association with RNAs. Examples of common RBDs include the RNA recognition motif (RRM), K-homology (KH) domain, RGG box/domain, Sm domain, zinc finger, and Piwi/Argonaute/Zwille domain [1]. These RBDs act as modules that can be combined and arranged to expand the functional ability of an RBP. This modular architecture provides versatility: RBP binding with high affinity and specificity to a specific target can be achieved by combining different RBDs that individually recognize shorter RNA sequences with weak affinity. Many RBPs contain additional posttranslational modifications that can influence their biochemical properties. One such modification that has been found in many RBPs is protein L-arginine methylation [2]. Recent findings have implicated this modification as a major regulator of RBP function within a cell [2], thereby pointing to the potential of this modification serving an important role in the cause or progression of human diseases.

Protein L-arginine methylation is catalyzed by members of an enzyme family known as protein L-arginine methyltransferases (PRMTs) using *S*-adenosyl-L-methionine (AdoMet) as the methyl donor in the methyltransferase reaction [2]. These enzymes possess a set of four conserved signature amino acid sequence motifs called I, post-I, II, and III, as well as a THW loop. The AdoMet-binding pocket is formed by motifs I, post-I, and the THW loop. Structural function analyses of PRMT catalysis indicate a pair of conserve glutamate residues, opposite the interface between AdoMet and the methyl-acceptor, and prime nucleophilic attack through polarizing the guanidine group of L-arginine. There are four types of PRMTs based on the forms of methylarginine they generate (Fig. 15.1). Type I PRMTs transfer one or two methyl groups to a terminal ω-nitrogen on a protein-incorporated L-arginine residue, forming either monomethylarginine (MMA) or asymmetric dimethylarginine (aDMA). Type II PRMTs catalyze monomethylation, but are also able to add a second methyl group to the opposing ω-nitrogen within the L-arginine residue, forming symmetric dimethylarginines (sDMA). Type III PRMTs catalyze only MMA formation of a ω-nitrogen, and type IV PRMTs, currently found only in the budding yeast, catalyze the formation of MMA within the δ-nitrogen atom [3]. Currently, there are nine canonical mammalian PRMTs identified [2], but 34 additional genes have been identified that share sequence homology to the known PRMT sequences [4]. For some PRMTs, there are alternatively spliced isoforms, but specific function with regard to these isoforms is mostly unknown [5]. Of the canonical PRMTs identified, six have shown type I activity (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6, and PRMT8), two have shown type II activity (PRMT5 and PRMT9), and one (PRMT7) has shown type III activity. PRMT1 is the most highly conserved type I PRMT and is responsible for the bulk of methylation in a cell. For type II PRMTs, PRMT5 is the most highly conserved member of the family. PRMTs are expressed in various tissues in mammals, including

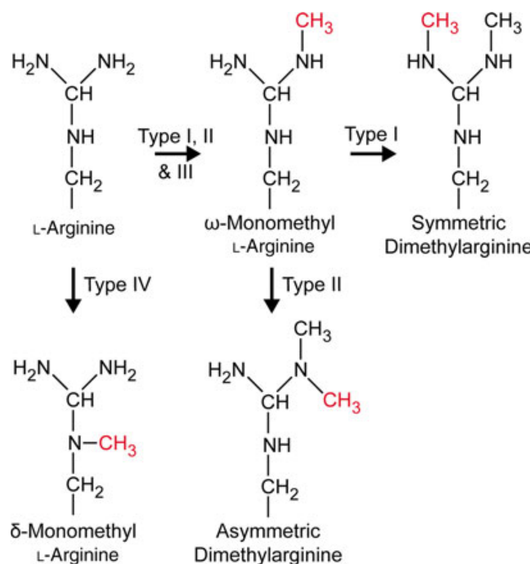

Fig. 15.1 The process of protein L-arginine methylation and the types of methylarginines generated

different parts of the brain, ciliary body, colon, esophagus, heart, immature dendritic cell, kidney, liver, lung, mature dendritic cell, ovary, pancreas, placenta, prostate, retina, stomach, testis, thyroid, urinary bladder, and gallbladder [6, 7]. PRMT1 and PRMT5 are expressed in the majority of these tissues. Other PRMTs, such as PRMT8, have only been identified in a single tissue (brain) [8]. Unlike other more dynamic posttranslational modifications such as phosphorylation, L-arginine methylation appears to be quite stable. JMJD6, a Jumonji domain-containing protein, was initially described as a putative L-arginine demethylase [9]. However, subsequent investigations from other labs have indicated that JMJD6 is not likely to act as an L-arginine demethylase but rather as lysine hydroxylase [10, 11]. PADI4 is a member of the enzyme family called peptidylarginine deiminases (PADIs) [12]. PADI4 converts MMA into citrulline by nonreversible deimination, thus removing the methylation mark and blocking further methylation on the citrulline residue. To date, no *bona fide* demethylase has been shown that is similar to the lysine demethylase family.

The preferred methylation target sequence for PRMT1 is an L-arginine residue flanked by one or more glycine residues or the GAR (glycine and L-arginine-rich) motifs [13], although findings from in vitro substrate profiling suggest that this enzyme may have additional target sequences [14]. RGG box/domains, which are a common RBD known to participate in protein–protein and protein–RNA interactions [1], contain this L-arginine–glycine motif and are often targets for L-arginine methylation. The aDMA-generating enzymes PRMT3, PRMT6, and PRMT8 also preferentially methylate GAR motifs. PRMT4 (also known as CARM1) has an affinity for methylating PGM motifs (proline-, glycine-, and methionine-rich) as opposed to the GAR motifs preferred by PRMT1 [15, 16]. PRMT5 has been shown to catalyze the formation of sDMA in both GAR and PGM motifs [16, 17]. These general rules regarding methylation target sites are subject to many exceptions, and the identification of additional PRMT substrates may warrant a revision of these notions in the future. The compendium of RBPs that contain mono- and dimethylated L-arginines, as established by the analysis using both L-arginine dimethyl-specific antibodies and proteomic identification technologies, is substantial. However, in most of the cases, it remains to be determined how these methylated L-arginines actually influence the molecular activities of these RBPs. In this review, I will discuss our current understanding for a number of human diseases in which L-arginine methylation of a specific RBP has been shown to be critical in the etiology or progression of a specific disease. It should be noted that while this modification has been found in many RBPs, only those with characterized or identified functional link for methylation and the cause for a specific disease are discussed in this review.

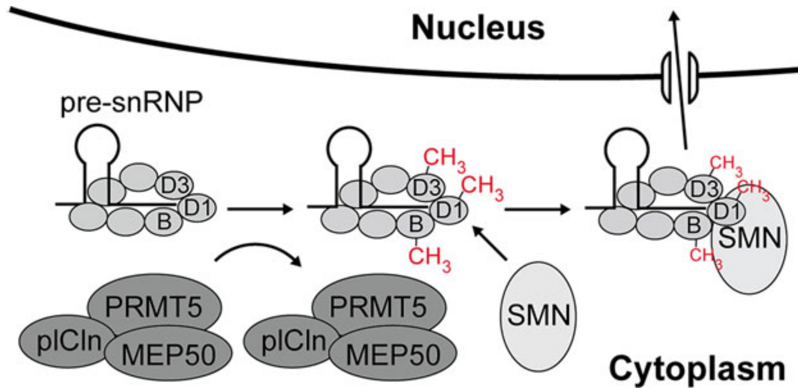

Fig. 15.2 Formation of spliceosomal U-snRNPs by PRMT5 complexes and SMN

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a multisystem neuromuscular disorder of lower motor neurons due to extremely low levels of cellular protein SMN [18]. Proximal SMA is the leading genetic cause of infant mortality and is the most common neuromuscular disease after muscular dystrophy. The cause of this disease stems from the homozygous deletion or mutation of the survival of motor neuron 1 (*SMN1*) gene. The SMN protein contains a single Tudor domain, which has been established as methylarginine-binding protein modules. The Tudor domain is a structural motif that consists of approximately 60 amino acids forming four antiparallel β-strands [19]. These β-strands form a barrel-like structure with an aromatic binding pocket at the surface to allow binding specifically to methylated target proteins [19]. Binding of SMN to the Sm proteins is required for the cytoplasmic assembly of functional U-snRNPs, which are the essential components of a working spliceosome, the macromolecular machinery that carries out pre-mRNA splicing in cells [20, 21]. In mammalian cells, each spliceosomal snRNP is first assembled in the cytoplasm from a seven-membered ring of core Sm proteins (SmB/B', SmD1, SmD2, SmD3, SmE, SmF, and SmG) and a newly exported snRNA (U1, U2, U4, or U5). The mature snRNPs in the cytoplasm are then imported into the nucleus, where they function in pre-mRNA splicing. A properly assembled Sm core is required for the nuclear import of mature cytoplasmic U-snRNPs, which enables the spliceosome to carry out the catalytic step in pre-mRNA splicing. SMN preferentially binds to symmetrically dimethylarginine-modified RG domains of SmD1 and SmD3, which are catalyzed by PRMT5 (Fig. 15.2). This interaction is necessary for the formation of a mature, functionally competent U-snRNPs. Deficiency in SMN, as seen in severe SMA, results in cell-type-specific effects on the amount of snRNAs and mRNAs, resulting in altered stoichiometry of snRNAs and functional U-snRNPs [22]. In SMN-deficient mouse tissues, there is a prevalent pre-mRNA splicing defects for many transcripts, implicating a key role for SMN complex in the regulation of splicing [22].

Recent work has also demonstrated that SMN interacts with another RBP, HuD, through its Tudor domain [23]. Hu proteins recognize and bind to AU-rich elements found within the 3′-untranslated regions (UTRs) in about 5 % of human genes [24]. Binding of specific RNAs by Hu proteins has been shown to be critical for RNA turnover. Like SMN, HuD can also be L-arginine methylated, but the enzyme that catalyzes this methylation is PRMT4/CARM1. Methylation of HuD has been demonstrated to be important in the switch between proliferation and differentiation in the neuronal tumor cell line PC12, through regulating specific RNA turnover events [25]. Thus, dysregulation of methylation on HuD likely impacts neuronal development and function given the critical role neuronal Hu proteins exert on the posttranscriptional control over a number of important genes involved in neuronal differentiation and function.

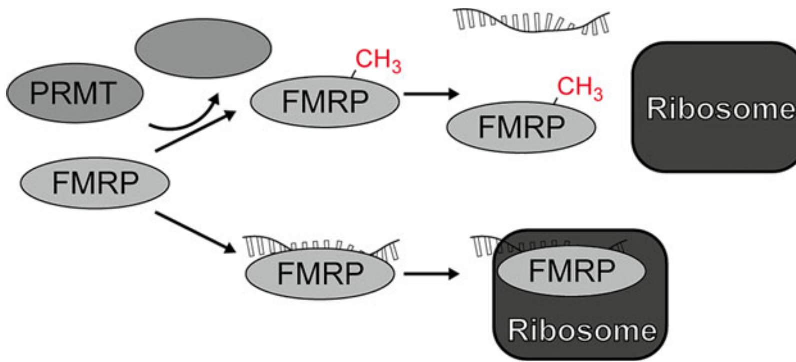

Fig. 15.3 Effects of protein L-arginine methylation on FMRP function

Fragile X Syndrome

Fragile X syndrome is an X-linked disease and the most common form of inherited mental retardation [26]. This disease is mainly caused by the abnormal expansion of the trinucleotide sequence CGG located in the 5′ UTR, or the first exon, of the *FMR1* gene located on the X chromosome. This expansion leads to hypermethylation and subsequent silencing of the *FMR1* gene. The *FMR1* gene encodes fragile X mental retardation protein (FMRP), a high-affinity RNA-binding protein found to bind to approximately 4 % of fetal brain mRNAs in vitro and regulate their translation. Its role in RNA metabolism is critical for synaptic plasticity, in the development of dendrites and axons, and in learning and memory. The FMRP is encoded by 17 exons of the *FMR1* gene, and alternative splicing at the 3′ end of the gene generates multiple isoforms of FMRP. FMRP harbors two KH domains in exons 8 and 10 and one RGG box, which contains L-arginine methylation. This RGG box is the major mRNA-binding domain of FMRP and it binds to the intramolecular G-quartet found in some mRNAs. Together with fragile X-related proteins 1 (FXR1P) and 2 (FXR2P), FMRP associates with polyribosomes in an RNA-dependent manner. PRMT1 is the enzyme that mediates this methylation event, and methylation of L-arginines 533 and 538 in FMRP is required for normal polyribosome association [27]. However, in vitro work has shown that PRMT3 and PRMT4 can also methylate the C-terminal domain in addition to PRMT1 [28]. Methylation of the RGG box in the FMRP reduces its ability to bind RNAs as well as decreasing the binding of FMRP to other proteins [29, 30] (Fig. 15.3). Given that FMRP associates with the polyribosomes and methylation of the RGG box in the FMRP reduces its RNA-binding capacity, it can be inferred that L-arginine methylation of the FMRP serves as a mean to regulate the amount of translatable RNAs in a cell [31, 32].

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease in which the dysfunction and loss of motor neurons from the brain and spinal cord lead to progressive muscle weakness, paralysis, and eventual death [33]. While the first gene to be linked to ALS was *SOD1* (which encodes Cu/Zn-superoxide dismutase), another gene called *FUS* (fused in sarcoma) has been implicated in a subset of familial ALS cases. Normally, FUS functions as a heterogeneous ribonuclear protein (it was identified as hnRNP P2) and plays a role in transcription, RNA processing, and local translation of mRNAs. In neurons, FUS is required for formation of dendritic spines and for the transport of mRNA along the dendrites. The localization of FUS is predominantly nuclear for most cell types, and this localization is important in the pathophysiological cause as cytoplasmic accumulation of FUS is found in ALS.

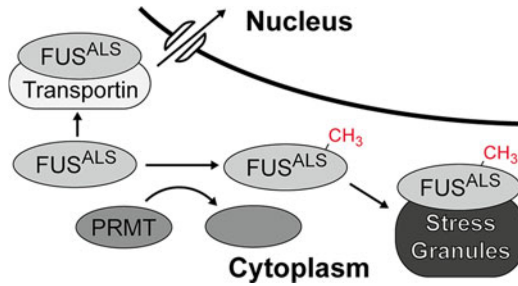

Fig. 15.4 Methylation of FUS alters its cellular localization

Like many PRMT substrates, FUS contains three GAR motifs that are typical motifs for harboring aDMAs and has been shown to interact with and be methylated by PRMT1 [34]. Proteomic analysis has identified 20 dimethylated L-arginine residues within the RGG domain of FUS [35]. Methylation of FUS by PRMT1 is critical for its cellular location and likely its pathophysiological role in ALS (Fig. 15.4), as aberrant localization of FUS to the cytoplasm has been demonstrated in ALS-linked FUS mutants and contributes to their cellular toxicity [34]. In some cases and other familial forms of ALS, FUS is found in the cytoplasm [36], implicating a role for the dysregulation of FUS methylation in the pathophysiology of ALS.

Oculopharyngeal Muscular Dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is a late-onset, autosomal dominant disorder caused by the abnormal expansion of a $(GCN)_N$ trinucleotide repeat in the coding region of a nuclear poly(A)-binding protein PABPN1 [37]. How such change in a ubiquitously expressed protein impacts a specific subset of muscles in this muscle disease remains largely unknown [37]. The PABPN1 protein contains three major domains: an acidic N-terminus, a central ribonucleoprotein-type RRM, and a basic L-arginine-rich C-terminus where PRMT-mediated methylation occurs. There are no obvious methylation motifs within the C-terminal domain of PABPN1, but, together with the RRM domain, the C-terminal domain is required to facilitate both RNA binding and oligomerization of PABPN1. Unlike many of the mentioned RBPs, which are substrates of a single PRMT, three different PRMTs (PRMT1, PRMT3, and PRMT6) have been found to be able to methylate the L-arginines within the C-terminal domain. Methylation of PABPN1 does not impact its ability to bind to RNA, but controls the nuclear accumulation of PABPN1 via weakening interaction between PABPN1 and transportin, the nuclear import receptor for PABPN1 (Fig. 15.5). Since mutant PABPN1 that displays abnormal aggregation has been shown to result in the pathophysiological phenotype of OPMD, methylation may serve as a control to prevent formation of such aggregates.

Cancer

Many L-arginine-methylated proteins play a role in the etiology of various human cancers, as this modification is found in a number of important cancer-related transcriptional regulators [e.g., p53, estrogen receptor alpha (ERα)], histones (e.g., H4R3), as well as in proteins that play a role in the DNA damage response pathway [2]. Here, we will focus on known methylation of RBPs in which methylation itself plays a role in the function of such RBP during tumorigenesis or cancer progression.

Src associated in mitosis, of 68 kDa (Sam68), is a KH domain-containing RBP that belongs to the signal transduction and activation of RNA (STAR) family of RBPs [38]. These proteins have known

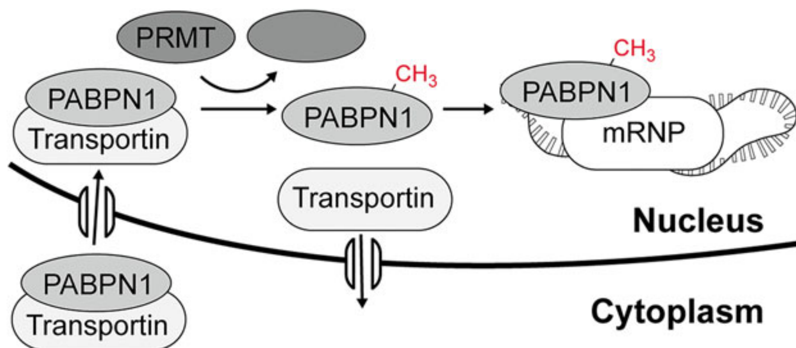

Fig. 15.5 Nuclear accumulation of PABPN1 is controlled by protein L-arginine methylation

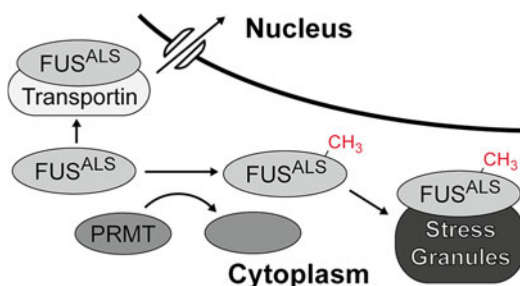

Fig. 15.6 (**a**) Methylation of Sam68 affects its cellular localization. (**b**) Methylation of hnRNP K increases its affinity for transcriptional activator p53

roles in cell proliferation and differentiation. Sam68 is methylated by PRMT1 and this modification impairs its interaction with SH3 domains [39] (Fig. 15.6a). Treating cells with the methyltransferase inhibitor adenosine dialdehyde (AdOx) results in increased cytoplasmic localization of Sam68. Notably, increased nuclear localization of Sam68 has been linked to poor clinical prognosis of colorectal cancer [40]. Methylation of Sam68 and its homologues, SLM-1 and SLM-2 (Sam68-like mammalian proteins), also results in a decreased affinity for binding some RNAs [41].

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is composed of protein–protein interaction domains and of domains that allow it to associate with both DNA and RNA [42]. Methylation of hnRNP K (Arg296 and Arg299) by PRMT1 during DNA damage response increases its affinity for tumor suppressor p53, whereas inhibiting methylation of hnRNP K attenuates the recruitment of tumor suppressor protein p53 to its promoter, thereby reducing its transcriptional activity [43] (Fig. 15.6b). Recently, it was demonstrated that hnRNP K methylation at Arg296 and Arg299 directly abolished PKC delta-mediated phosphorylation of Ser302 on hnRNP K, and this results in the negative modulation of cellular apoptosis [44]. Furthermore, methylation of hnRNP K affects its interaction with tyrosine kinase c-Src, which plays an important role in cancer progression and development. Thus, there clearly exists a regulatory role of methylation that controls the biological function of hnRNP K that are important for cancer etiology.

Mechanisms by Which L-Arginine Methylation Modulates RBP Function

Posttranslational modification of proteins is a major level of regulation of biological processes within a cell. These modifications affect protein structure and act as molecular switches, which can be very fast and reversible to slow or irreversible. Protein L-arginine methylation, one of the most extensive protein methylation reactions in mammals, represents a major regulator of protein functions. In general, the mechanisms by which L-arginine methylation modulates RBP function can be categorized below:

Protein–Protein Interactions

Addition of each methyl group to the L-arginine removes a hydrogen bond donor, decreases the electrostatic surface potential at the L-arginine residue, and results in a change of size and hydrophobicity that impacts how it binds to certain partners [2]. This change to protein–protein interactions is a major molecular mechanism by which protein L-arginine methylation modulates physiological function. In the case of Sam68, L-arginine methylation prevents the association between Sam68 and SH3 domains [39]. Methylation of hnRNP K by PRMT1 reduces its ability to interact with tyrosine kinase c-Src, consequently leading to inhibition of c-Src activation and the c-Src phosphorylation of hnRNP K [45].

A major protein domain that controls the ability for a protein to act as methylation "reader" is the Tudor domain, which recognizes and binds both methylarginines and methyllysines [46]. In humans, at least 36 proteins have been found to harbor Tudor domains. The Tudor domain contacts sDMA with an aromatic cage, the structure of which has been resolved.

Protein–RNA Interactions

L-Arginines and lysines are often important residues in RNA–protein interactions due to their basic side chains. Methylation, by perturbing hydrogen bonding between the L-arginine residue and RNA, may sterically hinder the association between RNA and protein. In other cases, methylation can promote stacking with the RNA bases by increasing the hydrophobicity of the L-arginine residue, thereby enhancing an association between RNA and protein. In the case with FMRP, for example, methylation serves to regulate its ability to target specific RNAs [27, 31].

Cellular Protein Localization

Localization of protein to specific subcellular compartments is another mechanism for protein regulation. Thus, activation or repression of a biological pathway can be controlled by allowing or preventing trafficking of a specific protein to a target location. The first biological role associated with L-arginine methylation is modulating nucleocytoplasmic shuttling of hnRNPs in the budding yeast *Saccharomyces cerevisiae* [47]. Regulation of intracellular localization has been shown as a conserved mechanism in mammalian proteins as well. This is illustrated by the example of SMA, where spliceosomal U-snRNPs are incorrectly retained in the cytoplasm in the absence of Sm protein methylation. Similarly, PRMT1-mediated L-arginine methylation on FUS determines its intracellular localization, with aberrant cytoplasmic localization of ALS-linked FUS mutants contributing to their toxicity [34].

Protein Stability

Protein degradation is controlled primarily by another posttranslational modification called ubiquitination, which specifically marks a protein for degradation by the proteasome [48]. Ubiquitin-mediated degradation can be altered by L-arginine methylation to control how much of a protein gets marked for degradation by the proteasome. PRMT5-mediated L-arginine methylation has been shown to regulate the stability of E2F-1 transcription factor through this type of interplay [49]. E2F is a family of

transcription factors that play a major role in proliferation and apoptosis [50]. Depleting PRMT5 results in a reduction of ubiquitination on wild-type E2F-1 and a consequent increase in protein abundance [49]. Additionally, methylation of hnRNP K affects its own phosphorylation status and thus negatively regulates the cellular apoptosis [44]. This type of protein modification crosstalk is likely to emerge as one of the major molecular mechanisms through which L-arginine methylation acts on the protein function.

RNA Stability

RNA abundance is controlled in part by regulating RNA stability. AU-rich elements (AREs) are regulatory *cis*-element found in the 3′ UTR of many short-lived mRNAs [24]. AREs direct the destabilization of transcripts in response to various intracellular and extracellular signals. Binding of these elements by the Hu family proteins, such as HuD, stabilizes these mRNAs. Methylation of HuD affects its association with the RNA and, as a result, a change in the overall stability of such RNA and its abundance, which is critical for the switch between proliferation and differentiation in the neuronal tumor cell line PC12 [25].

Conclusions

Recent work has illuminated the importance of L-arginine methylation in the control of RBP function and how misregulation of such modification on specific RBPs links to the etiology of a number of human diseases. High-throughput studies aimed at identifying cellular proteins containing this modification have further identified the immense potential for how this modification may regulate important cellular pathways based on the known function of these proteins. Clearly, RBPs represent a major class of proteins that are targeted by PRMT for further posttranslational modification. However, obtaining the direct evidence that connects the misregulation of L-arginine methylation on these proteins to the cause of a specific disease state is greatly lacking. Therefore, a major challenge in this field would be to provide the molecular basis that connects these two aspects.

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References

1. Lunde BM, Moore C, Varani G. RNA-binding proteins: modular design for efficient function. Nat Rev Mol Cell Biol. 2007;8(6):479–90.
2. Bedford MT, Clarke SG. Protein L-arginine methylation in mammals: who, what, and why. Mol Cell. 2009;33(1):1–13.
3. Zobel-Thropp P, Gary JD, Clarke S. delta-N-methylarginine is a novel posttranslational modification of L-arginine residues in yeast proteins. J Biol Chem. 1998;273(45):29283–6.
4. Richon VM, Johnston D, Sneeringer CJ, et al. Chemogenetic analysis of human protein methyltransferases. Chem Biol Drug Des. 2011;78(2):199–210.
5. Baldwin RM, Morettin A, Cote J. Role of PRMTs in cancer: could minor isoforms be leaving a mark? World J Biol Chem. 2014;5(2):115–29.
6. Hong E, Lim Y, Lee E, Oh M, Kwon D. Tissue-specific and age-dependent expression of protein L-arginine methyltransferases (PRMTs) in male rat tissues. Biogerontology. 2012;13(3):329–36.

7. Wu TT, Tsai TW, Shen YT, Hsu JD, Yang LC, Li C. Analyses of PRMT1 proteins in human colon tissues from Hirschsprung disease patients. Neurogastroenterol Motil. 2010;22(9):984–90. e254.
8. Taneda T, Miyata S, Kousaka A, et al. Specific regional distribution of protein L-arginine methyltransferase 8 (PRMT8) in the mouse brain. Brain Res. 2007;1155:1–9.
9. Chang B, Chen Y, Zhao Y, Bruick RK. JMJD6 is a histone L-arginine demethylase. Science. 2007;318(5849):444–7.
10. Webby CJ, Wolf A, Gromak N, et al. Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. Science. 2009;325(5936):90–3.
11. Unoki M, Masuda A, Dohmae N, et al. Lysyl 5-hydroxylation, a novel histone modification, by Jumonji domain containing 6 (JMJD6). J Biol Chem. 2013;288(9):6053–62.
12. Wang Y, Wysocka J, Sayegh J, et al. Human PAD4 regulates histone L-arginine methylation levels via demethylimination. Science. 2004;306(5694):279–83.
13. Gary JD, Clarke S. RNA and protein interactions modulated by protein L-arginine methylation. Prog Nucleic Acid Res Mol Biol. 1998;61:65–131.
14. Wooderchak WL, Zang T, Zhou ZS, Acuna M, Tahara SM, Hevel JM. Substrate profiling of PRMT1 reveals amino acid sequences that extend beyond the "RGG" paradigm. Biochemistry. 2008;47(36):9456–66.
15. Cheng D, Cote J, Shaaban S, Bedford MT. The L-arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. Mol Cell. 2007;25(1):71–83.
16. Lee J, Bedford MT. PABP1 identified as an L-arginine methyltransferase substrate using high-density protein arrays. EMBO Rep. 2002;3(3):268–73.
17. Branscombe TL, Frankel A, Lee JH, et al. PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J Biol Chem. 2001;276(35):32971–6.
18. Li DK, Tisdale S, Lotti F, Pellizzoni L. SMN control of RNP assembly: from post-transcriptional gene regulation to motor neuron disease. Semin Cell Dev Biol. 2014;32C:22–9.
19. Selenko P, Sprangers R, Stier G, Buhler D, Fischer U, Sattler M. SMN tudor domain structure and its interaction with the Sm proteins. Nat Struct Biol. 2001;8(1):27–31.
20. Chari A, Paknia E, Fischer U. The role of RNP biogenesis in spinal muscular atrophy. Curr Opin Cell Biol. 2009;21(3):387–93.
21. Paushkin S, Gubitz AK, Massenet S, Dreyfuss G. The SMN complex, an assemblyosome of ribonucleoproteins. Curr Opin Cell Biol. 2002;14(3):305–12.
22. Zhang Z, Lotti F, Dittmar K, et al. SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell. 2008;133(4):585–600.
23. Hubers L, Valderrama-Carvajal H, Laframboise J, Timbers J, Sanchez G, Cote J. HuD interacts with survival motor neuron protein and can rescue spinal muscular atrophy-like neuronal defects. Hum Mol Genet. 2011;20(3):553–79.
24. Wu X, Brewer G. The regulation of mRNA stability in mammalian cells: 2.0. Gene. 2012;500(1):10–21.
25. Fujiwara T, Mori Y, Chu DL, et al. CARM1 regulates proliferation of PC12 cells by methylating HuD. Mol Cell Biol. 2006;26(6):2273–85.
26. Maurin T, Zongaro S, Bardoni B. Fragile X syndrome: from molecular pathology to therapy. Neurosci Biobehav Rev. 2014;46(Pt 2):242–55.
27. Blackwell E, Zhang X, Ceman S. L-Arginine of the RGG box regulate FMRP association with polyribosomes and mRNA. Hum Mol Genet. 2010;19(7):1314–23.
28. Dolzhanskaya N, Merz G, Denman RB. Alternative splicing modulates protein L-arginine methyltransferase-dependent methylation of fragile X syndrome mental retardation protein. Biochemistry. 2006;45(34):10385–93.
29. Denman RB. Methylation of the L-arginine-glycine-rich region in the fragile X mental retardation protein FMRP differentially affects RNA binding. Cell Mol Biol Lett. 2002;7(3):877–83.
30. Stetler A, Winograd C, Sayegh J, et al. Identification and characterization of the methyl arginines in the fragile X mental retardation protein Fmrp. Hum Mol Genet. 2006;15(1):87–96.
31. Dolzhanskaya N, Merz G, Aletta JM, Denman RB. Methylation regulates the intracellular protein-protein and protein-RNA interactions of FMRP. J Cell Sci. 2006;119(Pt 9):1933–46.
32. Denman RB, Dolzhanskaya N, Sung YJ. Regulating a translational regulator: mechanisms cells use to control the activity of the fragile X mental retardation protein. Cell Mol Life Sci. 2004;61(14):1714–28.
33. Deng H, Gao K, Jankovic J. The role of FUS gene variants in neurodegenerative diseases. Nat Rev Neurol. 2014;10(6):337–48.
34. Tradewell ML, Yu Z, Tibshirani M, Boulanger MC, Durham HD, Richard S. L-Arginine methylation by PRMT1 regulates nuclear-cytoplasmic localization and toxicity of FUS/TLS harbouring ALS-linked mutations. Hum Mol Genet. 2012;21(1):136–49.
35. Rappsilber J, Friesen WJ, Paushkin S, Dreyfuss G, Mann M. Detection of L-arginine dimethylated peptides by parallel precursor ion scanning mass spectrometry in positive ion mode. Anal Chem. 2003;75(13):3107–14.

36. Sasaki S, Takeda T, Shibata N, Kobayashi M. Alterations in subcellular localization of TDP-43 immunoreactivity in the anterior horns in sporadic amyotrophic lateral sclerosis. Neurosci Lett. 2010;478(2):72–6.
37. Banerjee A, Apponi LH, Pavlath GK, Corbett AH. PABPN1: molecular function and muscle disease. FEBS J. 2013;280(17):4230–50.
38. Lukong KE, Richard S. Sam68, the KH domain-containing superSTAR. Biochim Biophys Acta. 2003;1653(2):73–86.
39. Bedford MT, Frankel A, Yaffe MB, Clarke S, Leder P, Richard S. L-Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. J Biol Chem. 2000;275(21):16030–6.
40. Liao WT, Liu JL, Wang ZG, et al. High expression level and nuclear localization of Sam68 are associated with progression and poor prognosis in colorectal cancer. BMC Gastroenterol. 2014;14(1):126.
41. Rho J, Choi S, Jung CR, Im DS. L-Arginine methylation of Sam68 and SLM proteins negatively regulates their poly(U) RNA binding activity. Arch Biochem Biophys. 2007;466(1):49–57.
42. Bomsztyk K, Denisenko O, Ostrowski J. hnRNP K: one protein multiple processes. BioEssays. 2004;26(6):629–38.
43. Chen Y, Zhou X, Liu N, et al. L-Arginine methylation of hnRNP K enhances p53 transcriptional activity. FEBS Lett. 2008;582(12):1761–5.
44. Yang JH, Chiou YY, Fu SL, et al. L-Arginine methylation of hnRNPK negatively modulates apoptosis upon DNA damage through local regulation of phosphorylation. Nucleic Acids Res. 2014;42(15):9908–24.
45. Ostareck-Lederer A, Ostareck DH, Rucknagel KP, et al. Asymmetric L-arginine dimethylation of heterogeneous nuclear ribonucleoprotein K by protein-L-arginine methyltransferase 1 inhibits its interaction with c-Src. J Biol Chem. 2006;281(16):11115–25.
46. Lu R, Wang GG. Tudor: a versatile family of histone methylation 'readers'. Trends Biochem Sci. 2013;38(11):546–55.
47. Shen EC, Henry MF, Weiss VH, Valentini SR, Silver PA, Lee MS. L-Arginine methylation facilitates the nuclear export of hnRNP proteins. Genes Dev. 1998;12(5):679–91.
48. Devoy A, Soane T, Welchman R, Mayer RJ. The ubiquitin-proteasome system and cancer. Essays Biochem. 2005;41:187–203.
49. Cho EC, Zheng S, Munro S, et al. L-Arginine methylation controls growth regulation by E2F-1. EMBO J. 2012;31(7):1785–97.
50. Chen HZ, Tsai SY, Leone G. Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer. 2009;9(11):785–97.

Chapter 16
DNA–L-Arginine Adducts and Implications in Disease

Haseeb Ahsan

Key Points

- The polybasic compounds and histones proteins are rich in basic amino acids, lysine and L-arginine.
- The basic amino acid L-arginine can be covalently cross-linked with DNA and polynucleotides resulting in the formation of photoadduct.
- The DNA–L-arginine photoadducts may have important implications in various pathophysiological and immunopathological conditions.
- A strong recognition of DNA–L-arginine and polynucleotide–L-arginine photoadducts was observed with antibodies from the sera of SLE patients.
- The research suggests the possible involvement of such photoadducts as a potential trigger for anti-DNA autoantibody production in autoimmunity.
- The chapter presents studies to elucidate the underlying mechanism by which pathogenic autoantibodies develop in autoimmune disorders, such as SLE and RA.

Keywords L-Arginine • DNA–L-arginine adducts • Polynucleotide–L-arginine adducts • Photoadducts • Autoantibodies

Abbreviations

Arg	L-Arginine
SLE	Systemic lupus erythematosus
UV	Ultraviolet
Tm	Thermal melting temperature
NO	Nitric oxide
O_2^-	Superoxide anion

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Introduction

L-Arginine

Among the different amino acids that make up proteins in the body, L-arginine, histidine, and lysine are classified as basic amino acids due to the fact that they contain side groups that are positively charged at neutral pH (Fig. 16.1). L-Arginine (R, Arg, 2-Amino-5-guanidinopentanoic acid) contains a guanidinium group and has been characterized as a semi-essential amino acid. It is encoded by the triplet bases (codons), namely AGA and AGG (Table 16.1). It is nonessential in the healthy organisms of most mammals, but has to be supplemented in the growing organisms, after trauma or during disease [1–4]. Normal level of L-arginine in plasma is in the range of 100–200 μM [5–8]. The de novo L-arginine formation, which contributes about 10–15 % of the total body L-arginine production, under normal conditions [9, 10] involves the conversion of citrulline to L-arginine which is catalyzed by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) [11–14]. Free L-arginine in vivo is obtained from the diet, endogenous synthesis, and turnover of proteins [15] (Fig. 16.2). Although synthesis of L-arginine from citrulline can occur in many cell types [16–18], a major part of endogenous synthesis occurs via a collaboration between the epithelial cells of the small intestine and proximal tubule cells of the kidney [14, 19–21]. In healthy adults, the level of endogenous synthesis is sufficient such that L-arginine is not a dietary essential amino acid. Accordingly, L-arginine is classified as a semi-essential or conditionally essential amino acid [3, 22, 23]. However, in cases of catabolic stress (e.g., inflammation or infection) or conditions involving dysfunction of the kidneys or small intestine, levels of endogenous synthesis may not be sufficient to meet metabolic demands. Plasma levels of L-arginine in healthy adults are 80–120 mmol/L. Therefore, L-arginine homeostasis is modulated by L-arginine catabolism rather than of L-arginine synthesis [24].

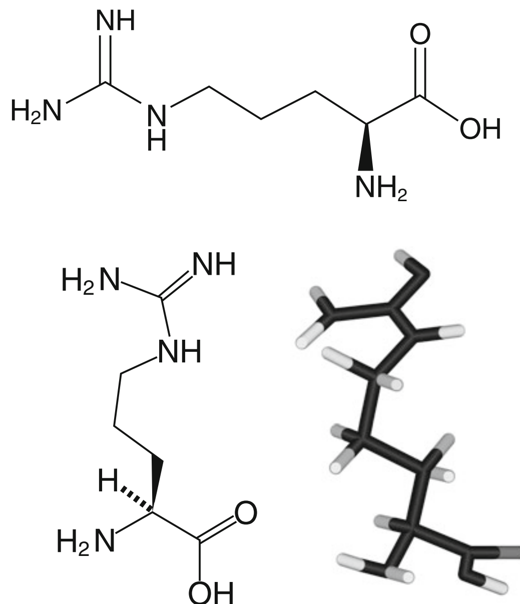

Fig. 16.1 Chemical structure of L-arginine, a basic amino acid

Table 16.1 Characteristic features of L-arginine, a basic amino acid

Feature/property (lexicographical order)	Characteristics
1. Appearance	Powder
2. Chemical formula	$C_6 H_{14} N_4 O_2$
3. Formula/molecular weight	174.2
4. Isoelectric point (pH)	11.1
5. IUPAC name	2-Amino-5-guanidinopentanoic acid
6. Melting point (°C)	222
7. One- and three-letter abbreviation	R, Arg
8. pKa value	2.1, 9.0, 13.2
9. Solubility	Water
10. Triplet codons	AGA, AGG

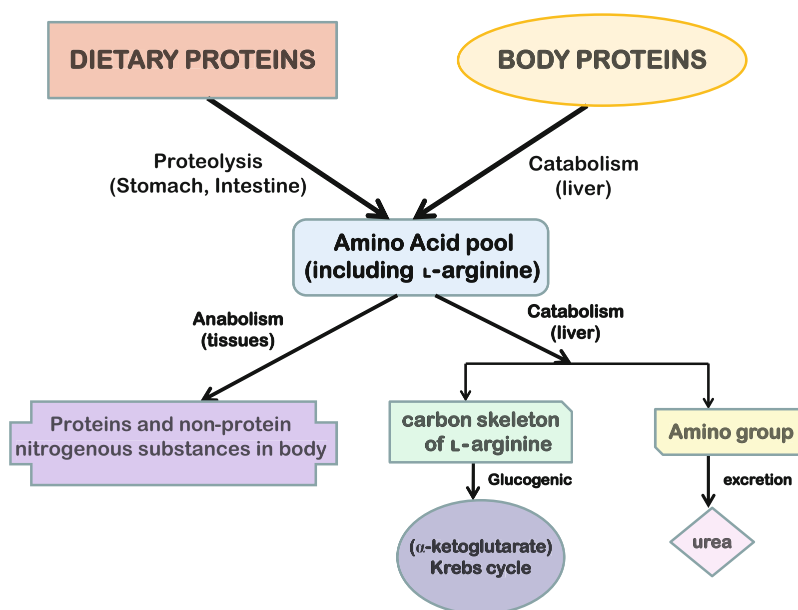

Fig. 16.2 The fate of proteins and amino acids (L-arginine) in the body

In addition to dietary sources and endogenous synthesis, the availability of L-arginine for metabolic functions is also determined by activities of its transporters in membranes [25–27]. L-Arginine is transported into the cells by a family of specific cationic amino acid transporter (CAT) proteins consisting of four members (CAT-1, -2A, -2B, and 3) with CAT-2A and 2B being splicing variants of the same gene. Failure to induce cationic amino acid transporter-2 (CAT-2) can limit L-arginine availability for induced NO synthesis to varying degrees, depending on the cell type [28–30]. CAT-2 deficiency in mice has been shown to result in spontaneous inflammation in lungs, and the studies suggest that CAT-2 regulates anti-inflammatory processes in lung via its impact on NO production by alveolar macrophages, which in turn is required for suppression of the activation of dendritic cells [31].

L-Arginine–Nucleic Acid Adducts

The studies in our laboratory have investigated the photochemical addition of lysine and L-arginine to native DNA in view of its potential importance in the photo-cross-linking of histones to DNA in chromatin [32, 33]. On UV irradiation, the C-2 carbon atom of thymine undergoes a covalent photoaddition reaction with the e-amino group of lysine to form a DNA–lysine photoconjugate or photoadduct [34]. Nearly 60 % of thymine and cytosine bases in DNA are modified due to lysine photoaddition. Every helical turn of DNA contains about one lysine molecule in the bound state [35]. The UV spectroscopic analysis of the DNA–lysine photoadduct showed hyperchromism, indicating either the formation of single-stranded breaks in DNA or opening of the double-stranded polymer at the site of lysine conjugation [34].

The UV spectral analysis of the DNA–L-arginine photoadduct shows changes in DNA spectra as a result of photomodification. A hyperchromic effect was observed due to increase in absorbance for the DNA–L-arginine photoadduct when compared with that of native DNA and the unirradiated DNA–L-arginine complex as control (Fig. 16.3a). Hyperchromicity represents the formation of single-stranded regions due to adduct formation. The UV absorbance ratios (A260/A280) of DNA–L-arginine adduct decreased from those of native DNA [33]. The results of these studies have also been summarized in Table 16.2 [33]. The melting profile of the DNA–L-arginine adduct reveals the UV radiation-induced incorporation of L-arginine into the native DNA (Fig. 16.4a). The photoaddition of L-arginine to DNA might have favorable A=T and G≡C pairing interaction of double helical native DNA, thus decreasing the duplex melting temperature (Tm) as compared with native DNA [33].

We have also investigated the photochemical addition of L-arginine to 200 base pair (bp) fragments of calf thymus DNA [36]. The UV spectroscopic analysis of 200 bp DNA–L-arginine photoadduct showed hypochromism, which may not be due to photoinduced denaturation of DNA. Evidence for the structural changes in 200 bp DNA as a result of L-arginine photoaddition was revealed by analyzing the data of thermal helix coil denaturation. The 200 bp DNA–L-arginine complex showed an increase in Tm value as compared to 200 bp DNA alone. Hence, the positively charged L-arginine on electrostatic interaction with negatively charged phosphate backbone of DNA renders it thermodynamically more stable [36] (Table 16.3, Fig. 16.3b).

The poly(dC) was covalently photolinked with either lysine or L-arginine by UV irradiation at 200–400 nm. The UV absorption spectroscopic analysis showed a substantial decrease in absorbance for poly(dC)-lysine photoadduct at 266 nm in comparison to native poly(dC) (Fig. 16.3c). This appreciable degree of hypochromism observed could be attributed to the formation of diadducts/cross-links in a major proportion than monoadducts. Poly(dC)-L-arginine photoadduct exhibited a higher magnitude of hypochromicity (37 %) compared with poly(dC)-lysine adduct [37].

By the photoaddition of lysine or L-arginine to DNA, the native B-conformation is considerably altered, resulting in the generation of high-affinity neo-epitopes. The unusual DNA conformations are most likely immunogenic because they exist transiently in cells and therefore not subject to tolerance. Thus, when administered to animals in a stabilized form, they can stimulate antibody production. Animals immunized with covalently/non-covalently modified DNA induce high titer antibodies, which are exclusively directed towards modified structures. Amino acids linked by ionic/covalent interactions might generate conformational epitopes producing antibodies of diverse antigenic specificity [36]. The 200 bp DNA–L-arginine photoadduct was used as an antigen for the induction of antibodies in rabbits. The photoadduct was found to be immunogenic inducing high titer antibodies. The results suggest that most of the immune IgG recognized the modified portion of DNA [36] (Fig. 16.5).

Several studies have suggested modified forms of DNA and polynucleotides as immunogens reacting with high titer antibodies whose antigen recognition resembles that of human anti-DNA antibody binding characteristics. The autoantibodies to DNA and its various animal models are heterogeneous in respect to antigen binding specificities and their reactivities range from single bases to dsDNA. Monoclonal representatives of anti-DNA antibodies are found to react more strongly with

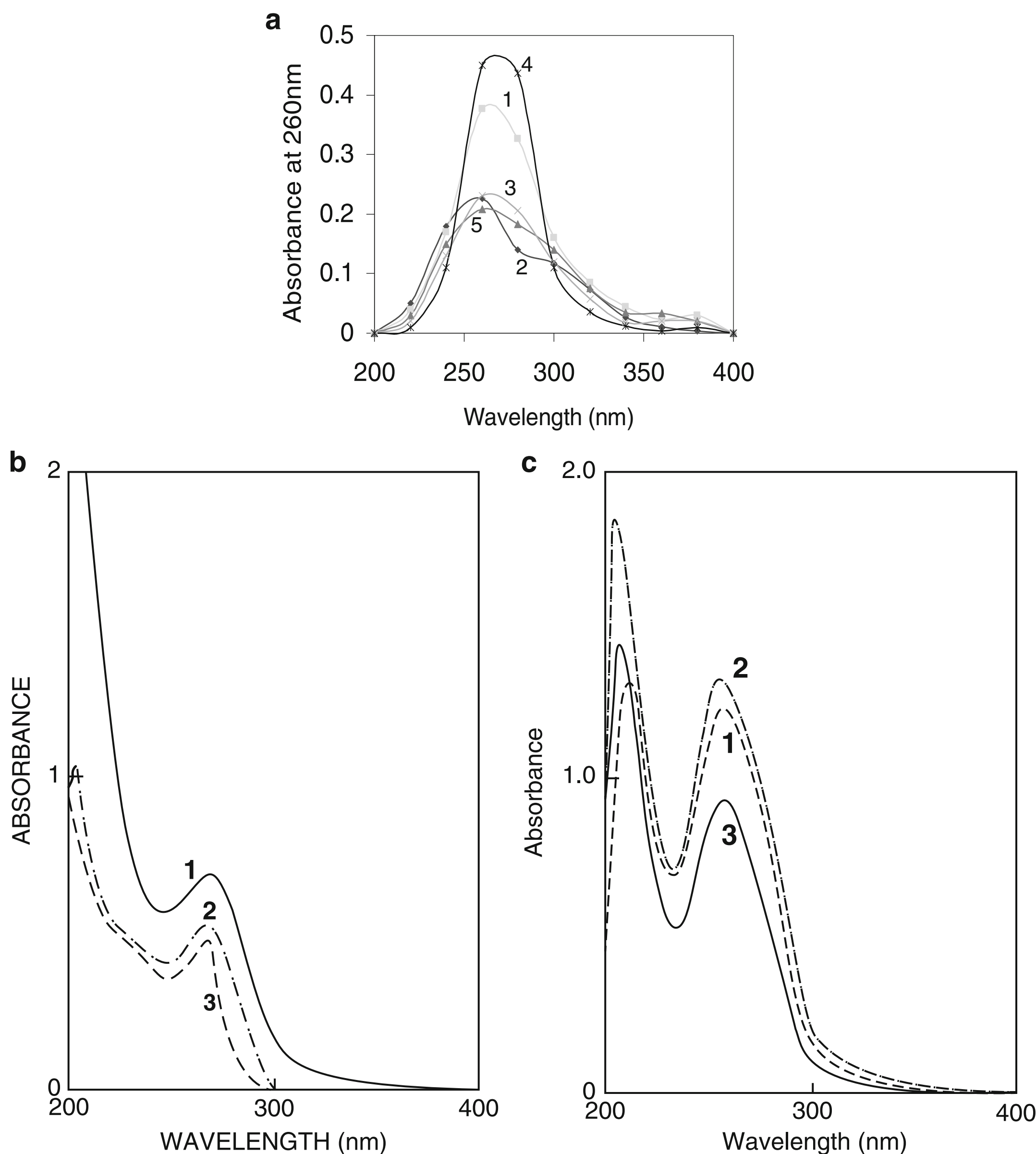

Fig. 16.3 (**a**) Ultraviolet absorption spectra of DNA–L-arginine photoadduct (*filled square*), native unirradiated DNA (*filled diamond*), irradiated L-arginine (*slash*), unirradiated L-arginine (*multiplication*), and unirradiated DNA–L-arginine adduct (*filled triangle*) (adapted from Ahmad et al. [33]). (**b**) UV absorption of 200 bp DNA–L-arginine photoadduct. 200 bp DNA alone (*dotted lines*), irradiated 200 bp DNA (*dashed lines*), and 200 bp DNA–L-arginine adduct (*solid lines*). (**c**) UV absorption spectra of polydeoxyribonucleotide–L-arginine photoadduct. Polynucleotide alone (1, *solid lines*), polynucleotide–lysine (2, *dashed lines*), and polynucleotide–L-arginine (3, *dotted lines*) (adapted from Dixit et al. [37])

denatured DNA than nDNA and are also found to react with synthetic polynucleotides, such as poly(G) and poly(I) [38]. Other specificities include RNA–DNA hybrids, triple helical RNA, poly(A), poly (U), poly(I), and poly(C). On the basis of reactivity with guanine, hypoxanthine, and adenine containing polymers, it has been suggested that polymers having 6,7,8 positions of the purine exposed in the major groove are likely to be involved [39].

Table 16.2 Biochemical properties of human DNA–L-arginine adduct

Property	L-Arginine–DNA photoadduct	NO-modified L-arginine—DNA photoadduct
$A_{260/280}$	1.1	1.1
Tm (°C)	61	69.5
Percent hyperchromicity	66	69

Adapted and modified from Ahmad and Ahsan [42]

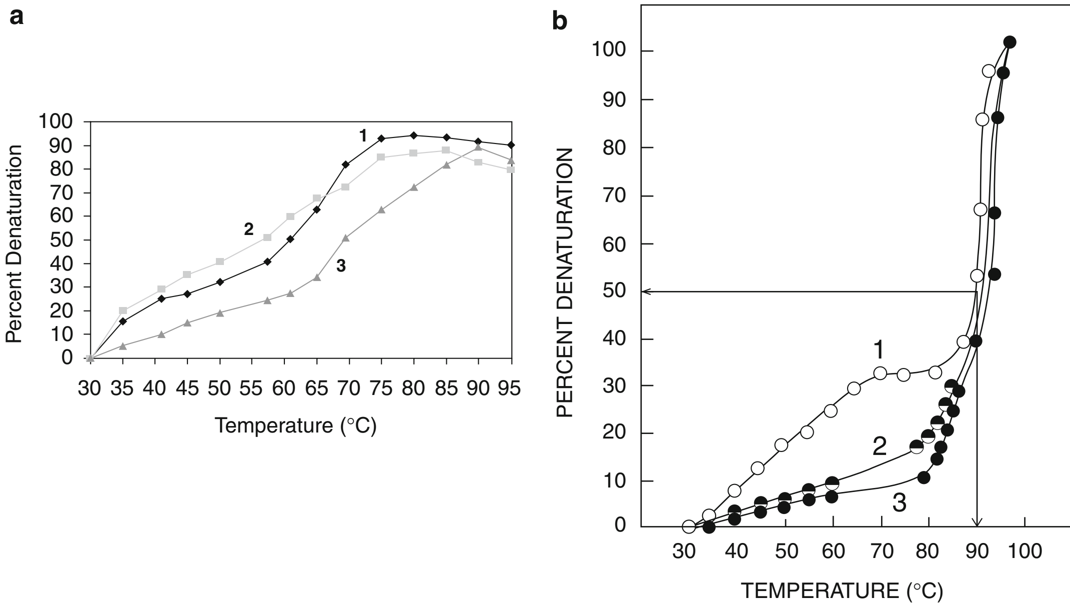

Fig. 16.4 (**a**) Thermal melting profile of native DNA (*filled diamond*), DNA–L-arginine adduct (*filled square*), and reactive nitrogen species modified DNA–L-arginine adduct (*filled triangle*) (adapted from Ahmad et al. [33]). (**b**) Thermal melting profile of native polydeoxyribonucleotides (1, *opened circle*), polydeoxyribonucleotide–lysine (3, *filled circle*), and polynucleotide–L-arginine (2, *half-filled circle*) photoadducts (adapted from Dixit et al. [37])

Table 16.3 Biochemical properties of 200 bp DNA–L-arginine adduct

Characteristics	Native 200 bp DNA	200 bp DNA–L-arginine photoadduct
A 260 nm	1.24	0.94
A 280 nm	0.68	0.52
$A_{260/280}$	1.82	1.80
Tm (°C)	80	83

Adapted and modified from Ahsan et al. [36]

Lysine- and L-arginine-rich histone H1 in nucleosome on modification by physical, chemical, or environmental agents might form histone–DNA adducts making it immunogenic and, thus, resulting in the production of autoantibodies [40, 41]. During the process of apoptosis, the release of excessive quantities of nucleosome debris may be a source of nuclear antigens that drives an immune response, inducing antioligonucleotide and antipolypeptide antibody production. Also, antinucleosome-specific antibodies have been reported in lupus prone mice. Only the pathogenic T-helper cells of these mice

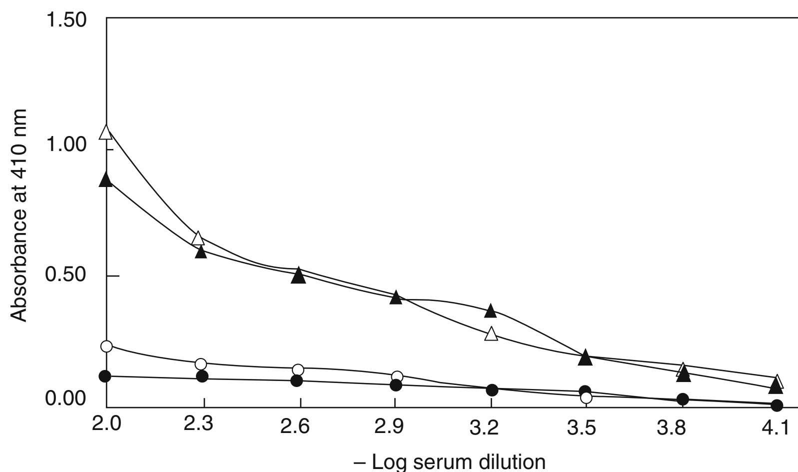

Fig. 16.5 Direct binding of ELISA of serum antibodies to 200 bp DNA–L-arginine photoadduct (*opened triangle*, *filled triangle*) generated in rabbits. The pre-immune sera show negligible binding (*filled circle*, *opened circle*)

responded to nucleosomal antigens and their stimulation was specific. Therefore, it appears that pathogenic anti-DNA autoantibodies are generated through some conformational epitope(s) of nucleic acids and polypeptides [36, 42–44].

Implications of L-Arginine Adducts in Immunopathology

Exposure to ultraviolet (UV) light, particularly the UVB (280–315 nm) component, has several harmful effects on humans. The photoconjugation of proteins and amino acids to DNA is one of the lesions produced in biologic systems by UV light. The role of DNA–protein cross-links in aging, carcinogenesis, and radiation-induced manifestations has been well documented. There are many compounds in the vicinity of DNA that may react upon irradiation, such as polyamines, histones, and nuclear matrix elements. The potential motifs in histones that are strongly involved in the binding of DNA to the nucleosome core in chromatin are believed to be those that are rich in lysine and L-arginine. The thymine–lysine conjugate is involved in photolinking histones to DNA in calf thymus nucleohistones and chicken erythrocyte nuclei. UV light can damage DNA by producing thymine dimers, which are cross-links between pyrimidine bases [45]. On the other hand, oxidants such as free radicals or hydrogen peroxide produce multiple forms of damage, including base modifications, particularly of guanosine and double-strand breaks. In human cells, the nitrogenous bases suffer oxidative damage every day and the most hazardous are double-strand breaks, as they are difficult to repair and can produce point mutations, insertions, and deletions from the DNA sequence as well as translocations. Many mutagens fit into the spaces between two adjacent base pairs by intercalation. Most intercalators are aromatic and planar molecules, such as ethidium bromide, aflatoxin, acridines, benzopyrene diol epoxide, daunomycin, and doxorubicin. For an intercalator to fit between base pairs, the bases must separate, distorting the DNA strands by unwinding the double helix. This inhibits both DNA replication and transcription, causing mutations [46]. Adduct formation results in increased secretion of messenger molecules such as cytokines and chemokines that mediate communication among cells and promote inflammation, e.g., tumor necrosis factor (TNF). Antibodies have been developed against an array of carcinogen–DNA adducts as well as UV damaged and oxidized bases [47].

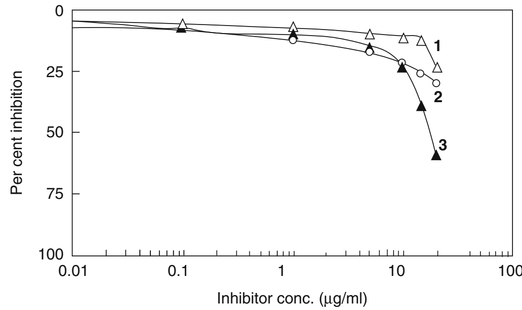

Fig. 16.6 Inhibition ELISA of immune serum with 200 bp DNA alone (*opened triangle*), native calf thymus DNA (*opened circle*), and 200 bp DNA–L-arginine adduct (*filled triangle*) (adapted from Ahsan et al. [36])

One of the most interesting aspects of autoimmune diseases is the spontaneous occurrence of anti-nucleic acid antibodies in some diseases. DNA against which most of the antibodies are detected in lupus and other autoimmune diseases is no longer regarded as an antigen initiating the disease, mainly because immunization with DNA does not induce SLE [48]. Although the correlation of SLE with the immune system is well documented, neither the origin nor the etiology of the disease is known. Polybasic molecules, such as polyamines and histones found in cells, have a great affinity for acidic constituents such as DNA. The SLE antibodies react with dsDNA from a wide range of species. Furthermore, they also react with single-strand DNA, RNA, and synthetic polymers, suggesting that they are not exclusively directed against the helical backbone of DNA. It appears that SLE antibodies recognize minor determinants in nucleic acid structure. The production of autoantibodies in certain autoimmune disorders, particularly SLE, has been attributed to the selective stimulation of autoreactive B lymphocytes by self-antigens or the cross-reaction of antigens with self [49]. In autoimmune prone individuals, B cells are hyperreactive to various polyclonal activators, which bypass the T-cell regulatory mechanism. These and several other factors cause immune dysfunction, leading to polyclonal B-cell activation [50]. An increased rate of apoptosis, correlating to disease activity, has been demonstrated in lymphocytes from SLE patients [51]. During the process of apoptosis, the release of excessive quantities of intact nucleosomes may be a source of nuclear antigens that drives an immune response, inducing anti-histone and anti-DNA antibody production. The UV-induced alteration of nucleic acids might result in altered conformations of DNA, which could be an alternative or additional mechanism for the production of anti-DNA autoantibodies. Polynucleotide antibodies may combine with circulating antigens at any stage of the disease process and contribute to deposition of immune complex(es) in renal glomeruli. It has been suggested that the site of immune deposition and the resulting pathologic and clinical abnormalities may be dependent on properties unique to subset of anti-DNA autoantibodies [52, 53].

In our studies on sera from SLE patients, the 200 bp DNA–L-arginine photoadduct exhibited increased binding as compared to 200 bp DNA alone (Fig. 16.6). The results were further confirmed by IgG isolated from sera of these patients, indicating increased recognition of the 200 bp DNA–L-arginine photoadduct by the immunoglobulins [36] (Fig. 16.7). Furthermore, the poly(dC)-lysine and poly(dC)-L-arginine photoadducts were also found to be effective inhibitors and their relative affinity was substantially higher than that of native poly(dC) (Fig. 16.8). These results point to the likelihood of modifications to polynucleotides in DNA for its better recognition by SLE autoantibodies as a causative agent for the induction of circulating anti-DNA or antipolynucleotide antibodies. Therefore, it appears that lysine and L-arginine may have an important role in the generation of these antibodies [36].

Our results suggest that on UV irradiation, L-arginine can induce certain conformational alterations in DNA, rendering it immunogenic. The recognition of DNA–L-arginine photoadduct by autoantibodies might be helpful in understanding its origin in SLE and the role of positively charged amino acids in the pathogenesis of autoimmune disorders. The greater binding of anti-DNA autoantibodies to

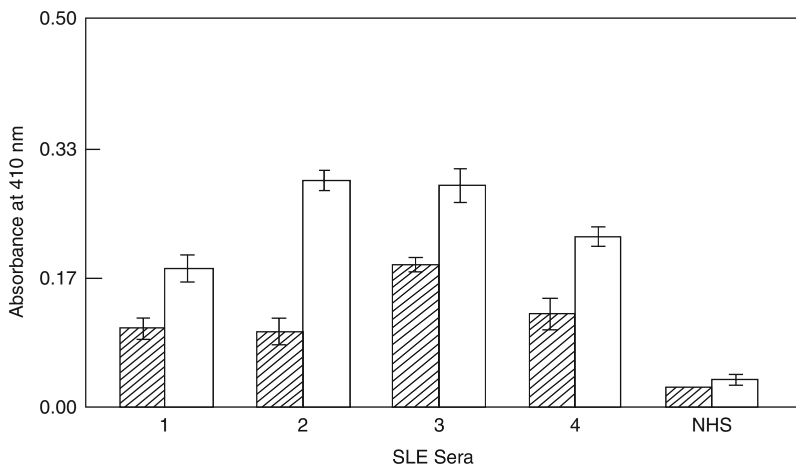

Fig. 16.7 Binding of SLE anti-DNA antibodies to 200 bp DNA (*dashed bar*) and 200 bp DNA–L-arginine photoadduct (*opened bar*). NHS represents normal human serum (adapted from Ahsan et al. [36])

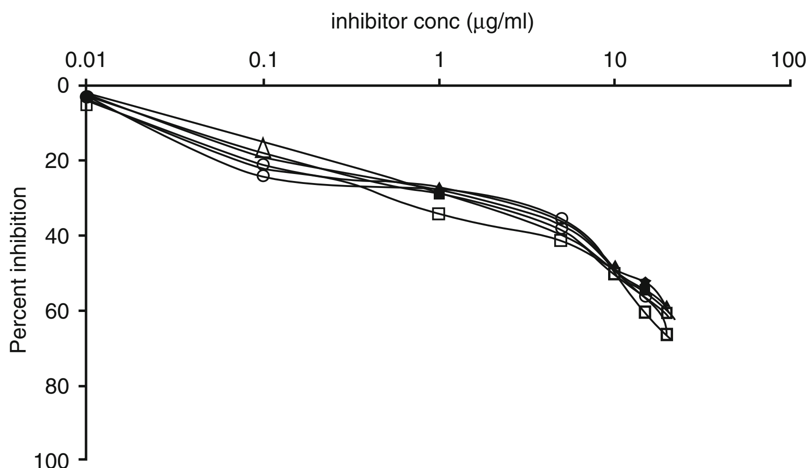

Fig. 16.8 Enzyme-linked immunosorbent assay of IgGs from SLE patients. The inhibitor used was polydeoxyribonucleotide–L-arginine adduct (adapted from Dixit et al. [37])

L-arginine photoadduct indicates the possible involvement of L-arginine in the etiopathogenesis of autoimmune diseases such as SLE and others [36].

It has been suggested that DNA released from the epidermal cells as a consequence of UV-induced cellular injury may react with circulating antibodies. Since a higher incidence of antibody deposits appears in sunlight-exposed areas, UV-induced alteration of cellular DNA may be involved in stimulating the formation of certain immune complexes in cutaneous lesions. Studies have implicated UV-induced damage of cellular DNA as an important factor in SLE. The deposition of antibodies occurs at the site of cutaneous lesions and in the skin of SLE patients [54].

Photoadduct formation and immune modulation are some factors responsible for induction or exacerbation of an immune response in SLE [55]. Studies have shown that SLE and autoantibodies bind furocoumarin–DNA and spermine–DNA conjugates [56, 57]. There are concerns about the possible risk of autoimmune diseases in psoralen and UVA light (PUVA)-treated patients. Hence,

alteration in DNA resulting from photoreaction could lead to the development of antibodies to DNA or could precipitate SLE. Since there are many polybasic compounds in the vicinity of DNA, there exists the possibility of their interaction with DNA on exposure to radiation. The retention of immunoreactivity of autoantibodies with isolated thymine–lysine and cytosine–lysine photoconjugate after immunoaffinity purification has strengthened the perception of an alternate antigen for the induction antibodies cross-reactive with native DNA [44].

Conclusion

L-Arginine, a basic amino acid, is one of the amino acids produced in the human body by the digestion or hydrolysis of proteins. L-Arginine methylation of non-histone proteins is used in transcriptional regulation. Protein–L-arginine methylation is used for regulation of transcriptional and various physiological pathological processes. Protein methylation may affect protein–protein, protein–DNA, or protein–RNA interaction. L-Arginine has an effect on the DNA-binding activity of NF-κB, a dominant transcriptional factor in inflammation. Adduct formation results in increased secretion of messenger molecules such as cytokines and chemokines that mediate communication among cells and promote inflammation. L-Arginine and lysine amino acid-rich histones in nucleosomes on modification by environmental agents form histone–DNA adducts, making it immunogenic. Alteration of DNA resulting from photomodification could lead to the development of antibodies or mutations to modified DNA.

Native DNA (nDNA) is a poor immunogen, whereas its modified forms have been demonstrated to be immunogenic. Many studies have suggested modified DNA and polynucleotides as immunogens reacting with high-titer antibodies whose antigen recognition resembles human anti-DNA antibody binding characteristics. The autoantibodies to DNA in human SLE are heterogeneous in respect to antigen binding specificity and their reactivities range from single bases to dsDNA. SLE is a prototype autoimmune and multifactorial disease characterized by the presence of autoantibodies to a diverse array of nuclear antigens including DNA, RNA, and histones. These naturally occurring autoantibodies are heterogeneous, exhibiting a wide heterogeneity in the recognition of nucleosides and nucleotides.

There are many compounds in the vicinity of DNA that may react with it upon irradiation. These compounds include polyamines, histones, and nuclear matrix elements. Polybasic molecules found in the cells have great affinity for acidic constituents, such as DNA and polynucleotides, and their interaction might play a role in the pathogenesis of SLE. Ultraviolet radiation has an effect on the immune system. It has been reported that certain autoimmune diseases can be induced or exacerbated by exposure to UV radiation. Flare of disease activity in some patients following exposure to UV light is a well-recognized phenomenon. Exposure of DNA to UV radiation results in the formation of photoproducts that are antigenic and can induce the formation of antibodies that react specifically with modified DNA. Hence, the DNA–L-Arginine photoadducts could have important implications in the etiopathogenesis of various conditions such as toxicity, carcinogenicity, and autoimmunity.

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References

1. Rose WC, Haines WJ, Warner DT. The amino acid requirements of man. V. The role of lysine, L-arginine, and tryptophan. J Biol Chem. 1954;206:421–30.
2. Ha YH, Milner JA, Corbin JE. L-Arginine requirements in immature dogs. J Nutr. 1978;108:203–10.
3. Barbul A. L-Arginine: biochemistry, physiology, and therapeutic implications. J Parenter Enteral Nutr. 1986;10:227–38.
4. Heird WC. Amino acids in pediatric and neonatal nutrition. Curr Opin Clin Nutr Metab Care. 1998;1:73–8.
5. Pieper GM, Dondlinger LA. Plasma and vascular tissue L-arginine are decreased in diabetes: acute L-arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. J Pharmacol Exp Ther. 1997;283:684–91.
6. Tangphao O, Chalon S, Coulston AM, Moreno Jr H, Chan JR, Cooke JP, et al. L-Arginine and nitric oxide-related compounds in plasma: comparison of normal and L-arginine-free diets in a 24-h crossover study. Vasc Med. 1999;4:27–32.
7. Tangphao O, Grossmann M, Chalon S, Hoffman BB, Blaschke TF. Pharmacokinetics of intravenous and oral L-arginine in normal volunteers. Br J Clin Pharmacol. 1999;47:261–6.
8. Morris CR, Poljakovic M, Lavrisha L, Machado L, Kuypers FA, Morris Jr SM. Decreased L-arginine bioavailability and increased serum arginase activity in asthma. Am J Respir Crit Care Med. 2004;170:148–53.
9. Castillo L, Sanchez M, Vogt J, Chapman TE, DeRojas-Walker TC, Tannenbaum SR, et al. Plasma L-arginine, citrulline, and ornithine kinetics in adults, with observations on nitric oxide synthesis. Am J Physiol Endocrinol Metab. 1995;268:E360–7.
10. Dejong CH, Welters CF, Deutz NE, Heineman E, Soeters PB. Renal L-arginine metabolism in fasted rats with subacute short bowel syndrome. Clin Sci (Lond). 1998;95:409–18.
11. Dhanakoti SN, Brosnan JT, Herzberg GR, Brosnan ME. Renal L-arginine synthesis: studies in vitro and in vivo. Am J Physiol Endocrinol Metab. 1990;259:E437–42.
12. Dioguardi FS. To give or not to give? Lessons from the L-arginine paradox. J Nutrigenet Nutrigenomics. 2011;4:90–8.
13. Domenico R. Pharmacology of nitric oxide: molecular mechanisms and therapeutic strategies. Curr Pharm Des. 2004;10:1667–76.
14. Featherston WR, Rogers QR, Freedland RA. Relative importance of kidney and liver in synthesis of L-arginine by the rat. Am J Physiol. 1973;224:127–9.
15. Luiking YC, Ten Have GAM, Wolfe RR, Deutz NEP. L-Arginine de novo and nitric oxide production in disease states. Am J Physiol Endocrinol Metab. 2012;303:E1177–89.
16. Eagle H. Amino acid metabolism in mammalian cell cultures. Science. 1959;130:432–7.
17. Jackson MJ, Beaudet al, O'Brien WE. Mammalian urea cycle enzymes. Annu Rev Genet. 1986;20:431–64.
18. Morris Jr SM. L-Arginine synthesis, metabolism, and transport: regulators of nitric oxide synthesis. In: Laskin JD, Laskin DL, editors. Cellular and molecular biology of nitric oxide. New York: Marcel Dekker, Inc.; 1999. p. 57–85.
19. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. Am J Physiol. 1981;241:E473–80.
20. Ryall J, Nguyen M, Bendayan M, Shore GC. Expression of nuclear genes encoding the urea cycle enzymes, carbamoyl-phosphate synthetase I and ornithine carbamoyl transferase, in rat liver and intestinal mucosa. Eur J Biochem. 1985;152:287–92.
21. Levillain O, Hus-Citharel A, Morel F, Bankir L. Localization of L-arginine synthesis along rat nephron. Am J Physiol. 1990;259:F916–23.
22. Morris RM. L-Arginine metabolism: boundaries of our knowledge. J Nutr. 2007;137:1602s–9.
23. Abumrad NN, Barbul A. The use of L-arginine in clinical practice. In: Cynober LA, editor. Metabolic and therapeutic aspects of amino acids in clinical nutrition. Boca Raton: CRC Press; 2004. p. 595–611.
24. Castillo L, Chapman TE, Sanchez M, Yu YM, Burke JF, Ajami AM, et al. Plasma L-arginine and citrulline kinetics in adults given adequate and L-arginine-free diets. Proc Natl Acad Sci U S A. 1993;90:7749–53.
25. Deves R, Boyd CAR. Transporters for cationic amino acids in animal cells: discovery, structure, and function. Physiol Rev. 1998;78:487–545.
26. Closs EI, Simon A, Vekony N, Rotmann A. Plasma membrane transporters for L-arginine. J Nutr. 2004;134:2752S–9.
27. Closs EI. Expression, regulation and function of carrier proteins for cationic amino acids. Curr Opin Nephrol Hypertens. 2002;11:99–107.
28. Manner CK, Nicholson B, MacLeod CL. CAT2 L-arginine transporter deficiency significantly reduces iNOS-mediated NO production in astrocytes. J Neurochem. 2003;85:476–82.
29. Nicholson B, Manner CK, Kleeman J, MacLeod CL. Sustained nitric oxide production in macrophages requires the L-arginine transporter CAT2. J Biol Chem. 2001;276:15881–5.

30. Nicholson B, Manner CK, MacLeod CL. Cat2 L-arginine transporter-deficient fibroblasts can sustain nitric oxide production. Nitric Oxide. 2002;7:236–43.
31. Rothenberg ME, Doepker MP, Lewkowich IP, Chiaramonte MG, Stringer KF, Finkelman FD, et al. Cationic amino acid transporter 2 regulates inflammatory homeostasis in the lung. Proc Natl Acad Sci U S A. 2006;103: 14895–900.
32. Ahmad R, Rasheed Z, Kaushal E, Singh D, Ahsan H. Biochemical evaluation of human DNA-lysine photoadduct treated with peroxynitrite. Toxicol Mech Method. 2008;18:589–95.
33. Ahmad R, Yadav N, Chaudhary K, Heming T, Ahsan H. Analysis of human DNA-L-arginine photoadduct modified with peroxynitrite. Nucleosides Nucleotides Nucleic Acids. 2012;31:377–87.
34. Ahmad R, Ahsan H. Contribution of peroxynitrite, a reactive nitrogen species, in the pathogenesis of autoimmunity. In: Mavragani CP, editor. Autoimmune disorders-pathogenetic aspects. Croatia: Intech Open Access; 2011. p. 141–96.
35. Islam N, Ali R. Formation of thymine-lysine and cytosine-lysine adducts in DNA-lysine photoconjugate. J Photochem Photobiol B. 1995;27(2):109–15.
36. Ahsan H, Abdi S, Ali A. Recognition of DNA-L-arginine photoadduct by anti-DNA autoantibodies in systemic lupus erythematosus. Ind J Med Res. 2002;115:201–11.
37. Dixit K, Ahsan H, Ali A. Polydeoxyribonucleotide C photoconjugated with lysine or L-arginine present unique epitopes for human anti-DNA autoantibodies. Hum Immunol. 2003;64(9):880–6.
38. Stollar BD, Zon G, Pastor RW. A recognition site on synthetic helical oligonucleotides for monoclonal antinative DNA autoantibody. Proc Natl Acad Sci U S A. 1986;83:4469.
39. Stollar BD. Antibodies to DNA. CRC Crit Rev Biochem. 1986;20:1.
40. Habib S, Moinuddin AR. Acquired antigenicity of DNA after modification with peroxynitrite. Int J Biol Macromol. 2005;35(3–4):221–5.
41. Habib S, Moinuddin AR. Peroxynitrite modified DNA: a better antigen for systemic lupus erythematosus anti-DNA autoantibodies. Biotechnol Appl Biochem. 2006;43(2):65–70.
42. Ahmad R, Ahsan H. Role of peroxynitrite-modified biomolecules in the etiopathogenesis of systemic lupus erythematosus. Clin Exp Med. 2014;14:1–11.
43. Ahmad R, Rasheed Z, Ahsan H. Biochemical and cellular toxicology of peroxynitrite: implications in cell death and autoimmune phenomenon. Immunopharmacol Immunotoxicol. 2009;31(3):388–96.
44. Islam N, Ali R. Immunological studies on DNA-lysine photoadduct. Biochem Mol Biol Int. 1998;45(3):453–64.
45. Pfeifer GP, You YH, Besaratinia A. Mutations induced by ultraviolet light. Mutat Res. 2005;571(1–2):19–31.
46. Werner MH, Gronenborn AM, Clore GM. Intercalation, DNA kinking, and the control of transcription. Science. 1996;271(5250):778–84.
47. Liehr JG, Gladek A, Macatee T, Randerath E, Randerath K. DNA adduct formation in liver and kidney of male Syrian hamsters treated with estrogen and/or alpha-naphthoflavone. Carcinogenesis. 1991;12(3):385–9.
48. Pisetsky DS, Grudier JP, Gilkeson GS. A role for immunogenic DNA in the pathogenesis of systemic lupus erythematosus. Arthritis Rheum. 1990;33:153.
49. Hardin JA. The lupus autoantigens and the pathogenesis of systemic lupus erythematosus. Arthritis Rheum. 1986;29:457–60.
50. Klinman DM, Steinberg AD. Inquiry into murine and human lupus. Immunol Rev. 1995;144:157.
51. Emlen W, Neibur J, Kadera R. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. J Immunol. 1994;152(7):3685–92.
52. Vlahakos DV, Foster MH, Adams S, Katz M, Ucci AA, Banett KJ, et al. Anti-DNA antibodies from immune deposits at distinct glomerular and vascular sites. Kidney Int. 1992;41:1690.
53. Raz E, Brezis M, Rosenmann E, Eilat D. Anti-DNA antibodies bind directly to renal antigens and induce kidney dysfunction in the isolated perfused rat kidney. J Immunol. 1989;142:3076.
54. Zamansky GD. Sunlight-induced pathogenesis in systemic lupus erythematosus. J Invest Dermatol. 1985;85:179–80.
55. Vermeer BJ, Hurks M. The clinical relevance of immunosuppression by UV irradiation. J Photochem Photobiol B. 1994;24:149–54.
56. Arjumand S, Moinuddin AA. Binding characteristics of SLE anti-DNA autoantibodies to modified DNA analogs. Biochem Mol Biol Int. 1997;43:643–53.
57. Moinuddin AA. SLE autoantibodies recognize spermine induced Z-conformation of native calf thymus DNA. Biochem Mol Biol Int. 1996;40:787–97.

Chapter 17
Homoarginine and L-Arginine/Glycine Amidinotransferase in Stroke

Chi-un Choe, Edzard Schwedhelm, and Dorothee Atzler

Key Points

- L-Arginine/glycine amidinotransferase synthesizes creatine precursors and homoarginine.
- Creatine deficiency protects from metabolic syndrome, i.e., obesity, diabetes, and hypercholesterinemia.
- Homoarginine is a marker of cardiovascular outcome and stroke in humans.
- Homoarginine reduces cerebral infarct size in experimental stroke model.
- Homoarginine influences NO metabolism.

Keywords L-Arginine/glycine amidinotransferase • Creatine • Homoarginine • Genome-wide association studies • Stroke • Cardiovascular disease • Metabolic syndrome

Abbreviations

4D	Die Deutsche Diabetes Dialyse
AGAT	L-Arginine/glycine amidinotransferase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BMI	Body mass index

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DCM	Dilatative cardiomyopathy
eGFR	Estimated glomerular filtration rate
eNOS	Endothelial NO synthase
GAMT	Guanidinoacetate-*N*-methyltransferase
GHS	Gutenberg Health Study
GWA	Genome-wide association
ICM	Ischemic cardiomyopathy
iNOS	Inducible NO synthase
LURIC	Ludwigshafen Risk and Cardiovascular Health
MRS	Magnetic resonance spectroscopy
NIHSS	National Institutes of Health Stroke Scale
nNOS	Neuronal NO synthase
NO	Nitric oxide
NOS	NO synthases
SEARCH	Study of effectiveness of additional reduction in cholesterol and homocysteine
SHIP	Study of Health in Pomerania
NT-proBNP	N-terminal pro-B-type natriuretic peptide
YFS	Young Finns Study

Introduction

Stroke is the second most frequent cause of death and leading cause of disability worldwide [1]. A third of stroke patients die within 1 year and more than half of the stroke patients are dead after 5 years [2, 3]. The most common causes of death are recurrent stroke and cardiovascular disease. In general, large and small vessel arteriosclerosis underlies both vascular entities. Consequently, cardiovascular disease and stroke pathology involve the same vascular risk factors. Therapeutic strategies and research have focused especially on modifiable risk factors, i.e., hypertension, diabetes, hyperlipidemia, and obesity. The combination of these four risk factors is known as metabolic syndrome, which is the consequence of supernutrition and excess body fat. Interestingly, reduced body weight is also associated with vascular mortality eliciting a bell-shaped association of body mass index (BMI) with vascular death [4, 5]. Therefore, balanced caloric intake and expenditure are necessary to optimize nutrition and health. In addition to established and conventional metabolic risk factors, much effort has been spent to identify novel metabolic pathways, which could influence vascular pathology and outcome. The long known, but until recently neglected, endogenous amino acid homoarginine has emerged as a significant marker and even more importantly as potential mediator of vascular disease [6, 7]. The enzyme, which catalyzes the synthesis of homoarginine, is L-arginine/glycine amidinotransferase (AGAT, EC 2.1.4.1). So far, AGAT was only known as the first and rate-limiting enzyme of creatine synthesis. To understand the role of AGAT in stroke pathology, it is mandatory to differentiate effects of AGAT metabolites creatine and homoarginine.

Creatine: Old Product of AGAT with New Functions

On the cellular level, energy-producing and consuming processes involve phosphorylation and hydrolysis of adenosine triphosphate (ATP), respectively. Therefore, ATP has to be generated by glycolysis, oxidative phosphorylation, and β-oxidation or replenished by high-energy-phosphate donors. Organs of fluctuating energy demand (i.e., the skeletal muscle, heart, and brain) possess the creatine/phosphocreatine system, which functions as a rapidly available energy buffer for ATP replenishment [8].

Creatine concentrations are about 40 mM in skeletal muscle, about 80 mM in heart, and about 10 mM in brain [9–11]. Creatine is subject to continued nonenzymatic degradation to creatinine at a constant rate of 1.7 % per day [12]. Creatinine cannot be recycled and is eliminated by renal excretion [8]. Therefore, creatine stores have to be refilled consecutively. Given its importance for whole-body energy metabolism, two alternative pathways exist. In addition to exogenous dietary creatine intake, vertebrates are able to synthetize creatine endogenously. Creatine biosynthesis is a two-step enzymatic process occurring mainly in the kidney and liver [8]. First, AGAT transfers the guanidino group of L-arginine to glycine producing guanidinoacetate and ornithine. Secondly, guanidinoacetate is methylated by guanidinoacetate-*N*-methyltransferase (GAMT, EC 2.1.1.2) using *S*-adenosylmethionine as methyl donor (Fig. 17.1). The first step of creatine biosynthesis mainly takes place in the kidney, whereas GAMT is highly expressed in liver [8]. Patients with AGAT and GAMT deficiency reveal reduced cerebral levels of creatine and phosphocreatine measured by magnetic resonance spectroscopy (MRS). Furthermore, the clinical presentation of patients with creatine synthesis defects includes muscular hypotonia and mental retardation development [13, 14]. These findings of human creatine deficiency underline the importance of creatine for organs of high energy demand, like the skeletal muscle and brain.

Creatine deficiency due to AGAT deficiency in mice results in a striking metabolic phenotype characterized by reduced body weight and decreased fat deposition in adipose and non-adipose tissue [11, 15]. Further analysis of creatine-deficient AGAT$^{-/-}$ mice revealed attenuated gluconeogenesis, improved glucose tolerance, and reduced cholesterol levels. Biochemical studies uncovered chronic activation of AMP-activated protein kinase (AMPK) in AGAT$^{-/-}$ mice as possible underlying cellular mechanism [15]. Oral creatine supplementation in AGAT$^{-/-}$ mice completely replenished creatine/phosphocreatine stores, rescued the metabolic phenotype, and normalized AMPK activation, proving creatine dependency [15]. Further analyses revealed that the complex metabolic phenotype of creatine-deficient AGAT$^{-/-}$ mice depends on the neuroendocrine axis [16]. Taken together, creatine deficiency attenuates the development of vascular risk factors, like diabetes, hyperlipidemia, and obesity.

Although protection from metabolic syndrome by AGAT deficiency implies beneficial effects for stroke risk reduction, we have also demonstrated that intracellular energy depletion impairs proper muscle ultrastructure and function [12]. AGAT$^{-/-}$ mice exhibited enhanced muscular atrophy and decreased grip strength [11, 15]. Metabolic analysis revealed an impaired oxidative phosphorylation in mitochondria associated with reduced ATP and increased inorganic phosphate levels. Hind limb ischemia resulted in an immediate decrease of pH indicating absence of an adequate buffering system and decreased tolerance to ischemia [12]. AGAT deficiency reduces intracellular energy stores, thereby protecting from metabolic syndrome but also decreasing ischemic tolerance, e.g., in skeletal muscle.

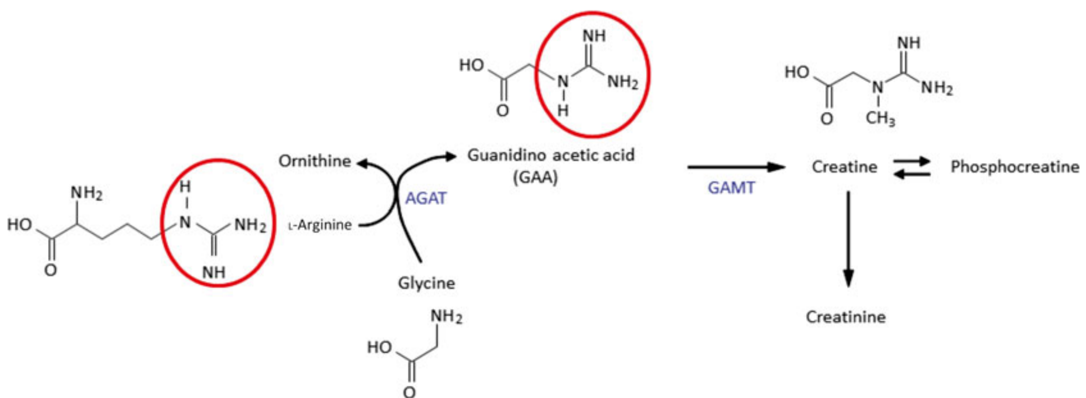

Fig. 17.1 Metabolic scheme of creatine synthesis by AGAT and GAMT. Creatine synthesis is a two-step enzymatic process, consisting of AGAT and GAMT. AGAT transfers the amidino group of L-arginine to glycine producing guanidinoacetate. GAMT methylates guanidino acetate resulting in creatine formation

Homoarginine: The New Product of AGAT

Homoarginine Metabolism and Function

As expected from human patients, AGAT deficiency in mice resulted in complete absence of guanidinoacetate, creatine, and phosphocreatine [13–15]. Extensive analysis of guanidino compounds in AGAT$^{-/-}$ mice revealed also undetectable levels of homoarginine—an endogenous non-proteinogenic amino acid with structural similarity to L-arginine [6]. Additional in vitro experiments using stable isotope-labeled L-arginine and lysine proved that AGAT is not only able to catalyze the transfer of the guanidino group from L-arginine to glycine producing guanidinoacetate but is also capable of facilitating the addition of the guanidino group of L-arginine to lysine catalyzing the synthesis of homoarginine (Fig. 17.2) [6]. This recent finding is in line with studies performed almost 50 years ago. Ryan et al. have shown that homoarginine is synthetized from lysine and that an amidinotransferase in kidney tissue is responsible for homoarginine synthesis [17, 18]. Most recently, two independent genome-wide association (GWA) analyses revealed that the *AGAT* gene locus is strongly and significantly associated with homoarginine levels in humans (see below). Consistently, in vitro studies with lymphoblasts from AGAT-deficient patients confirmed that AGAT is not only associated with homoarginine levels but responsible for homoarginine synthesis from L-arginine and lysine [19]. Circulating concentrations of homoarginine are about 2–3 μM in healthy humans with material age- and sex-related differences. Reference ranges for homoarginine obtained from healthy participants of the population-based Study of Health in Pomerania (SHIP) were 1.41–5.00 and 1.20–5.53 μmol/L for men and women, respectively (2.5th–97.5th percentile) [20]. In C57BL/6 mice, circulating homoarginine concentrations are approximately 0.15 μM and are therefore substantially lower than in humans [6, 21] (Figs. 17.3 and 17.4).

In the 1960s homoarginine was detected as a major component of about 1 % in different species of *Lathyrus sativus* L. (grass pea) [22–24]. Early functional studies in the 1970s have identified homoarginine as inhibitor of human liver and bone alkaline phosphatase [25–27]. More recent studies have suggested an involvement in vascular function and disease. Given its structural similarity with L-arginine, homoarginine is suspected to influence L-arginine metabolism. Probably the most important function of L-arginine is to serve as substrate for nitric oxide (NO) synthesis. Three subtypes of NO synthases (NOS) have been described so far, i.e., neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). Homoarginine has been shown to act as an alternative substrate for NOS also leading to NO formation [28–30]. Whereas maximal activity (V_{max})

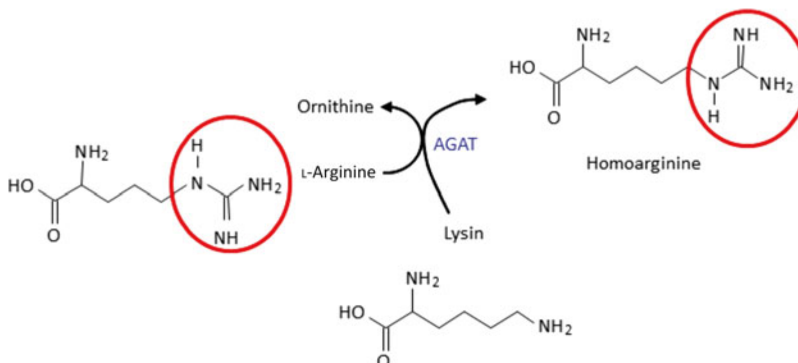

Fig. 17.2 Metabolic scheme of homoarginine synthesis by AGAT. Creatine synthesis is a two-step enzymatic process, consisting of AGAT and GAMT. AGAT transfers the amidino group of L-arginine to glycine producing guanidinoacetate. GAMT methylates guanidino acetate resulting in creatine formation

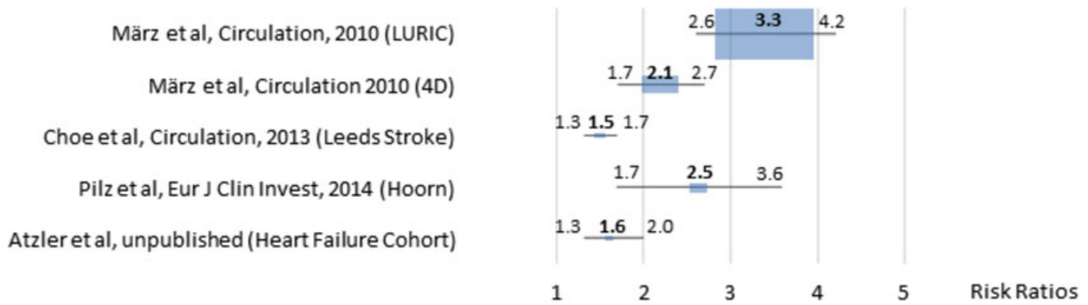

Fig. 17.3 Schematic forest plot of homoarginine levels and all-cause mortality risk (unadjusted risk ratios). Decreased homoarginine levels were associated with all-cause mortality in the LURIC study, 4D study, Hoorn study, Leeds Stroke Cohort, and Heart Failure Cohort (unadjusted risk ratios). Studies are plotted according to the last name of the first author followed by the journal name, publication year, and name of the study in parentheses. *Horizontal lines* represent 95 % confidence intervals. Each *square* represents the proportional weight of the study

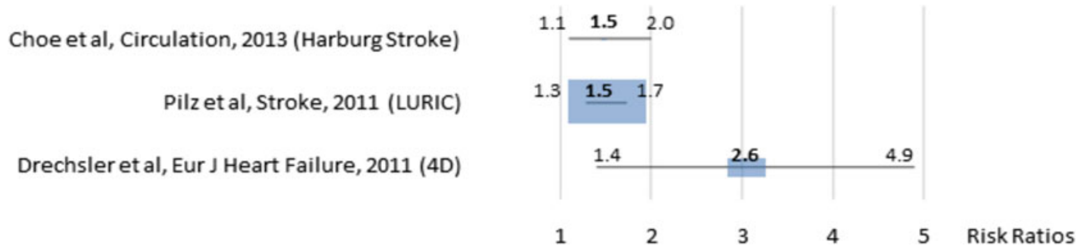

Fig. 17.4 Schematic forest plots of homoarginine levels and risk of cerebrovascular outcome (unadjusted risk ratios). Decreased homoarginine levels were associated with cerebrovascular outcome, i.e., fatal stroke in LURIC study, stroke incidence in 4D study, and NIHSS score in Harburg Stroke Cohort (unadjusted risk ratios). Studies are plotted according to the last name of the first author followed by the journal name, publication year, and name of the study in parentheses. *Horizontal lines* represent 95 % confidence intervals. Each *square* represents the proportional weight of the study

for NOS-dependent NO formation was similar between L-arginine and homoarginine, binding affinity (K_m) was 10- to 20-fold decreased for homoarginine compared with L-arginine [28]. Therefore, homoarginine is generally considered a weak substrate for NOS. In addition to biochemical analysis, mouse studies revealed lower maximal NO levels, but more sustained NO formation after homoarginine supplementation compared with L-arginine [30]. Nitrate/nitrite concentrations in the blood—an indirect parameter for NOS activity—were increased even 8 h after homoarginine treatment, whereas nitrate/nitrite levels in L-arginine supplemented mice returned to normal after 4 h [30]. High concentrations of homoarginine (2 mg/g body weight) were injected for this purpose. Therefore the physiological relevance of these findings needs to be validated. In addition to serving as a substrate for NOS, several groups have shown inhibition of the L-arginine-degrading enzyme arginase by homoarginine suggesting increased L-arginine levels and subsequently increased NO formation [28–30]. In the light of homoarginine being a weak substrate for NOS, but potentially increasing L-arginine availability by arginase inhibition, the net effect on NO production remains still unclear. Motivated by the structural similarity of homoarginine and L-arginine, Radomsky et al. already studied in the 1990s the regulation of human platelet aggregation by homoarginine. In this context, homoarginine, similar to L-arginine, was found to inhibit aggregation of human platelets, both being applied in supraphysiological concentrations [31]. Taken together, these biochemical, pharmacodynamic, and physiological studies have indicated an interference of homoarginine with pathways related to cardiovascular disease. However, at this point further experimental (animal) studies will be necessary to define precisely the physiological significance of homoarginine.

Homoarginine and Cardiovascular Disease

During the last 5 years, numerous observational and epidemiological studies have been published associating circulating homoarginine levels with clinical parameters and outcomes (Tables 17.1 and 17.2). Data from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study—including patients undergoing coronary angiography—and 4D (*Die Deutsche Diabetes Dialyse*) study—comprising patients with type 2 diabetes mellitus receiving maintenance hemodialysis—established significant associations between low homoarginine serum levels and increased cardiovascular and all-cause mortality [7]. Consistently, longitudinal analyses in the Hoorn study reported the association of homoarginine with overall mortality and especially cardiovascular death in an older population [32].

Table 17.1 Negative correlations of homoarginine levels with clinical parameters

Clinical parameter	References
All-cause mortality	März et al. [7], Pilz et al. [32, 33], Ravani et al. [34], Tomaschitz et al. [35]
Cardiovascular mortality	März et al. [7], Pilz et al. [32]
Onset of dialysis	Ravani et al. [34]
Fatal stroke	Pilz et al. [36]
Post-stroke mortality	Choe at al. [6]
NIHSS score	Choe et al. [6]
Death due to heart failure	Drechsler et al. [37], Pilz et al. [33]
Sudden cardiac death	Drechsler et al. [37]
Myocardial infarction	Pilz et al. [33]
Peripheral vascular disease	Ravani et al. [34]
NYHA stages	Atzler et al. [38], Pilz et al. [33]
Left ventricular ejection fraction	Pilz et al. [33]
NT-proBNP	Atzler et al. [38], Drechsler et al. [37], Pilz et al. [33]
CRP	Choe et al. [6], Drechsler et al. [37], Pilz et al. [33], Ravani et al. [34],
Proteinuria	Drechsler et al. [39], Ravani et al. [34]
Smoking	Pilz et al. [32, 33], Sobczak et al. [40], van der Zwan et al. [41]
Coagulation parameter	Choe et al. [6], Marz et al. [7], Pilz et al. [33]
Alkaline phosphatase	März et al. [7], Pilz et al. [32]
Systolic blood pressure	Kayacelebi et al. [42]

Negative correlations of homoarginine levels were described with all-cause and CV mortality, onset of dialysis, fatal stroke, post-stroke mortality, NIHSS score, death due to heart failure, sudden cardiac death, myocardial infarction, peripheral vascular disease, NYHA stages, left ventricular ejection fraction, NT-proBNP, CRP, proteinuria, smoking, coagulation parameter, alkaline phosphatase, and systolic blood pressure

Table 17.2 Positive correlations of homoarginine levels with clinical parameters

Clinical parameter	References
BMI	Drechsler et al. [37, 39], März et al. [7], Pilz et al. [32, 33], Ravani et al. [34], van der Zwan et al. [41]
Triglycerides	Pilz et al. [33]
Fasting glucose	Pilz et al. [32], van der Zwan et al. [41]
HbA1c	Pilz et al. [32], van der Zwan et al. [41]
Diabetes mellitus type 2	Ravani et al. [34], van der Zwan et al. [41]
eGFR	Choe et al. [6], Drechsler et al. [39], März et al. [7], Tomaschitz et al. [35], Ravani et al. [34]
Flow-mediated dilatation	Valtonen et al. [43]
Arterial hypertension	Pilz et al. [32]
Systolic blood pressure	Pilz et al. [32, 33], van der Zwan et al. [41]

Positive correlations of homoarginine levels were described with BMI, triglycerides, fasting glucose, HbA1c, diabetes mellitus type 2, eGFR, flow-mediated dilatation, arterial hypertension, and systolic blood pressure

Further studies tried to target the reasons of death followed by impaired homoarginine. Tellingly, low homoarginine serum levels were shown to predict fatal strokes in the LURIC study and were associated with sudden cardiac death or death due to heart failure in the 4D study [36, 37]. To unravel this link to cardiac and vascular function, circulating homoarginine levels were associated with angiographic parameters and laboratory parameters of heart failure and endothelial dysfunction. Angiographic ejection fraction was positively and N-terminal pro-B-type natriuretic peptide (NT-proBNP) inversely correlated with circulating homoarginine suggesting a positive linkage between homoarginine and myocardial function. In line with the latter findings, another cohort of 282 heart failure patients confirmed the correlation with NT-proBNP levels [38]. This study additionally showed that low homoarginine levels were also associated with clinical impairment as demonstrated by patients suffering from moderate or severe heart failure (i.e., New York Heart Association classifications 3 and 4) exhibited decreased homoarginine levels [33, 38]. Furthermore, homoarginine plasma levels were similar in dilatative (DCM) and ischemic cardiomyopathy (ICM) patients, and subgroup analysis did not reveal a correlation with heart failure etiology [38]. Several studies link a worsening of endothelial function to myocardial dysfunction. In this context, März et al. reported an inverse association of homoarginine with markers of impaired endothelial function (i.e., intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) suggesting a potential link to endothelial function. Taken together, angiographic, laboratory, and clinical parameters of heart failure and vascular function are linked to homoarginine levels, but strong causal relationships have yet to be established.

Some authors suggest that low homoarginine levels might indicate reduced intracellular energy stores, which is a hallmark of heart failure [44]. Clinical studies have confirmed cellular and molecular causal associations of homoarginine with metabolites of energy metabolism—namely, creatine [6, 33]. As described above, phosphorylated creatine serves as a spatial and temporal energy buffer. And key components of the creatine/phosphocreatine system are downregulated in the failing heart [45]. In line with this hypothesis, creatine-deficient mouse models reveal left ventricular hypertrophy, a reduced inotropic reserve, and increased susceptibility to cardiac ischemic injury [46–48]. Murine studies and cell culture experiments have shown that levels of creatine and homoarginine are dependent on AGAT and therefore positively correlated with each other. Association studies in humans have validated this finding [6, 33]. Therefore, homoarginine might play the role of an indicator of intracellular energy stores in heart failure. But recent results suggest that the association of creatine and heart failure is not as trivial as presumed. For example, increased levels of myocardial creatine and phosphocreatine also resulted in left ventricular hypertrophy and myocardial dysfunction, therefore suggesting rather a bell-shaped association of creatine with left ventricular hypertrophy [49]. And creatine-deficient mice revealed an unaltered response to chronic myocardial infarction suggesting that it might be a dispensable metabolite for left ventricular remodeling and development of chronic heart failure following myocardial infarction [50]. Therefore, the exact association of creatine with cardiac energetics in heart failure remains unsolved [44]. This is even more the case for homoarginine's role in heart failure.

Homoarginine and Cardiovascular Risk Factors

Numerous studies have suggested an increased risk of stroke in patients with chronic kidney disease [51]. A large meta-analysis revealed that the overall risk ratio for patients with reduced estimated glomerular filtration rate (eGFR) of <60 ml/min/1.73 m^2 was increased by 43 % (HR 1.43; 95 % confidence interval 1.31–1.57, $p < 0.001$) [52]. Analysis of the Leeds Stroke cohort, LURIC study, and 4D study revealed a positive correlation of circulating homoarginine with parameters of kidney function, i.e., creatinine levels and eGFR [7, 15, 33]. Given that AGAT is mainly expressed in the kidney, renal dysfunction and damage might be associated with reduced AGAT expression and therefore reduced homoarginine production. A further explanation for the association of homoarginine with renal

function might also be an involvement of homoarginine in renal NO metabolism. All three NOS subtypes are expressed in the kidney and regulate renal hemodynamics and damage in response to injury [53]. Therefore, homoarginine might influence renal hemodynamics and kidney damage.

Major modifiable vascular risk factors are arterial hypertension and smoking, which double the risk of stroke and cardiovascular disease. In a recent cross-sectional study of 231 healthy males, smoking was associated with decreased homoarginine plasma levels [40]. A similar association has been described in the LURIC and Hoorn studies [32, 33]. It is tempting to speculate that the increased risk for vascular events in smokers is partly mediated by decreased homoarginine levels. In the population-based Hoorn cohort of older participants and in the LURIC study, circulating homoarginine was positively linked to systolic and diastolic blood pressure [32, 41]. A 0.5 μmol/L increase of homoarginine levels was associated with an increase of systolic blood pressure by 3.9 mmHg, which would increase the risk of stroke by about 40 % [54]. Therefore, it seems unlikely that blood pressure changes are involved in mediating the beneficial effects of homoarginine in vascular disease [55]. In addition to smoking and hypertension, associations between homoarginine and metabolic parameters have been described. Positive correlations were found with BMI and triglyceride levels, but not with LDL or HDL cholesterol levels. In the LURIC study, no substantial correlation was found between homoarginine and parameters of glucose metabolism (i.e., hemoglobin A1c and diabetes), whereas results from the Hoorn study indicated a positive correlation between homoarginine with hemoglobin A1c [41]. Given the discrepancy between these studies, at present it remains unclear if homoarginine has a direct effect on blood pressure and metabolic parameters.

Homoarginine and Genes

To date, circulating homoarginine concentrations have been related to gene polymorphisms in three large population-based studies. GWA from the Gutenberg Health Study (GHS) revealed a strong link between plasma homoarginine and single nucleotide polymorphisms (SNPs) located in or in the vicinity of the *AGAT* gene on chromosome 15 [6]. Among them, one of the top SNPs, i.e., rs12887765, coded the exchange of A -> T, resulting in a missense transition of Gln110His within the second exon of the *AGAT* gene. Carriers of two TT alleles showed substantially lower plasma concentrations, i.e., 1.80 (1.40, 2.28) μM, whereas homoarginine was higher in AT carriers (2.08 (1.55, 2.54) μM) and highest in AA carriers (2.24 (1.75, 2.95) μM), representing a gene-dose dependent increase by 16 % and 24 %, respectively. Allele frequency was 54 % for TT carriers in this population. GWA from patients of the LURIC study and from participants of the Young Finns Study (YFS) confirmed the strong association between circulating homoarginine and AGAT [56]. Of note, three other loci were identified within the latter cohorts: (1) on chromosome 2 at the carbamoyl phosphate synthetase I locus, (2) on chromosome 5 at the alanine-glyoxylate aminotransferase 2 locus, and (3) on chromosome 6 at the Homo sapiens mediator complex subunit 23 gene/arginase I locus. Recently, SNPs of *AGAT* locus were associated with statin-induced myopathy in humans underlining the importance of AGAT for muscle physiology [57]. *AGAT* SNPs revealed one of the most significant and strongest associations of expression quantitative trait loci (eQTL) with simvastatin exposure in human lymphoblastoid cell lines. But in contrast to $AGAT^{-/-}$ mouse models presenting with muscular atrophy (see above), reduced AGAT expression was linked with protection from statin-induced myopathy in two clinical cohorts of patients with statin treatment (Marshfield cohort and *Study of Effectiveness of Additional reduction in Cholesterol and Homocysteine* (SEARCH) cohort) [57]. The authors suggested that reduced AGAT expression could modify cellular energy stores and AMPK signaling, which would protect from statin-induced myopathy. Arguing against this hypothesis, AGAT-deficient patients suffer from myopathy, which improves upon oral creatine supplementation [58]. The impact of this association is not completely clear since the AGAT expression levels in circulating blood cells

are very low and may not reflect the local expression levels, i.e., in skeletal muscle. The kidney is the tissue with the highest expression of AGAT and several markers of kidney function and failure have been conclusively related to *AGAT* gene polymorphisms [59].

Conclusions

On the one hand, AGAT deficiency improves the metabolic risk profile, which is associated with a better outcome after stroke [15, 60]. On the other hand, AGAT deficiency results in diminished intracellular energy stores (i.e., ATP and phosphocreatine), which are reduced under conditions of cerebral ischemia [12, 61, 62]. Therefore, animal experiments and controlled randomized clinical trials are necessary to differentiate in which cases homoarginine is just a marker or in which other cases it functions as a causal mediator.

References

1. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. Lancet. 2008;371(9624):1612–23.
2. Hankey GJ, Jamrozik K, Broadhurst RJ, et al. Long-term risk of first recurrent stroke in the Perth Community Stroke Study. Stroke. 1998;29(12):2491–500.
3. Hankey GJ, Jamrozik K, Broadhurst RJ, et al. Five-year survival after first-ever stroke and related prognostic factors in the Perth Community Stroke Study. Stroke. 2000;31(9):2080–6.
4. Chen Y, Copeland WK, Vedanthan R, et al. Association between body mass index and cardiovascular disease mortality in east Asians and south Asians: pooled analysis of prospective data from the Asia Cohort Consortium. BMJ. 2013;347:f5446.
5. Hankey GJ. Nutrition and the risk of stroke. Lancet Neurol. 2012;11(1):66–81.
6. Choe CU, Atzler D, Wild PS, et al. Homoarginine levels are regulated by L-arginine:glycine amidinotransferase and affect stroke outcome: results from human and murine studies. Circulation. 2013;128(13):1451–61.
7. März W, Meinitzer A, Drechsler C, et al. Homoarginine, cardiovascular risk, and mortality. Circulation. 2010;122(10):967–75.
8. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80(3):1107–213.
9. Kushmerick MJ, Moerland TS, Wiseman RW. Mammalian skeletal muscle fibers distinguished by contents of phosphocreatine, ATP, and Pi. Proc Natl Acad Sci U S A. 1992;89(16):7521–5.
10. Lygate CA, Bohl S, ten Hove M, et al. Moderate elevation of intracellular creatine by targeting the creatine transporter protects mice from acute myocardial infarction. Cardiovasc Res. 2012;96(3):466–75.
11. Schmidt A, Marescau B, Boehm EA, et al. Severely altered guanidino compound levels, disturbed body weight homeostasis and impaired fertility in a mouse model of guanidinoacetate N-methyltransferase (GAMT) deficiency. Hum Mol Genet. 2004;13(9):905–21.
12. Nabuurs CI, Choe CU, Veltien A, et al. Disturbed energy metabolism and muscular dystrophy caused by pure creatine deficiency are reversible by creatine intake. J Physiol. 2013;591(Pt 2):571–92.
13. Item CB, Stockler-Ipsiroglu S, Stromberger C, et al. L-Arginine:glycine amidinotransferase deficiency: the third inborn error of creatine metabolism in humans. Am J Hum Genet. 2001;69(5):1127–33.
14. Stockler S, Isbrandt D, Hanefeld F, Schmidt B, von Figura K. Guanidinoacetate methyltransferase deficiency: the first inborn error of creatine metabolism in man. Am J Hum Genet. 1996;58(5):914–22.
15. Choe CU, Nabuurs C, Stockebrand MC, et al. L-Arginine:glycine amidinotransferase deficiency protects from metabolic syndrome. Hum Mol Genet. 2013;22(1):110–23.
16. Stockebrand M, Sauter K, Neu A, Isbrandt D, Choe CU. Differential regulation of AMPK activation in leptin- and creatine-deficient mice. FASEB J. 2013;27(10):4147–56.
17. Ryan WL, Johnson RJ, Dimari S. Homoarginine synthesis by rat kidney. Arch Biochem Biophys. 1969;131(2):521–6.
18. Ryan WL, Wells IC. Homocitrulline and homoarginine synthesis from lysine. Science. 1964;144(3622):1122–7.
19. Davids M, Ndika JD, Salomons GS, Blom HJ, Teerlink T. Promiscuous activity of L-arginine:glycine amidinotransferase is responsible for the synthesis of the novel cardiovascular risk factor homoarginine. FEBS Lett. 2012;586(20):3653–7.

20. Atzler D, Schwedhelm E, Nauck M, Ittermann T, Böger RH, Friedrich N. Serum reference intervals of homoarginine, ADMA, and SDMA in the Study of Health in Pomerania. Clin Chem Lab Med. 2014;52:1835–42.
21. Atzler D, Mieth M, Maas R, Böger RH, Schwedhelm E. Stable isotope dilution assay for liquid chromatography-tandem mass spectrometric determination of L-homoarginine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2011;879(23):2294–8.
22. Bell EA. Alpha, gamma-Diaminobutyric acid in seeds of twelve species of Lathyrus and identification of a new natural amino-acid, L-homoarginine, in seeds of other species toxic to man and domestic animals. Nature. 1962;193:1078–9.
23. Rao SL, Ramachandran LK, Adiga PR. The isolation and characterization of l-homoarginine from seeds of Lathyrus sativus. Biochemistry. 1963;2:298–300.
24. Bell EA. The isolation of L-homoarginine from seeds of Lathyrus cicera. Biochem J. 1962;85:91–3.
25. Fishman W, Sie HG. L-homoarginine; an inhibitor of serum “bone and liver” alkaline phosphatase. Clin Chim Acta. 1970;29(2):339–41.
26. Fishman WH, Sie HG. Organ-specific inhibition of human alkaline phosphatase isoenzymes of liver, bone, intestine and placenta; L-phenylalanine, L-tryptophan and L homoarginine. Enzymologia. 1971;41(3):141–67.
27. Lin CW, Fishman WH. L-Homoarginine. An organ-specific, uncompetitive inhibitor of human liver and bone alkaline phosphohydrolases. J Biol Chem. 1972;247(10):3082–7.
28. Moali C, Boucher JL, Sari MA, Stuehr DJ, Mansuy D. Substrate specificity of NO synthases: detailed comparison of L-arginine, homo-L-arginine, their N omega-hydroxy derivatives, and N omega-hydroxynor-L-arginine. Biochemistry. 1998;37(29):10453–60.
29. Reczkowski RS, Ash DE. Rat liver arginase: kinetic mechanism, alternate substrates, and inhibitors. Arch Biochem Biophys. 1994;312(1):31–7.
30. Pentyala J, Rao SLN. Sustained nitric oxide generation with L-homoarginine. Res Commun Biochem Cell Mol Biol. 1999;3(3–4):223–32.
31. Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA. 1990;87(13):5193–7.
32. Pilz S, Teerlink T, Scheffer PG, et al. Homoarginine and mortality in an older population: the Hoorn study. Eur J Clin Invest. 2014;44:200–8.
33. Pilz S, Meinitzer A, Tomaschitz A, et al. Low homoarginine concentration is a novel risk factor for heart disease. Heart. 2011;97(15):1222–7.
34. Ravani P, Maas R, Malberti F, et al. Homoarginine and mortality in pre-dialysis chronic kidney disease (CKD) patients. PLoS One. 2013;8(9):e72694.
35. Tomaschitz A, Meinitzer A, Pilz S, et al. Homoarginine, kidney function and cardiovascular mortality risk. Nephrol Dial Transplant. 2014;29(3):663–71.
36. Pilz S, Tomaschitz A, Meinitzer A, et al. Low serum homoarginine is a novel risk factor for fatal strokes in patients undergoing coronary angiography. Stroke. 2011;42(4):1132–4.
37. Drechsler C, Meinitzer A, Pilz S, et al. Homoarginine, heart failure, and sudden cardiac death in haemodialysis patients. Eur J Heart Fail. 2011;13(8):852–9.
38. Atzler D, Rosenberg M, Anderssohn M, et al. Homoarginine-an independent marker of mortality in heart failure. Int J Cardiol. 2013;168(5):4907–9.
39. Drechsler C, Kollerits B, Meinitzer A, et al. Homoarginine and progression of chronic kidney disease: results from the Mild to Moderate Kidney Disease Study. PLoS One. 2013;8(5):e63560.
40. Sobczak A, Prokopowicz A, Radek M, et al. Tobacco smoking decreases plasma concentration of the emerging cardiovascular risk marker, L-homoarginine. Circ J. 2014;78(5):1254–8.
41. van der Zwan LP, Davids M, Scheffer PG, Dekker JM, Stehouwer CD, Teerlink T. L-Homoarginine and L-arginine are antagonistically related to blood pressure in an elderly population: the Hoorn study. J Hypertens. 2013;31(6):1114–23.
42. Kayacelebi AA, Beckmann B, Gutzki FM, Jordan J, Tsikas D. GC-MS and GC-MS/MS measurement of the cardiovascular risk factor homoarginine in biological samples. Amino Acids. 2014;46:2205–17.
43. Valtonen P, Laitinen T, Lyyra-Laitinen T, et al. Serum L-homoarginine concentration is elevated during normal pregnancy and is related to flow-mediated vasodilatation. Circ J. 2008;72(11):1879–84.
44. Lygate CA, Schneider JE, Neubauer S. Investigating cardiac energetics in heart failure. Exp Physiol. 2013;98(3):601–5.
45. Lygate CA, Fischer A, Sebag-Montefiore L, Wallis J, ten Hove M, Neubauer S. The creatine kinase energy transport system in the failing mouse heart. J Mol Cell Cardiol. 2007;42(6):1129–36.
46. Nahrendorf M, Spindler M, Hu K, et al. Creatine kinase knockout mice show left ventricular hypertrophy and dilatation, but unaltered remodeling post-myocardial infarction. Cardiovasc Res. 2005;65(2):419–27.
47. Spindler M, Meyer K, Stromer H, et al. Creatine kinase-deficient hearts exhibit increased susceptibility to ischemia-reperfusion injury and impaired calcium homeostasis. Am J Physiol Heart Circ Physiol. 2004;287(3):H1039–45.

48. ten Hove M, Lygate CA, Fischer A, et al. Reduced inotropic reserve and increased susceptibility to cardiac ischemia/reperfusion injury in phosphocreatine-deficient guanidinoacetate-N-methyltransferase-knockout mice. Circulation. 2005;111(19):2477–85.
49. Wallis J, Lygate CA, Fischer A, et al. Supranormal myocardial creatine and phosphocreatine concentrations lead to cardiac hypertrophy and heart failure: insights from creatine transporter-overexpressing transgenic mice. Circulation. 2005;112(20):3131–9.
50. Lygate CA, Aksentijevic D, Dawson D, et al. Living without creatine: unchanged exercise capacity and response to chronic myocardial infarction in creatine-deficient mice. Circ Res. 2013;112(6):945–55.
51. Ninomiya T. Risk of stroke in kidney disease. Contrib Nephrol. 2013;179:58–66.
52. Lee M, Saver JL, Chang KH, Liao HW, Chang SC, Ovbiagele B. Low glomerular filtration rate and risk of stroke: meta-analysis. BMJ. 2010;341:c4249.
53. Prabhakar SS. Regulatory and functional interaction of vasoactive factors in the kidney and extracellular pH. Kidney Int. 2004;66(5):1742–54.
54. Collins R, Peto R, MacMahon S, et al. Blood pressure, stroke, and coronary heart disease. Part 2, Short-term reductions in blood pressure: overview of randomised drug trials in their epidemiological context. Lancet. 1990;335(8693):827–38.
55. Liu L, Wang Z, Gong L, et al. Blood pressure reduction for the secondary prevention of stroke: a Chinese trial and a systematic review of the literature. Hypertens Res. 2009;32(11):1032–40.
56. Kleber ME, Seppala I, Pilz S, et al. Genome-wide association study identifies 3 genomic loci significantly associated with serum levels of homoarginine: the AtheroRemo Consortium. Circ Cardiovasc Genet. 2013;6(5):505–13.
57. Mangravite LM, Engelhardt BE, Medina MW, et al. A statin-dependent QTL for GATM expression is associated with statin-induced myopathy. Nature. 2013;502(7471):377–80.
58. Edvardson S, Korman SH, Livne A, et al. L-arginine:glycine amidinotransferase (AGAT) deficiency: clinical presentation and response to treatment in two patients with a novel mutation. Mol Genet Metab. 2010;101(2–3):228–32.
59. Atzler D, Schwedhelm E, Zeller T. Integrated genomics and metabolomics in nephrology. Nephrol Dial Transplant. 2014;29(8):1467–74.
60. Lu Y, Hajifathalian K, Ezzati M, Woodward M, Rimm EB, Danaei G. Metabolic mediators of the effects of body-mass index, overweight, and obesity on coronary heart disease and stroke: a pooled analysis of 97 prospective cohorts with 1.8 million participants. Lancet. 2014;383(9921):970–83.
61. Ljunggren B, Schutz H, Siesjo BK. Changes in energy state and acid-base parameters of the rat brain during complete compression ischemia. Brain Res. 1974;73(2):277–89.
62. Pulsinelli WA, Duffy TE. Regional energy balance in rat brain after transient forebrain ischemia. J Neurochem. 1983;40(5):1500–3.

Chapter 18
The L-Arginine/Asymmetric Dimethylarginine (ADMA) Ratio in Health and Disease: An Overview

Murat Celik and Hilmi Umut Unal

Key Points

- Nitric oxide deficiency plays a crucial role in the pathogenesis of endothelial dysfunction and asymmetric dimethylarginine (ADMA) levels have been interested as a priority in nitric oxide deficiency.
- Since ADMA inhibits nitric oxide production by competing with L-Arginine for nitric oxide synthase binding, the L-Arginine/ADMA ratio (substrate/inhibitor ratio) might be a good predictor of the net amount of nitric oxide production than ADMA or L-Arginine concentration separately.
- Although increased plasma ADMA level has been demonstrated several times, only a few studies have measured plasma concentration of L-Arginine and reported the L-Arginine/ADMA ratio.
- However, it is essential to obtain further insights into the consequences of alterations of L-Arginine/ADMA ratio in patients with various diagnosed diseases.

Keywords L-Arginine • Asymmetric dimethylarginine • L-Arginine/asymmetric dimethylarginine ratio • Nitric oxide • ADMA

Abbreviations

ADMA	Asymmetric dimethylarginine
Aix	Augmentation index
aPWV	Aortic pulse wave velocity
BNP	Brain natriuretic peptide
CAT	Cationic amino acid transporter

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CCD	Coronary collateral development
CIMT	Carotid wall intima–media thickness
CVD	Cardiovascular disease
DDAH	Dimethylarginine dimethylaminohydrolase
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
L-NMMA	NG-monomethyl-L-arginine
NEC	Necrotizing enterocolitis
NO	Nitric oxide
NOS	Nitric oxide synthase
NYHA	New York Heart Association
PRMT	Protein L-arginine methyltransferase
RAAS	Renin–angiotensin–aldosterone system
SDMA	Symmetric dimethylarginine
TIPS	Transjugular intrahepatic portosystemic shunt

Introduction

Endothelium is acting a vital role in the control of tonus of vasculature and homeostasis [1]. Although the significance of endothelial dysfunction has been well described by many published studies, especially in cardiovascular diseases, so far, there are some challenges in the evaluation of endothelial function due to lack of a standardization. Nitric oxide (NO) is mainly released by the endothelium and plays a critical role in the maintenance of normal endothelial function by regulating the vascular tone [2]. Inactivation and/or reduced synthesis of NO leads to endothelial dysfunction, whereas an excess of NO may cause circulatory shock [3]. In addition, NO modulates the interactions of circulating blood cells with the vascular wall, which are the key processes involved in the pathogenesis of atherosclerosis such as adhesion of inflammatory cells to the vascular wall, the aggregation of platelets, and the proliferation of smooth muscle cells [4]. Therefore, NO bioavailability is more important and has recently been used as an indicator of endothelial function.

Nitric Oxide Bioavailability

NO is synthesized from the amino acid precursor L-Arginine in a multistep reaction controlled by the enzyme NO synthase (NOS) [5]. NOS activity is regulated at many levels, including transcription, translation, phosphorylation, posttranslational modifications, interactions with other proteins and the plasma membrane, binding of cofactors (tetrahydrobiopterin), production of reactive oxygen species capable of NO inactivating, and the balance between NOS substrate and its endogenous inhibitor [6]. Compared to other regulatory mechanisms, NO production is mainly determined by both substrate availability (L-Arginine) and the presence of endogenous inhibitor [asymmetric dimethylarginine (ADMA)], and thereby, the L-Arginine/ADMA ratio has been accepted as an important determinant of NO production [7]. Since both L-Arginine and ADMA play a regulatory role in the synthesis of NO, it is critical to get perception into the consequences of changes of L-Arginine, ADMA, and L-Arginine/ADMA ratio.

L-Arginine

L-Arginine, an essential amino acid, is a natural component of dietary proteins, with the relative quantity varying between 3 and 15 %. It has gained a main scientific attention in recent years due to its most versatile roles in metabolism. The most noticeable role is its role as a physiological substrate for NOS [8]. Besides, L-Arginine contributes in various metabolic pathways independent of NO production such as glucose metabolism and the synthesis of creatinine, urea, L-ornithine, L-glutamate, proline, agmatine, and polyamines [9, 10]. The homeostasis of L-Arginine involves dietary intake, L-arginine synthesis in the kidney, transport mechanisms across vascular cellular membranes, and its utilization and degradation by arginase.

Intracellular L-Arginine level is significantly higher than those in the extracellular fluid or in plasma. However, plasma L-Arginine can be quickly hold by endothelial cells via the cationic amino acid transporter 1 (CAT1) and can directly contribute to NO production [11]. D-Arginine has a same structure to L-Arginine but does not share the similar transport system with L-Arginine. Arginase is an enzyme that expressed by endothelial cells catalyzes the hydrolysis of L-Arginine in the final step of the urea cycle to ornithine [12]. Arginase competes with NOS for substrate (L-Arginine) and increased arginase levels have been proposed to limit NO formation through increased L-Arginine consumption [3]. L-Arginine remains in proteins are posttranslationally methylated by the action of protein L-arginine methyltransferase (PRMT) enzyme during the normal protein turnover in many tissues, including vascular endothelial cells [13]. *N*-monomethyl-L-arginine (L-NMMA), ADMA, and symmetric dimethylarginine (SDMA) are three methylated analogs of L-Arginine, which released as free amino acids during proteolysis. Although ADMA and NMMA are direct inhibitors of NOS, SDMA is an indirect inhibitor of NOS.

Asymmetric Dimethylarginine

Among methylated analogs of L-Arginine, ADMA is the only one that can compete with L-Arginine for the active site of all isoforms of NOS. Normal physiological levels of ADMA only have an unassertive influence on NOS activity; however under pathological conditions, intracellular ADMA is elevated and may reach adequate levels to inhibit the activity of NOS and impair vascular function [14]. Thence, current understandings into NO metabolism has shown an important role of endogenously produced inhibitors of NOS, in particular ADMA. ADMA not only blocks NOS activity but also limits the cellular uptake of L-Arginine via y^+ transport system and competes with the other methylated arginines, SDMA and L-NMMA [15].

Among different biomarkers of endothelial dysfunction, elevated plasma ADMA level has received a considerable notice during the last two decades. The combination of increased generation and/or decreased clearance or altered dissemination between the extracellular and intracellular components could theoretically elucidate the higher plasma levels of ADMA. Among these, reduced activity of clearance of ADMA has been proposed as the most likely pathophysiologic mechanism. ADMA is largely metabolized by the dimethylarginine dimethylaminohydrolase (DDAH) into mono- or dimethylamine and citrulline. Thus, the activity of DDAH intensely affects the circulating ADMA levels. The accumulation of ADMA increases oxidative stress by uncoupling of electron transport between NOS and L-Arginine resulting in increased production of radical oxygen species, decreases the production of NO, reduces vascular compliance, increases vascular resistance, limits the blood flow, and thereby contributes to impairment of endothelial function [16].

L-Arginine Paradox

L-Arginine was revealed either to improve endothelial function or fail to do so. This discrepancy has previously been termed as the "L-Arginine paradox" [7]. The underlying molecular mechanism remains confusing. Namely, at physiological extracellular levels of L-Arginine and in the presence of normal range of plasma ADMA concentrations, NOS is well-saturated with the substrate L-Arginine, leading to physiological production of NO. Under such conditions, the addition of exogenous L-Arginine does not alter the enzyme's activity. In the presence of pathophysiologically relevant concentrations of plasma ADMA and physiological concentration of L-Arginine (relatively low L-Arginine/ADMA ratio), the rate of NO formation remains below the physiological levels by decreasing of the NOS activity. Under these conditions, exogenous L-Arginine supplementation replaces with the competitive inhibitor and restores the physiological L-Arginine/ADMA ratio to a level that fixes eNOS activity [7]. The L-Arginine paradox suggests that NOS activity is linked to extracellular levels of L-Arginine, ADMA, and thus L-Arginine/ADMA ratio.

L-Arginine/ADMA Ratio

Elevated ADMA and normal or low L-Arginine levels may contribute to the inhibition of NOS through NOS uncoupling. Namely, when ADMA levels are high, NO synthesis might still be achievable when L-Arginine levels are adequately high to dislocate ADMA. The L-Arginine/ADMA ratio (substrate/inhibitor ratio) reflects the local concentration of NOS substrate in relation to its endogenous inhibitor and suggested to be more appropriate to define the degree of NOS activity than L-Arginine or ADMA concentration alone [7]. The measurement of both arginase and DDAH also might be important in determining the NOS activity. It is likely that the decreased levels of L-Arginine and increased levels of ADMA are caused by upregulation of arginase and inhibition of DDAH activity, respectively [6]. Both pathways result in lowering of the NOS substrate/inhibitor ratio, leading to a diminished NO production and endothelial dysfunction. In the infarcted rat heart, the increased levels and activity of both DDAH-1 and DDAH-2 were associated with a decreased L-Arginine/ADMA ratio [17]. The L-Arginine/ADMA ratio has been proved to be of more prognostic value in the recent studies. Whereas an increase in the L-Arginine/ADMA ratio leads to an elevation in NO bioavailability, a decreased L-Arginine/ADMA ratio is associated with endothelial dysfunction. In a prospective Framingham Offspring Cohort, L-Arginine/ADMA ratio has been inversely associated with all-cause mortality [18].

Despite several studies, there is still a discrepancy to define a limit for the L-Arginine/ADMA ratio between healthy subjects and patients. Lüneburg et al. [18] report that the reference limit (2.5th and 97.5th percentiles) for the L-Arginine/ADMA ratio was 74.3–225 mmol/L from a healthy reference sample of participants from the Framingham Offspring Cohort (similar results for men and women). In their valuable research, Bode-Böger et al. [7] analyzed 14 studies including 588 healthy subjects and 23 studies involving a total of 3070 patients with various diseases from 1980 to 2006. They reported that the mean L-Arginine/ADMA ratio varies between 54.3 ± 6.6 and 227 ± 32 (median, 183) in healthy subject and 13.7 ± 1.4 and 194.0 ± 55.3 (median, 73) in patients with various diseases. A wide range of the L-Arginine/ADMA ratio in studied populations might result from used methodological techniques, such as an enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), or mass spectrometry-based analytical methods [19]. Because, the ELISA technique might overestimate the concentration of ADMA compared to other methods. Also, the intracellular L-arginine concentration values are found between 60% and 70% higher than in corresponding plasma samples [20]. Nonetheless, outcomes from existing studies revealed that there was an evident reduction of the L-Arginine/ADMA ratios with diagnosed diseases and the etiology seems to have no influence on the L-Arginine/ADMA ratio because of similar results between different patients groups.

The Role of L-Arginine/ADMA Ratio in Diseases

While substantial information exists about factors affecting plasma L-Arginine and ADMA levels separately in health and disease, L-Arginine/ADMA ratio has not been so extensively studied. In most clinical conditions associated with endothelial dysfunction, circulating L-Arginine concentrations have been observed to be within the normal range. Clinical and experimental evidences suggest that elevation of ADMA may cause a relative L-Arginine deficiency, even in the presence of "normal" L-Arginine levels [21]. Nevertheless, low L-Arginine plasma levels in combination with high ADMA plasma levels more deteriorate systemic hemodynamics and reduce blood flow through the vital organs such as the kidney, spleen, and liver [22].

L-Arginine/ADMA ratio may serve as better marker of endothelial functional status than each individual parameter for disease activity and progression, and thereby, involvement of L-Arginine/ADMA ratio in clinical studies has been required in recent papers. Since L-Arginine/ADMA ratio is superior to the levels of L-Arginine and ADMA alone, knowledge about conditions associated with lower L-Arginine/ADMA ratio as an indicator of endothelial dysfunction is therefore important.

Inflammation

L-Arginine enhances wound healing, improves immune function (T-cell antitumor immunity), and has anti-catabolic effects during the healing process [23]. However, elevated inflammatory markers such as c-reactive protein, myeloperoxidase, erythrocyte sedimentation rate, TNF-α, and IL-6 have been suggested to correlate with reduced L-Arginine levels, increased circulatory ADMA levels, and unfavorable change in the L-Arginine/ADMA ratio in several patient populations [24–26]. Van der Zwan et al. [6] found that even low-grade systemic inflammation was associated with a decreased L-Arginine/ADMA ratio. Moreover, recent studies have proved that experimental acute inflammation reduces the L-Arginine/ADMA ratio. *Escherichia coli* endotoxin (lipopolysaccharide) administration had resulted in a significant reduction of L-Arginine and ADMA plasma concentrations, thereby a reduction in the L-Arginine/ADMA ratio in rat [27] and healthy human [28]. Also, oxidative stress seems to enhance the association between inflammation and increased ADMA levels. These inflammation-associated decreased levels of L-Arginine and increased levels of ADMA and thereby reduced L-Arginine/ADMA ratios are leading to a diminished NO production and may contribute to the vascular pathology.

Traditional Cardiovascular Risk Factors

The development of atherosclerotic lesions is a multifactorial process that includes many traditional cardiovascular risk factors. These cardiovascular risk factors associated with endothelial dysfunction mediate their deleterious effects on the vascular wall by dysfunction of the endothelial NO production. A decreased L-Arginine/ADMA ratio (relatively increased plasma ADMA level) has been demonstrated to be associated with many of these risk factors despite circulating L-Arginine concentration being found to be in the normal range [29]. Main ones are as follows.

Female gender has been found in association with lower L-Arginine/ADMA ratio [30]. L-Arginine/ADMA ratio is also associated with women with previous gestational diabetes [31] and pre-eclampsia [32]. The inverse association between body mass index and endothelial NO has been also explained by an imbalance between L-Arginine and ADMA [33]. Despite Maas et al. [34] demonstrated that smokers tended to have lower plasma ADMA levels compared to nonsmokers, smokers widely have a tendency

to higher plasma ADMA levels, lower L-Arginine levels, and lower L-Arginine/ADMA ratio. A reduction of the L-Arginine/ADMA ratio has been demonstrated in subjects with hypertension [35]. It has been suggested that the association between reduced NO bioavailability and the development of hypertension develop before the onset of hypertension [36]. Oxidized low-density lipoprotein cholesterol and lysophosphatidylcholine decrease L-Arginine transport into endothelial cells and have been shown to attenuate DDAH activity, allowing L-Arginine/ADMA ratio to reduce and block NO synthesis in patients with hypercholesterolemia [37]. Böger et al. [38] reported increased plasma homocysteine levels had resulted in a redox-mediated inhibition of DDAH and thereby a decreased L-Arginine/ADMA ratio.

Coronary Artery Disease

Although L-Arginine has anti-atherogenic effects [39], increased plasma ADMA level has been shown to a critical predisposing factor in development of atherosclerotic heart disease. Presumably because of the adverse effect on endothelial function, a decreased L-Arginine/ADMA ratio has been proposed more accurately to explain the possible underlying mechanism of the development of coronary artery disease [40]. L-Arginine/ADMA ratio might predict myocardial infarctions in the general population [41] and cardiovascular events in patients who had undergone percutaneous coronary intervention [42]. Sahinarslan et al. [43] showed that a decreased L-Arginine/ADMA ratio is associated with the presence and extensive of coronary artery disease, as visualized by coronary angiography. L-Arginine/ADMA ratio might also have a diagnostic potential for the identification of patients with coronary artery ectasia and slow coronary flow. Koc et al. [44] showed that the L-Arginine/ADMA ratio was lower in the coronary artery ectasia group compared with the control group whereas the plasma ADMA levels were similar. Selcuk et al. [45] demonstrated patients with slow coronary flow have higher plasma concentrations of plasma ADMA level and lower L-Arginine/ADMA ratio, which further support the consideration that vascular endothelial function is disrupted in patients with slow coronary flow. In contrast, in a study of patients with stable angina pectoris, Walker et al. [46] demonstrated that the L-Arginine/ADMA ratio did not associate with endothelial function.

Heart Failure

NO has a protective role on the deteriorating myocardium of patients with dilated cardiomyopathy. The levels of dimethylarginines rose with the degree of heart failure and the prognostic value of the L-Arginine/ADMA ratio is related to myocardial NOS inhibition [47]. Anderssohn et al. [48] showed that the L-Arginine/ADMA ratio is an independent predictor of all-cause mortality in patients with chronic heart failure due to dilated cardiomyopathy but not in patients with ischemic cardiomyopathy. Seljeflot et al. [49] showed that endothelial dysfunction evaluated by the L-Arginine/ADMA ratio was significantly associated with measures of the severity of heart failure, such as NT-proBNP, the 6-min walking distances, and exercise capacity, and was more pronounced than those with plasma L-Arginine and ADMA levels alone in patients with chronic heart failure.

Atrial Fibrillation

The most comprehensive assessment of patients with atrial fibrillation includes the subset analysis of the Candesartan in the Prevention of Relapsing Atrial Fibrillation (CAPRAF) study. In this study, the authors found no relationship between risk of atrial fibrillation recurrence and L-Arginine levels,

ADMA levels, or the L-Arginine/ADMA ratio except a higher risk of recurrence in the highest L-Arginine quartile when compared with the three lowest quartiles grouped together [50]. Interestingly, they observed that restoration and maintenance of sinus rhythm for 6 months were associated with an increase in the L-Arginine/ADMA ratio and argued that this increased L-Arginine/ADMA ratio might resulted from improved endothelial function in the vasculature due to improved hemodynamics in patients who remain in sinus rhythm.

Diabetes

Plasma ADMA level is apparently elevated in advanced stages of diabetes mellitus [29, 51] and the L-Arginine/ADMA ratio was significantly lower in hyperglycemic groups compared to normoglycemic ones [52]. Several studies focusing on diabetic subjects reported that elevated plasma ADMA levels predict cardiovascular morbidity and mortality [53] and in some of these studies plasma ADMA level also affected progression of diabetic nephropathy [54]. Nonetheless, Ellger et al. [52] suggested that maintaining normoglycemia during critical illness preserves the physiological regulation of substrate availability for NOS. It is noteworthy that possible link between plasma ADMA level and cardiovascular disease in diabetic patients is insulin resistance. Eid et al. [55] demonstrated that endothelial dysfunction evaluated by L-Arginine/ADMA ratio has been linked to insulin resistance and glucometabolic disturbances.

Peripheral Artery Disease

Peripheral artery diseases, as well as coronary artery disease, have been found in association with diminished NO bioavailability. A decreased L-Arginine/ADMA ratio may reduce the NO-mediated smooth muscle relaxation and the arteries' capacity of vasodilatation [14]. Reduction of L-Arginine/ADMA ratio after administration of ADMA has resulted in impairment in the aortic pulse wave velocity (aPWV) and the augmentation index (AIx), which are both associated with endothelial function [56]. Nonetheless, Angel et al. [57] demonstrated that improvement in the L-Arginine/ADMA ratio might have contributed to improvement in aPWV. Additionally, increased circulating ADMA levels have been independently in association with carotid-wall intima–media thickness (CIMT) and its progression even in the absence of clinical manifestations of arteriosclerosis [26].

Renal Failure

Since the kidney releases more L-Arginine into plasma than it extracts, renal L-Arginine synthesis outweighs L-Arginine consumption [58]. Renal failure may result in a mean decrease in the L-Arginine plasma concentration (0.05 mmol/L per 1 mL/min decline of eGFR) [18]. Besides its key role in maintaining a constant supply of L-Arginine, the kidneys also provide a significant route for clearance of dimethylarginines (ADMA and SDMA), which can be explained by renal DDAH activity [36]. Additionally, Yilmaz et al. [59] demonstrated that altered insulin signaling might contribute to increase plasma ADMA level in patients with renal parenchymal damage. The degree of proteinuria independently influences circulating levels of ADMA [59]. Nonetheless, plasma ADMA and SDMA concentrations have increased in chronic kidney disease patients with proteinuria [60] and without proteinuria [61]. Also, ADMA acts as a potent predictor of diabetic nephropathy progression independent on

subject's renal function [54]. Numerous studies demonstrated that increased plasma ADMA level is as an independent risk factor for cardiovascular morbidity and mortality in patients with both end stage [62] and in milder stages of chronic kidney disease [63].

Although increased plasma ADMA level has been demonstrated several times in patients with chronic kidney disease, only a few studies have measured plasma concentration of L-Arginine and reported the L-Arginine/ADMA ratio. However, a reduced L-Arginine/ADMA ratio might increase cardiovascular risk and worsen the prognosis in patients with chronic kidney disease than ADMA alone [64]. In their study, Celik et al. [65] found that L-Arginine/ADMA ratio was higher in patients with GFR > 60 mL/min/1.73 m^2 and good coronary collateral development (CCD) than in patients with GFR < 60 mL/min/1.73 m^2 and poor CCD. It is essential to obtain further insights into the consequences of alterations of L-Arginine/ADMA ratio in patients with renal failure.

Hepatic Failure

The liver contains large amounts of arginase and plays an important role in the regulation of plasma L-Arginine concentrations. Liver failure is associated with high plasma levels of L-Arginine [66]. In their study, Siroen et al. [67] showed that besides a rise in L-Arginine levels, the L-Arginine/ADMA ratio is also increased significantly after transjugular intrahepatic portosystemic shunt (TIPS) placement and confirmed the major role of the liver as an ADMA clearing organ. The authors suggested that since blood does not arrive at the hepatocyte, but shunts directly from a portal branch into the hepatic vein after TIPS placement, an increase in L-Arginine plasma levels and an enhancement in renal excretion of dimethylarginines due to increased renal blood flow are provided. Furthermore, ADMA levels have been reported in increased critically ill patients with hepatic dysfunction. A recent study of Mookerjee et al. [68] exposed reduced DDAH expression and increased ADMA levels in liver tissue of patients with severe alcoholic hepatitis. Also, ADMA has been used as a possible indicator of acute allograft rejection in patients undergoing liver transplantation [69].

Erectile Dysfunction

Paroni et al. [70] observed that ADMA and SDMA concentrations are significantly higher and L-Arginine/ADMA and L-Arginine/SDMA ratios are lower in patients who have arteriogenic erectile dysfunction compared with both patients with non-arteriogenic erectile dysfunction and controls. Chen et al. [71] reported that sexual function is improved significantly when L-Arginine/ADMA ratio is elevated after treated with L-Arginine supplementation in combination with phosphodiesterase type 5 inhibitors.

Subarachnoid Hemorrhage

NO is also released from autonomic nerves and neurons and crucial for the regulation of cerebral blood flow [72]. After subarachnoid hemorrhage, Washington et al. [73] showed that circulating levels of plasma ADMA are increased and Staalso et al. [74] reported that low values of the L-Arginine/ADMA ratio are associated with mortality in patients with subarachnoid hemorrhage. A positive correlation between reduced values of reactive hyperemia index measured by fingertip arterial tonometry and diminished L-Arginine/ADMA ratio was found in stroke patients [75] and patients with subarachnoid hemorrhage [76].

Airway Diseases

Endogenous nitric oxide (NO) plays a key beneficial role in the physiological regulation of airway functions including the control of vascular tone, bronchial tone, and neuroendocrine regulation of airway mediator release [77]. Impaired L-Arginine uptake and the accumulation of plasma ADMA level in the lung might contribute to decrease NO production in respiratory airway, thereby increasing the airway resistance and reducing lung compliance [78]. This reduced compliance is linked to increased collagen deposition [78].

Necrotizing Enterocolitis

L-Arginine/ADMA ratio also plays an important role in regulating the development of gastrointestinal circulation and in preserving the integrity of the gastrointestinal mucosal barrier [79]. The animal studies indicate that inhibition of the L-Arginine-NO pathway could result in decreased gastrointestinal blood flow and impairment of the intestinal mucosal barrier [80]. Richir et al. [79] showed that premature infants with necrotizing enterocolitis (NEC) have both lower L-Arginine and ADMA levels for NO synthesis. Since the decrease of L-Arginine is larger than the decrease of ADMA, the L-Arginine/ADMA ratio is lower suggesting a reduced capacity for NO synthesis. Therefore, Richir et al. [79] suggested that lower L-Arginine/ADMA ratio could be a compensatory mechanism in patients with NEC.

Therapeutic Strategies

Theoretically, therapeutic strategies to increase L-Arginine concentration or decrease ADMA concentration may positively influence the endothelium by improving NOS function. Since it is difficult to demonstrate alters in plasma L-Arginine and ADMA concentrations, the L-Arginine/ADMA ratio (substrate/inhibitor ratio) may be used in assessing the treatment response and prognosis evaluation.

Increase L-Arginine/ADMA Ratio

The pharmacological arrangement of plasma ADMA concentration is an interesting area. Although there is no currently any specific ADMA-lowering therapy available, the effect of several pharmacological agents on ADMA metabolism has been investigated. The most studied drug group is renin–angiotensin–aldosterone system (RAAS) inhibitors. RAAS inhibitors have been shown to reduce plasma ADMA concentration in both cultured endothelial cells [81] and in clinical studies [82]. However, in the subset analysis of the CAPRAF study, treatment with candesartan did not affect the levels of ADMA, L-Arginine, or their ratio [50]. Hov et al. [83] observed a significant increase on the L-Arginine/ADMA ratio without any significant difference on plasma ADMA level after medication with RAAS inhibitors. Delles et al. [84] showed no effect of RAAS inhibitors on plasma L-Arginine concentration in a small study of young patients with essential hypertension. Therefore, the effect of RAAS inhibitors on the L-Arginine/ADMA ratio should be further examined.

There is a discrepancy among the results of clinical studies with cholesterol-lowering drugs, especially statins, on circulatory ADMA levels. Janatuinen et al. [85] found the beneficial vascular effect of pravastatin only in patients with low plasma ADMA level, whereas they did not found it in those with increased plasma ADMA level. Janatuinen et al. [85] tried to explain in part this inconsistent

result by blockade NOS with increased plasma ADMA level despite its upregulated gene expression after statin treatment. Therefore, Janatuinen et al. [85] recommend the use of L-Arginine supplementation combined with pravastatin in addition to overcome this blockade. Similarly, Böger et al. found that simvastatin enhanced endothelium-dependent vasodilation only when it was combined with supplemental nutritional L-Arginine in patients with an elevated ADMA concentration. Dierkers et al. [86] showed that fenofibrate raises L-Arginine and L-Citrulline, but not ADMA. Consequently, the L-Arginine/ADMA ratio increased. However, the addition of folic acid and vitamin B12 and B6 to fenofibrate had no significant effect on L-Arginine, ADMA, and their ratios. Similar results have been found in monkeys [38] and in patients with peripheral occlusive atherosclerotic disease [87].

Other drug research studies are as follows. Both Stühlinger et al. [88] and Wang et al. [89] demonstrated that administration of the rosiglitazone (a PPAR-gamma agonist, which reduce blood glucose levels by improving insulin sensitivity in organs) reduces the plasma ADMA level. O'Kane et al. [90] recently demonstrated that aspirin increased the activity of NOS type 3 in platelets and decreased L-Arginine concentration by increasing L-Arginine–citrulline turnover. This could explain why use of platelet inhibitors (mainly aspirin) was associated with lower L-Arginine/ADMA ratio. Setola et al. [91] demonstrated that growth hormone replacement decreases plasma ADMA levels and increases L-Arginine/ADMA ratio in patients with growth hormone deficiency. The management of inflammation and oxidative stress have also improved L-Arginine/ADMA ratio [6]. The favorable effect of anti-TNF-α therapy on the L-Arginine/ADMA ratio has been found in association with an increase in L-Arginine and without influencing ADMA levels [57].

Supplementing L-Arginine

Since ADMA is a competitive inhibitor of NOS, theoretically, L-Arginine supplementation (natural substrate of NOS) could be able to displace ADMA, reverse the competitive inhibition of NOS by ADMA, and increase NO levels in the body. Thus, the detrimental vascular effects of elevated plasma ADMA level might be counteracted. The effect of L-Arginine supplementation on the improvement of NO bioavailability is controversial. Dietary supplementation with L-Arginine has been reported to improve endothelial function [37], improve symptoms of claudication in patients with peripheral vascular disease [92], increase walking distance, increase flow-mediated dilation of the brachial artery [93], improve coronary microvascular flow and therefore myocardial ischemia [94], improve glucose tolerance and enhance insulin sensitivity [95], decrease endothelin-1 levels [96], reduce plasma levels of fatty acids and triglyceride [95] reduce endothelial adhesiveness for monocytes, inhibit platelet aggregation, and retard atherogenesis [97]. Despite these beneficial effects of L-Arginine supplementation, recent studies have reported some deleterious effects of L-Arginine on vascular function, especially in critically ill patients [98].

The beneficial effects of L-Arginine supplementation are mainly being attributed to increasing the plasma L-Arginine/ADMA ratio (approximately 60 %) in both experimental animal model and preliminary clinical studies [46]. Although L-Arginine administration does not affect renal ADMA clearance [37], the effect of L-Arginine supplementation might be influenced by the presence of ADMA. Böger et al. [21] proposed that nutritional L-Arginine supplementation has little or no effect on humans with no disturbed L-Arginine/ADMA balance. Therefore, it has been suggested that L-Arginine/ADMA ratio might help to identify patients that could receive the greatest benefit from L-Arginine supplementation [7].

The major part of dietary L-Arginine is metabolized in the liver and utilized in the hepatic urea cycle [34]. Because of its extensive first-pass metabolism in the liver and intestine, only a small portion of dietary L-Arginine is converted to NO. Thus, the use of L-Arginine supplementation as a therapeutic modality is limited. The standard dose for L-Arginine is 3–6 g (three times a day), with a total dose of 15–18 g. However, it should be kept in mind that taking more than 10 g of L-Arginine at once can result in gastrointestinal distress and diarrhea.

Another supplementation option is by increasing the concentration of the L-arginine precursor, L-Citrulline or glutamine. L-Citrulline is a nonessential amino acid but essential to detoxify and remove ammonia from muscle and liver cells. L-Citrulline is converted into L-Arginine by the L-Citrulline/L-Arginine cycle in the kidneys. L-Citrulline is not subjective to extensive first-pass metabolism by gut bacteria or liver arginases [99]. Since it has a better absorption rate, L-Citrulline is able to increase levels of plasma L-Arginine more effective than L-Arginine itself [70]. Nevertheless, the inhibition of the enzyme of arginase (an indirect strategy for elevating L-Arginine) could improve L-Arginine bioavailability. Tousoulis et al. [39] have suggested that the inhibition of the enzyme of arginase is more effective than exogenous L-Arginine administration. Plasma L-Arginine concentration can also be altered by pharmacotherapy. Dierkers et al. [86] showed that fenofibrate raises L-Arginine and Holven et al. [100] showed that folic acid treatment reduces L-Arginine levels.

Conclusion

The L-Arginine/ADMA ratio is a more important determinant of NOS bioavailability and endothelial function than plasma L-Arginine and ADMA concentrations alone. The alterations in L-Arginine/ADMA ratio have important insights in the understanding of the pathophysiology and the biochemical mechanisms of various diseases and metabolic conditions, which occur as a result of endothelial dysfunction. L-Arginine/ADMA ratio may also be a conceivable target for pharmacological approaches. A combined management of L-Arginine supplementation and decrease of ADMA level may best achieve the improvement of NOS bioavailability by an increase of the substrate/inhibitor ratio.

References

1. Widlansky ME, Gokce N, Keaney Jr JF, et al. The clinical implications of endothelial dysfunction. J Am Coll Cardiol. 2003;42:1149–60.
2. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424.
3. Forstermann U. Nitric oxide and oxidative stress in vascular disease. Pflugers Arch. 2010;459:923–39.
4. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol. 2006;147 Suppl 1:S193–201.
5. Anthony S, Leiper J, Vallance P. Endogenous production of nitric oxide synthase inhibitors. Vasc Med. 2005;10 Suppl 1:S3–9.
6. van der Zwan LP, Scheffer PG, Dekker JM, et al. Systemic inflammation is linked to low L-arginine and high ADMA plasma levels resulting in an unfavourable NOS substrate-to-inhibitor ratio: the Hoorn Study. Clin Sci (Lond). 2011;121:71–8.
7. Bode-Boger SM, Scalera F, Ignarro LJ. The L-arginine paradox: Importance of the L-arginine/asymmetrical dimethylarginine ratio. Pharmacol Ther. 2007;114:295–306.
8. Palmer RM, Rees DD, Ashton DS, et al. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem Biophys Res Commun. 1988;153:1251–6.
9. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(Pt 1):1–17.
10. Morris Jr SM. L-Arginine metabolism in vascular biology and disease. Vasc Med. 2005;10 Suppl 1:S83–7.
11. Zani BG, Bohlen HG. Transport of extracellular L-arginine via cationic amino acid transporter is required during in vivo endothelial nitric oxide production. Am J Physiol Heart Circ Physiol. 2005;289:H1381–90.
12. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357:593–615.
13. Teerlink T, Luo Z, Palm F, et al. Cellular ADMA: regulation and action. Pharmacol Res. 2009;60:448–60.
14. Cardounel AJ, Cui H, Samouilov A, et al. Evidence for the pathophysiological role of endogenous methylarginines in regulation of endothelial NO production and vascular function. J Biol Chem. 2007;282:879–87.
15. Closs EI, Basha FZ, Habermeier A, et al. Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. Nitric Oxide. 1997;1:65–73.
16. Vallance P, Leone A, Calver A, et al. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992;339:572–5.

17. Gray GA, Patrizio M, Sherry L, et al. Immunolocalisation and activity of DDAH I and II in the heart and modification post-myocardial infarction. Acta Histochem. 2010;112:413–23.
18. Luneburg N, Xanthakis V, Schwedhelm E, et al. Reference intervals for plasma L-arginine and the L-arginine:asymmetric dimethylarginine ratio in the Framingham Offspring Cohort. J Nutr. 2011;141:2186–90.
19. Horowitz JD, Heresztyn T. An overview of plasma concentrations of asymmetric dimethylarginine (ADMA) in health and disease and in clinical studies: methodological considerations. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;851:42–50.
20. Martens-Lobenhoffer J, Bode-Boger SM. Simultaneous detection of L-arginine, asymmetric dimethylarginine, symmetric dimethylarginine and citrulline in human plasma and urine applying liquid chromatography-mass spectrometry with very straightforward sample preparation. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;798:231–9.
21. Boger RH. Asymmetric dimethylarginine (ADMA) and cardiovascular disease: insights from prospective clinical trials. Vasc Med. 2005;10 Suppl 1:S19–25.
22. Richir MC, van Lambalgen AA, Teerlink T, et al. Low L-arginine/asymmetric dimethylarginine ratio deteriorates systemic hemodynamics and organ blood flow in a rat model. Crit Care Med. 2009;37:2010–7.
23. Barbul A, Lazarou SA, Efron DT, et al. L-Arginine enhances wound healing and lymphocyte immune responses in humans. Surgery. 1990;108:331–6. discussion 336–7.
24. Erre GL, Sanna P, Zinellu A, et al. Plasma asymmetric dimethylarginine (ADMA) levels and atherosclerotic disease in ankylosing spondylitis: a cross-sectional study. Clin Rheumatol. 2011;30:21–7.
25. Antoniades C, Demosthenous M, Tousoulis D, et al. Role of asymmetrical dimethylarginine in inflammation-induced endothelial dysfunction in human atherosclerosis. Hypertension. 2011;58:93–8.
26. Chirinos JA, David R, Bralley JA, et al. Endogenous nitric oxide synthase inhibitors, arterial hemodynamics, and subclinical vascular disease: the PREVENCION Study. Hypertension. 2008;52:1051–9.
27. Nijveldt RJ, Teerlink T, van Guldener C, et al. Handling of asymmetrical dimethylarginine and symmetrical dimethylarginine by the rat kidney under basal conditions and during endotoxaemia. Nephrol Dial Transplant. 2003;18:2542–50.
28. Mittermayer F, Namiranian K, Pleiner J, et al. Acute Escherichia coli endotoxaemia decreases the plasma L-arginine/asymmetrical dimethylarginine ratio in humans. Clin Sci (Lond). 2004;106:577–81.
29. Boger RH. The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. Cardiovasc Res. 2003;59:824–33.
30. Landray MJ, Emberson JR, Blackwell L, et al. Prediction of ESRD and death among people with CKD: the Chronic Renal Impairment in Birmingham (CRIB) prospective cohort study. Am J Kidney Dis. 2010;56:1082–94.
31. Mittermayer F, Mayer BX, Meyer A, et al. Circulating concentrations of asymmetrical dimethyl-L-arginine are increased in women with previous gestational diabetes. Diabetologia. 2002;45:1372–8.
32. Ellis J, Wennerholm UB, Bengtsson A, et al. Levels of dimethylarginines and cytokines in mild and severe preeclampsia. Acta Obstet Gynecol Scand. 2001;80:602–8.
33. Holguin F, Comhair SA, Hazen SL, et al. An association between L-arginine/asymmetric dimethyl arginine balance, obesity, and the age of asthma onset phenotype. Am J Respir Crit Care Med. 2013;187:153–9.
34. Maas R, Schulze F, Baumert J, et al. Asymmetric dimethylarginine, smoking, and risk of coronary heart disease in apparently healthy men: prospective analysis from the population-based monitoring of trends and determinants in cardiovascular disease/Kooperative Gesundheitsforschung in der Region Augsburg study and experimental data. Clin Chem. 2007;53:693–701.
35. Moss MB, Brunini TM, Soares De Moura R, et al. Diminished L-arginine bioavailability in hypertension. Clin Sci (Lond). 2004;107:391–7.
36. Hsu CN, Huang LT, Lau YT, et al. The combined ratios of L-arginine and asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats. Transl Res. 2012;159:90–8.
37. Boger RH, Bode-Boger SM, Szuba A, et al. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. Circulation. 1998;98:1842–7.
38. Boger RH, Bode-Boger SM, Sydow K, et al. Plasma concentration of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, is elevated in monkeys with hyperhomocyst(e)inemia or hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2000;20:1557–64.
39. Tousoulis D, Boger RH, Antoniades C, et al. Mechanisms of disease: L-arginine in coronary atherosclerosis—a clinical perspective. Nat Clin Pract Cardiovasc Med. 2007;4:274–83.
40. El-Tamimi H, Davies GJ, Crea F, et al. Response of human coronary arteries to acetylcholine after injury by coronary angioplasty. J Am Coll Cardiol. 1993;21:1152–7.
41. Leong T, Zylberstein D, Graham I, et al. Asymmetric dimethylarginine independently predicts fatal and nonfatal myocardial infarction and stroke in women: 24-year follow-up of the population study of women in Gothenburg. Arterioscler Thromb Vasc Biol. 2008;28:961–7.
42. Lu TM, Ding YA, Lin SJ, et al. Plasma levels of asymmetrical dimethylarginine and adverse cardiovascular events after percutaneous coronary intervention. Eur Heart J. 2003;24:1912–9.
43. Sahinarslan A, Cengel A, Biberoglu G, et al. Plasma asymmetric dimethylarginine level and extent of lesion at coronary angiography. Coron Artery Dis. 2006;17:605–9.

44. Koc F, Ardic I, Erdem S, et al. Relationship between L-arginine/asymmetric dimethylarginine, homocysteine, folic acid, vitamin B levels, and coronary artery ectasia. Coron Artery Dis. 2010;21:445–9.
45. Selcuk MT, Selcuk H, Temizhan A, et al. Asymmetric dimethylarginine plasma concentrations and L-arginine/asymmetric dimethylarginine ratio in patients with slow coronary flow. Coron Artery Dis. 2007;18:545–51.
46. Walker HA, McGing E, Fisher I, et al. Endothelium-dependent vasodilation is independent of the plasma L-arginine/ADMA ratio in men with stable angina: lack of effect of oral L-arginine on endothelial function, oxidative stress and exercise performance. J Am Coll Cardiol. 2001;38:499–505.
47. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol. 2006;40:16–23.
48. Anderssohn M, Rosenberg M, Schwedhelm E, et al. The L-arginine-asymmetric dimethylarginine ratio is an independent predictor of mortality in dilated cardiomyopathy. J Card Fail. 2012;18:904–11.
49. Seljeflot I, Nilsson BB, Westheim AS, et al. The L-arginine-asymmetric dimethylarginine ratio is strongly related to the severity of chronic heart failure. No effects of exercise training. J Card Fail. 2011;17:135–42.
50. Tveit A, Grundvold I, Olufsen M, et al. Candesartan in the prevention of relapsing atrial fibrillation. Int J Cardiol. 2007;120:85–91.
51. Boger RH, Cooke JP, Vallance P. ADMA: an emerging cardiovascular risk factor. Vasc Med. 2005;10 Suppl 1:S1–2.
52. Ellger B, Richir MC, van Leeuwen PA, et al. Glycemic control modulates arginine and asymmetrical-dimethylarginine levels during critical illness by preserving dimethylarginine-dimethylaminohydrolase activity. Endocrinology. 2008;149:3148–57.
53. Anderssohn M, Schwedhelm E, Luneburg N, et al. Asymmetric dimethylarginine as a mediator of vascular dysfunction and a marker of cardiovascular disease and mortality: an intriguing interaction with diabetes mellitus. Diab Vasc Dis Res. 2010;7:105–18.
54. Hanai K, Babazono T, Nyumura I, et al. Asymmetric dimethylarginine is closely associated with the development and progression of nephropathy in patients with type 2 diabetes. Nephrol Dial Transplant. 2009;24:1884–8.
55. Eid HM, Reims H, Arnesen H, et al. Decreased levels of asymmetric dimethylarginine during acute hyperinsulinemia. Metabolism. 2007;56:464–9.
56. Wilkinson IB, MacCallum H, Cockcroft JR, et al. Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. Br J Clin Pharmacol. 2002;53:189–92.
57. Angel K, Provan SA, Mowinckel P, et al. The L-arginine/asymmetric dimethylarginine ratio is improved by anti-tumor necrosis factor-alpha therapy in inflammatory arthropathies. Associations with aortic stiffness. Atherosclerosis. 2012;225:160–5.
58. Boger RH. The pharmacodynamics of L-arginine. J Nutr. 2007;137:1650S–5.
59. Yilmaz MI, Sonmez A, Saglam M, et al. ADMA levels correlate with proteinuria, secondary amyloidosis, and endothelial dysfunction. J Am Soc Nephrol. 2008;19:388–95.
60. Caglar K, Yilmaz MI, Sonmez A, et al. ADMA, proteinuria, and insulin resistance in non-diabetic stage I chronic kidney disease. Kidney Int. 2006;70:781–7.
61. Boger RH, Zoccali C. ADMA: a novel risk factor that explains excess cardiovascular event rate in patients with end-stage renal disease. Atheroscler Suppl. 2003;4:23–8.
62. Zoccali C, Bode-Boger S, Mallamaci F, et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet. 2001;358:2113–7.
63. Shi B, Ni Z, Zhou W, et al. Circulating levels of asymmetric dimethylarginine are an independent risk factor for left ventricular hypertrophy and predict cardiovascular events in pre-dialysis patients with chronic kidney disease. Eur J Intern Med. 2010;21:444–8.
64. Brunini TM, Moss MB, Siqueira MA, et al. Nitric oxide, malnutrition and chronic renal failure. Cardiovasc Hematol Agents Med Chem. 2007;5:155–61.
65. Celik M, Iyisoy A, Celik T, et al. The relationship between L-arginine/ADMA ratio and coronary collateral development in patients with low glomerular filtration rate. Cardiol J. 2012;19:29–35.
66. Nijveldt RJ, Siroen MP, van der Hoven B, et al. High plasma L-arginine concentrations in critically ill patients suffering from hepatic failure. Eur J Clin Nutr. 2004;58:587–93.
67. Siroen MP, Wiest R, Richir MC, et al. Transjugular intrahepatic portosystemic shunt-placement increases arginine/asymmetric dimethylarginine ratio in cirrhotic patients. World J Gastroenterol. 2008;14:7214–9.
68. Mookerjee RP, Malaki M, Davies NA, et al. Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. Hepatology. 2007;45:62–71.
69. Siroen MP, Warle MC, Teerlink T, et al. The transplanted liver graft is capable of clearing asymmetric dimethylarginine. Liver Transpl. 2004;10:1524–30.
70. Paroni R, Barassi A, Ciociola F, et al. Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and L-arginine in patients with arteriogenic and non-arteriogenic erectile dysfunction. Int J Androl. 2012;35:660–7.
71. Chen J, Wollman Y, Chernichovsky T, et al. Effect of oral administration of high-dose nitric oxide donor L-arginine in men with organic erectile dysfunction: results of a double-blind, randomized, placebo-controlled study. BJU Int. 1999;83:269–73.
72. Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: recent advances. Pharmacol Rev. 2009;61:62–97.

73. Washington CW, Zipfel GJ, Participants in the International Multi-disciplinary Consensus Conference on the Critical Care Management of Subarachnoid H. Detection and monitoring of vasospasm and delayed cerebral ischemia: a review and assessment of the literature. Neurocrit Care. 2011;15:312–7.
74. Staalso JM, Bergstrom A, Edsen T, et al. Low plasma L-arginine:asymmetric dimethyl L-arginine ratios predict mortality after intracranial aneurysm rupture. Stroke. 2013;44:1273–81.
75. Scherbakov N, Sandek A, Martens-Lobenhoffer J, et al. Endothelial dysfunction of the peripheral vascular bed in the acute phase after ischemic stroke. Cerebrovasc Dis. 2012;33:37–46.
76. Bergstrom A, Staalso JM, Romner B, et al. Impaired endothelial function after aneurysmal subarachnoid haemorrhage correlates with L-arginine:asymmetric dimethylarginine ratio. Br J Anaesth. 2014;112:311–8.
77. Gaston B, Drazen JM, Loscalzo J, et al. The biology of nitrogen oxides in the airways. Am J Respir Crit Care Med. 1994;149:538–51.
78. Holguin F. L-Arginine and nitric oxide pathways in obesity-associated asthma. J Allergy (Cairo). 2013;2013:714595.
79. Richir MC, Siroen MP, van Elburg RM, et al. Low plasma concentrations of L-arginine and asymmetric dimethylarginine in premature infants with necrotizing enterocolitis. Br J Nutr. 2007;97:906–11.
80. Kubes P. Nitric oxide modulates epithelial permeability in the feline small intestine. Am J Physiol. 1992;262:G1138–42.
81. Scalera F, Martens-Lobenhoffer J, Bukowska A, et al. Effect of telmisartan on nitric oxide—asymmetrical dimethylarginine system: role of angiotensin II type 1 receptor gamma and peroxisome proliferator activated receptor gamma signaling during endothelial aging. Hypertension. 2008;51:696–703.
82. Kawata T, Daimon M, Hasegawa R, et al. Effect of angiotensin-converting enzyme inhibitor on serum asymmetric dimethylarginine and coronary circulation in patients with type 2 diabetes mellitus. Int J Cardiol. 2009;132:286–8.
83. Hov GG, Sagen E, Hatlen G, et al. L-Arginine/asymmetric dimethylarginine ratio and cardiovascular risk factors in patients with predialytic chronic kidney disease. Clin Biochem. 2011;44:642–6.
84. Delles C, Jacobi J, Schlaich MP, et al. Assessment of endothelial function of the renal vasculature in human subjects. Am J Hypertens. 2002;15:3–9.
85. Janatuinen T, Laakso J, Laaksonen R, et al. Plasma asymmetric dimethylarginine modifies the effect of pravastatin on myocardial blood flow in young adults. Vasc Med. 2003;8:185–9.
86. Dierkes J, Westphal S, Martens-Lobenhoffer J, et al. Fenofibrate increases the L-arginine:ADMA ratio by increase of L-arginine concentration but has no effect on ADMA concentration. Atherosclerosis. 2004;173:239–44.
87. Sydow K, Schwedhelm E, Arakawa N, et al. ADMA and oxidative stress are responsible for endothelial dysfunction in hyperhomocyst(e)inemia: effects of L-arginine and B vitamins. Cardiovasc Res. 2003;57:244–52.
88. Stuhlinger MC, Abbasi F, Chu JW, et al. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. JAMA. 2002;287:1420–6.
89. Wang TD, Chen WJ, Cheng WC, et al. Relation of improvement in endothelium-dependent flow-mediated vasodilation after rosiglitazone to changes in asymmetric dimethylarginine, endothelin-1, and C-reactive protein in nondiabetic patients with the metabolic syndrome. Am J Cardiol. 2006;98:1057–62.
90. O'Kane P, Xie L, Liu Z, et al. Aspirin acetylates nitric oxide synthase type 3 in platelets thereby increasing its activity. Cardiovasc Res. 2009;83:123–30.
91. Setola E, Monti LD, Lanzi R, et al. Effects of growth hormone treatment on L-arginine to asymmetric dimethylarginine ratio and endothelial function in patients with growth hormone deficiency. Metabolism. 2008;57:1685–90.
92. Boger RH, Bode-Boger SM, Thiele W, et al. Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. J Am Coll Cardiol. 1998;32:1336–44.
93. Adams MR, McCredie R, Jessup W, et al. Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. Atherosclerosis. 1997;129:261–9.
94. Drexler H, Zeiher AM, Meinzer K, et al. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. Lancet. 1991;338:1546–50.
95. Lucotti P, Monti L, Setola E, et al. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism. 2009;58:1270–6.
96. Okyay K, Cengel A, Sahinarslan A, et al. Plasma asymmetric dimethylarginine and L-arginine levels in patients with cardiac syndrome X. Coron Artery Dis. 2007;18:539–44.
97. Tsao PS, Theilmeier G, Singer AH, et al. L-arginine attenuates platelet reactivity in hypercholesterolemic rabbits. Arterioscler Thromb. 1994;14:1529–33.
98. Wilson AM, Harada R, Nair N, et al. L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. Circulation. 2007;116:188–95.
99. Schwedhelm E, Maas R, Freese R, et al. Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: impact on nitric oxide metabolism. Br J Clin Pharmacol. 2008;65:51–9.
100. Holven KB, Haugstad TS, Holm T, et al. Folic acid treatment reduces elevated plasma levels of asymmetric dimethylarginine in hyperhomocysteinaemic subjects. Br J Nutr. 2003;89:359–63.

Chapter 19
L-Arginine and Its Transporters in Colorectal Cancer

Bingguan Chen, Weimin Wang, Kang Ding, Junchen Wang, Peng Gao, Guowang Xu, and Hai Hu

Key Points

- The L-arginine (Arg) catabolism pathway is overactive in colorectal cancer (CRC).
- Endothelial nitric oxide synthase (eNOS) and ornithine decarboxylase (ODC) are the main enzymes for Arg catabolism in cancer cells.
- Polyamines are known as oncometabolites.
- Arg transporter CAT-1 and ATB$^{(0,+)}$ (SLC6A14) are overexpressed in CRC cells.
- A cationic amino acid probe may be used to develop a novel molecular imaging diagnostic tool.
- Mass spectrometry analysis of oncometabolites may be useful for cancer screening.
- Several molecules in the Arg catabolism pathway may be potential targets for chemoprevention and targeted CRC therapy.

Keywords L-Arginine • L-Arginine transporter • Polyamines • Colorectal cancer • Biomarker • Metabolomics • Molecular imaging • Targeted therapy

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Abbreviations

APAO	Acetylpolyamine oxidase
APC	Adenomatous polyp
ADI	L-Arginine deiminase
Arg	L-Arginine
ASS	Argininosuccinate synthetase
ASL	Argininosuccinate lyase
ARG	Arginase
α-MT	α-methyl-dl-tryptophan
CAT	Cationic amino acid transporter
Cit	Citrulline
CRC	Colorectal cancer
eNOS	Endothelial nitric oxide synthase
EREG	Epiregulin
ER	Estrogen receptor
eIF5A	Eukaryotic translation initiation factor-5A
HATs	Heteromeric(di) amino acid transporters
HPLC	High-performance liquid chromatography
HUGO	Human genome organization
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
ODC	Ornithine decarboxylase
PCR	Polymerase chain reaction
PET	Positron emission tomography
PKC	Protein kinase C
RCC	Renal cell carcinoma
shRNA	Short hairpin RNA
SMO	Spermine oxidase
SAT1 or SSAT	Spermidine/spermine *N*1-acetyl transferase
SLC7A	Solute carrier family 7

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in the world. The current treatment protocol still heavily relies on early diagnosis and surgery. Treatment options remain limited for recurrent and metastatic CRC patients, and less than 20 % respond to the current targeted therapies [1, 2]. Obviously, it is very important to discover novel biomarkers of CRC to develop more effective targeted treatments. Researchers are focusing their efforts on cancer genomics, proteomics, and current metabolomics to identify novel cancer biomarkers to facilitate the development of new serological diagnostic tools and targeted CRC therapies [3–5].

Abnormal metabolism is one of the important characteristics of cancer; it could be an initiating factor of carcinogenesis or a consequence of cancer development and progress. With advancing progress in cancer metabolomics, there is new insight into tumor cell metabolism pathways of reprogramming and the effects of abnormal metabolism on tumors' biological behavior. Thanks to these advances, significant progress in tumor prevention has been achieved, especially in CRC. Among cancer metabolites, polyamines have gained increasing attention in recent years because of their association to the early-stage tumor development and colorectal carcinoma progression [3, 6]. It is well

known that L-arginine (Arg) is a substrate of polyamines. Recently, several lines of research confirmed that abnormal Arg catabolism pathway may be characteristic of tumor cell metabolism. We have focused on Arg metabolism in CRC and found elevated level of polyamines, the Arg terminal metabolite in cancer patient sera [7]. Based on this finding, we [8] established an analytical method of high-performance liquid chromatography (HPLC) for simultaneous determination of Arg and L-citrulline (Cit) and found abnormal Arg metabolism in the urea cycle and decreased Arg levels in the sera of CRC patients. Our subsequent study [9] demonstrated significantly higher Arg and Cit levels in CRC tissues compared with adjacent normal colorectal tissues, indicating increased Arg bioavailability in CRC tissues. Our results suggested that Arg catabolism pathway is overactive in the CRC cells. We [10] also found that the higher bioavailability of Arg in CRC cells was associated with higher expression of certain Arg transporters in CRC cells. In order to better understand Arg metabolism and develop novel diagnostic tools and therapies for CRC, this review details the recent findings regarding Arg and its transporters in CRC.

Arg Catabolism Pathway in Cancer Cells

There are two pathways to metabolize Arg in human colon epithelial cells and adenocarcinoma: the nitric oxide synthase (NOS) pathway and the ornithine polyamine pathway, as reviewed by Blachier et al. [11]. L-Arg is the substrate of endothelial nitric oxide synthase [eNOS or nitric oxide synthase 3 (NOS3)]. The metabolic products of the L-Arg–NO pathway are nitric oxide (NO) and Cit. The latter can be recycled to synthesize Arg in the cells by argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). Experimental studies have demonstrated that cancer cells express higher eNOS that is necessary for persistent Ras-driven PI3K–AKT signal pathway in tumor growth [12, 13]. Tumor cells express initiating oncogenes like *Ras*, while loss of oncogene expression in established tumors leads to tumor regression. In many cancers, HRas, NRas, or KRas is mutated to remain in the active GTP-bound oncogenic state. PI3K usually remains activated by oncogenic Ras through activation of protein kinase B (PKB; also known as AKT) to maintain tumor growth, and Ras activates several proteins to initiate human tumor growth. In 2008, Lim et al. [13] discovered that blocking phosphorylation of the AKT substrate (eNOS) inhibits tumor initiation and maintenance. In addition, eNOS enhances the nitrosylation and activation of endogenous wild-type Ras proteins, which are required throughout tumorigenesis. The existing evidence suggests that activation of the PI3K–AKT–eNOS (wild-type)-Ras pathway by oncogenic *Ras* in cancer cells is required to initiate and maintain tumor growth. As Arg is a substrate of eNOS, the activation of the PI3K–AKT–eNOS–Ras pathway will accelerate Arg catabolism in cancer. Thus, targeting Arg catabolism may substantially inhibit the pathway to suppress tumor growth.

In CRC cells, the ornithine polyamine pathway (i.e., the catabolism pathway of Arg for polyamine generation via ornithine) has received increasing attention [14]. L-Arg catabolized by arginase (ARG) produces ornithine that can be further catabolized by ornithine decarboxylase (ODC) to produce polyamines. One of the polyamines, putrescine, plays an important role in CRC development and proliferation [15]. It has been found that many tumors, such as CRC [16] and neuroblastoma [17], express higher levels of ODC. An experimental study [14] confirmed that ODC plays an important role in the carcinogenesis of CRC. Both the adenomatous polyp (APC) tumor suppressor and KRAS genes are involved in the regulation of polyamine generation and the initiation of intestinal tumorigenesis in APC mutant mice [Apc(Min/+) mice]. In this strain of mouse, ODC transcription increases, and acceleration of the polyamine synthesis process is c-Myc dependent. Notably, ODC inhibitors can significantly reduce the incidence of colon polyps. The KRAS gene can increase ODC activity and polyamine levels in cells and tissues, which results in carcinogenesis. In addition, ODC can replace c-Myc to cooperate with RAS for cell transformation. We also demonstrated higher ODC expression in CRC in a tissue microarray study (Fig. 19.1).

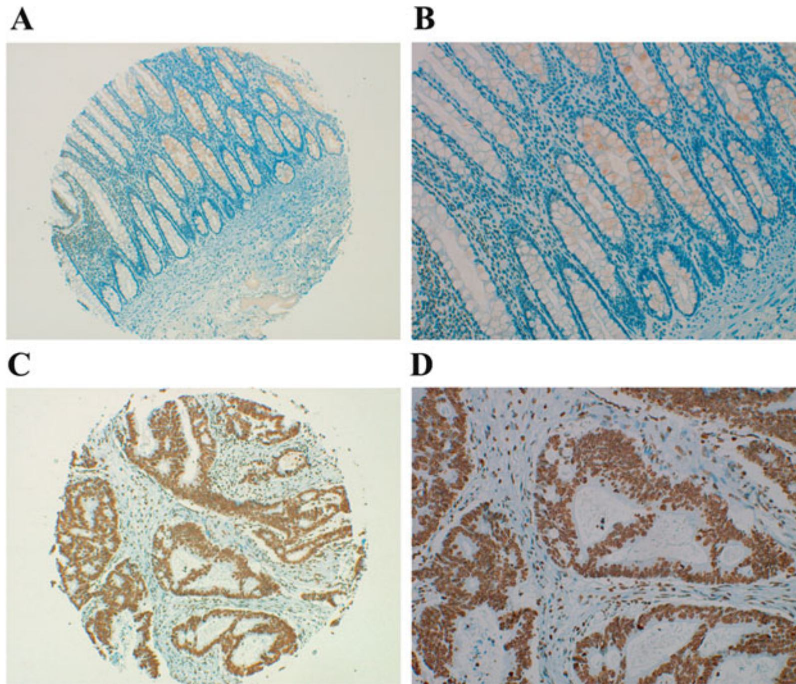

Fig. 19.1 Immunohistochemical labeling demonstrating strong ODC expression in human CRC tissue. Histochemistry of matched tissue specimens: (**c** and **d**) cancer tissue and (**a** and **b**) adjacent normal colon tissue. The pathological characteristics of colon adenocarcinoma (**c**, **d**) and adjacent normal colon tissue (**a**, **b**) in the tumor specimen are shown in the hematoxylin- and eosin-stained sections. The densities of ODC protein expression in colon adenocarcinoma (**c** and **d**) and adjacent normal colon tissue (**a** and **b**) were assessed following labeling with the ASS antibody at 10× (**a** and **c**) and 20× amplifications (**b** and **d**)

Recently Manna et al. [3] performed mass spectrometry-based metabolomic and gene expression analyses of urine and tissue samples from mice and humans to identify markers of colorectal carcinogenesis. In human studies, metabolic profiles were compared between colon tumor and adjacent non-tumor tissues from 39 patients. The results identified metabolites related to Arg/polyamine metabolism, nucleic acid metabolism, and methylation and distinguished tumor-bearing Apc(Min/+) mice with 100 % accuracy. The metabolites also accurately identified mice with polyps. These alterations in urinary metabolites were also observed in mice with azoxymethane-induced tumors and in mice with colon-specific β-catenin activation. Metabolite screening also revealed that human tumor tissues had stage-dependent increases in 12 metabolites associated with the same metabolic pathways identified in mice (including amino acid metabolism and Arg/polyamine metabolism). Ten metabolites (proline, threonine, glutamic acid, Arg, *N*1-acetylspermidine, xanthine, uracil, betaine, symmetric dimethylarginine, and asymmetric-dimethylarginine) were increased in tumor tissues compared with non-tumor tissues and urine from both human and tumor-bearing mice. The authors further demonstrated that gene expression and metabolomic profiles of urine and tissue samples from mice with colorectal tumors and CRC tumor samples from patients exhibited derangements of specific metabolic pathways that are indicative of early-stage tumor development. They concluded that these urine and tissue markers might be used for the early detection of CRC.

Polyamines are organic polycations with low molecular weights that can bind to negatively charged RNAs, microRNAs, and proteins to affect protein translation and transcription and support c-Myc-controlled gene function [6, 14, 18]. Polyamines can modify eukaryotic translation initiation

factor-5A (eIF5A) to become a very rare amino acid hypusine-containing protein that regulates gene function by affecting the synthesis and translation of proto-oncogene and tumor suppressor genes, as well as protein modification [18, 19]. However, the precise molecular involvement of hypusinated protein in the process of carcinogenesis is still unclear. The microRNA let-7 family has been known to inhibit the function of oncogenes, such as KRAS and the growth-associated transcription factor HMGA2. Polyamine depletion can significantly increase let-7 activity and inhibit tumor growth [18]. Thus, polyamines play an important role in CRC development and cell proliferation as carcinogenic metabolites (oncometabolites) [14, 18]. In order to confirm polyamine accumulation in CRC cells, we performed applied metabolomics (mass spectrometry) to analyze metabolites in human tumor tissues and found that Arg, ornithine, spermidine, spermine, and putrescine were significantly increased in CRC tissues (Fig. 19.2). It has been confirmed that the ODC inhibitor DFMO (eflornithine), which is used to treat toxoplasmosis, can be used for chemoprevention of colorectal adenoma, prostate, and skin cancers. The anti-inflammatory drug sulindac, which promotes polyamine excretion, can be combined with DFMO to effectively reduce the incidence of colonic adenomas [14]. Limiting Arg-rich meat consumption and inhibiting ODC activity can largely reduce polyamine synthesis and the incidence of CRC in certain instances [14, 20]. This strategy may also be applied for the prevention of other cancers, such as lung, breast, and prostate cancers. However, it is not clear if DFMO can be used in chemotherapy.

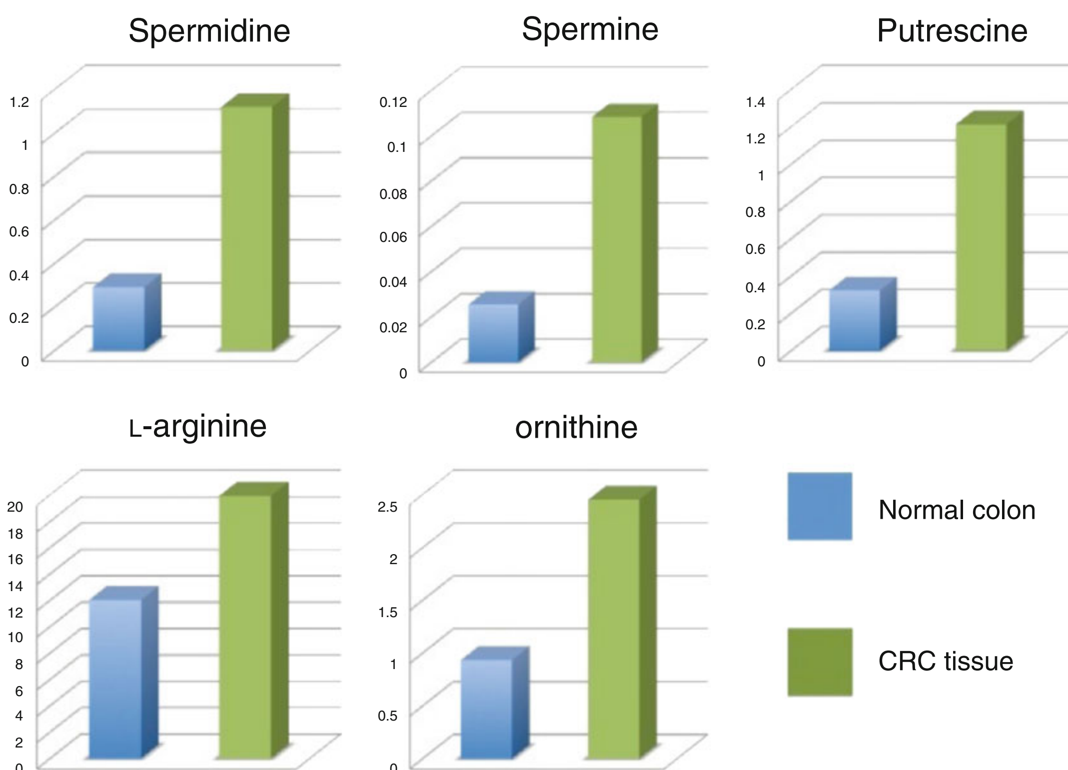

Fig. 19.2 Arg metabolites in human CRC tissues. The samples are matched tissue specimens from 11 CRC cases. The metabolites were analyzed by mass spectrometry, and 207 metabolites were detected. Among them, 153 metabolites showed significant differences between cancer tissue and adjacent healthy colon tissue (as $P < 0.05$). Specifically, five arginine metabolites were found to be significantly higher in CRC tissues. The number shown in the figure is indicated as mass intensity/g tissue dry weight ($P < 0.05$)

Polyamine Catabolic Pathways in CRC

Intracellular polyamine levels are controlled to maintain homeostasis via strict regulation of every step in the synthesis, absorption, and transport of polyamine metabolism pathway, and these events are disrupted in cancer cells. The catabolism of spermine/spermidine is usually via the one-step enzymatic reaction of spermine oxidase (SMO), or by two steps of spermidine/spermine N1-acetyl transferase (SAT1 or SSAT) coupled with *N*1-acetylpolyamine oxidase (APAO). Polyamine catabolism is mainly through easily induced SSAT, which can acetylate polyamines and promote the transport of acetylated polyamines out of the cells through specific transmembrane proteins, thus reducing intracellular polyamine levels. Studies in both human cells and mouse models have confirmed that sulindac and other nonsteroidal anti-inflammatory drugs induce SAT1 expression by unique transcriptional mechanisms, which promote the acetylation of polyamines and their subsequent transport out of the cells. This activity contributes to the therapeutic effect of these agents on neuroblastoma and inflammation-induced CRC [16, 17]. Recently, it was found that overexpressing SAT1 via an adenovirus can quickly remove intracellular spermidine and spermine, directly inhibit cell protein synthesis within 24 h, and halt cell growth [21]. Based on these results, the clinical application of SAT1 adenovirus for cancer treatment should be assessed in future studies. However, polyamine catabolism involves synergy among several catabolic enzymes, including SSAT, APAO, and SMO. Thus, it is not clear whether the use of SSAT/APAO and SMO to alter the total intracellular polyamine pool is a viable cancer treatment strategy.

Arg Transporters in CRC

Arg is a positively charged (cationic) amino acid that can be shuttled across the membrane by several transporters. The most common Arg transporter family is the family of Na^+-independent cationic amino acid transporters (CAT) comprised of four members, CAT-1, CAT-2A, CAT-2B, CAT-3, and CAT-4, which all exhibit a nearly identical substrate pattern for Arg. CAT-1 and CAT-3 are encoded for by separate genes, while CAT-2A and CAT-2B are splice variants that differ only over a 42-amino acid stretch. The human genome organization (HUGO) has assigned the solute carrier family 7 (SLC7) gene names SLC7A1, SLC7A2, SLC7A3, and SLC7A4 to human CAT-1, CAT-2, CAT-3, and CAT-4, respectively. The function and specificity of CAT-3 and CAT-4 are not clear in many ways [22]. The second branch of the SLC7 family is comprised of the light chains of heteromeric(di) amino acid transporters (HATs, SLC7A5-11). In contrast to the CAT proteins, these glycoprotein-associated transporters need to couple with a glycoprotein (SLC3A1 or SLC3A2) to be targeted to the plasma membrane. The HATs 4F2hc/y^+LAT1, 4F2hc/y^+LAT2 (SLC3A2/SLC7A7 and SLC7A6), and rBAT/$b^{0,+}$AT (SLC3A1/SLC7A9) accept both cationic and neutral amino acids (see more in detail in [22]). Another Arg transporter is the sodium- and chloride-dependent neutral and basic amino acid transporter $B^{(0+)}$ that is encoded by the SLC6A14 gene in humans. In normal tissues, SLC6A14 protein is expressed at a low level [23].

To better understand the molecular mechanism of Arg accumulation in CRC tissues, we studied the transport mechanism of Arg in CRC cells. We first performed quantitative polymerase chain reaction (PCR) to detect the expression level of each Arg transport subunit in CRC samples. We found that CAT-1 and SLC6A14 were uniquely overexpressed in more than 70 % of patients (Fig. 19.3). CAT-1 was overexpressed in 86/122 CRC samples. The positive rate was 70 %, while other Arg transporter molecules were only overexpressed in <10 % of CRC tissues [10]. The results were confirmed by immunohistochemistry in 25 CRC cases using commercial tissue microarray and another 90 CRC cases using a tissue microarray developed by our group [10]. Based on the pathological analysis of 25

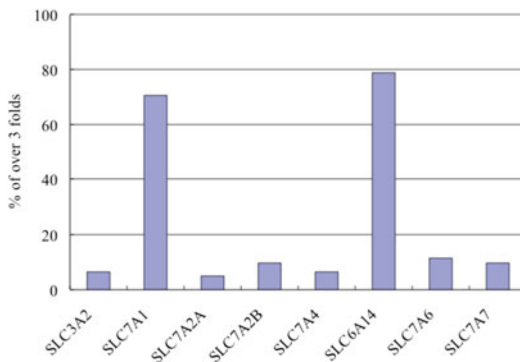

Fig. 19.3 The overexpression rate of each Arg transporter in human CRC tissue. The mRNA expression for each Arg transporter in CRC tissues was measured by qRT-PCR, and overexpression was defined as at least threefold higher expression than that in normal colon tissue. The figure shows the percentage of samples with overexpression (threefold) of individual L-arginine transporter genes among 122 CRC tissue samples. The CAT-1 and SLC6A14 genes were overexpressed in more than 70 % of CRC tissues

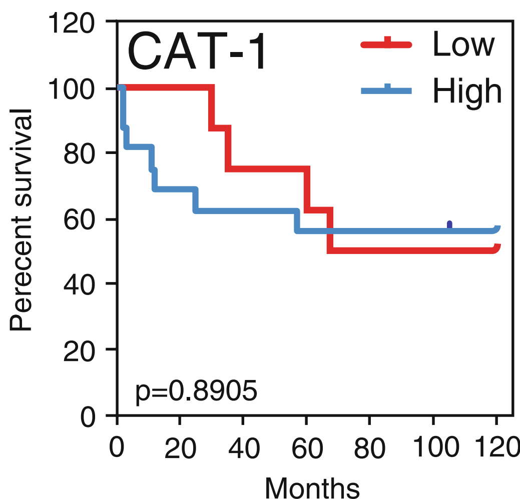

Fig. 19.4 The relationship between CAT-1 expression and CRC patient survival ($n = 25$)

cases, CAT-1 expression appears to be related to patient survival and pathological grade. CAT-1 was expressed at a higher level in poorly differentiated CRC compared to well-differentiated CRC [10]. Our results also indicated that higher CAT-1 expression might be related to poor survival of CRC patients, although this result was not significant (Fig. 19.4 shows the tissue microarray data of 25 cases with survival results). A study of the specificity and sensitivity of CAT-1 expression in CRC and other tumors is ongoing in our laboratory.

We also observed that the downregulation of CAT-1 by small-interfering RNA induced colon cancer cell death and apoptosis, suggesting that targeting CAT-1 in the treatment of CRC has potential clinical significance [10]. At the same time, Camps et al. [24] independently applied small-interfering RNA technology to CRC cell lines in a systemic gene expression research and found that SLC7A1/CAT-1 was one of eight maximally expressed genes. Although they did not focus on CAT-1, they found that downregulation of CAT-1 with small-interfering RNA reduced the cancer cell survival rate and significantly inhibited expression of the growth factor epiregulin (EREG) (supplementary

Fig. 6 in [24]), directly supported our findings [10]. EREG was shown to play a key role in colitis-associated carcinogenesis in a recent animal study [25]. Interestingly, the macrophages and other immune cells mainly input Arg via the CAT-2 transporter [26]. These results indicate that the uptake channel of Arg is different between immune cells and CRC cells, suggesting that CAT-1 may be specific to colon cancer cells. An early bioinformatics study also indicated the tissue-specific expression of CAT-1 in only a few cells, such as CRC cells, red cell leukemia cells, stem cells, and endothelial cells [27]. All of the above evidence supports the concept that CAT-1 could be a unique target for the treatment of CRC. Based on the results, it is hypothesized that the high CAT-1 expression in CRC cells is the result of increased Arg intake by cancer cells, which leads to Arg accumulation in cancer tissues and consequent decreases in CRC patient serum Arg levels due to higher consumption by cancer cells. In addition, because CAT-1 is a membrane protein, it may be an ideal therapeutic target for a monoclonal antibody that can theoretically inhibit Arg uptake by cancer cells, directly kill tumor cells, or induce cell apoptosis through complement-dependent cytotoxicity.

In our study of Arg transporters, we also found that SLC6A14 was frequently expressed in CRC tissues at a higher level than that measured in normal colon tissue. Among the 122 cases, 96 (78.7 %) CRC tissues showed positive SLC6A14 expression. Some CRC tissue expressed SLC6A14 at a level of 661-times higher than that in normal colorectal tissue (unpublished data). Moreover, we observed that there may be a relationship between SLC6A14 expression in colorectal carcinoma and pathological grade (unpublished data). Further study is ongoing to confirm this phenomenon. Gupta et al. [28] previously reported that SLC6A14 was highly expressed in 10 cases of CRC tissue. Recently, the same group [29] further demonstrated that the SLC6A14 is specifically upregulated in estrogen receptor (ER)-positive breast cancer tissues and human breast cancer cell lines, which result in the concentrative transport of leucine (an mTOR activator), glutamine (an essential amino acid for nucleotide biosynthesis), and Arg (an essential amino acid for tumor cells) to meet the increased demand for these amino acids. Moreover, they found a selective blocker of SLC6A14; α-methyl-dl-tryptophan (α-MT) induces amino acid deprivation in ER-positive breast cancer cells in vitro, inhibits mTOR, and activates autophagy. These effects of α-MT are specific to SLC6A14 in ER-positive breast cancer cells; the ability of α-MT to cause amino acid deprivation was significantly attenuated in the ER-positive breast cancer line MCF-7 when SLC6A14 was silenced with short hairpin RNA (shRNA). In their mouse xenograft studies, they further demonstrated that α-MT is sufficient to reduce the growth of the ER-positive ZR-75-1 breast cancer cells. Combined with our results, the evidence suggests that SLC6A14 may be a novel and effective drug target for the treatment of ER-positive breast cancer and CRC.

The leucine transported by SLC6A14 is an mTOR activator (see the review cited in 34 for more details). mTOR plays a very important role in the development of cancer, particularly in the metabolic disorder-related cancers, and it is now receiving increased research attention [30]. mTOR kinase has two unique protein complexes, namely, mTORC1 and mTORC2. mTORC1 is activated by certain amino acids, stress, oxygen, energy, and growth factors. Although the mechanism of mTORC1's response to intracellular amino acids remains a mystery, it is known that amino acids, particularly leucine and Arg, can activate mTORC1 and appear to be necessary for other upstream signals to activate mTORC1. Since SLC6A14 overexpression will result in higher intracellular concentrations of leucine and Arg, SLC6A14 is a necessary upstream molecule for mTORC1 pathway activation. As Karunakaran et al. reported [29], mTOR inhibition, selective blockade of SLC6A14 amino acid uptake, or SLC6A14 RNA silencing could inhibit growth of the ER-positive breast cancer cell line MCF-7 in vitro and in vivo. Our results further indicate that SLC6A14 may be an effective drug molecule target for many tumors, and blocking SLC6A14 activity may be effective for both tumor prevention and treatment. Due to the simultaneous transport of Arg and leucine, targeting SLC6A14 may both block Arg uptake and can inhibit the mTOR pathway, which may have potential clinical significance.

As demonstrated above, Arg transporters may play important roles in cancer development and progression. However, the molecular mechanism of high Arg transporter expression in cancer cells and their regulation is still not clear. In endothelial cells, the surface expression of CAT-1 can be upregulated by lipopolysaccharide, transforming growth factor-β, and other cytokines [31–34]. These cytokines are known to be present in cancer microenvironments and are associated with cancer development and immune suppression. The upregulation of CAT-1 on cancer cells may be one of the mechanisms to promote cancer progress and immune suppression in the cancer microenvironment. The molecular mechanism of CAT-1 expression involves endocytosis and degradation and is associated with the activation of the intracellular signal transduction molecule protein kinase C (PKC) in human embryonic kidney 293 (HEK293) cells [35]. The higher expression of CAT-1 on cancer cells may share the same mechanism as PKC is an important downstream molecule in the noncanonical WNT pathway and EGFR-PI3K-AKT pathway in CRC cells, which are strongly involved in regulating cancer cell proliferation and the cell cycle. Recently, Samluk et al. [36] demonstrated that ATB (0, +) (i.e., SLC6A14) expression and leucine transport are also regulated by PKC. Activation of PKC can enhance the relocation of PKCα to the membrane to facilitate binding to ATB (0, +), thus enhancing leucine transport by ATB (0, +). It is well known that PKC is a downstream molecule of mTORC2 and is the main signal molecule regulating tumor cell proliferation [30]. Therefore, mTORC1, mTORC2, PKC, and ATB (0, +) may cooperate to regulate cancer cell proliferation [30, 36]. Here, we hypothesize that CAT-1 and SLC6A14 overexpression increases leucine and Arg transport and activate mTOR to promote or maintain tumor cell proliferation and metastasis. Activated mTOR enhances the expression and functions of CAT-1 and SLC6A14 through upregulated PKCα (Fig. 19.5). This vicious cycle may thus promote cancer progression. However, further study is necessary to clarify the mechanisms of CAT-1 and SLC6A14 expression in cancer cells and the potential significance of therapies that target this pathway. It is also warranted to clarify whether different Arg transporters can compensate for each other, and if there is an association between individual Arg expression and the pathological classification of different types of cancers. The molecular mechanism of SLC6A14 upregulation in cancer cells and its role in cancer development and progression also require further clarification. Nevertheless, targeting either SLC6A14 or CAT-1 may block cancer cell development and progression, thus effectively preventing CRC.

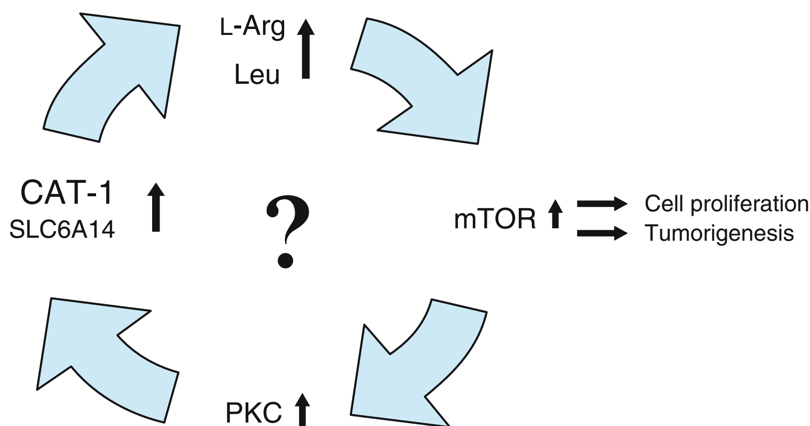

Fig. 19.5 Proposed loop of Arg transporter, CAT-1, and SLC6A14 in cancer development and progression. CAT-1 and SLC6A14 overexpression increases leucine and L-arginine transport. Both amino acids activate mTOR, which promotes or maintains tumor cell proliferation and metastasis. Activated mTOR enhances the expression and function of CAT-1 and SLC6A14 through upregulated PKCα. The vicious cycle of the regulatory loop accelerates cancer cell proliferation and metastasis

Although we did not find a strong relationship between CRC and other Arg transporters, some investigators [37, 38] reported that LAT1 and CD98hc (SLC3A2) may be useful biomarkers in lung cancer and renal cell carcinoma (RCC). Kaira et al. [38] demonstrated positive LAT1 expression in 163 of 321 patients (51 %), including 29 % adenocarcinoma (58 of 200), 91 % squamous cell carcinoma (91 of 100), and 67 % large cell carcinoma (14 of 21) patients. LAT1 expression was significantly associated with lymph node metastasis and disease stage. Importantly, the 5-year survival rate was significantly lower in the LAT1-positive patient population compared with LAT1-negative patients (51.8 vs. 87.8 %, $P<0.001$). Multivariate analysis confirmed that positive LAT1 expression was an independent factor for predicting poor prognosis. Therefore, LAT1 expression may be a promising biomarker to predict the prognosis of patients with resectable stage I–III non-small cell lung cancer (NSCLC). Prager et al. [37] demonstrated that CD98hc (SLC3A2) expression correlated with RCC pathological type. They found that the more malignant type II pRCC expressed significantly higher levels of CD98hc compared with the less malignant and more differentiated type I pRCC (type II 83.34 %, type I 4.76 % CD98hc positive, $P<0.00001$; $n=51$), indicating that in pRCCs, CD98hc may represent a novel and reliable marker for type II pRCC. Based on current findings, different types of cancer may utilize different types of Arg transporter to meet their own growth requirements. Additional studies are needed to confirm the specificity of each Arg transporter and develop novel targeted therapies for different cancers.

Targeting Arg Metabolism in Cancer Treatment

As mentioned above, overactive Arg catabolism may be a common phenomenon in tumor development and progression. Therefore, numerous attempts have been made to inhibit this pathway for cancer treatment. Figure 19.6 summarizes the current strategies of targeting the Arg metabolism pathway. Inhibition of ODC and induction of SSAT are all used for cancer prevention. However, it remains to be seen if these strategies can be applied as part of a chemotherapy regimen. In therapeutic research, Arg deprivation in tumor tissue and blood via treatment with L-arginine deiminase (ADI) to inhibit tumor growth is currently in clinical trials [39]. In vitro experiments have shown that the intracellular Arg synthesis enzyme ASS is defective in several types of cancer, such as RCC, hepatocellular carcinoma, pancreatic cancer, and prostate cancer. The growth of these cancer cells is dependent on external Arg supplementation [40–42]. However, Arg can also be synthesized by the intracellular enzymes ASS and ASL, so Arg deprivation may only be a viable treatment for cancers with defects in these enzymes, such as, liver, pancreatic, and prostate cancers. Even in liver cancer, which expresses low levels of ASS, Arg deprivation may need to be combined with ASL downregulation. Huang et al. [43] recently demonstrated that knockdown of ASL expression by shRNA in three liver cancer cell lines, ML-1, HuH-7, and HepG2, decreased colony formation in vitro, and lentiviral infection of ASL shRNA inhibited tumor growth in a therapeutic animal tumor model. They further revealed that the inhibitory effect was chiefly mediated by decreases of cyclin A2 and NO.

Moreover, in the human body, L-Arg is both an important raw material for protein synthesis and critical for appropriate physiological function, especially in immune cells [26, 44–46]. It is known that tumor-infiltrating immune cells cannot effectively uptake L-Arg, even though cancer-specific dendritic cells and T cells are present in the tumor tissues [45, 46]. In fact, our previous results [9] showed that L-Arg and L-Cit concentrations were significantly increased in colorectal carcinoma tissues compared to adjacent normal colorectal tissue. This suggests that L-Arg bioavailability is higher in the colorectal carcinoma tissue microenvironment and that the suppression of tumor-infiltrating immune cells may be related to their insufficient uptake of L-Arg, despite high local concentrations. Our results [10] further showed that CRC cells expressed higher levels of ASS and ASL. Thus, Arg deprivation could be a

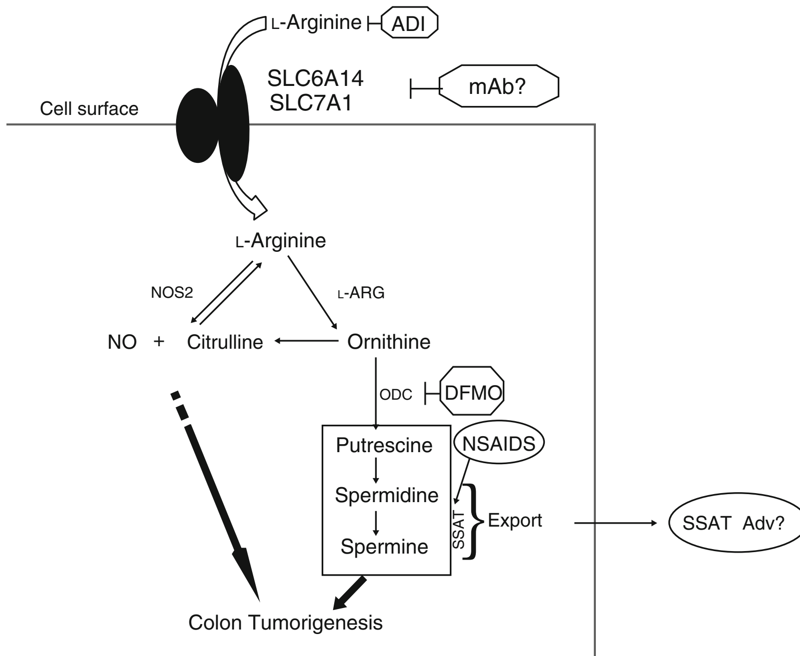

Fig. 19.6 Potential target of Arg catabolism pathway in the development of CRC prevention and therapy. As summarized in the figure, L-arginine deprivation by ADI and blocking ODC was clinically developed to reduce polyamine generation in tumor cells for chemoprevention and targeted therapy of cancers, such as CRC and liver cancer. Inducing or introducing SSAT may potentially decrease intracellular polyamine levels, resulting in cancer cell growth inhibition or arrest. Blocking Arg uptake by targeting Arg transporters may be another option for specifically inhibiting Arg catabolism to clinically treat cancer

double-edged sword in CRC treatment, in that it may decrease the effectiveness of tumor-infiltrating cells and have little or no effect on cancer cells. In contrast, Ma et al. [47] reported that L-Arg successfully improved cancer patient immunity and demonstrated the benefit of L-Arg supplementation in the treatment of CRC. As such, we hypothesize that blocking Arg uptake by specifically targeting Arg transporters may be a better option to prevent and treat cancers than systemic Arg deprivation. However, the regulatory mechanism of L-Arg transportation and the compensation of each Arg transporter in CRC cells should be studied further before targeting Arg transporters in the treatment of CRC. As shown in Fig. 19.6, it remains unclear which pathway would serve as the best target.

Other Potential Clinical Applications of the Arg Metabolism Pathway in Cancer

As discussed above, Arg is involved in a number of biosynthetic pathways that significantly influence carcinogenesis and tumor biology. Arg is transported by several cationic transport systems, including ATB$^{0,+}$ (SLC6A14), which is upregulated in several human cancers, such as cervical cancer, CRC, and ER-positive breast cancer. Cancer cells harbor unique metabolic characteristics relative to their healthy counterparts. Thus, Mirnezami et al. [48] characterized metabolic properties in CRC using HR-MAS NMR spectroscopy and identified Arg as one of the important metabolites in CRC tissues.

In their study of 44 consecutive patients with confirmed CRC, they acquired a total of 171 spectra (center of tumor, $n=88$; 5 cm from the tumor margin, $n=83$). They found that colon cancer samples ($n=49$) contained higher levels of acetate ($P<0.005$) and Arg ($p<0.005$) and lower levels of lactate ($p<0.005$) relative to rectal cancer samples ($n=39$). Collectively, HR-MAS NMR profiling demonstrated cancer-specific metabolic signatures in CRC and revealed metabolic differences between colonic and rectal cancers, which may be useful in future staging and therapeutic approaches. Another report [3] showed that metabolomic and gene expression analyses of urine and tissue samples from humans may offer robust noninvasive methods for CRC screening and diagnosis.

Based on higher expression of ATB(0,+) (SLC6A14) in cancer cells, Müller et al. [49, 50] demonstrated that ATB(0,+) activity can be imaged in vivo by positron emission tomography (PET) with [(18)F]FEMAET. They synthesized an imaging agent *O*-(2-[^{18}F]fluroethyl)-L-tyrosine ([^{18}F]FET), namely, *O*-2((2-[^{18}F]fluoroethyl)methylamino) ethyltyrosine ([^{18}F]FEMAET) as an ATB(0,+)-selective PET probe. They then demonstrated that [(18)F]FEMAET was accumulated in PC-3 and NCI-H69 cancer cells in vitro, and probe uptake was inhibited by LAT/ATB(0,+) inhibitors and dibasic amino acids. Moreover, [(18)F]FEMAET efflux was only moderately stimulated by extracellular amino acids. Further in vivo experiments confirmed that PET revealed accumulation of the tracer in ATB(0,+)-positive PC-3, and NCI-H69 xenografts and significant reduction of this accumulation were observed following ATB(0,+) inhibition. Conversely, uptake was negligible in ATB(0,+)-negative MDA-MB-231 xenografts. Due to these successful preclinical studies, the specificity and sensitivity of individual Arg transporter expression in different cancers needs to be further characterized to develop reliable novel diagnostic tools and targeting treatments.

Conclusion

CRC is one of the most common malignant diseases in the world, but less than 20 % metastatic CRCs respond to currently available targeted therapies. With considerable advancements in cancer metabolomic research, Arg catabolism pathway abnormalities have received increasing attention. The findings have consistently confirmed that Arg catabolism accelerates to initiate or maintain tumor cell growth in CRC, and possibly many other, or all cancers. Among the Arg metabolites, polyamines may be the most important oncometabolites. Thus, metabolomic profiling may potentially be applied in cancer screening and diagnostic tests. Targeting the polyamine pathway has been confirmed to be an effective way to prevent CRC in clinical trials. This strategy may potentially be applicable in other types of cancer. Because tumor growth is Arg-dependent, Arg deprivation may be a viable option to treat cancer, and clinical trials are ongoing to assess its utility in liver cancer. However, Arg deprivation is only one point to consider; it is necessary to consider host immunity and the presence of endogenous Arg synthesis pathways in cancer cells. Recent findings have demonstrated that different cancer cells may utilize different Arg transporters for uptaking Arg to meet the requirement of cancer cells. CAT-1 and SLC6A14 may be dominantly expressed in CRC cells, while SLC3A2 and LAT1 may be dominantly expressed in other cancers. A pioneering study has demonstrated that a specific probe for Arg transporters may be useful for cancer imaging diagnosis. Since blocking Arg transporters can inhibit Arg uptake, thus inhibiting tumor growth, Arg transporter blockade may be another potential anticancer strategy. Although research into Arg/polyamine metabolism and the involved transporters appears promising for understanding carcinogenesis and developing potential diagnostic tools and therapeutics in CRC and other cancers, more studies are necessary to fully elucidate the regulation of this metabolism loop in cancer cells.

References

1. Cunningham D, Atkin W, Lenz HJ, et al. Colorectal cancer. Lancet. 2010;375:1030–47.
2. Pritchard CC, Grady WM. Colorectal cancer molecular biology moves into clinical practice. Gut. 2011;60:116–29.
3. Manna SK, Tanaka N, Krausz KW, et al. Biomarkers of coordinate metabolic reprogramming in colorectal tumors in mice and humans. Gastroenterology. 2014;146:1313–24.
4. Jimenez CR, Knol JC, Meijer GA, Fijneman RJA. Proteomics of colorectal cancer: overview of discovery studies and identification of commonly identified cancer-associated proteins and candidate CRC serum markers. J Proteomics. 2010;73:1873–95.
5. Denkert C, Budczies J, Weichert W, et al. Metabolite profiling of human colon carcinoma-deregulation of TCA cycle and amino acid turnover. Mol Cancer. 2008;7:72.
6. Gerner EW, Meyskens FL. Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer. 2004;4:781–92.
7. Mao HM, Chen BG, Qian X, Liu Z. Simultaneous determination of twelve biogenic amines in serum by high performance liquid chromatography. Microchemistry. 2009;91:176–80.
8. Mao HM, Wei W, Xiong WJ, Lu Y, Chen BG, Liu Z. Simultaneous determination of L-citrulline and L-arginine in plasma by high performance liquid chromatography. Clin Biochem. 2010;43:1141–7.
9. Mao HM, Chen BG, Wei W, et al. Simultaneous analysis of citrulline and L-arginine in serum and tissue. Microchem J. 2011;97:291–5.
10. Lu Y, Wang W, Wang J, et al. Overexpression of L-arginine transporter CAT-1 is associated with accumulation of L-arginine and cell growth in human colorectal cancer tissue. PLoS One. 2013;8:e73866.
11. Blachier F, Davila AM, Benamouzig R, Tome D. Channeling of L-arginine in NO and polyamine pathways in colonocytes and consequences. Front Biosci. 2011;16:1331–43.
12. Fukumura D, Kashigawa S, Jain RK. The role of nitric oxide in tumor progression. Nat Rev Cancer. 2006;6:521–34.
13. Lim KH, Ancrile BB, Kashatus DF, Counter CM. Tumour maintenance is mediated by eNOS. Nature. 2008;452:646–9.
14. Battaglia V, Shields CD, Murray-Stewart T, Casero Jr RA. Polyamine catabolism in carcinogenesis: potential targets for chemotherapy and chemoprevention. Amino Acids. 2014;46:511–9.
15. Gerner EW, Meyskens Jr FL. Combination of chemoprevention for colon cancer targeting polyamine synthesis and inflammation. Clin Cancer Res. 2009;15:758–61.
16. Goodwin AC, Destefano Shields CE, Wu S, et al. Polyamine catabolism contributes to enterotoxigenic *Bacteroides fragilis*-induced colon tumorigenesis. Proc Natl Acad Sci U S A. 2011;108:15354–9.
17. Evageliou NF, Hogarty MD. Molecular pathways: disrupting polyamine homeostasis as a therapeutic strategy for neuroblastoma. Clin Cancer Res. 2009;15:5956–61.
18. Paz EA, LaFleur B, Gerner EW. Polyamines are oncometabolites that regulate the LIN28/let-7 pathway in colorectal cancer cells. Mol Carcinog. 2014;53 Suppl 1:E96–106.
19. Scuoppo C, Miething C, Lindqvist L, et al. A tumour suppressor network relying on the polyamine–hypusine axis. Nature. 2012;487:244–8.
20. Zell JA, Lin BS, Ziogas A, Anton-Culver H. Meat consumption, ornithine decarboxylase gene polymorphism, and outcomes after colorectal cancer diagnosis. J Carcinog. 2012;11:17.
21. Mandala S, Mandala A, Johansson HE, Orjalo AV, Park MH. Depletion of cellular polyamines, spermidine and spermine, causes a total arrest in translation and growth in mammalian cells. Proc Natl Acad Sci U S A. 2013;110:2169–74.
22. Closs EI, Biossel JP, Habermeier A, Rotmann A. Structure and function of cationic amino acid transporters (CATs). J Membr Biol. 2006;213:67–77.
23. Sloan JL, Mager S. Cloning and functional expression of a human Na(+) and Cl(−)-dependent neutral and cationic amino acid transporter B(0+). J Biol Chem. 1999;274:23740–5.
24. Camps J, Pitt JJ, Emons G, et al. Genetic amplification of the NOTCH modulator LNX2 upregulates the WNT/b--catenin pathway in colorectal cancer. Cancer Res. 2013;73(6):2003–13.
25. Neufert C, Becker C, Türeci Ö, et al. Tumor fibroblast-derived epiregulin promotes growth of colitis-associated neoplasms through ERK. J Clin Invest. 2013;123(4):1428–43.
26. Morris Jr SM. L-Arginine: master and commander in innate immune responses. Sci Signal. 2010;3:pe27.
27. Su AI, Wiltshire T, Batalov S, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci U S A. 2004;101:6062–7.
28. Gupta N, Miyauchi S, Martindale RG, et al. Upregulation of the amino acid transporter $ATB^{0,+}$ (SLC6A14) in colorectal cancer and metastasis in humans. Biochim Biophys Acta. 2005;1741:215–23.

29. Karunakaran S, Ramachandran S, Coothankandaswamy V, et al. SLC6A14 ($ATB^{0,+}$) protein, a highly concentrative and broad specific amino acid transporter, is a novel and effective drug target for treatment of estrogen receptor-positive breast cancer. J Biol Chem. 2011;286:31830–8.
30. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149:274–93.
31. Vásquez R, Farías M, Vega JL, et al. D-glucose stimulation of L-arginine transport and nitric oxide synthesis results from activation of mitogen-activated protein kinases p42/44 and Smad2 requiring functional type II TGF-β receptors in human umbilical vein endothelium. J Cell Physiol. 2007;212:626–32.
32. Abdelmagid SA, Rickard JA, McDonald WJ, et al. CAT-1-mediated L-arginine uptake and regulation of nitric oxide synthases for the survival of human breast cancer cell lines. J Cell Biochem. 2011;112:1084–92.
33. González M, Gallardo V, Rodríguez N, et al. Insulin-stimulated L-arginine transport requires SLC7A1 gene expression and is associated with human umbilical vein relaxation. J Cell Physiol. 2011;226:2916–24.
34. Guzmán-Gutiérrez E, Westermeier F, Salomón C, et al. Insulin-increased L-Arginine transport requires A_{2A} adenosine receptors activation in human umbilical vein endothelium. PLoS One. 2012;7:e41705.
35. Vina-Vilaseca A, Bender-Sigel J, Sorkina T, et al. Protein kinase C dependent ubiquitination and clathrin-mediated endocytosis of the cationic amino acid transporter CAT-1. J Biol Cell. 2011;286:8697–706.
36. Samluk L, Czeredys M, Skowronek K, Nałęcz KA. Protein kinase C regulates amino acid transporter ATB(0,+). Biochem Biophys Res Commun. 2012;422:64–9.
37. Prager GW, Poettler M, Schmidinger M, et al. CD98hc (SLC3A2), a novel marker in renal cell cancer. Eur J Clin Invest. 2009;39:304–10.
38. Kaira K, Oriuchi N, Imai H, et al. Prognostic significance of L-type amino acid transporter 1 expression in resectable stage I-III nonsmall cell lung cancer. Br J Cancer. 2008;98:742–8.
39. Phillips MM, Sheaff MT, Szlosarek PW. Targeting L-arginine-dependent cancers with L-arginine-degrading enzymes: opportunities and challenges. Cancer Res Treat. 2013;45:251–62.
40. Kim RH, Coates JM, Bowles TL, et al. L-Arginine deiminase as a novel therapy for prostate cancer induces autophage and caspase-independent apoptosis. Cancer Res. 2009;69:700–8.
41. Cheng PN, Lam TL, Lam WM, et al. Pegylated recombinant human arginase (rhArg-peg5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through L-arginine depletion. Cancer Res. 2007;67:309–17.
42. Delage B, Fennell DA, Nicholson L, et al. L-Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. Int J Cancer. 2010;126:2762–72.
43. Huang HL, Hsu HP, Shieh SC, et al. Attenuation of argininosuccinate lyase inhibits cancer growth via cyclin A2 and nitric oxide. Mol Cancer Ther. 2013;12:2505–16.
44. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
45. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood. 2007;109:1568–73.
46. Norian LA, Rodriguez PC, O'Mara LA, et al. Tumor-infiltrating regulatory dendritic cells inhibit CD8+ T cell function via L-arginine metabolism. Cancer Res. 2009;69:3086–94.
47. Ma Q, Wang Y, Gao X, et al. L-arginine reduces cell proliferation and ornithine decarboxylase activity in patients with colorectal adenoma and adenocarcinoma. Clin Cancer Res. 2007;13:7407–12.
48. Mirnezami R, Jiménez B, Li JV, et al. Rapid diagnosis and staging of colorectal cancer via high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy of intact tissue biopsies. Ann Surg. 2014;259:1138–49.
49. Müller A, Chiotellis A, Keller C, et al. Imaging tumour ATB0,+ transport activity by PET with the cationic amino acid O-2((2-$^{[18F]}$fluoroethyl)methyl-amino) ethyltyrosine. Mol Imaging Biol. 2014;16:412–20.
50. Chiotellis A, Müller A, Weyermann K, et al. Synthesis and preliminary biological evaluation of O-2((2-$^{[18F]}$fluoroethyl)methylamino)ethyltyrosine ($^{[18F]}$FEMAET) as a potential cationic amino acid PET tracer for tumor imaging. Amino Acids. 2014;46:1947–59.

Chapter 20
L-Arginine Uptake and Its Role in the Survival of Breast Cancer Cells

Catherine K.L. Too and Salma A. Abdelmagid

Key Points

- Breast cancer cells express several cationic amino acid transporter (CAT) isoforms.
- CAT-1 plays a significant role in the uptake of L-arginine in human MCF-7 and T47D breast cancer cells.
- Carboxypeptidase-D (CPD), bound to the plasma membrane, cleaves C-terminal L-arginine from extracellular substrates.
- CPD-released L-arginine is transported into the cell for the intracellular production of nitric oxide (NO).
- CPD plays a primary role in NO production in MCF-7 cells.
- CPD gene transcription is stimulated by prolactin and androgens in MCF-7 cells.
- The CPD gene promoter contains active elements that bind prolactin-activated transcription factors Stat5a/Stat5b and the ligand-bound androgen receptor.
- Hormonal upregulation of CPD increases NO production to increase viability and decrease apoptosis of MCF-7 cells.
- The CPD–arginine–NO pathway is a potential therapeutic target for modulation of NO levels in cancer cells.
- Inhibition of the CPD–arginine–NO pathway through prolactin/androgen ablation is an alternate strategy in endocrine therapy for breast cancers.

Keywords L-Arginine • CAT-1 • Nitric oxide • Carboxypeptidase-D • Hormones • Breast cancer • Cell viability • Apoptosis

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Abbreviations

CAT	Cationic amino acid transporter
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CP	Carboxypeptidase
CPA	Carboxypeptidase-A
CPB	Carboxypeptidase-B
CPD	Carboxypeptidase-D
CPE	Carboxypeptidase-E
CPM	Carboxypeptidase-M
DAF-2DA	4,5-Diaminofluorescein diacetate (cell permeable)
DAF-2	4,5-Diaminofluorescein (non-permeable)
DAF-T	Triazolofluorescein (non-permeable, fluorescent)
DMEM	Dulbecco's Modified Eagles Medium
Fa–Ala–Arg	Furylacryloyl–alanine–arginine
Fa–Ala–Lys	Furylacryloyl–alanine–lysine
HUVEC	Umbilical vein endothelial cells
MGTA	DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (CPD inhibitor)
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (to assay cell viability)
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RT-PCR	Reverse transcription followed by polymerase chain reaction
siRNA	Small interfering RNA

Introduction

L-Arginine is an essential amino acid that plays a critical role in the growth of breast cancer cells. L-Arginine may be derived intracellularly through biosynthesis or extracellularly from diverse sources such as the culture medium in vitro, the diet in vivo, and proteolysis of extracellular polypeptide substrates both in vitro and in vivo. L-Arginine, transported into the cell by cationic amino acid transporters (CATs), is the common substrate of two enzymes, arginase and nitric oxide synthase, for the production of ornithine and nitric oxide (NO), respectively (Fig. 20.1). Ornithine is the precursor of polyamines that are essential for cell proliferation. NO has many physiological and pathophysiological functions, including modulation of cancer cell growth. We have reported that breast cancer cells express several System y^+ CATs [1]. We have also reported that a plasma membrane-bound metalloproteinase, carboxypeptidase-D (CPD), cleaves C-terminal L-arginine residues from extracellular substrates for NO production in cancer cells [2, 3] (Fig. 20.1). This chapter presents our studies on the role of System y^+ carrier CAT-1 in the uptake of L-arginine in breast cancer cells. Our studies also show that CPD, through its extracellular cleavage of L-arginine, plays a significant role in the intracellular production of NO for the survival of cancer cells. Furthermore, this CPD–arginine–NO pathway is stimulated by hormones, 17β-estradiol, prolactin, and androgens in breast cancer cells, thereby implicating its potential usefulness as a therapeutic target, not only for the modulation of NO levels but also for endocrine therapy in breast cancer.

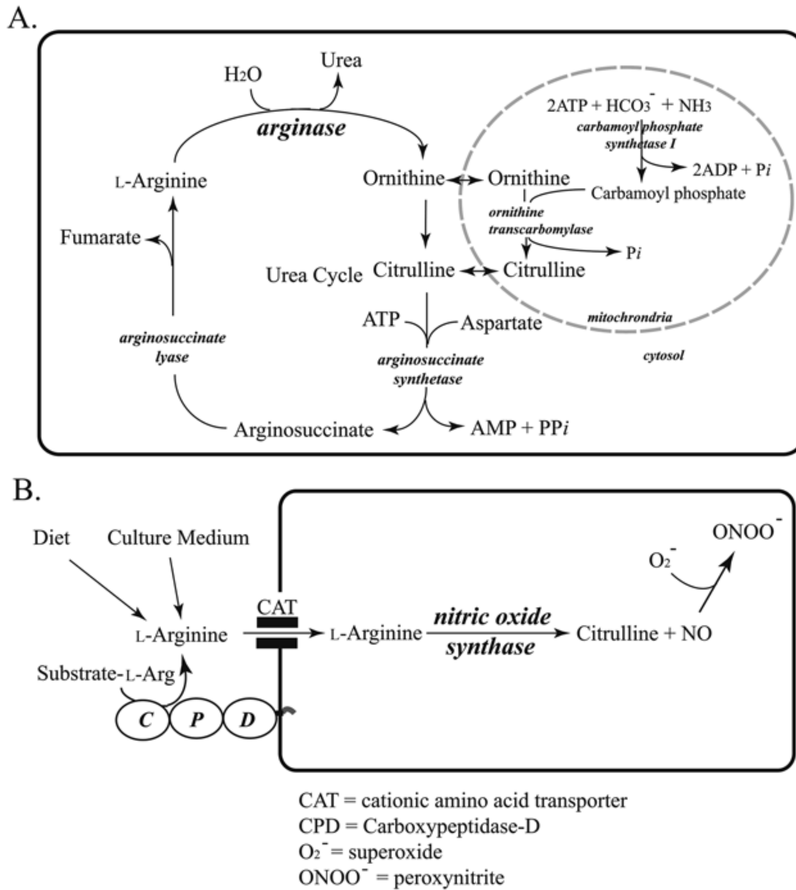


Fig. 20.1 Sources of L-arginine. (**a**) L-Arginine biosynthesis: Arginine is produced in the urea cycle and is the substrate of arginase for the production of ornithine, the precursor of polyamines. (**b**) Extracellular L-arginine can be derived from the diet in vivo and culture medium in vitro or released from extracellular substrates. Carboxypeptidase-D (CPD) cleaves C-terminal arginine both in vivo and in vitro. L-Arginine, transported into the cell by cationic amino acid transporters, is the substrate of nitric oxide synthase for the production of citrulline and nitric oxide (NO). NO reacts with superoxides to produce peroxynitrites which trigger or modulate cellular responses

L-Arginine Metabolism: Role of Arginase and Nitric Oxide Synthase

L-Arginine is a semi-essential amino acid that may be derived intracellularly from L-arginine biosynthesis, such as its production from argininosuccinate in the urea cycle, or extracellularly from dietary intake, whole body protein catabolism, and proteolysis of extracellular substrates (Fig. 20.1). L-Arginine plays a variety of roles in cellular metabolism and is the substrate of two enzymes, arginase and nitric oxide synthase. Arginase in the cytosol converts L-arginine to urea and ornithine, and this reaction occurs in the urea cycle (Fig. 20.1a). Ornithine, through the action of ornithine decarboxylase, is the precursor of polyamines which bind DNA and, by altering chromatin structure and gene expression, promotes cell proliferation [4]. An increase in polyamine levels has been reported in several human cancers including breast cancer which contains two- to threefold higher polyamine levels than adjacent normal mammary tissues [5], and this increase likely reflects increased tumor cell proliferation. Conversely, inhibition of arginase activity, such as in the human MDA-MB-468 breast cancer cell line which contains high arginase activity, can cause intracellular depletion of polyamines and cell apoptosis [6].

Nitric oxide synthase converts L-arginine to L-citrulline and NO (Fig. 20.1b). There are three isoforms of nitric oxide synthase, namely, neuronal nNOS (NOSI), inducible iNOS (NOSII), and endothelial eNOS (NOSIII), which are expressed in a variety of cells for the production of NO. NO is described as a pleiotropic regulator of many physiological processes such as vasodilation, neurotransmission, and immunomodulation, but NO also plays a complex role in human diseases such as cancers [7, 8]. NO reacts with superoxides to produce peroxynitrites which react with lipids, DNA, and proteins to trigger or modulate cellular processes such as cell signaling, cell necrosis or apoptosis, cell proliferation and survival, neoplastic transformation, and tumor development [8]. The pathophysiological actions of NO are dependent on a variety of determinants such as the expression, activity, and localization of the various nitric oxide synthase isoforms, the intracellular levels of NO, the duration of NO production, and the local microenvironment [7]. Therefore, depending on these determinants, endogenous NO may exert opposing effects on the metastatic progression of tumor cells and to either stimulate or suppress cell growth, including that of breast cancer cells [9–11].

Cationic Amino Acid Transporters

The cellular uptake of L-arginine and the other cationic amino acids, L-lysine and L-ornithine, is mediated by several different transmembrane carrier proteins that belong to the Na^+-independent systems y^+, y^+L, and $b^{0,+,}$ and the Na^+-dependent system $B^{0,+}$ [12, 13]. System y^+ is selective for cationic amino acids only, whereas systems y^+L, $b^{0,+}$, and $B^{0,+}$ also accept neutral amino acids. System y^+ is the principal cationic amino acid transport system expressed in NO-producing cells and is believed to play a key role in regulating an intracellular supply of L-arginine as a substrate for the nitric oxide synthases [12, 13].

System y^+ consists of five carrier proteins, CAT-1, CAT-2A, CAT-2B, CAT-3, and CAT-4, which are all classified as members of the solute carrier family 7 (SLC7) of transporters [12, 13]. CAT-1, CAT-3, and CAT-4 are encoded by genes named SLC7A1, SLC7A3, and SLC7A4, respectively. CAT-2A and CAT-2B are splice variants of SLC7A2. CAT-1, CAT-2A, CAT-2B, and CAT-3 proteins are glycosylated, suggesting localization in the plasma membrane. A cDNA, SLC7A4, encoding CAT-4 and with ~42 % sequence identity to the CAT family members, was first identified in a human placental cDNA library. The CAT-4 transcript was detected in the human placenta, the brain, and testicular tissues using Northern blot analysis [14]. However, expression of CAT-4 in the plasma membrane did not induce L-arginine transport activity in *Xenopus laevis* oocytes nor in several human tumor cell lines, suggesting that CAT-4 may not be a true transporter or that it requires additional factors to be functional [15].

System y^+ CAT-1-mediated transport of L-arginine, L-lysine, and L-ornithine is pH independent and is stimulated by substrates on the *trans* side of the cell membrane to produce an effect called *trans*-stimulation and by membrane hyperpolarization [12, 13]. CAT-1 activity is present in all cell types except the liver, and the transmembrane transport of L-arginine, mediated primarily by CAT-1, is a rate-limiting step for NO production in cytokine-stimulated cells. Formation of a caveolar complex comprising CAT-1 and eNOS further implicates an association between CAT-1-mediated uptake of L-arginine and the nitric oxide synthase-catalyzed production of NO in the cell [16]. CAT-2B is often induced under inflammatory conditions in a variety of cell types. CAT-2A is a low-affinity carrier for cationic amino acids and is relatively insensitive to *trans*-stimulation [13]. CAT-3 is brain specific in rat and mouse [17, 18], whereas human CAT-3 is expressed preferentially in peripheral tissues such as the mammary gland, uterus, testis, and thymus where it is most abundant, as well as in the brain [19].

System y^+L transports cationic amino acids in a Na^+-independent manner as well as neutral amino acids with high affinity in a Na^+-dependent manner [12, 13]. There is evidence that System y^+L is also involved in NO synthesis [20]. High activity of System y^+L can result in L-arginine depletion, suggesting that it may serve as an efflux pathway for cationic amino acids [21].

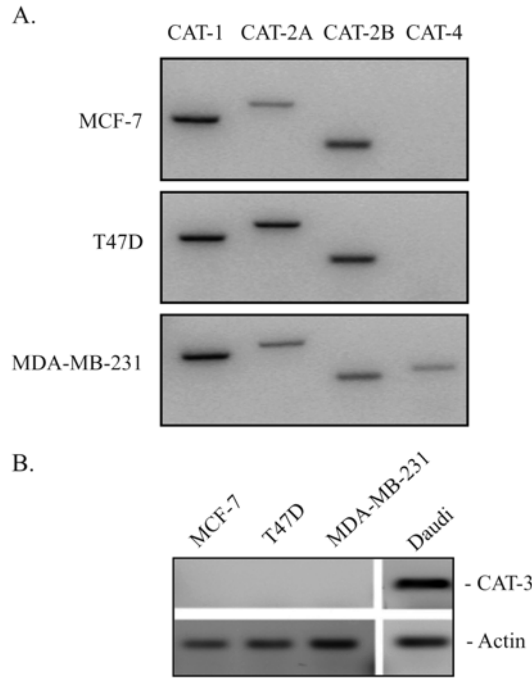


Fig. 20.2 CAT isoforms in breast cancer cells. Total RNA, isolated from actively growing human breast cancer cell lines MCF-7, T47D, and MDA-MB-231, was used for RT-PCR analysis. (**a**) Detection of CAT-1, CAT-2A, CAT-2B, and/or CAT-4 transcripts. (**b**) Detection of CAT-3. Daudi B-lymphoma cells were used as a positive control. Actin was used as a loading control (From Ref. [1]: Abdelmagid et al., 2011; with copyright permission from publisher John Wiley and Sons)

Expression of Cationic Amino Acid Transporters in Breast Cancer Cells

Using reverse transcription and polymerase chain reaction (RT-PCR) analysis, we have detected CAT-1, CAT-2A, and CAT-2B transcripts in the human MCF-7, T47D, and MDA-MB-231 breast cancer cell lines [2] (Fig. 20.2). The CAT-4 transcript is expressed in MDA-MB-231 cells only, whereas CAT-3 is undetectable in all three cell lines. However, others have reported CAT-3 mRNA expression in normal human mammary tissues, using multiple tissue expression arrays containing poly(A+) RNA of human tissues and cell lines [19].

The CAT-mediated uptake of L-arginine in a variety of cells, including breast cancer cells, is often coordinated with the expression and/or activities of the three nitric oxide synthase isoforms, and the NO produced may either stimulate or suppress cell growth [9–11]. We have reported that elevated production of NO promotes viability and inhibits apoptosis of human breast [2] and prostate cancer cells [3]. Since CAT-1 is believed to conform best to System y^+ and to be the main L-arginine transporter [12, 13], we sought to determine its role in L-arginine uptake in human breast cancer cells. Therefore, MCF-7 and T47D breast cancer cells were transfected with small interfering RNAs (siRNAs) to specifically knockdown CAT-1 gene expression or with nontargeting siRNA as controls. MCF-7 and T47D cells transfected with siCAT-1 showed approximately 50 % reduction in CAT-1 protein levels, as compared to the controls, and this was accompanied by a significant decrease in the uptake of L-[2,3,4,5-H^3]-arginine by ~35 % and ~40 % in MCF-7 and T47D cells, respectively [1] (Fig. 20.3). Treatment of siCAT-1-transfected cells with L-lysine, which inhibits total L-arginine transport activities, further decreased L-arginine uptake by

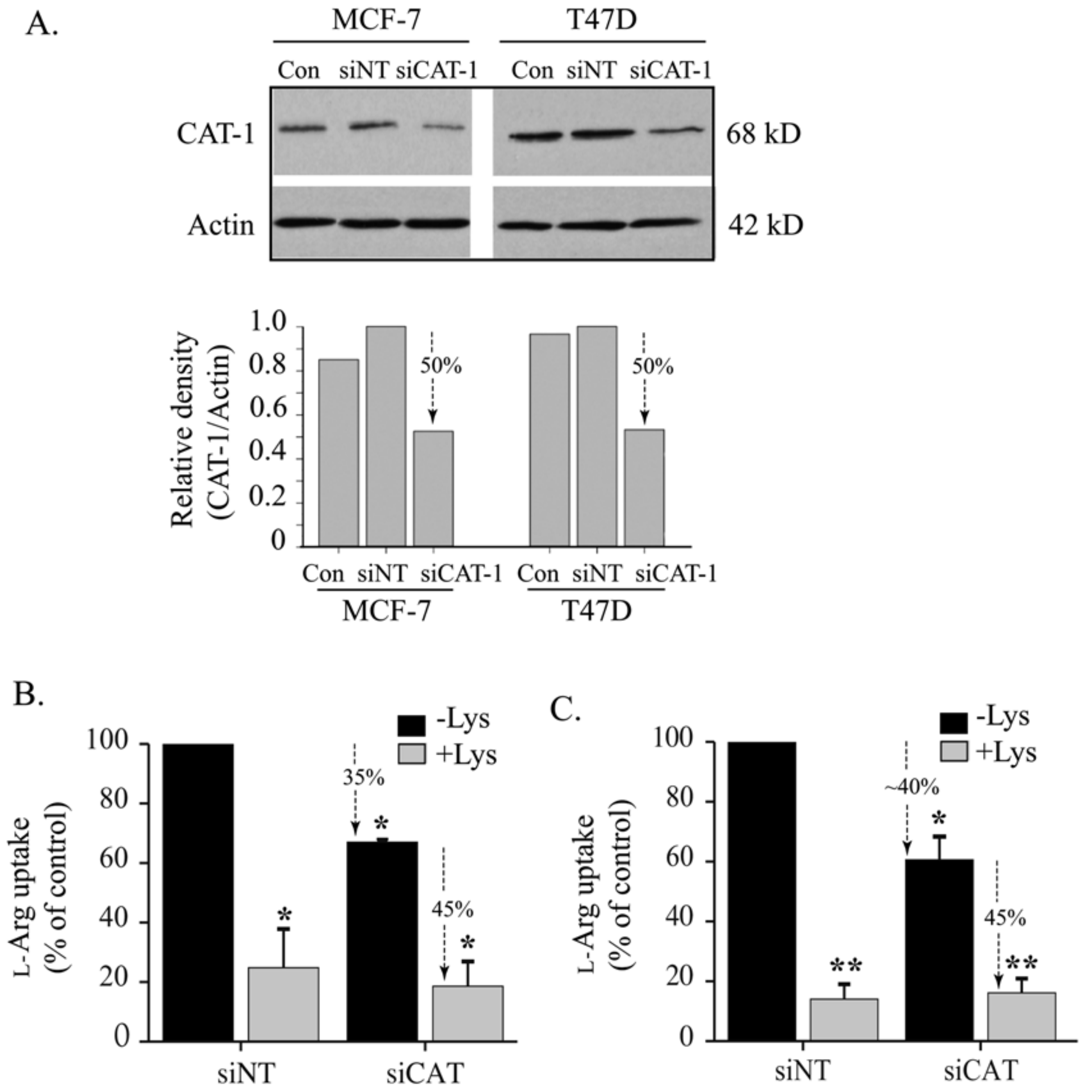


Fig. 20.3 siCAT-1 decreases L-arginine uptake. MCF-7 and T47D cells were transfected with siRNA-targeting CAT-1 (siCAT-1) or nontargeting siNT control. (**a**) Knockdown of CAT-1 gene expression at 48 h was confirmed using Western analysis (upper panel), and the CAT-1/actin ratio was determined by densitometry (lower panel). (**b**, **c**) Uptake of L-[2,3,4,5-H^3]-arginine was measured in (**b**) MCF-7 and (**c**) T47D cells, ±L-lysine, the latter to inhibit total L-arginine uptake. Cells transfected with siNT/Lys were set at 100 %. Mean ± SEM of three separate experiments, each in duplicate. $^{**}P<0.0001$; $^{*}P<0.05$ showed significant decrease compared to siNT/Lys cells (Part of original figure from Ref. [1]: Abdelmagid et al. 2011; with copyright permission from publisher John Wiley and Sons)

another 45 % compared to controls [1] (Fig. 20.3), which could be attributed to the remaining CAT-1 protein and/or the presence of additional L-arginine transport systems, such as y^+L, $b^{0,+}$, and/or $B^{0,+}$, in these cells. The decreased L-arginine uptake in siCAT-1-transfected MCF-7 was accompanied by a significant decrease in cell viability and a robust increase in apoptosis, measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and annexin-V fluorescent staining, respectively [1] (Fig. 20.4). Similarly, siCAT-1 decreased viability and increased apoptosis in T47D cells (Fig. 20.4). Therefore,

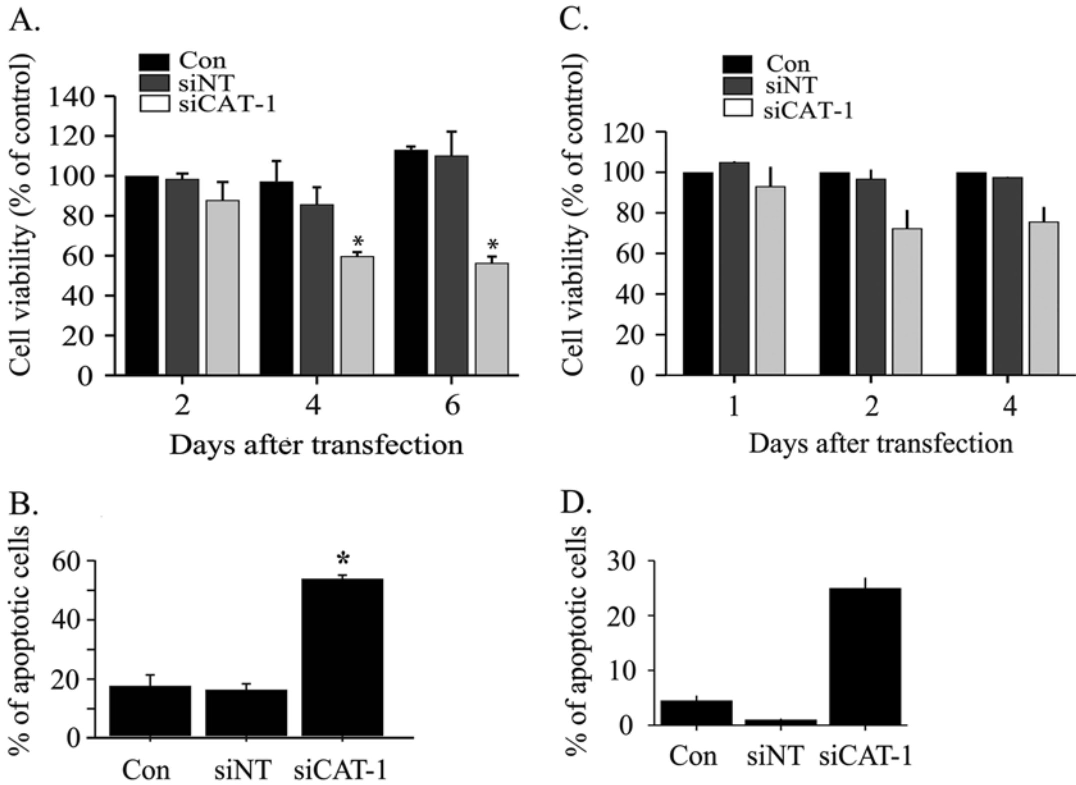


Fig. 20.4 siCAT-1 decreases cell viability and promotes apoptosis. MCF-7 (**a**, **b**) and T47D (**c**, **d**) cells were transfected with siCAT-1 or nontargeting siNT. (**a**, **c**) MTS assays, to measure cell viability, were performed on the indicated days after transfection. The viability of untransfected cells (Con) was set as 100 % (day 2 for MCF-7, day 1 for T47D). Compared to siNT-transfected cells, cells with siCAT-1 were less viable. (**a**) Mean ± SEM ($n = 3$); $^{*}P < 0.0001$ showed significant decrease compared to Con. (**c**) Mean ±range of two experiments, each in triplicate. (**b**, **d**) Annexin-V staining, to measure cell apoptosis, was performed after 4 days. The percent of apoptotic cells was plotted and showed that siCAT-1 increased cell apoptosis. (**b**) Mean ± SEM ($n = 3$); $^{*}P < 0.0001$ as compared to Con or siNT-transfected cells. (**d**) Mean ± range of two experiments, each in triplicate (Part of original figure from Ref. [1]: Abdelmagid et al. 2011; with copyright permission from publisher John Wiley and Sons)

CAT-1 plays a significant role in L-arginine uptake for the survival of MCF-7 and T47D breast cancer cells [1].

Glucose Modulates L-Arginine Uptake

Glucose is known to modulate the transport of L-arginine and the expression of the various CAT isoforms; hence, the glucose concentration used in the culture systems for the study of L-arginine uptake is important. In our studies, we routinely maintain the human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 in 10 % fetal bovine serum and high D-glucose (25 mM)-containing Dulbecco's Modified Eagles Medium [1]. Others have reported that 25 mM D-glucose induces maximal L-arginine transport in human umbilical vein endothelial cells (HUVEC), which may or may not be accompanied by altered expression of CAT-1 and CAT-2B [13]. Another study showed that high extracellular doses of D-glucose increase L-arginine transport, CAT-1 mRNA expression, and eNOS activity in HUVEC cells [22]. Conversely, glucose depletion was reported to induce a dramatic increase in CAT-1 levels and to stimulate L-arginine uptake in human C6 glioma cells [23].

Intracellular and Extracellular Sources of L-Arginine

L-Arginine from the Culture Medium

For NO synthesis, cells in culture may readily obtain L-arginine from the culture medium, whereas the intracellular production of L-arginine, such as through the recycling of citrulline in the urea cycle, may not be adequate. For example, inflammatory cytokine interferon-γ and bacterial endotoxin lipopolysaccharide both stimulate L-arginine uptake and intracellular NO production in the murine EMT-6 breast cancer cell line. This stimulatory action of interferon-γ/lipopolysaccharide is critically dependent on extracellular L-arginine, suggesting that intracellular L-arginine sources are inadequate in this cell line [24].

L-Arginine from the Diet

The diet is a natural source of L-arginine for cells in vivo, and there has been much debate on the beneficial effects of dietary supplements of L-arginine in health and disease. In breast cancer patients, oral supplements of L-arginine can activate the immune system to enhance host defenses [25] and potentiate responses to cell cycle-specific cytotoxic agents administered during chemotherapy [26]. On the other hand, L-arginine supplements have also been shown to stimulate tumor protein synthesis and tumor growth [27, 28].

L-Arginine from Extracellular Substrates: Action of Carboxypeptidase-D

The proteolytic digestion of extracellular substrates also provides L-arginine to cells cultured in vitro or to cells in vivo. One such example is the proteolytic cleavage of polypeptide substrates by membrane-bound CPD (Fig. 20.1b). CPD is a metalloproteinase that was first isolated from bovine pituitary [29], and it acts by cleaving C-terminal L-arginine and lysine residues from polypeptide substrates [30]. Whereas other members of the carboxypeptidase (CP) family, such as digestive CPA and CPB (30–40 kDa) and the peptide-processing CPE and CPM (50–60 kDa), have single carboxypeptidase domains, the exceptionally large CPD (180 kDa) has three highly conserved carboxypeptidase domains, a single transmembrane domain, and a short C-terminal cytoplasmic tail [30] (see Fig. 20.1b). Similar to other members of the metalloproteinase family, CPD has a tightly bound zinc atom as an essential cofactor. The CPD domains I and II are enzymatically active, whereas domain III plays a role in substrate binding and presentation [31, 32].

CPD is found in the *trans*-Golgi network where it processes polypeptides and prohormones that transit the secretory pathway [29] and it also trafficks to the plasma membrane [30, 33]. We have detected CPD in the nuclei of breast cancer cells using confocal immunofluorescent microscopy [34]. We and others have used a synthetic substrate, furylacryloyl–alanine–arginine (Fa–Ala–Arg), to demonstrate the activity of the plasma membrane CPD. Fa–Ala–Arg itself remains extracellular and is not taken into cells, but the CPD-released C-terminal L-arginine is transported into the cells for the production of NO [2, 3, 35, 36] (Fig. 20.1b).

There are many potential physiological substrates for cell surface CPD in cancer cells. For example, human lactoferrin (…Arg–Lys–C), erythropoietin (…Arg–C), and oxytocin (…Arg–C) are produced and secreted by both normal and malignant cells of the breast and prostate [37–41]. Epidermal growth factor, a paracrine/autocrine growth factor in prostate cancer cells [42] and also produced by some breast cancer cells [43], has a C-terminal Arg. Activation of circulatory proteolytic cascades (e.g., fibrinolytic, coagulation, or complement systems) also results in cleavage of Arg–*X* bonds to generate new protein chains with C-terminal Arg [44]; therefore, each step of activation produces a potential substrate for CPD.

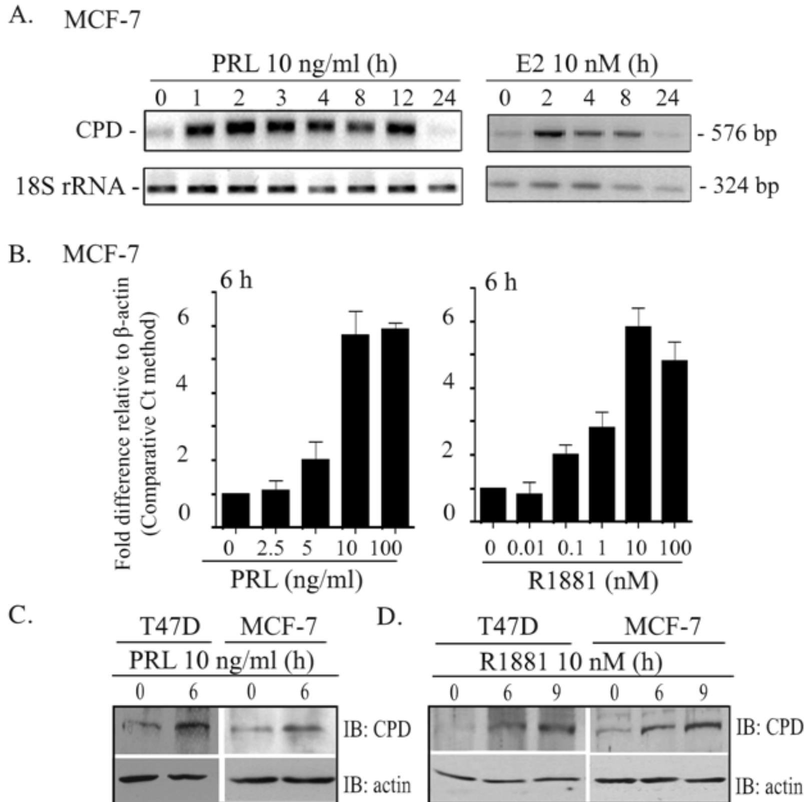
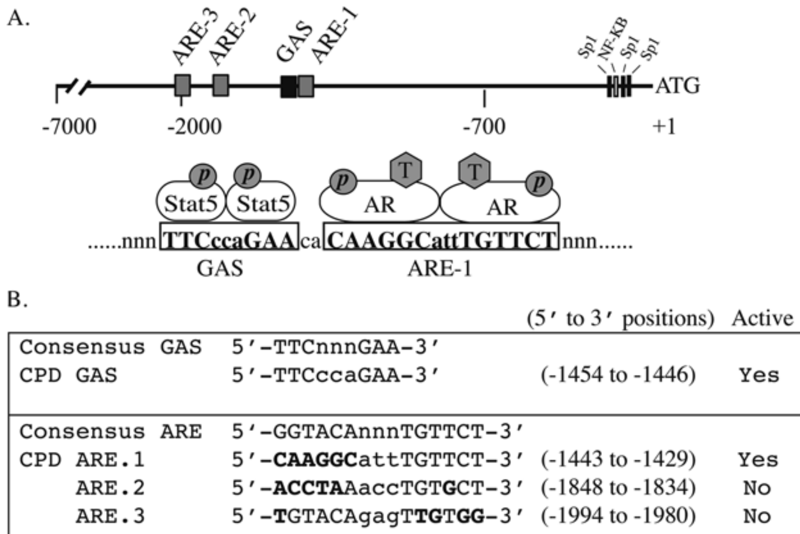


Fig. 20.5 Prolactin (PRL), 17β-estradiol (E2), and R1881 upregulate CPD gene expression. MCF-7 and T47D cells were made quiescent for 48 h in phenol red-free DMEM containing either 1 % lactogen-free horse serum (prior to PRL treatment) or 1 % charcoal-stripped FBS (prior to PRL or steroid treatment) before the addition of PRL, E2, or synthetic androgen R1881, with doses and times as indicated. (**a**, **b**) Total RNA was isolated for (**a**) semiquantitative RT-PCR or (**b**) qPCR analysis using the $2^{-\Delta\Delta Ct}$ method. (**c**, **d**) Cell lysates were prepared for SDS-polyacrylamide gel electrophoresis and Western blotting. (**a**–**d**) All experiments were performed at least three times. (**a**) Representative RT-PCR. (**b**) Mean ± SEM ($n = 3$). (**c**, **d**) Representative Western blot using 20–40 μg protein per lane, with β-actin as a loading control (Part of original figures from Ref. [2]: Abdelmagid and Too, 2008, and Ref. [46]: Koirala et al., 2014; with copyright permission from the Endocrine Society)

Hormonal Regulation of CPD

CPD is a hormone-responsive gene in several cancer cell types. We initially reported that the pituitary hormone prolactin upregulates CPD gene expression in human HepG2 hepatoma and MCF-7 breast cancer cells [45]. In addition to prolactin, 17β-estradiol and synthetic androgen R1881 also elevate CPD mRNA and/or protein levels in MCF-7 and T47D breast cancer cells, in a time- and dose-dependent manner [2, 46] (Fig. 20.5). Hormonal upregulation of CPD also occurs in prostate cancer cells. We have reported that CPD levels are elevated by prolactin and testosterone in several prostate cancer cell lines, as compared to benign prostate cells [3, 47].

The CPD gene promoter has been reported to contain several potential binding sites for transcription factors Sp1 and NF-κB [48]. Using luciferase reporter assays, we have shown that the CPD gene promoter is activated by prolactin and synthetic androgen R1881 in MCF-7 cells [46]. Using chromatin immunoprecipitation qPCR (ChIP-qPCR) assays, we have identified in the CPD gene promoter a consensus and active γ-interferon-activated sequence that binds to the prolactin-activated transcription factors Stat5a and Stat5b, as well as a non-consensus but active



		(5′ to 3′ positions)	Active
Consensus GAS	5′-TTCnnnGAA-3′		
CPD GAS	5′-TTCccaGAA-3′	(-1454 to -1446)	Yes
Consensus ARE	5′-GGTACAnnnTGTTCT-3′		
CPD ARE.1	5′-**CAAGGC**attTGTTCT-3′	(-1443 to -1429)	Yes
ARE.2	5′-**ACCTA**AaccTGT**G**CT-3′	(-1848 to -1834)	No
ARE.3	5′-**T**GTACAgagT**TGTGG**-3′	(-1994 to -1980)	No

Fig. 20.6 Diagrammatic presentation of the CPD gene promoter. (**a**) The CPD gene promoter contains a consensus γ-interferon-activated sequence (GAS) and several non-consensus androgen response elements (ARE.1, ARE.2, and ARE.3). ChIP-qPCR analysis showed that GAS and ARE.1 bind to prolactin-activated Stat5 and the ligand-bound androgen receptor (AR), respectively [46]. Transcription start site ATG is shown as +1; *T*, testosterone; *p*, phosphate. The CPD gene promoter also contains binding sites for transcription factors Sp1 and NF-κB [48]. (**b**) The 5′ and 3′ positions of the GAS and AREs. Non-consensus nucleotides are in *bold* (Part of original figure from Ref. [46]: Koirala et al., 2014; with copyright permission from the Endocrine Society)

androgen response element that binds to the ligand-bound androgen receptor [46] (Fig. 20.6). Prolactin is a recognized autocrine/paracrine growth factor in both breast and prostate cancer cells [49], suggesting that locally produced prolactin plays a role in the activation of CPD gene transcription in both these cancer cell types.

Although 17β-estradiol increases CPD gene expression [2], the CPD gene promoter does not contain a consensus estrogen response element. The activation of CPD gene expression by 17β-estradiol is under further investigation.

The CPD–Arginine–NO Pathway in Breast Cancer Cells

Growth of the human MCF-7 breast cancer cell line is highly dependent on L-arginine, and this has been attributed to the irreversible conversion of L-arginine to L-ornithine and urea by the enzyme arginase. The depletion of L-arginine from the culture medium could potentially inhibit protein biosynthesis and cell growth [50]. Another probable explanation is that L-arginine is required for the production of NO, and NO promotes breast cancer cell growth.

We have shown that MCF-7 cells, cultured in L-arginine-free medium and treated with prolactin to induce CPD gene expression, produce low or undetectable levels of NO. However, the addition of L-arginine (1 mM) to the culture medium restores NO production and increases MCF-7 cell viability, measured using the 4,5-diaminofluorescein diacetate (DAF-2DA) and MTS assays, respectively [2] (Fig. 20.7). In MCF-7 cells cultured in L-arginine-free and hormone-free medium, NO production is also restored upon addition of the synthetic CPD substrate Fa–Ala–Arg and further increased by treatment of these cells with prolactin or 17β-estradiol, which stimulates CPD gene expression [2] (Fig. 20.8a). In contrast, addition of Fa–Ala–Lys or Fa–Ala–Arg plus L-lysine

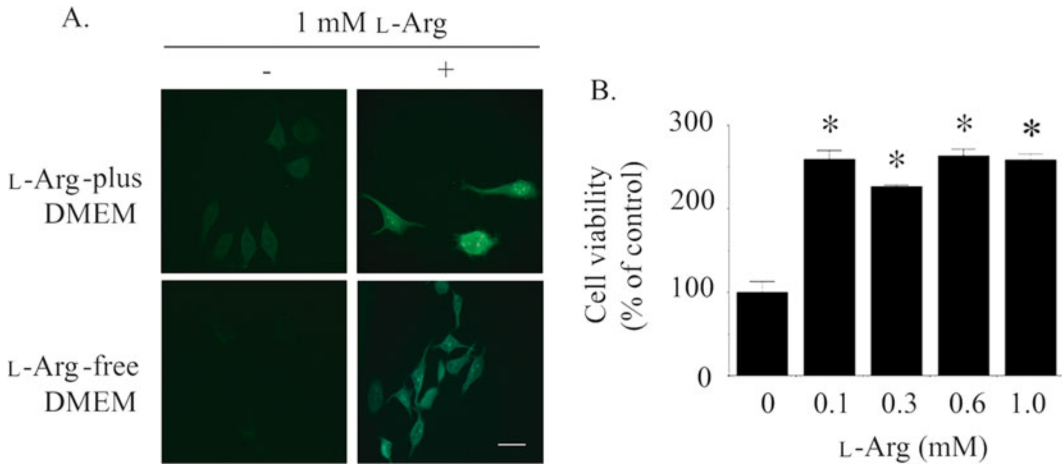


Fig. 20.7 L-Arginine promotes NO production and MCF-7 cell viability. (**a**) MCF-7 cells in Arg-containing DMEM (Arg plus, 0.398 mM L-Arg-HCL, Invitrogen) or Arg-free DMEM for 16–24 h were treated with prolactin (100 ng/ml, PRL) for 2 h. The cells were then washed in phosphate-buffered saline before loading with 5 μM DAF-2DA for 30 min at 37 °C. L-Arg (1 mM) was added for a 15-min incubation. In this DAF-2DA assay, nonfluorescent DAF-2DA (Sigma-Aldrich) was taken into the cell and hydrolyzed by intracellular esterases to form membrane-impermeable DAF-2. DAF-2 reacted with intracellular NO to produce the fluorescent triazole derivative DAF-2 T, which was detected by fluorescent microscopy. Arg-treated cells were noticeably more robust. Bar, 20 μm. (**b**) MCF-7 cells in Arg-free medium for 16–24 h were treated with increasing doses of L-Arg. After 3 days, MTS assay was performed to measure cell viability. Representative of at least three separate experiments, each in triplicate. Mean ± SEM, triplicates of one experiment. $^{*}P<0.0001$ versus control (0 mM Arg) (From Ref. [2]: Abdelmagid and Too, 2008; with copyright permission from the Endocrine Society)

(to inhibit L-arginine uptake) fails to restore NO production [2], showing that the CPD-released C-terminal L-arginine is essential for NO production. The CPD inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) inhibits NO production and decreases MCF-7 cell viability [2] (Fig. 20.8b, c), further implicating CPD in NO production for the growth of these cells. Furthermore, synthetic NO donor diethylamine/nitric oxide decreases apoptosis of MCF-7 cells, showing that NO itself promotes MCF-7 cell growth [2]. Together, our studies show that the hormonal stimulation of the CPD–arginine–NO pathway promotes growth and inhibits apoptosis in human breast and prostate cancer cells [2, 3].

Critical Role of CPD in NO Production in Breast Cancer Cells

Prolactin and 17β-estradiol also stimulate expression of the eNOS and iNOS isoforms in MCF-7 cells [1], suggesting that NO levels may increase through the hormonal upregulation of CPD and/or the nitric oxide synthases. However, NO production in prolactin-stimulated MCF-7 cells is abrogated by siRNA-targeting CPD [2] (Fig. 20.9a, b), implicating CPD, not eNOS nor iNOS, as having the primary role in NO production in these breast cancer cells (see Fig. 20.9c).

Another metalloproteinase, CPM, is also found in the plasma membrane [30], but is not involved in NO production in breast cancer cells. Our studies showed that CPM gene expression is not increased by prolactin or 17β-estradiol. Unlike CPD-targeting siRNA, CPM-targeting siRNA has no effect on NO production in MCF-7 cells [2].

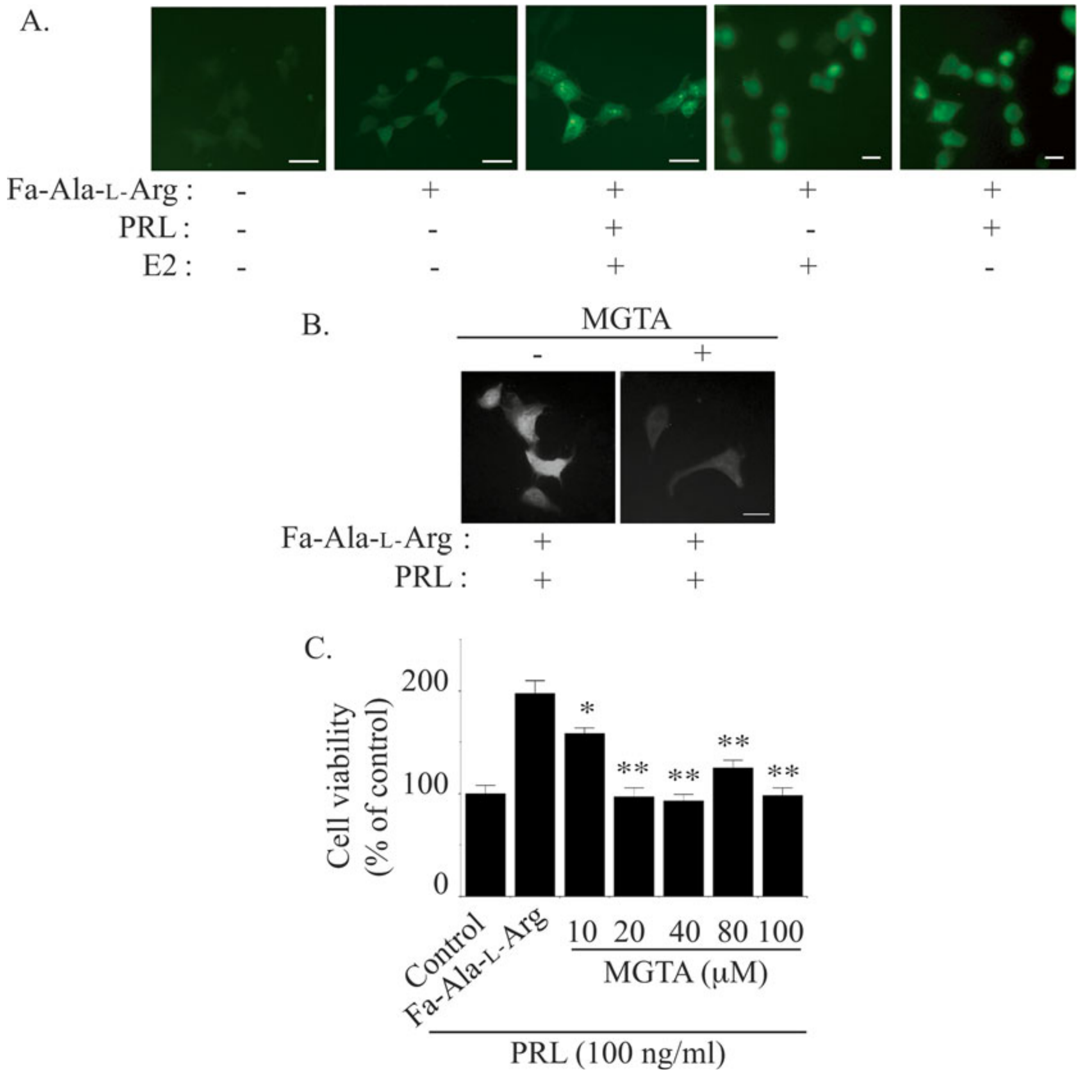


Fig. 20.8 Prolactin (PRL) and 17β-estradiol (E2) increase, but MGTA decreases NO and cell viability. (**a**) Quiescent MCF-7 cells, in Arg-free and hormone-free medium, were treated with PRL (100 ng/ml) and/or E2 (10 nM) for 4 h to upregulate CPD or had no hormone treatment. All the cells were preloaded with DAF-2DA, and all but controls were given Fa–Ala–Arg (4 mM) before fluorescent microscopy to determine NO levels. (**b**) MCF-7 cells, in Arg-free medium, were treated with 10 μM MGTA (+) or left untreated (−) for 16 h. This was followed by addition of PRL (2 h), then DAF-2DA and Fa–Ala–Arg, and fluorescent microscopy. Bar, 20 μm. (**c**) PRL-stimulated MCF-7 cells were left without further treatment (Control) or given Fa–Ala–Arg (1 mM, positive control) or Fa–Ala–Arg with increasing doses of MGTA. MTS assay was performed on day 2. Mean ± SEM ($n = 3$). $^{**}P < 0.0001$; $^{*}P < 0.05$ versus positive control (Fa–Ala–Arg). All experiments were repeated two to three times (From Ref. [2]: Abdelmagid and Too, 2008; with copyright permission from the Endocrine Society)

Prolactin and 17β-estradiol also have no effect on the expression of the System y^+ carrier CAT-1 nor on the uptake of L-[2,3,4,5-H^3]-arginine in MCF-7 cells [1]. Therefore, our studies suggest that CPD activity, not CAT-1 or the NOS isoforms, is critical for NO production in hormone-stimulated MCF-7 cells (Fig. 20.9c for model).

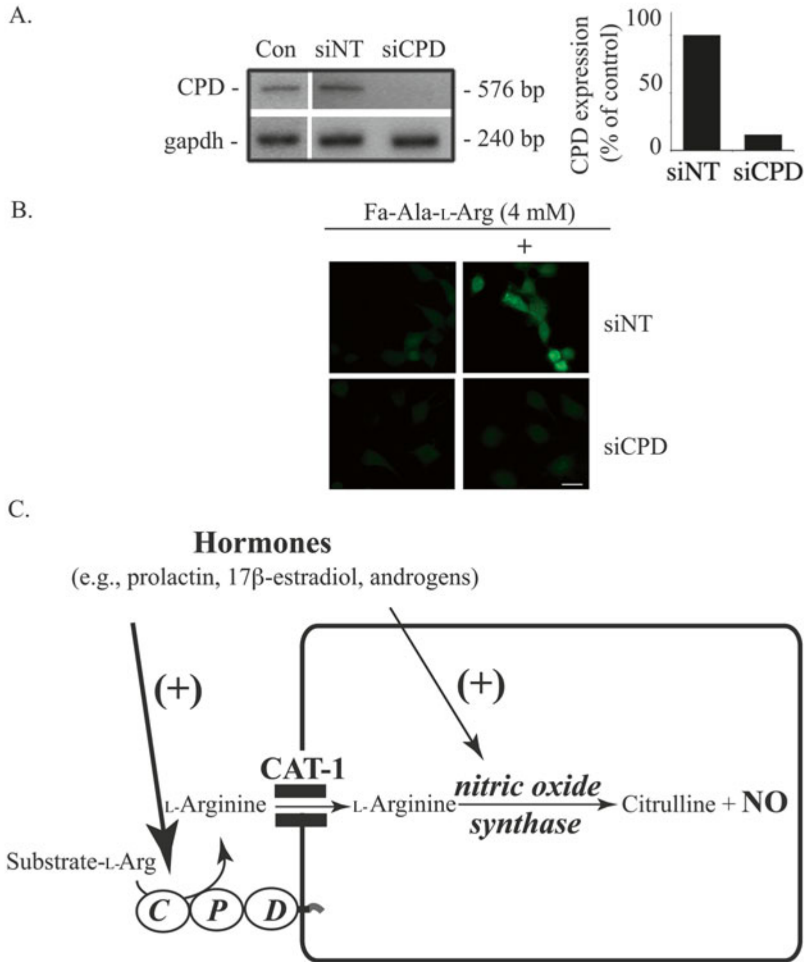


Fig. 20.9 siCPD decreases NO production. (**a**, **b**) MCF-7 cells were transfected with siRNA-targeting CPD (siCPD, 10 nM) or nontargeting siNT. Control (Con) cells were untransfected. After 24 h, cells were seeded in medium containing prolactin for another 24 h. (**a**) Total RNA was isolated for semiquantitative RT-PCR 48 h after transfection (*left panel*). The CPD/GAPDH ratio in transfected cells was analyzed by densitometry (*right panel*) which showed ~80 % decrease in CPD mRNA levels. (**b**) Intracellular NO production in transfected cells was measured using DAF-2DA, ±Fa–Ala–Arg. Bar, 20 µm. Each is a representative of two to three separate experiments (From Ref. [2]: Abdelmagid and Too, 2008; with copyright permission from the Endocrine Society). (**c**) *Summary*: Prolactin and 17β-estradiol increase CPD and iNOS/eNOS gene expression and increase NO production in breast cancer cells. Abrogation of NO production by siCPD in MCF-7 cells suggests that CPD plays the primary role (*thick arrow*) in NO production in these hormone-treated cells. Neither hormone has any effect on CAT-1 gene expression nor CAT-1 transporter activity

Conclusions

L-Arginine is necessary for the synthesis of polyamines and for the production of nitric oxide, each of which promotes growth and survival of breast cancer cells. Several CATs are expressed in breast cancer cells, and the system y^+ carrier CAT-1 plays a key role in the uptake of L-arginine in the MCF-7 breast cancer cell line. Breast cancer cells are responsive to hormones such as 17β-estradiol and prolactin, but neither has any effect on CAT-1 gene expression nor activity. Extracellular L-arginine can be derived from the culture medium in vitro as well as from the proteolytic activity of plasma

membrane-bound CPD in vitro and in vivo. CPD cleaves C-terminal L-arginine from extracellular polypeptide substrates, and a number of autocrine/paracrine growth factors found in breast cancer cells are potential CPD substrates. Prolactin, androgens, and 17β-estradiol upregulate CPD mRNA and protein expression in MCF-7 cells, whereas reporter assays show that only prolactin and androgens activate CPD gene transcription. Although prolactin also stimulates expression of the eNOS and iNOS isoforms in MCF-7 cells, CPD plays the primary role in NO production in these cells. The CPD-mediated production of NO promotes viability and inhibits apoptosis of MCF-7 cells, implicating a critical role for the CPD–arginine–NO pathway for the survival of breast cancer cells.

Therefore, the CPD–arginine–NO pathway is a potential therapeutic target for modulation of NO levels in cancer cells. Inhibition of this pathway to decrease NO production may be useful for the treatment of breast cancers. Furthermore, since CPD gene transcription is upregulated by prolactin and androgens, prolactin/androgen ablation is an alternate strategy in endocrine therapy for breast cancers.

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References

1. Abdelmagid SA, Rickard JA, McDonald WJ, Thomas LN, Too CKL. CAT-1-mediated L-arginine uptake and regulation of nitric oxide synthases for the survival of human breast cancer cell lines. J Cell Biochem. 2011;112:1084–92.
2. Abdelmagid SA, Too CKL. Prolactin and estrogen upregulate carboxypeptidase-D to promote nitric oxide production and survival of MCF-7 breast cancer cells. Endocrinology. 2008;149:4821–8.
3. Thomas LN, Morehouse TJ, Too CKL. Testosterone and prolactin increase carboxypeptidase-D and nitric oxide levels to promote survival of prostate cancer cells. Prostate. 2012;72:450–60.
4. Casero Jr RA, Marton LJ. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. Nat Rev Drug Discov. 2007;6:373–90.
5. Chandra R, Ganguly AK. Polyamines in relation to human breast, rectal and squamous cell carcinoma. Cancer Lett. 1988;39:311–8.
6. Singh R, Pervin S, Karimi A, Cederbaum S, Chaudhuri G. Arginase activity in human breast cancer cell lines: N(omega)-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. Cancer Res. 2000;60:3305–12.
7. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer. 2006;6:521–34.
8. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424.
9. Mortensen K, Holck S, Christensen IJ, Skouv J, Hougaard DM, Blom J, et al. Endothelial cell nitric oxide synthase in peritumoral microvessels is a favorable prognostic indicator in premenopausal breast cancer patients. Clin Cancer Res. 1999;5:1093–7.
10. Reveneau S, Arnould L, Jolimoy G, Hilpert S, Lejeune P, Saint-Giorgio V, et al. Nitric oxide synthase in human breast cancer is associated with tumor grade, proliferation rate, and expression of progesterone receptors. Lab Invest. 1999;79:1215–25.
11. Pance A. Nitric oxide and hormones in breast cancer: allies or enemies? Future Oncol. 2006;2:275–88.
12. Deves R, Boyd CA. Transporters for cationic amino acids in animal cells: discovery, structure, and function. Physiol Rev. 1998;78:487–545.
13. Mann GE, Yudilevich DL, Sobrevia L. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiol Rev. 2003;83:183–252.
14. Sperandeo MP, Borsani G, Incerti B, Zollo M, Rossi E, Zuffardi O, et al. The gene encoding a cationic amino acid transporter (SLC7A4) maps to the region deleted in the velocardiofacial syndrome. Genomics. 1998;49:230–6.
15. Wolf S, Janzen A, Vekony N, Martine U, Strand D, Closs EI. Expression of solute carrier 7A4 (SLC7A4) in the plasma membrane is not sufficient to mediate amino acid transport activity. Biochem J. 2002;364:767–75.
16. McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "L-arginine paradox". J Biol Chem. 1997;272:31213–6.
17. Hosokawa H, Sawamura T, Kobayashi S, Ninomiya H, Miwa S, Masaki T. Cloning and characterization of a brain-specific cationic amino acid transporter. J Biol Chem. 1997;272:8717–22.

18. Ito K, Groudine M. A new member of the cationic amino acid transporter family is preferentially expressed in adult mouse brain. J Biol Chem. 1997;272:26780–6.
19. Vekony N, Wolf S, Boissel JP, Gnauert K, Closs EI. Human cationic amino acid transporter hCAT-3 is preferentially expressed in peripheral tissues. Biochemistry. 2001;40:12387–94.
20. Arancibia-Garavilla Y, Toledo F, Casanello P, Sobrevia L. Nitric oxide synthesis requires activity of the cationic and neutral amino acid transport system y+L in human umbilical vein endothelium. Exp Physiol. 2003;88:699–710.
21. Mendes Ribeiro AC, Brunini TM, Yaqoob M, Aronson JK, Mann GE, Ellory JC. Identification of system y+L as the high-affinity transporter for L-arginine in human platelets: up-regulation of L-arginine influx in uraemia. Pflugers Arch. 1999;438:573–5.
22. Sobrevia L, Gonzalez M. A role for insulin on L-arginine transport in fetal endothelial dysfunction in hyperglycaemia. Curr Vasc Pharmacol. 2009;7:467–74.
23. Fernandez J, Bode B, Koromilas A, Diehl JA, Krukovets I, Snider MD, et al. Translation mediated by the internal ribosome entry site of the cat-1 mRNA is regulated by glucose availability in a PERK kinase-dependent manner. J Biol Chem. 2002;277:11780–7.
24. Cendan JC, Topping DL, Pruitt J, Snowdy S, Copeland 3rd EM, Lind DS. Inflammatory mediators stimulate L-arginine transport and L-arginine-derived nitric oxide production in a murine breast cancer cell line. J Surg Res. 1996;60:284–8.
25. Brittenden J, Park KG, Heys SD, Ross C, Ashby J, Ah-See A, et al. L-arginine stimulates host defenses in patients with breast cancer. Surgery. 1994;115:205–12.
26. Brittenden J, Heys SD, Miller I, Sarkar TK, Hutcheon AW, Needham G, et al. Dietary supplementation with L-arginine in patients with breast cancer (>4 cm) receiving multimodality treatment: report of a feasibility study. Br J Cancer. 1994;69:918–21.
27. Park KG, Heys SD, Blessing K, Kelly P, McNurlan MA, Eremin O, et al. Stimulation of human breast cancers by dietary L-arginine. Clin Sci (Lond). 1992;82:413–7.
28. Garlick PJ, McNurlan MA. Protein metabolism in the cancer patient. Biochimie. 1994;76:713–7.
29. Song L, Fricker LD. Purification and characterization of carboxypeptidase D, a novel carboxypeptidase E-like enzyme, from bovine pituitary. J Biol Chem. 1995;270:25007–13.
30. Skidgel RA, Erdos EG. Cellular carboxypeptidases. Immunol Rev. 1998;161:129–41.
31. Tan F, Rehli M, Krause SW, Skidgel RA. Sequence of human carboxypeptidase D reveals it to be a member of the regulatory carboxypeptidase family with three tandem active site domains. Biochem J. 1997;327:81–7.
32. Novikova EG, Eng FJ, Yan L, Qian Y, Fricker LD. Characterization of the enzymatic properties of the first and second domains of metallocarboxypeptidase D. J Biol Chem. 1999;274:28887–92.
33. Varlamov O, Fricker LD. Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. J Cell Sci. 1998;111:877–85.
34. O'Malley PG, Sangster SM, Abdelmagid SA, Bearne SL, Too CKL. Characterization of a novel, cytokine-inducible carboxypeptidase D isoform in haematopoietic tumour cells. Biochem J. 2005;390:665–73.
35. Hadkar V, Skidgel RA. Carboxypeptidase D is up-regulated in RAW 264.7 macrophages and stimulates nitric oxide synthesis by cells in L-arginine-free medium. Mol Pharmacol. 2001;59:1324–32.
36. Hadkar V, Sangsree S, Vogel SM, Brovkovych V, Skidgel RA. Carboxypeptidase-mediated enhancement of nitric oxide production in rat lungs and microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2004;287:L35–45.
37. Rossiello R, Carriero MV, Giordano GG. Distribution of ferritin, transferrin and lactoferrin in breast carcinoma tissue. J Clin Pathol. 1984;37:51–5.
38. Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA, Varia MA, et al. Functional significance of erythropoietin receptor expression in breast cancer. Lab Invest. 2002;82:911–8.
39. Cassoni P, Marrocco T, Sapino A, Allia E, Bussolati G. Oxytocin synthesis within the normal and neoplastic breast: first evidence of a local peptide source. Int J Oncol. 2006;28:1263–8.
40. Arcasoy MO, Amin K, Vollmer RT, Jiang X, Demark-Wahnefried W, Haroon ZA. Erythropoietin and erythropoietin receptor expression in human prostate cancer. Mod Pathol. 2005;18:421–30.
41. Whittington K, Assinder S, Gould M, Nicholson H. Oxytocin, oxytocin-associated neurophysin and the oxytocin receptor in the human prostate. Cell Tissue Res. 2004;318:375–82.
42. Tillotson JK, Rose DP. Endogenous secretion of epidermal growth factor peptides stimulates growth of DU145 prostate cancer cells. Cancer Lett. 1991;60:109–12.
43. Murphy LC, Murphy LJ, Dubik D, Bell GI, Shiu RP. Epidermal growth factor gene expression in human breast cancer cells: regulation of expression by progestins. Cancer Res. 1988;48:4555–60.
44. Barrett A, Rawlings N, Woessner J. Handbook of proteolytic enzymes. London: Academic; 1998.
45. Too CKL, Vickaryous N, Boudreau RT, Sangster SM. Identification and nuclear localization of a novel prolactin and cytokine-responsive carboxypeptidase D. Endocrinology. 2001;142:1357–67.
46. Koirala S, Thomas LN, Too CKL. Prolactin/Stat5 and androgen R1881 coactivate carboxypeptidase-D gene in breast cancer cells. Mol Endocrinol. 2014;28:331–43.

47. Thomas LN, Merrimen J, Bell DG, Rendon R, Goffin V, Too CKL. Carboxypeptidase-D is elevated in prostate cancer and its anti-apoptotic activity is abolished by combined androgen and prolactin receptor targeting. Prostate. 2014;74:732–42.
48. Timblin B, Rehli M, Skidgel RA. Structural characterization of the human carboxypeptidase D gene and its promoter. Int Immunopharmacol. 2002;2:1907–17.
49. Goffin V, Binart N, Touraine P, Kelly PA. Prolactin: the new biology of an old hormone. Annu Rev Physiol. 2002;64:47–67.
50. Caso G, McNurlan MA, McMillan ND, Eremin O, Garlick PJ. Tumour cell growth in culture: dependence on L-arginine. Clin Sci (Lond). 2004;107:371–9.

Part IV
L-*Arginine* Status and Use in Healthy Individuals

Chapter 21
L-Arginine Production During Pregnancy

Farook Jahoor, Jean W. Hsu, Pratibha Dwarkanath, Minerva M. Thame, and Anura V. Kurpad

Key Points

- L-Arginine becomes an essential amino acid during pregnancy because of an increased demand to sustain faster synthesis of maternal and fetal proteins, NO, numerous biochemicals and growth factors, and proline.
- To meet this increased demand, there is an upregulation in L-arginine flux and NO synthesis starting in early pregnancy in adult American and Jamaican women and in Jamaican adolescent girls.
- Pregnant Indian women, with low and normal BMI, have L-arginine fluxes that are less than 50 % the rate of their American and Jamaican counterparts during pregnancy.
- Pregnant Indian women also have high rates of low birth weight babies.
- An inadequate maternal supply of L-arginine is associated with delivery of a low birth weight baby.

Keywords L-Arginine • Nitric oxide • Pregnancy • Adult women • Adolescent girls • Body mass index

Abbreviations

BMI	Body mass index
FSR	Fractional synthesis rate
IUGR	Intrauterine growth retardation

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LBW Low birth weight
NO Nitric oxide
NOx Nitrite plus nitrate
wk Week

Introduction

Pregnancy is a period of net tissue deposition involving maternal tissues and the growth and development of the fetus. In an optimal pregnancy, maternal health is maintained and a healthy normal weight baby is born. This requires successful hormonal changes which induce metabolic/physiologic adaptations that enable the mother to provide adequate nutrients and growth factors necessary for her own anatomical changes and optimal growth of the fetus. Major changes in macronutrient metabolism ensure a continuous supply of nutrients to the fetus despite intermittent maternal food intake. These include increased maternal fat synthesis and deposition in early pregnancy and increased breakdown of maternal fat stores in midpregnancy to late pregnancy to supply fatty acids for maternal energy production and fetal synthesis of lean tissues, the brain, and fat deposition. There is increased maternal glucose production to provide the extra glucose needed by the fetus for energy production, de novo fatty acid, and glycogen syntheses, and there is increased maternal protein turnover but decreased protein oxidation to provide an adequate supply of amino acids to synthesize new proteins for maternal and fetal tissue formation and to support placental and fetal energy production and increased maternal glucose synthesis [1, 2].

As pregnancy progresses, the requirement for amino acids rises in order to sustain faster rates of protein synthesis and gluconeogenesis [3, 4], and the significant lowering of plasma concentrations of amino acids after a brief fast suggests that the balance between maternal amino acid supply and utilization is very tight, more so for the gluconeogenic amino acids [5, 6]. That is, the supply of dispensable amino acids from breakdown of body proteins plus de novo synthesis is not sufficient to meet the maternofetal requirements after a brief fast, as these amino acids represent the largest source of maternal amino acid nitrogen transferred to the fetus [7]. Apart from their unique individual biochemical functions, dispensable amino acids, such as L-arginine, are synthesized de novo because there is a high demand for them as precursors for the synthesis of proteins and peptides plus numerous biochemicals and metabolites necessary for maintenance of physiologic/metabolic homeostasis.

Possible Essentiality of L-Arginine in Human Pregnancy

L-Arginine is considered to be a dietary semi-essential amino acid because during periods of rapid tissue deposition, such as in childhood and wound healing, its rate of endogenous production is not sufficient to meet overall requirements. This is due to the fact that besides being one of the 20 amino acids needed to synthesize proteins and peptides in the body, it is a substrate for the synthesis of numerous biochemical compounds that are necessary for maintenance of homeostasis [8–10]. The most important among these are creatine, necessary for ATP production in cardiac and skeletal muscle cells; the polyamines, e.g., putrescine, spermidine, and spermine, which are important in the regulation of cell proliferation and differentiation; and nitric oxide (NO), an important mediator of vascular

tone and blood flow, which also stimulates angiogenesis, leukocyte adhesion, platelet aggregation, superoxide generation, and the expression of vascular cell adhesion molecules and monocyte chemotactic peptides [9]. It is also a precursor for the synthesis of proline, an amino acid present in large quantities in cartilage. As pregnancy is a period of increased maternal and fetal tissue deposition and increased NO synthesis has been implicated as a major contributor to the maternal vascular expansion of pregnancy and placental blood flow [11, 12], it is a distinct possibility that L-arginine becomes an essential amino acid in the pregnant woman.

L-Arginine Supply May Impact Pregnancy Outcome

An adequate L-arginine supply from endogenous sources and the diet will be critical for a successful pregnancy in terms of increased tissue deposition, maternal vascular expansion, and increased maternal-fetal blood flow, hence nutrient supply to the fetus. This will be especially true in trimester 3 when large quantities of proline-rich fetal cartilage are being synthesized. On the other hand, an inadequate maternal supply of L-arginine will impair both maternal and fetal protein syntheses, hence new tissue formation, leading to intrauterine growth retardation (IUGR) and delivery of a low birth weight (LBW) baby. It may also impair NO synthesis resulting in gestational hypertension [11].

Estimating L-Arginine Requirement During Pregnancy

The amount of L-arginine required during pregnancy can be estimated from the amount of protein deposited and the L-arginine content of maternal and fetal protein. It has been estimated that about 992 g of protein is deposited in a normal pregnancy with a median maternal weight gain of 12.5 kg and infant birth weight of 3.3 kg [13]. Of this, the fetus accounts for 42 % and maternal tissues 58 %. Based on a 7.2 % L-arginine content of fetal protein [14], it can be calculated that fetal protein deposition will require 30 g of L-arginine or an average of 107 mg/day, and based on a 6.6 % L-arginine content of adult human body protein [15], maternal protein deposition will require 38 g L-arginine or an average of 136 mg/day during pregnancy, that is, an average increase in maternal requirement for L-arginine of 243 mg/day for net protein deposition during pregnancy. Of course this is likely to be lower in the first half of pregnancy and much higher in the second half when the rate of tissue deposition is greater. For example, from 26 to 40 weeks of gestation, it can be calculated that total maternal/fetal protein deposition is 10 g/day [16] which translates to 660 mg/day of L-arginine.

This average L-arginine requirement, however, represents a minimum estimation of daily requirement during pregnancy. For example, in studies in the sheep [17], it has been shown that only ~40 % of fetal L-arginine uptake is used for protein accretion, indicating that the actual fetal requirement is 60 % greater. If the same holds true for the human fetus, then fetal requirement for protein deposition will be about 267.5 mg/day for a minimum average maternal requirement of 404 mg/day. This excess fetal L-arginine uptake in all likelihood is used to produce fetal energy, growth factors, and other amino acids necessary for protein synthesis and deposition, especially proline in trimester 3 when large quantities of proline-rich cartilage is synthesized. Indeed, fetal protein accretion of proline is markedly greater than uptake indicating significant de novo synthesis from L-arginine [17].

First In vivo Evidence of Increased L-Arginine Flux and NO Synthesis in Human Pregnancy

To determine how L-arginine flux and its rate of conversion to NO respond during different phases of normal human pregnancy compared to the nonpregnant state, a stable isotope tracer method was used to measure and compare L-arginine flux and NO synthesis at 18–20 and 36–39 weeks of gestation and at 8–10 weeks postpartum [18]. A constant infusion of guanidino-[$^{15}N_2$]-arginine was used to measure L-arginine flux and its rate of conversion to plasma nitrite/nitrate (NOx) as an index of the fractional rate of synthesis (FSR) of NO in five healthy pregnant American adult women aged 18–31 years. All of the participants had singleton pregnancies, and none had any history of medical diseases or pre-eclampsia. As shown in Fig. 21.1a, L-arginine flux was significantly higher ($P<0.01$) in mid-gestation than late gestation and postpartum (107.8 ± 5.7 vs. 72.5 ± 6.6 vs. 82 ± 3.4 μmol/kg/h, respectively). This increase in L-arginine flux at 18–20 weeks was associated with a significantly higher ($P<0.05$) plasma L-arginine concentration (Fig. 21.1b) and with a significantly faster ($P<0.02$) FSR of NO (Fig. 21.1c). The FSR of NO at 18–20 weeks was 44 % and 67 % greater than the rates at 36–39 weeks and postpartum, respectively (6.2 ± 0.4 vs. 4.3 ± 0.3 vs. 3.7 ± 0.85 % pool/h). There was no statistically significant difference between values obtained in late gestation compared with postpartum values. These findings show for the first time that L-arginine flux and NO synthesis increase in mid-gestation and start to fall as gestation approaches term, suggesting that increased maternal requirement for L-arginine and NO starts early, probably in trimester 1, and peaks around mid-gestation. In addition, the finding that peak L-arginine supply and NO synthesis parallel the vascular changes observed at

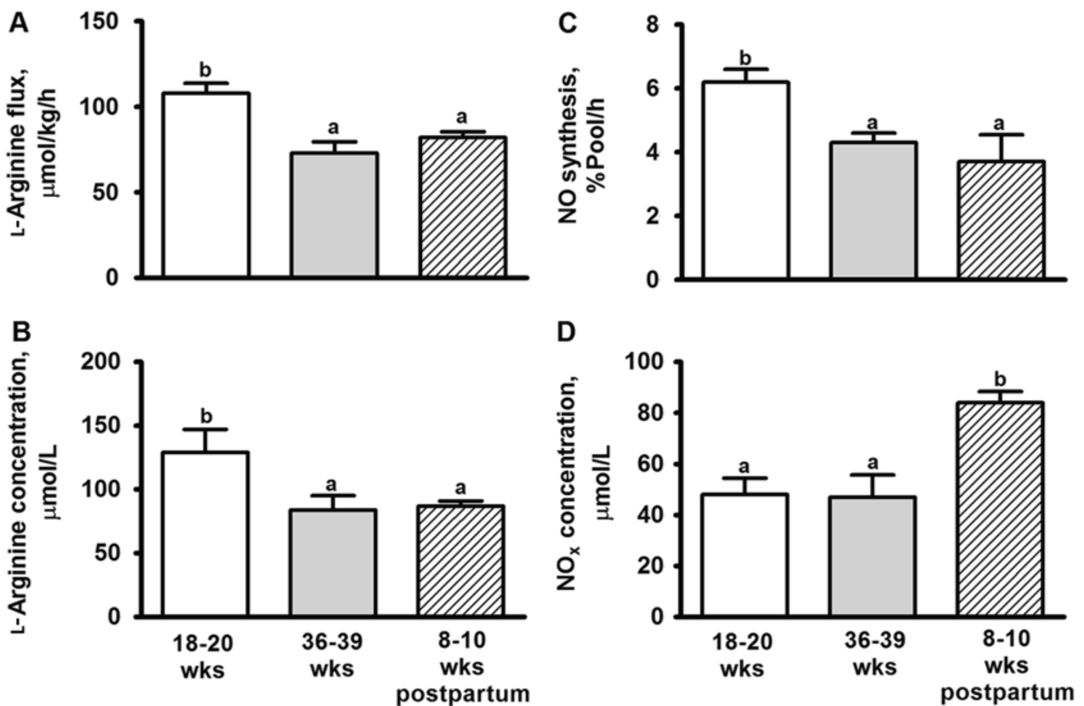


Fig. 21.1 L-Arginine flux (**A**), L-arginine concentration (**B**), NO synthesis (**C**), and plasma NOx concentration (**D**) in American women during pregnancy and postpartum. Values are mean ± SE; $n=5$ in each group. Values with different superscript letters are significantly different, $P<0.05$. Figure drawn from data first published in Goodrum et al. [18]

midpregnancy suggests that increasing L-arginine supply and its conversion to NO may be important in facilitating the vascular expansion of pregnancy [18].

Despite the higher rate of conversion of L-arginine to NO at 18–20 weeks and 36–39 weeks, the concentrations of plasma NOx were significantly lower than postpartum values (Fig. 21.1d). The lower plasma NOx concentrations at midpregnancy and late pregnancy compared with the postpartum value agree with the findings of Conrad et al. [19] who reported lower or unchanged plasma and urinary NOx during pregnancy in women on a reduced nitrate diet. Possible explanations for the lower plasma NOx include marked expansion of the maternal intravascular volume, thereby decreasing NOx concentration and/or increased urinary excretion of NOx.

L-Arginine Flux and NO Synthesis in Pregnant Adolescent Girls Versus Adult Women

The incidence of LBW babies and gestational hypertension is higher in the pregnant adolescent population [20, 21]. The finding that both L-arginine flux and NO synthesis rose steeply at midpregnancy in normal healthy adult women suggests that an inability to increase L-arginine flux and NO synthesis may explain the higher incidence of LBW, gestational hypertension, and preeclampsia observed in pregnant adolescent girls [21, 22] for the following reason. In fasting humans, the major source of L-arginine is whole-body protein breakdown as de novo L-arginine synthesis constitutes only ~15 % of flux [10]. Based on the report that whole-body protein breakdown increases to a greater extent in pregnant women whose BMI exceeds 25 kg/m^2 [23] and pregnant adolescent girls are known to have less lean body mass than adult women [24], it is a distinct possibility that pregnant adolescent girls will have slower endogenous L-arginine flux which may negatively affect their ability to increase the synthesis of maternal and fetal proteins and NO. To determine whether adolescent girls can increase L-arginine flux and NO synthesis to the same extent as their adult counterparts as pregnancy progresses to late gestation, L-arginine flux and NO synthesis were measured by intravenous infusions of guanidino-$^{15}N_2$-argininine and $^2H_2$-citrulline in eight pregnant adolescent Jamaican girls and eight pregnant adult Jamaican women [25]. All of the study participants were studied after fasting overnight for 8 h on two occasions, at the end of the first trimester (12.8 ± 0.39 weeks of gestation) and the beginning of the third trimester (27.8 ± 0.4 weeks of gestation).

As shown in Table 21.1, both the adult women and adolescent girls had BMIs within the normal range, 22.6 ± 0.4 and 20.8 ± 0.3 kg/m^2, respectively, indicating that they were well nourished at the trimester 1 study. Maternal weight and BMI at the trimester 1 study, however, were significantly lower ($P < 0.05$) in the adolescent girls compared with their adult counterparts, indicating that the adolescents had less lean body mass than the adults. On the other hand, weight gain from week 12 to 36 of gestation was significantly greater in the adolescents compared to the adults ($P < 0.01$). Among the 16 study participants, there was one fetal loss in the adolescent group (Table 21.1). Although there was no significant difference in gestational age between the groups, the adolescent girls had two premature deliveries while the adults had none. The mean birth weights of the groups were not significantly different, and each group had one LBW infant. Similarly, there were no significant differences in placental weight, newborn head circumference, and crown-heel length, though the latter trended shorter in the adolescent group.

There was no difference between the groups in L-arginine flux expressed both per unit of body weight and per whole body in trimester 1 (Fig. 21.2a, b). However, L-arginine flux decreased significantly ($P < 0.05$) from trimester 1 to 3 in the adolescents but not in the adult women (Fig. 21.2a, b). Similarly, plasma L-arginine concentration increased significantly in the adults ($P = 0.02$) but was

Table 21.1 Maternal characteristics at recruitment, pregnancy outcome, and newborn characteristics of Jamaican adult women and adolescent girls[a]

Variables[b]	Adult women	Adolescent girls[c]
Maternal characteristics		
Age (*y*)	26.1 ± 0.4	16.1 ± 0.4[d]
Height (cm)	165.0 ± 2.2	162.0 ± 1.9
Body mass index (kg/m^{2})	22.6 ± 0.4	20.8 ± 0.3[d]
Trimester 1 study weight[e] (kg)	61.9 ± 2.2	54.9 ± 1.3[d]
Trimester 3 study weight[e] (kg)	64.9 ± 1.7	61.1 ± 2.1
Weight at 36 weeks (kg)	71.2 ± 2.7	69.1 ± 1.8
Weight gain from 12 to 36 weeks (kg)	9.0 ± 1.9	15.8 ± 1.1[d]
Hemoglobin at trimester 1 study (g/dL)	12.3 ± 0.9	11.4 ± 1.7
Pregnancy outcome and newborn characteristics		
Fetal loss (*n*)	0	1
Gestational age at birth (weeks)	38.4 ± 0.4	37.6 ± 0.8
Premature delivery (<37 weeks) (*n*)	0	2
Birth weight (kg)	3.14 ± 0.2	2.87 ± 0.1
Low birth weight (<2.5 kg) (*n*)	1	1
Placenta weight (g)	631.3 ± 48.2	645.7 ± 1.7
Head circumference (cm)	34.2 ± 0.7	33.8 ± 0.5
Crown-heel length (cm)	49.2 ± 1.2	46.2 ± 1.2

[a]Data adapted from Thame et al. [25]
[b]Values are mean ± SE; $n = 8$ in each group
[c]For the adolescent group, all newborn values are for $n = 7$
[d]Different from adult women, $P < 0.001$; unpaired *t*-test
[e]Trimester 1 study: performed at 12.8 ± 0.39 weeks of gestation; trimester 3 study: performed at 27.8 ± 0.4 weeks of gestation

unchanged in the adolescents from trimester 1 to 3 (Fig. 21.2c). These findings suggest that after a brief period of food deprivation such as an overnight fast, the pregnant adolescent cannot maintain L-arginine flux like her adult counterpart in trimester 3. This inability to maintain L-arginine flux seems to be related to her younger age as there was a positive association ($r = 0.55$, $P = 0.02$) between trimester 3 L-arginine flux and age of the study participants, indicating that flux was slower in the adolescent group (Fig. 21.3a). The slower L-arginine flux did not, however, have a negative effect on the adolescent mother's ability to increase NO synthesis in trimester 3 or on her baby's birth weight.

Though weight-specific NO synthesis rate increased by 18 % in adults and 7 % in adolescents from trimester 1 to 3, these changes were not statistically different (Fig. 21.2d). However, when expressed per whole body, NO synthesis increased significantly in the adolescents ($P < 0.05$) (Fig. 21.2e). These changes in NO synthesis from trimester 1 to 3 were associated with significant increases ($P < 0.05$) in plasma nitrite concentrations in both groups (Fig. 21.2f). Although L-arginine flux correlated positively with NO synthesis at the start of trimester 3 ($r = 0.62$, $P = 0.01$) (Fig. 21.3b), there was no difference in NO synthesis between the groups at this time. That is, despite having a slower L-arginine flux at the start of trimester 3, the adolescents were still able to increase NO synthesis to the same extent as their adult counterparts. This finding implies that the relatively smaller amount of L-arginine being produced by the adolescents at the start of trimester 3 does not negatively affect their ability to increase NO synthesis.

It is interesting that at the time of the trimester 3 study, weekly maternal weight gain, which was greater in the adolescent group, correlated positively with both NO synthesis ($r = 0.51$, $P = 0.04$) (Fig. 21.4) and plasma NO_2 concentrations in trimester 3 ($r = 0.58$, $P = 0.01$) suggesting that L-arginine supply in the adolescents was still adequate to maintain both NO synthesis and maternal protein synthesis and, hence, lean tissue deposition.

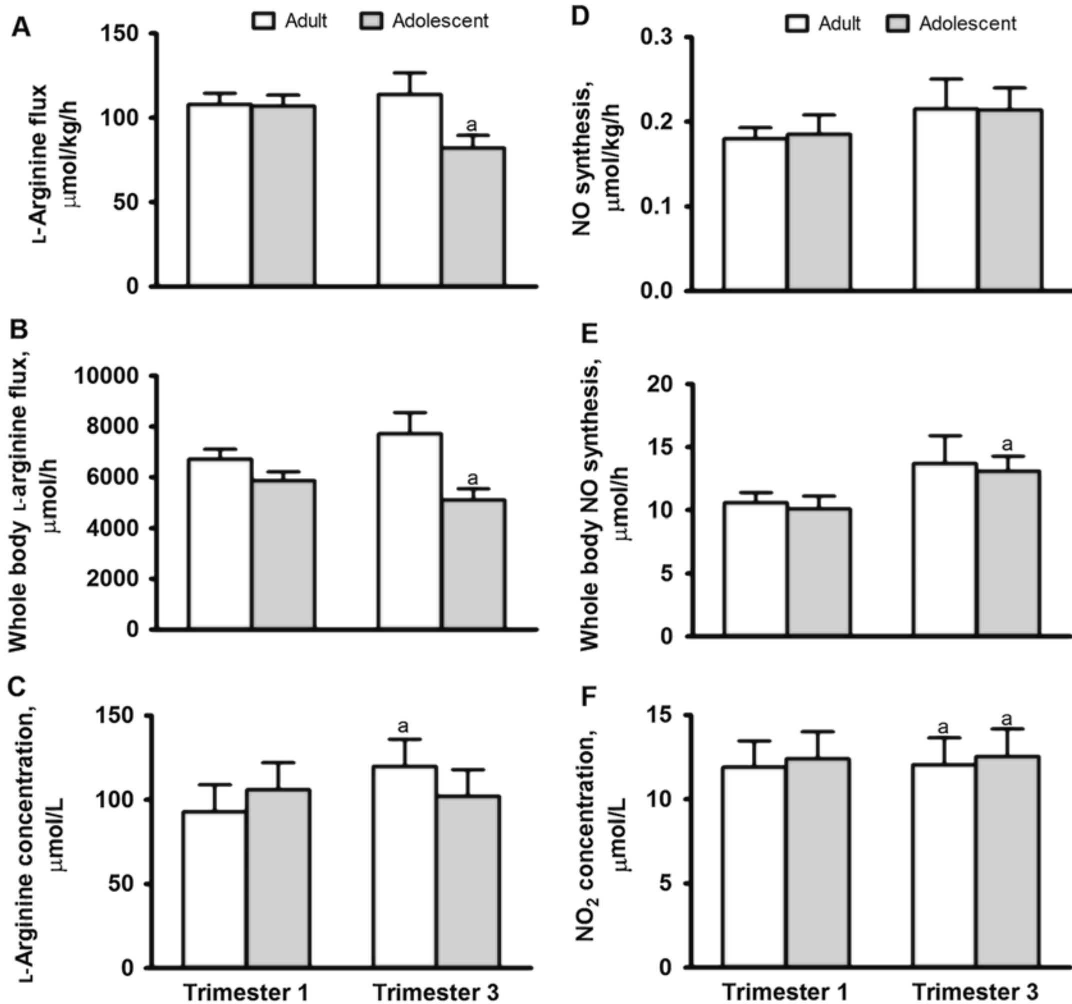


Fig. 21.2 L-Arginine flux (**A**), whole-body L-arginine flux (**B**), plasma L-arginine concentration (**C**), NO synthesis (**D**), whole-body NO synthesis (**E**), and plasma nitrite concentration (**F**) in pregnant Jamaican adult women and adolescent girls at trimester 1 (12.8 gestational weeks) and trimester 3 (28 gestational weeks). Values are mean ± SE; $n = 8$ in each group. [a]Different from trimester 1, $P < 0.05$. Figure drawn from data first published in Thame et al. [25]

L-Arginine Flux and NO Synthesis in Pregnant Women with Normal BMI Versus Low BMI

As in the adolescent mother, there are several reports that the incidence of LBW and small for gestational age babies is higher in adult women with low prepregnancy BMI [26], suggesting an inability to provide sufficient nutrients to sustain fetal protein synthesis and, hence, fetal tissue deposition. An inadequate maternal supply of L-arginine will impair both maternal and fetal protein syntheses, hence new tissue formation, leading to IUGR and delivery of an LBW baby. On the other hand, lower BMI correlates with lower systolic blood pressure [27] and a decreased risk for preeclampsia in pregnant women [28], suggesting that women with low BMI can synthesize NO at a greater rate than their normal BMI counterparts during pregnancy. First, to determine whether Indian women with low BMI can increase L-arginine supply to the same extent as their normal BMI counterparts during pregnancy

Fig. 21.3 Correlation between L-arginine flux at trimester 3 (28 gestational weeks) and subject's age in pregnant Jamaican adult women and adolescent girls (**A**); correlation between NO synthesis and L-arginine flux at trimester 3 (28 gestational weeks) in pregnant Jamaican adult women and adolescent girls (**B**). (**A**) Pearson $r=0.55$; $P=0.026$; $n=16$. (**B**) Pearson $r=0.62$; $P=0.01$; $n=16$. Figure drawn from data first published in Thame et al. [25]

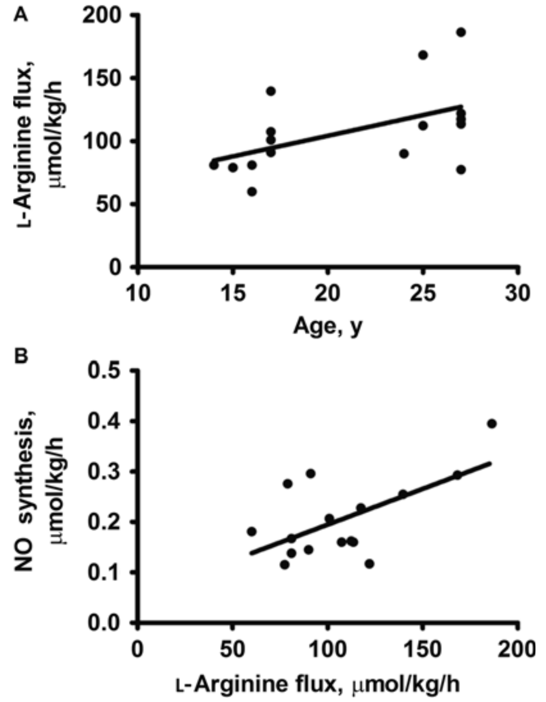
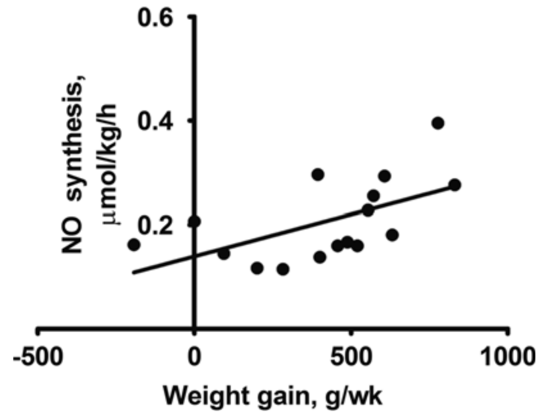


Fig. 21.4 Correlation between NO synthesis at trimester 3 (28 gestational weeks) and weekly weight gain at that time in pregnant Jamaican adult women and adolescent girls. Pearson $r=0.51$; $P=0.04$; $n=16$. Unpublished data



and second, whether they can synthesize NO at a faster rate than their normal BMI counterparts, L-arginine flux and NO synthesis were measured in two groups ($n = 10$ each) of pregnant adult Indian women with normal BMI (>18.5 < 25) and low BMI (≤18.5) after an 8 h fast. Measurements were made with constant infusions of guanidino-$^{15}N_2$-arginine and ^{13}C, $^{2}H_4$-citrulline on two occasions, at the end of the first trimester (12 ± 1 weeks of gestation) and toward the end of the second trimester (24 ± 1 weeks of gestation) [29].

As shown in Table 21.2, the low BMI group had significantly lower weight, BMI, fat free mass, and fat mass at the time of recruitment into the study. Between the groups, the increase in body weight (normal BMI = 3.26 ± 0.62 kg; low BMI = 3.83 ± 1.34 kg) and BMI (normal = 1.53 ± 0.28 kg/m^2, low = 1.82 ± 0.39 kg/m^2) from trimester 1 to trimester 2 were not different. There were no differences in age, height, parity, and hemoglobin concentration between groups. Diastolic blood pressure was significantly lower in the low BMI group at both 12 and 24 weeks of gestation (Table 21.3).

Table 21.2 Maternal characteristics at recruitment and pregnancy outcomes of pregnant Indian women[a]

Variables[b]	Normal BMI (>18.5 < 25)	Low BMI (≤18.5)
Maternal characteristics		
Age (y)	24.5 ± 1.2	21.4 ± 1
Weight (kg)	49.1 ± 1.8	42.2 ± 1.4[c]
Height (cm)	151 ± 3.2	153 ± 1.34
BMI (kg/m^2)	21.46 ± 0.4	17.9 ± 0.4[c]
Fat-free mass (kg) (% of body weight)	34.7 ± 1.4 (71 %)	32.9 ± 0.9 (77.8 %)[c]
Fat mass (kg) (% of body weight)	14.4 ± 0.86 (29 %)	9.2 ± 0.61 (21.8 %)[c]
Hemoglobin (g/dL)	12.3 ± 1.1	11.9 ± 1.8
Pregnancy outcomes		
Gestational Wt gain (g/day)	54.7 ± 9.3	69 ± 5.3
Birth weight (kg)	2.35 ± 0.49	2.65 ± 0.33
Gestational age at birth (weeks)	37 ± 3.1	38.1 ± 1.5
Premature delivery (<37 weeks) (*n*)	1	1
Low birth weight (<2.5 kg) (*n*)	6	3[d]

[a]Data adapted from Kurpad et al. [29]
[b]Values are mean ± SE; $n = 10$ in each group
[c]Different from normal BMI, $P < 0.001$; unpaired *t*-test
[d]Different from normal BMI, $P < 0.05$; Fisher's exact test

Table 21.3 Blood pressures of pregnant Indian women with normal and low BMI[a, b]

	Normal BMI		Low BMI	
	Gestation week			
	12	24	12	24
Systolic (mm)	105.5 ± 2.7	104 ± 3.1	100 ± 2.9	101.9 ± 2.3
Diastolic (mm)	68 ± 2.0	65.8 ± 2.4	60 ± 1.6[c]	60.4 ± 1.6[c]

[a]Data adapted from Kurpad et al. [29]
[b]Values are mean ± SE; $n = 10$ in each group
[c]Different from the normal BMI group, $P < 0.05$

There were no significant differences in gestational age at birth, and one mother in each group had a premature delivery (35th and 36th week in the normal and low BMI group, respectively), followed by death of the baby (Table 21.2). Similarly, there were no significant differences in birth weight, although the low BMI mothers gave birth to babies that were on average ~300 g heavier. The low BMI mothers also gave birth to only 3/10 LBW babies compared to 6/10 LBW babies in the normal BMI group ($P<0.01$).

L-Arginine flux when expressed per unit of body weight was significantly faster ($P<0.01$) in the low BMI group compared with the normal BMI group in trimester 1, and there was a significant reduction in flux from trimester 1 to 2 in the low BMI group ($P<0.01$) (Fig. 21.5a). When expressed per whole body, this reduction in L-arginine flux in the low BMI group from trimester 1 to 2 did not achieve statistical significance. There was no difference in plasma L-arginine concentrations between the groups at trimesters 1 and 2 and within each group from trimesters 1 to 2 (Fig. 21.5c).

NO synthesis expressed per unit of body weight was faster in the low BMI group compared with the normal BMI group in both trimesters; however, the difference was only significant in trimester 2

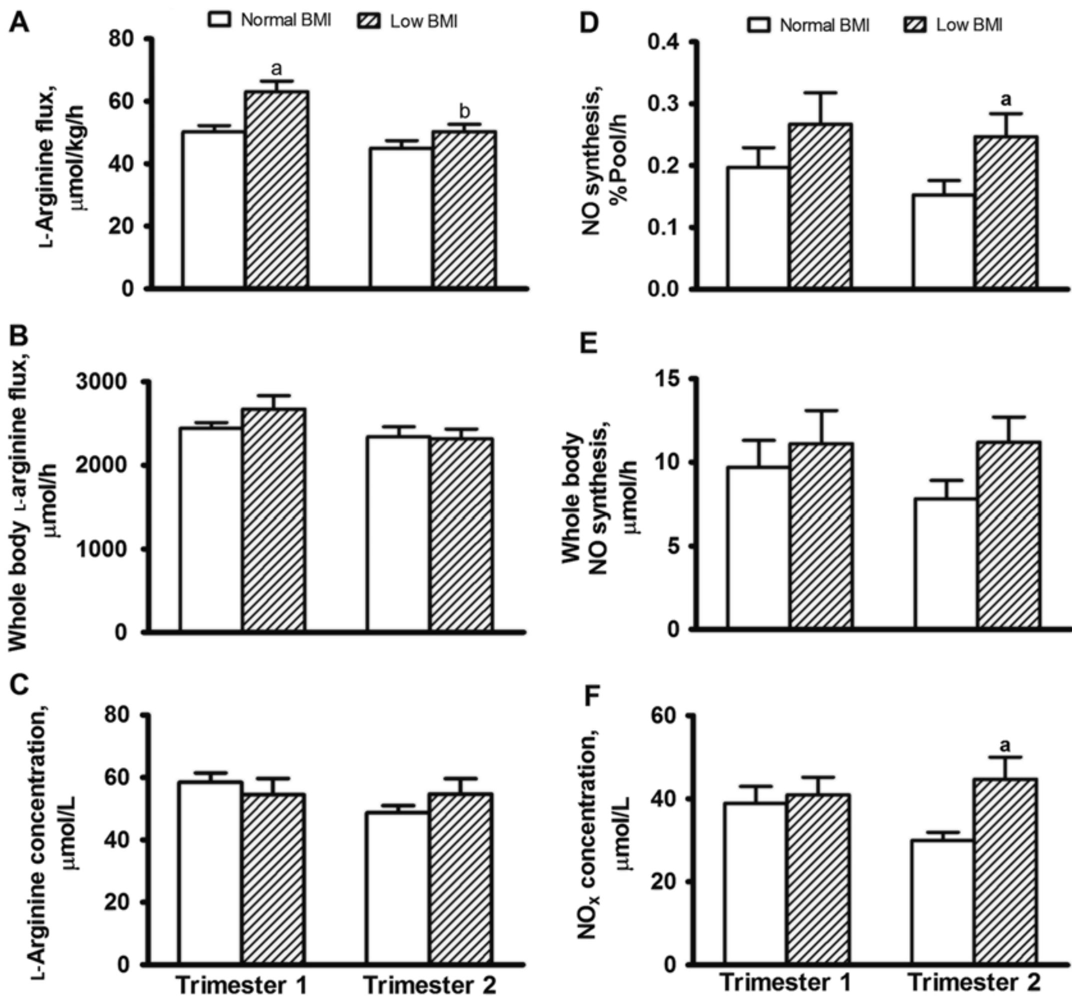


Fig. 21.5 Weight-specific L-arginine flux (**A**), whole-body L-arginine flux (**B**), L-arginine concentration (**C**), weight-specific NO synthesis (**D**), whole-body NO synthesis (**E**), and NOx concentration (**F**) in Indian women with normal and low BMI at trimester 1 (12 gestational weeks) and trimester 2 (24 gestational weeks). Values are mean ± SE; $n=10$ in each group. [a]Different from normal BMI, $P<0.01$. [b]Different from trimester 1, $P<0.01$. Figure drawn from data first published in Kurpad et al. [29]

($P<0.05$) (Fig. 21.5d). This was associated with a significantly higher ($P=0.03$) plasma NOx concentration in the low BMI group in trimester 2 (Fig. 21.5f). NO synthesis, when expressed either per unit of body weight or per whole body, did not change in any group from trimester 1 to 2 (Fig. 21.5d, e).

These findings show that pregnant Indian women with low BMI produce L-arginine and NO at rates that are faster than their normal BMI counterparts. Both groups of women, however, fail to increase L-arginine flux and NO synthesis as pregnancy progresses to the end of trimester 2. It is possible that the consistently faster NO synthesis of the low BMI women may explain their relatively better LBW outcome and their lower diastolic blood pressure at both trimesters.

Comparing the L-Arginine and NO Kinetics of American, Indian, and Jamaican Women and Adolescent Girls

When the data obtained from the three different nationalities were compared, the adult Jamaican women produced L-arginine at the end of trimester 1 and beginning of trimester 3 at about the same rate as the Americans at midpregnancy, and the rates of both groups were significantly greater ($P<0.01$) than the rate of the nonpregnant American women at 8–10 weeks postpartum (Fig. 21.6). The same was true of the Jamaican adolescent girl at trimester 1, though she had a 28 % reduction in L-arginine flux as her pregnancy progressed to trimester 3. Both groups of Indian women, however, had L-arginine fluxes that were markedly slower by ~50 % at both trimesters 1 and 2 compared to the rates of the Jamaicans and Americans. These L-arginine fluxes of the Indian women were even slower ($P<0.01$) than that of the nonpregnant American women at postpartum, indicating that the Indian women were unable to increase L-arginine supply to meet the demands of pregnancy. Further, there were significant positive associations between birth weight and L-arginine flux at the end of trimester 1 ($r=0.54$, $P=0.0031$) and beginning of trimester 3 ($r=0.53$, $P=0.0037$) (Fig. 21.7), suggesting that suboptimal L-arginine availability results in a lower birth weight. Not surprisingly the Indian women delivered 9/20 (45 %) LBW babies compared to 2/15 (13.3 %) for all Jamaicans (Table 21.4). Though there were no differences in NO synthesis among the four groups of Jamaicans and Indians in trimester 1, in trimester 2 Indian women with normal BMI had slower NO synthesis compared to the other groups (Fig. 21.8). Interestingly, this group had the worst outcome in terms of gestational age and

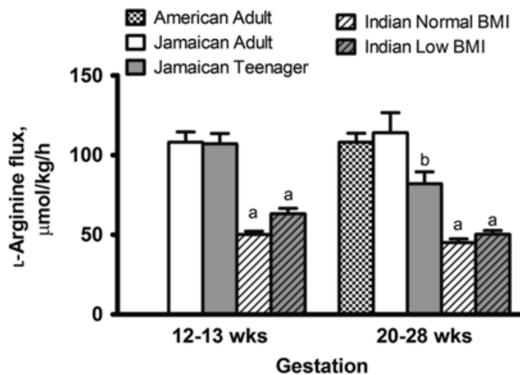


Fig. 21.6 Comparing L-arginine fluxes of Jamaican, American, and Indian women during pregnancy. Values are mean ± SE, $n=5$ in American women, $n=8$ in Jamaican adult women and adolescent girls, $n=10$ in Indian women with normal and low BMI. [a]Significantly slower comparing to American and Jamaican values, $P<0.01$. [b]Significantly slower comparing to Jamaican adults, $P<0.01$. Figure drawn from data first published in Goodrum et al. [18], Thame et al. [25], and Kurpad et al. [29]

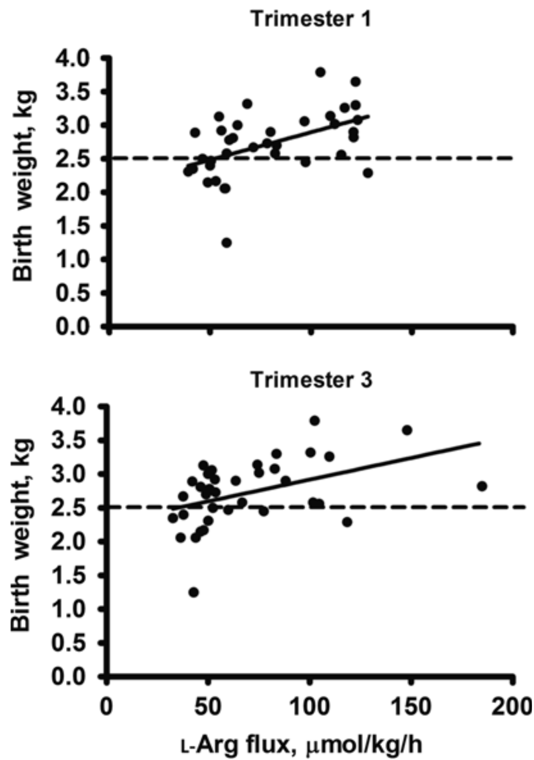


Fig. 21.7 Correlation between birth weight and L-arginine flux at trimester 1 and trimester 3 in pregnant Jamaican and Indian women. Trimester 1, Pearson $r=0.54$, $P=0.0031$; trimester 3, Pearson $r=0.53$, $P=0.0037$; $n=36$. Figure drawn from data first published in Thame et al. [25] and Kurpad et al. [29]

Table 21.4 Comparing gestational age and birth weight in Jamaican women and adolescent girls versus Indian women[a]

Group[b]	Gestational age (weeks)	Birth weight[c] (kg)	LBW baby (%)
Jamaican adult	38.4 ± 0.4	3.14 ± 0.1[d]	12.5 (1/8)
Jamaican adolescent	37.6 ± 0.8	2.87 ± 0.17[d]	14.3 (1/7)
Indian BMI <18.5	38.1 ± 1.5	2.64 ± 0.11[d]	30 (3/10)
Indian BMI >18.5 < 25	37 ± 3.1	2.3 ± 0.05[e]	60 (6/10)

[a]Data adapted from Thame et al. [25] and Kurpad et al. [29]
[b]Values are mean ± SE; $n=8$ in Jamaican adult and adolescent girl groups; $n=10$ in Indian normal BMI and low BMI groups
[c]One-way ANOVA, $P<0.0001$; [d,e]values with different superscript letters are significantly different, $P<0.05$

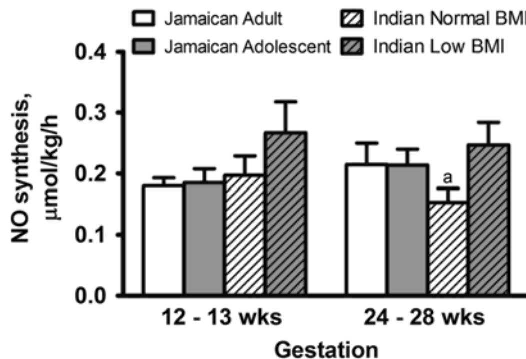


Fig. 21.8 Comparing NO syntheses of Jamaican and Indian women during pregnancy. Values are mean ± SE, $n=8$ in Jamaican adult women and adolescent girls, $n=10$ in Indian women with normal and low BMI. [a]Significantly slower comparing to other group values at weeks 24–28, $P<0.01$. Figure drawn from data first published in Thame et al. [25] and Kurpad et al. [29]

baby birth weight (Table 21.4), indicating that inability to increase L-arginine supply and NO synthesis during pregnancy may both be contributing to the high incidence of LBW babies in Indian women. This may also explain the observation that even affluent well-nourished Indian women have a high incidence of LBW babies [30].

Our data do not provide an explanation for the slower L-arginine flux of Indian women during pregnancy. Because the endogenous flux of a dispensable amino acid consists of its release from whole-body protein breakdown plus its de novo synthesis, the slower L-arginine flux of the Indian women could have been due to a decrease in either one or in both mechanisms. Evidence from studies in pregnant normal weight healthy adult women suggests that the extra amino acids required for increased maternal protein synthesis are provided by increased release from body protein breakdown in the fasted state and an overall decrease in amino acid catabolism [3, 23]. Duggleby and Jackson [23] also reported that protein breakdown rose to a greater extent in pregnant women whose BMI exceeded 25 kg/m^2 compared to those with lower BMI, suggesting that amino acid supply is directly related to maternal BMI. Because the BMIs of the two groups of Indian women were lower than those of the Americans and Jamaicans, this may be one factor contributing to their slower L-arginine flux. Another explanation is that the pregnant Indian women may not be able to downregulate the rate of amino acid oxidation to conserve nitrogen for the de novo synthesis of dispensable amino acids such as L-arginine. A definitive answer to this question can only be provided by more detailed studies of protein and amino acid metabolism in pregnant Indian women.

Conclusions

As seen in adult American and Jamaican women and in adolescent girls, there is an upregulation in L-arginine flux and NO synthesis starting in early pregnancy, in all likelihood a necessary adaptation to meet the increased demands of protein synthesis and maternal vascular expansion. Although the pregnant adolescent girl was unable to maintain a higher L-arginine flux like her adult counterpart in late pregnancy, this inability to maintain L-arginine flux does not seem to affect her ability to synthesize NO or to affect her baby's birth weight. This is not the case in pregnant Indian women who, regardless of their body weight and BMI, had L-arginine fluxes that were less than 50 % the rate of their American and Jamaican counterparts during pregnancy. Further, in the Indian woman, this slower L-arginine flux is associated with slower NO synthesis in those women with normal BMI but not in those with low BMI. Finally, the slower L-arginine flux and NO synthesis of Indian women seem to have a negative effect on fetal growth resulting in a high rate of LBW babies.

References

1. Hay Jr WW. Development of the fetus: carbohydrate and lipid metabolism. In: Duggan CP, Watkins JB, Walker WA, editors. Nutrition in pediatrics. 4th ed. Shelton: People's Medical Publishing House; 2009. p. 311–25.
2. Regnault TRH, Battaglia FC. Amino acid nutrition in utero: placental function and metabolism. In: Duggan CP, Watkins JB, Walker WA, editors. Nutrition in pediatrics. 4th ed. Shelton: People's Medical Publishing House; 2009. p. 327–39.
3. Duggleby SL, Jackson AA. Protein, amino acid and nitrogen metabolism during pregnancy: how might the mother meet the needs of her fetus? Curr Opin Clin Nutr Metab Care. 2002;5(5):503–9.
4. Kalhan S, Rossi K, Gruca L, Burkett E, O'Brien A. Glucose turnover and gluconeogenesis in human pregnancy. J Clin Invest. 1997;100(7):1775–81.
5. Felig P, Kim YJ, Lynch V, Hendler R. Amino acid metabolism during starvation in human pregnancy. J Clin Invest. 1972;51(5):1195–202.
6. Fitch WL, King JC. Plasma amino acid, glucose, and insulin responses to moderate-protein and high-protein test meals in pregnant, nonpregnant, and gestational diabetic women. Am J Clin Nutr. 1987;46(2):243–9.

7. Lemons JA, Adcock 3rd EW, Jones Jr MD, Naughton MA, Meschia G, Battaglia FC. Umbilical uptake of amino acids in the unstressed fetal lamb. J Clin Invest. 1976;58(6):1428–34.
8. Morris Jr SM. L-Arginine: beyond protein. Am J Clin Nutr. 2006;83(2):508S–12.
9. Wu G, Meininger CJ. L-Arginine nutrition and cardiovascular function. J Nutr. 2000;130(11):2626–9.
10. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(Pt 1):1–17.
11. Molnar M, Hertelendy F. N omega-nitro-L-arginine, an inhibitor of nitric oxide synthesis, increases blood pressure in rats and reverses the pregnancy-induced refractoriness to vasopressor agents. Am J Obstet Gynecol. 1992;166(5): 1560–7.
12. Sladek SM, Magness RR, Conrad KP. Nitric oxide and pregnancy. Am J Physiol. 1997;272(2 Pt 2):R441–63.
13. Prentice AM, Spaaij CJ, Goldberg GR, et al. Energy requirements of pregnant and lactating women. Eur J Clin Nutr. 1996;50 Suppl 1:S82–110. discussion S110-111.
14. Wu G, Ott TL, Knabe DA, Bazer FW. Amino acid composition of the fetal pig. J Nutr. 1999;129(5):1031–8.
15. Block RJ, Weiss KW. Amino acid handbook. Springfield, IL: Charles C. Thomas; 1956.
16. King JC, Calloway DH, Margen S. Nitrogen retention, total body 40 K and weight gain in teenage pregnant girls. J Nutr. 1973;103(5):772–85.
17. Chung M, Teng C, Timmerman M, Meschia G, Battaglia FC. Production and utilization of amino acids by ovine placenta in vivo. Am J Physiol. 1998;274(1 Pt 1):E13–22.
18. Goodrum LA, Saade GR, Belfort MA, Moise Jr KJ, Jahoor F. L-Arginine flux and nitric oxide production during human pregnancy and postpartum. J Soc Gynecol Investig. 2003;10(7):400–5.
19. Conrad KP, Kerchner LJ, Mosher MD. Plasma and 24-h NO(x) and cGMP during normal pregnancy and pre-eclampsia in women on a reduced NO(x) diet. Am J Physiol. 1999;277(1 Pt 2):F48–57.
20. Fraser AM, Brockert JE, Ward RH. Association of young maternal age with adverse reproductive outcomes. N Engl J Med. 1995;332(17):1113–7.
21. Orvos H, Nyirati I, Hajdu J, Pal A, Nyari T, Kovacs L. Is adolescent pregnancy associated with adverse perinatal outcome? J Perinat Med. 1999;27(3):199–203.
22. Saftlas AF, Olson DR, Franks AL, Atrash HK, Pokras R. Epidemiology of preeclampsia and eclampsia in the United States, 1979–1986. Am J Obstet Gynecol. 1990;163(2):460–5.
23. Duggleby SL, Jackson AA. Relationship of maternal protein turnover and lean body mass during pregnancy and birth length. Clin Sci (Lond). 2001;101(1):65–72.
24. Thame M, Trotman H, Osmond C, Fletcher H, Antoine M. Body composition in pregnancies of adolescents and mature women and the relationship to birth anthropometry. Eur J Clin Nutr. 2007;61(1):47–53.
25. Thame MM, Fletcher HM, Baker TM, Marini JC, Kao CC, Jahoor F. L-Arginine flux, but not nitric oxide synthesis, decreases in adolescent girls compared with adult women during pregnancy. J Nutr. 2011;141(1):71–4.
26. Ehrenberg HM, Dierker L, Milluzzi C, Mercer BM. Low maternal weight, failure to thrive in pregnancy, and adverse pregnancy outcomes. Am J Obstet Gynecol. 2003;189(6):1726–30.
27. Miller RS, Thompson ML, Williams MA. Trimester-specific blood pressure levels in relation to maternal pre-pregnancy body mass index. Paediatr Perinat Epidemiol. 2007;21(6):487–94.
28. Bodnar LM, Catov JM, Klebanoff MA, Ness RB, Roberts JM. Prepregnancy body mass index and the occurrence of severe hypertensive disorders of pregnancy. Epidemiology. 2007;18(2):234–9.
29. Kurpad AV, Kao C, Dwarkanath P, et al. In vivo L-arginine production and nitric oxide synthesis in pregnant Indian women with normal and low body mass indices. Eur J Clin Nutr. 2009;63(9):1091–7.
30. Deshmukh JS, Motghare DD, Zodpey SP, Wadhva SK. Low birth weight and associated maternal factors in an urban area. Indian Pediatr. 1998;35(1):33–6.

Chapter 22
L-Arginine in the Uterus and Placenta and During Gestation in Mammals

Jonathan M. Greene and Peter L. Ryan

Key Points

- L-arginine is a component of the uterine histotroph.
- L-arginine is transported across uterine and placental tissues.
- L-arginine is metabolized in uterine and placental tissues.
- L-arginine stimulates cell signaling in uterine and placental cells.
- L-arginine stimulates contractility, vasoactivity, and proliferation in uterine cells and reduces apoptosis.
- L-arginine stimulates cell migration, proliferation, and angiogenesis in placental cells.
- L-arginine has in vivo effects during pregnancy including stimulating angiogenesis, increasing fecundity, and reducing hypertension.

Keywords L-arginine • Nitric oxide • Polyamines • Uterus • Placenta • Pregnancy • Gestation

Introduction

Often considered to be one of the most versatile amino acids, L-arginine is classified as a basic, cationic amino acid with three amine groups comprising a guanidino group in the side chain. L-arginine was first isolated from lupin seedlings by Schulze and Steiger [1], and shortly thereafter, Hedin [2] discovered that L-arginine is a component of animal proteins (as reviewed by [3]). Following the discovery of L-arginine, many efforts to determine its essentiality or dispensability were undertaken with a definitive answer still being debated today. The results from Scull and Rose [4] suggested that L-arginine was a dispensable or nonessential amino acid. This finding was repeated in humans by Rose and colleagues [5] who reported that removal of L-arginine from the diet did not result in a negative nitrogen balance in adult males.

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Like many observations in science, the classification of L-arginine as a dispensable amino acid does not hold true under all circumstances. Shortly after Scull and Rose [4] reported their findings in the rat, Arnold and colleagues [6] demonstrated that the simple addition of L-arginine at 1 % of the diet enhanced the growth of 1-day-old chicks, and Klose and colleagues [7] observed that removal of L-arginine from the diet caused weight loss of chicks, while the addition of L-arginine stimulated weight gain. Together, these two sets of experiments [6, 7] established L-arginine as a dispensable amino acid for the growing chick and set forth the concept that essentiality of L-arginine may differ among species and even within species depending upon the physiological circumstances.

Possibly one of the most compelling arguments regarding the discrepancy surrounding the essentiality of L-arginine was offered by Borman and colleagues [8], who fed an L-arginine-free diet, and the same diet supplemented with L-arginine to growing rats. Borman and colleagues observed that while L-arginine restriction did not result in weight loss, the addition of L-arginine to the diet increased growth, suggesting a role in tissue growth. More importantly, Borman and colleagues [8] conjectured that the essentiality of L-arginine should not solely be based upon its role in growth but also its role in other bodily functions such as reproduction.

Much of the renewed interest regarding the role of L-arginine in bodily functions other than growth stems from the fact that in addition to being incorporated into many proteins and having a role in ammonia detoxification [9], L-arginine is also a precursor for many important molecules in cellular physiology, including proline, glutamate, creatine, and, most notably, polyamines and nitric oxide [3]. To this end, the argument set forth by Borman and colleagues, along with the versatility of L-arginine, supports the modern efforts to determine the role of L-arginine in other bodily functions such as reproduction. This chapter will explore the effects and role of L-arginine in the tissues of the uterus and placenta and during gestation in mammals. Information has been gathered from various species including humans, rodents, and domestic animals.

Transport and Metabolism of L-Arginine in the Uterus and Placenta

The Uterus

As a reproductive organ, the uterus has been designated as the organ of pregnancy as it provides the maternal environment for the developing offspring. The uterus provides nourishment for the conceptus throughout gestation. During the preimplantation and peri-implantation periods, the uterus provides essential nutrients and growth factors in a secretion known as uterine histotroph. Additionally, the uterus continues this supportive role for the offspring following embryo attachment by acting as the point of interface with the placenta and by providing the environment in which the conceptus will grow and develop [10, 11].

Before discussing the transport and metabolism of L-arginine in the uterus, a brief description of uterine anatomy is provided. The uterus can be described in terms of three layers: the perimetrium, the myometrium, and the endometrium. Serving as the serosal layer of the uterus, the perimetrium forms a tunic around the uterus and is comprised of a thin layer of squamous epithelial cells that is continuous with the visceral peritoneum and is suspended by the broad ligament [11, 12]. Underneath the perimetrium lies the myometrium or the muscular wall of the uterus [13]. The endometrium is the layer that forms the uterine lumen. Histologically, the endometrium consists of glandular and luminal epithelium and stroma (fibroblasts). The endometrium epithelium of viviparous mammals contains both simple cuboidal and simple columnar epithelial cells which form the surface to which the developing embryo attaches as well as the coiled tubular glands which secrete the histotroph that is vital for early embryonic survival [10].

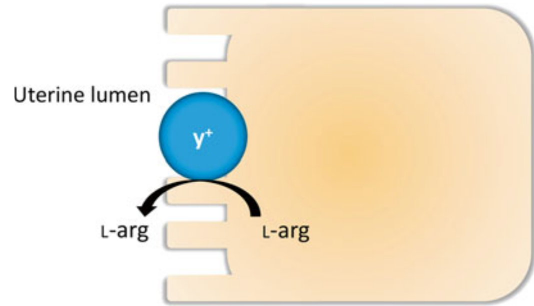

Fig. 22.1 Transport of L-arginine into the uterine lumen. L-arginine is transported across the uterine epithelial cell through the y^+ amino acid transporter system which is accomplished independent of sodium

L-arginine is transported across uterine endometrial tissue and secreted into the uterine lumen (Fig. 22.1). The y^+ amino acid transport system, which transports L-arginine, is comprised of CAT1, CAT2, and CAT3, which are encoded by the genes SLC7A1, SLC7A2, and SLC7A3. During the ovine estrous cycle, SLC7A2 mRNA expression in the endometrium does not change; however, SLC7A1 mRNA does exhibit temporal changes with an increase being observed on day 16 of the cycle [14]. L-arginine has been reported to be present in the uterine flushes of sheep [15], cows [16], rats [17], and humans [18]. In women, uterine lumen concentrations of L-arginine fluctuate during the menstrual cycle, with the greatest amounts being observed during the proliferative phase [18], suggesting that L-arginine may have a role in the regeneration of the endometrial epithelium that is shed during menstruation. Indeed, we have recently reported that L-arginine enhances human endometrial cell proliferation and reduces apoptosis using an endometrial carcinoma cell line model [19]. During the ovine estrous cycle, the uterine lumen concentration of L-arginine is also dynamic and exhibits an increase following estrus with the peak being observed on day 15 of the cycle [15]. Similarly, in cow, the concentration of L-arginine in the uterine lumen also increases following estrus and is elevated at day 14 of the estrous cycle [16]. Interestingly, in both the cow and ewe, L-arginine is elevated at a time when a potential embryo would be recognized by the maternal environment and attach to the endometrial lining. Furthermore, the positive influence that L-arginine has on cell signaling, proliferation, hypertrophy, hyperplasia, and migration of ovine trophectoderm cells [20, 21] suggests that L-arginine is a component of the uterine histotroph that is transported into the uterine lumen to support growth and development of the peri-implantation embryo.

The endometrium also has the ability to metabolize L-arginine in numerous species, including sheep [22–24], pigs [25], mice [26], rats [27], and humans [28], due to the presence of NOS and/or arginase enzymes. Thus, the endometrium produces the necessary enzymes to convert L-arginine into nitric oxide and polyamines. Nitric oxide is produced in the endometrium [29] and is involved in embryo implantation and development [30–32]. Additionally, the endometrium is a site of polyamine synthesis [33, 34] which has been shown to be necessary for embryo implantation, as inhibition of polyamine synthesis reduced pregnancy rates in mice [33]. Nitric oxide synthase is expressed in endometrial tissue throughout the menstrual cycle in women [28]. In addition, the ovine endometrium has been used extensively as a model for studying endometrial metabolism of L-arginine. Endometrial neuronal nitric oxide synthase I (nNOS) mRNA and protein exhibit temporal changes during the ovine estrous cycle, while NOS II (iNOS) is weakly expressed and NOS III (eNOS) expression does not fluctuate [35]. The mRNA expression of ornithine decarboxylase (ODC), a key enzyme needed for polyamine synthesis, also exhibits temporal changes in the endometrium during the ovine estrous cycle; however, ODC protein expression does not appear to fluctuate [35]. During ovine gestation, endometrial NOS enzymatic activity peaks at days 40 and 60 and then declines during the remainder of gestation, with NO synthesis following the same pattern [24]. Arginase activity, which is involved in polyamine synthesis, peaks at days 40 and 60 of gestation in the ovine endometrium followed by declining activity during the remainder of gestation, and ODC activity peaks at day 40 of gestation

and then declines [23]. Moreover, polyamine concentrations in the ovine endometrium peak at days 40 and 60 of gestation and then decline [23]. Interestingly, day 60 of ovine gestation represents a time point during which uterine milk protein mRNA expression begins to increase [36], suggesting that the peak synthesis of NO and polyamines (L-arginine metabolites) may be involved in endometrial signaling that promotes uterine gland secretory function.

The Placenta

Eutherian mammals have a distinct mechanism that imparts them with a reproductive advantage over other species: the placenta. Non-eutherian mammals lay eggs in which their offspring develop prior to birth. In sharp contrast, eutherian mammals exhibit intrauterine growth and development of offspring, and this phenomenon is made possible by a transient organ known as the placenta. Intrauterine growth and the placenta ensure that the developing offspring has the best opportunity to receive nutritional support and protection during a critical time [11]. As a transient metabolic organ, the placenta serves as the point of exchange between the dam and developing fetus [11, 37]. Moreover, the placenta provides a means for respiratory gasses, nutrients, and waste products to be exchanged between the dam and fetus. In addition to a point of nutrient exchange, the placenta provides structural support for the developing conceptus, serves as a transient endocrine gland, and provides an immunological barrier [37]. In this way, the placenta exerts significant control over the normal and successful development of the fetus, and its proper development and function are paramount for normal pregnancy.

Understanding the transport of L-arginine in the placenta first requires a brief description of the microscopic layers that separate the maternal and fetal circulation. Placentae can be classified based upon the number of microscopic layers that separate the maternal and fetal circulation, which differs greatly between species. As such, this classification provides a comparative point of view in terms of the placental invasiveness. There are a possible total of six tissue layers that may separate the maternal and fetal circulation: (1) uterine endothelium, (2) uterine stroma (connective tissue), (3) uterine epithelium, (4) chorionic (fetal) epithelium, (5) fetal connective tissue, and (6) fetal endothelium [37]. According to this classification system, there are three types of placentation: (1) epitheliochorial (Fig. 22.2), (2) endotheliochorial (Fig. 22.3), and (3) hemochorial (Fig. 22.4), with the number

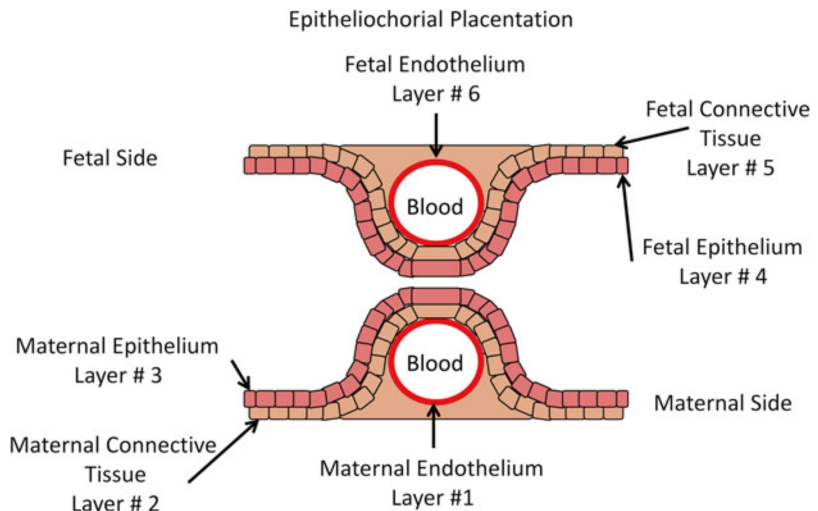

Fig. 22.2 Representation of the tissue layers that separate maternal and fetal circulation in epitheliochorial placentation: (1) maternal endothelium, (2) maternal connective tissue, (3) maternal epithelium, (4) fetal epithelium, (5) fetal connective tissue, and (6) fetal endothelium

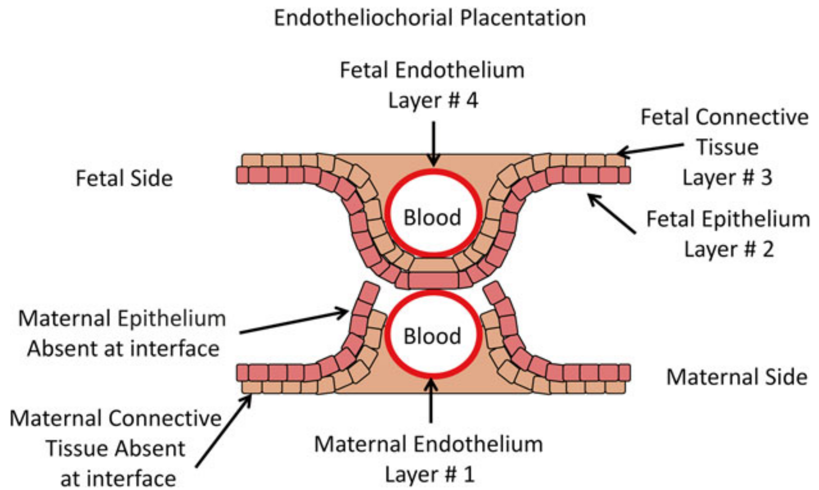

Fig. 22.3 Representation of the tissue layers that separate maternal and fetal circulation in endotheliochorial placentation: (1) maternal endothelium, (2) fetal epithelium, (3) fetal connective tissue, and (4) fetal endothelium

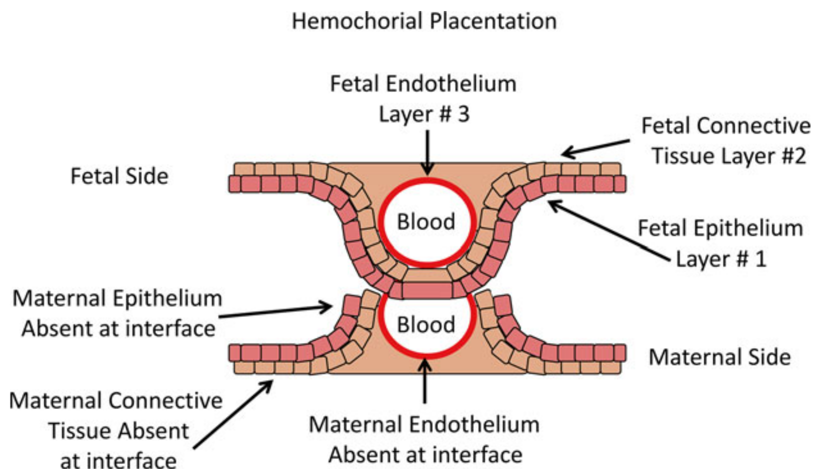

Fig. 22.4 Representation of the tissue layers that separate maternal and fetal circulation in hemochorial placentation: (1) fetal epithelium, (2) fetal connective tissue, and (3) fetal endothelium

of microscopic tissue layers separating maternal and fetal circulation being six, four, and three, respectively.

Amino acids, including L-arginine, partially satisfy the carbon and nitrogen demands of the developing placenta and fetus [38]. In addition to providing carbon and nitrogen substrates, amino acids also serve as constituents of proteins and are precursors for nonprotein substances such as signaling molecules and nucleotides [39]. Concentrations of most essential amino acids are greater in the fetal circulation compared to maternal circulation [38], indicating that active transport of amino acids is an important process during gestation. In terms of nutrient transfer across the placenta, an interesting phenomenon occurs as the pregnancy progresses. In the human, fetal weight increases by approximately 20-fold between weeks 16 and 40 [38]. However, during a similar time period (weeks 25 and 36), the villous surface area of the placenta increases by only a factor of 9.5 [40]. This would suggest that nutrient transfer across the placenta increases in efficiency as gestation progresses [38, 40]. This increased efficiency could easily be achieved as there is an approximately tenfold increase in villous volume occupied by vasculature [41], which would enhance the transfer of nutrients.

Recalling the anatomy of the placenta, amino acids that are to undergo placental transport would be present either in the extracellular space at the maternal-fetal interface (i.e., epitheliochorial and

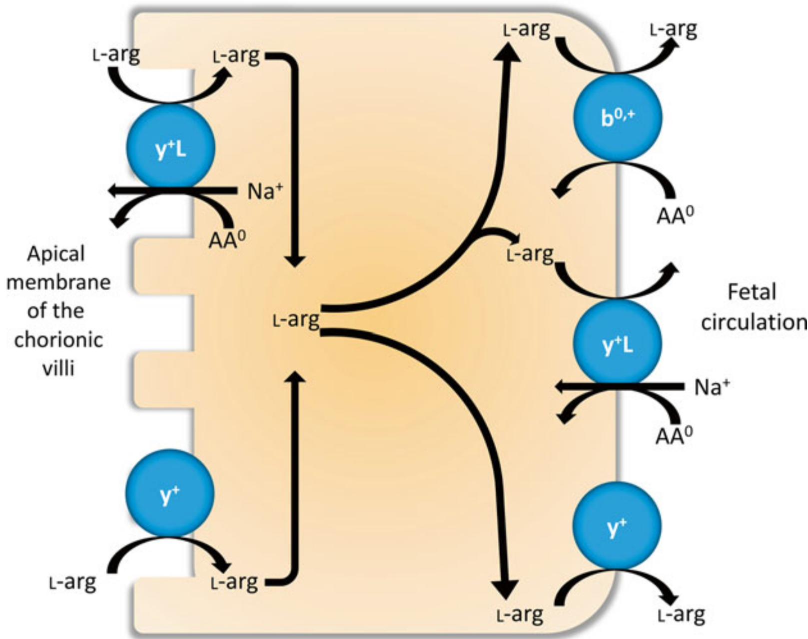

Fig. 22.5 Transport of L-arginine into the placental epithelium and efflux into fetal circulation. In the human placenta, L-arginine is transported across the apical membrane of the chorionic membrane via either the y^+ or y^+L amino acid transporters. On the basolateral membrane, L-arginine is transported by either the $b^{0,+}$, y^+L, or y^+ systems. In the y^+ system, L-arginine is transported across the membrane independent of sodium. L-arginine is transported by the y^+L system in an antiport fashion with sodium and neutral amino acids. L-arginine transport by the $b^{0,+}$ system involves the antiport movement of a neutral amino acid.

endotheliochorial placentas) or in the pools of maternal blood (i.e., hemochorial placentas). As such, placental transport of amino acids involves three general steps: (1) uptake across the apical membrane of the chorionic villi into the trophoblasts, (2) transport through the cytoplasm of the trophoblasts, and (3) efflux of amino acids across the basal lateral membrane into fetal circulation [38] (Fig. 22.5). The transport of L-arginine across the placenta involves three systems: $b^{0,+}$, y^+L, and y^+. Studies in the human placenta have localized these systems on trophoblasts, with systems y^+ and y^+L functioning on the apical membrane of the chorionic villi and all three systems being present on the basal membrane of the chorionic villi [42, 43]. Additionally, the activities of these transport systems appear to be temporally regulated during gestation. In the human placenta, the apical transport of L-arginine by system y^+ increases with gestational age; however, y^+L transport of L-arginine has an inverse relationship with gestational age [44].

In addition to being delivered to the fetal circulation, L-arginine can also be metabolized by trophoblasts. As previously described, polyamines and NO are two L-arginine metabolites that are synthesized through the actions of arginase and ODC (polyamines) and NOS (NO). Nitric oxide synthesis in the ovine placenta peaks at day 60 and is followed by a decline in both the intercotyledonary chorioallantoic membranes and placentome, while NOS activity in the placentome increases with a peak at day 60 and remains elevated for the duration of pregnancy [24]. Arginase is expressed throughout gestation in the human placenta but decreases as pregnancy progresses [45]. This corresponds well to the porcine and ovine placenta in which polyamines and the activity of ODC, the rate-limiting enzyme for polyamine synthesis, decrease after day 40 of gestation [23]. Interestingly, the elevated polyamine production in the early ovine placenta suggests a role for polyamines in cell proliferation of the trophoblasts as it has been demonstrated that polyamine

biosynthesis enhances cell proliferation and protein synthesis in early ovine trophectoderm cells [20, 21].

Nitric oxide synthase is produced in the human [46], sheep [24], pig [25], rat [27], and mouse [47] placenta. Immunolocalization techniques have revealed that NOS is present in the human umbilical artery and vein endothelium as well as the trophoblasts of the chorionic villi [46]. In the ovine placenta, NOS activity and NO synthesis are greatest in the placentome compared to the intercotyledonary spaces of the chorioallantoic membranes. Nitric oxide synthase activity in the intercotyledonary chorioallantoic membranes increases with a peak at day 60 of gestation followed by a decline through the remainder of gestation, while activity in the placentome increases with a peak at day 60 and remains elevated for the duration of pregnancy [24]. Nitric oxide synthesis in the ovine placenta peaks at day 60 and is followed by a decline in both the intercotyledonary chorioallantoic membranes and placentome [24].

In the pig placenta, polyamine synthesis is present throughout gestation, increasing with a peak being observed around day 40 which is followed by a decline for the remainder of term. Likewise, the activity of ODC, the rate-limiting enzyme in polyamine synthesis, increases until day 40 of gestation and then declines [48]. Polyamine concentrations are greater in the ovine placentome compared to the intercotyledonary chorioallantoic membranes. In the placentome, polyamine concentrations increase and peak at day 40 of gestation and then decline, while the polyamine concentration in the intercotyledonary chorioallantoic membranes increases and peaks at day 80 and declines during the remainder of gestation [23].

Cellular Effects of L-Arginine and Its Metabolites in the Uterus and Placenta

The Uterus

The uterus is largely regarded as an organ of pregnancy. As such, it must expand rapidly to accommodate the growing fetus. Also, proliferation of the uterus is necessary throughout the menstrual cycle as the uterine epithelium proliferates in preparation for an implanting embryo. There appears to be a scarce amount of information available on the cellular effects that L-arginine has on uterine tissues. Much of the information focuses on the effect that the L-arginine metabolite nitric oxide has on contractility and vascular control in the uterus. In strips of myometrial tissue, L-arginine and a nitric oxide donor both increased the secondary messenger cyclic guanosine monophosphate, while a nitric oxide synthase inhibitor decreased its production. Furthermore, spontaneous contractility was increased by the nitric oxide synthase inhibitor and decreased by the nitric oxide donor [49], suggesting that L-arginine, through a nitric oxide-cGMP pathway, regulates contractility of the uterine myometrium. In addition to affecting contractility of the myometrium, L-arginine also appears to exert an effect on vascular control in uterine tissues. In human uterine artery rings, L-arginine induced contraction-dependent relaxation, and this result continued even after the removal of the endothelium. Exposure of uterine artery rings to methylene blue, an inhibitor of nitric oxide synthase [50], inhibited the relaxation induced by L-arginine [51], indicating that L-arginine's effect on uterine artery relaxation is a result of it being metabolized into nitric oxide. In addition to its effects on vascular control and contractility of the uterine cells, L-arginine also appears to stimulate cell proliferation and reduce apoptosis in uterine-derived cells. Using a human endometrial carcinoma cell line as a model, we have recently demonstrated that L-arginine enhances endometrial epithelial proliferation, and it achieves this through a mechanism mediated by nitric oxide and polyamine synthesis. Moreover, L-arginine reduced the incidence of mitochondrial mediated apoptosis in endometrial epithelial cells, and this

was associated with an increase in phosphorylation of BAD protein. Thus, L-arginine seems to promote the survival and proliferation of the cells that line the lumen of the uterus [19]. L-arginine has been reported to be present in the uterine flushes of humans, with the concentration depending upon the phase of the menstrual cycle [18]. Proliferation of the endometrium has been implicated as a vital process which provides an optimal environment for embryo adhesion and implantation [52], and this argument is further supported by the observation that increasing endometrial thickness is associated with improved implantation rates in humans [53–55]. Interestingly, the uterine lumen concentration of L-arginine is greatest during the proliferative phase of the menstrual cycle [18], suggesting that L-arginine may have a role in the proliferation of the endometrial epithelium which must regenerate following menstruation. In addition, the uterus must expand to accommodate the growing fetus during early pregnancy. To this end, L-arginine might represent a crucial amino acid in terms of uterine biology during the peri-implantation period and early gestation.

The Placenta

As a transient endocrine organ, the placenta originates from the trophoblast cells from the early developing embryo. As such, much of the work involving L-arginine has determined the cellular effects that this particular amino acid has on trophoblast cells. As one of the earliest developing tissues of the preimplantation and peri-implantation embryo, proliferation and migration of these trophoblast cells are two critical processes that occur. These processes are largely driven by a collection of growth factors, hormones, cytokines, and nutrients, known as histotroph, which is secreted by the uterine epithelium. Several studies have reported the presence of L-arginine in the uterine lumen of several species, including humans [18], sheep [15], and cattle [16]. These findings would suggest that L-arginine is a component of uterine histotroph and, thus, would exert an effect of trophoblast cells.

In mouse trophoblast cells, the presence of amino acids is necessary for outgrowth of these cells to occur [56]. A similar effect has been observed with only L-arginine in ovine trophoblast cells. Trophectoderm cells, isolated from day 15 ovine conceptuses, displayed enhanced cellular migration when cultured with L-arginine [21]. Migration of ovine trophectoderm cells is an important aspect of the peri-implantation period of sheep as the early conceptus elongates to a length of 25 cm or more by day 17 of pregnancy [57]. Accompanying this migration of trophectoderm cells is an increase in cell proliferation to support the growing trophectoderm. It appears that L-arginine also supports ovine trophoblast cell proliferation, and this effect is abrogated when nitric oxide and polyamine synthesis are inhibited. In addition, L-arginine enhances protein synthesis, reduces protein degradation, and stimulates MTOR signaling in ovine trophoblast cells [20]. L-arginine also appears to have a similar proliferative effect in porcine trophoblast cells and achieves this through activation of MTOR signaling as well [58].

Distinct from its effects on stimulating cell migration and proliferation, L-arginine may also have a role in the recognition of pregnancy in ruminant species. In ruminants, the embryo produces interferon tau which stimulates a series of events that result in reduced expression of endometrial oxytocin receptors. In this way, oxytocin, produced by the corpus luteum, cannot stimulate endometrial production of PGF2α, which normally would cause lysis of the corpus luteum and lead to the initiation of a new estrous cycle [59]. Thus, interferon tau is considered critical in the early maintenance and recognition of pregnancy in ruminant species. Interestingly, L-arginine stimulates interferon-tau production in ovine trophoblast cells. It appears that L-arginine's stimulatory effect on interferon tau is mediated through the production of polyamines and their effect on the TSC2-MTOR signaling pathway [60].

In addition to promoting cell migration, cell proliferation and interferon-tau production in trophoblast cells, L-arginine, through the action of its metabolite NO, may also represent a stimulant of placental angiogenesis. Angiogenesis is particularly important for properly functioning placental tissues as one of the primary roles of the placenta is to facilitate the efficient nutrient, gas, and waste

exchange between the dam and the fetus [61]. Nitric oxide directly affects placental angiogenesis by stimulating the production of placental endothelial cells which is a critical process in angiogenesis. In vitro exposure of ovine fetoplacental artery endothelial cells and human placental artery endothelial cells to an exogenous source of NO results in enhanced cell proliferation through the activation of the mitogen-activated protein kinase pathway [62]. Interestingly, this is the same pathway through which vascular endothelial growth factor (VEGF) elicits its pro-angiogenic effects, leaving one to speculate a potential relationship between these two molecules. Moreover, dietary supplementation of *N*-carbamylglutamate, which increases endogenous synthesis of L-arginine [63], increases VEGF gene expression in the umbilical vein during porcine gestation [64]. Thus, as more information emerges on the role of L-arginine in reproductive processes, it appears that it influences the cells of the placenta by stimulating migration, proliferation, interferon-tau production, and angiogenesis.

Physiological Effects and Roles of L-Arginine and Its Metabolites During Gestation

In a comprehensive review, Lefèvre and colleagues [65] provide an update on the general cellular and molecular functions of polyamines during embryonic development, implantation, and post-implantation formation of the placenta. Critical to the synthesis of polyamines is the availability of essential substrates that include L-arginine, among others, whose main source is dietary. To ensure early conceptus development, successful embryo implantation, and establishment of the pregnancy, it is essential that these compounds be available in the uterine environment [66, 67]. Endogenously produced polyamines may be synthesized from amino acids such as L-arginine or L-proline through the L-ornithine pathway [3]. L-Arginine is regarded as nutritionally essential among amino acids for gestation in animals [68]. It serves not only as a building block for proteins but also as an important precursor in the synthesis of many biologically active molecules including nitric oxide, which is thought to mediate placental vascular development and function [69]. A number of studies have reported that reproductive success in women and in several domestic species is closely associated with maternal nutrition that includes dietary supplementation with L-arginine: pigs [70–72], sheep [73], cattle [74], rodent [75, 76], and women [77, 78].

Wu and colleagues [79] have provided convincing evidence that the L-arginine family of amino acids play an important role in the development and vascularization of the placenta, in particular, during the early stages of development in swine. In a series of studies, they observed that dietary supplementation with L-arginine (0.08–1.0 %) not only increased the plasma concentrations of L-arginine in gestating sows but also the concentrations in allantoic fluid, leading to enhanced embryonic development, increased litter birth weight, and a greater number of live-born piglets at delivery [67]. Similar observations have been made in gestating ewes where L-arginine concentrations were found to increase in the uterine lumen early in gestation between days 10 and 15 [66] and rats where dietary supplementation enhanced embryonic survival [75]. There is also evidence that amino acid concentrations, including L-arginine, in the bovine uterine lumen of cloned versus in vitro fertilized pregnancies, were lower leading to the suggestion that the higher incidence of developmental abnormalities seen in cloned embryos may be, in impart, due to disturbed embryo-maternal interactions [80].

A major focus of recent research efforts is the impact dietary L-arginine supplementation may have on the angiogenesis (development of new blood vessels from preexisting vascular structures) of the developing fetal membranes, embryonic tissue, and the endometrial wall where it is thought to possibly play an angiogenic and/or vasodilatory role in the endometrial tissue lining the lumen of the uterus. What is of interest here is the possible mechanism of action through which dietary L-arginine may influence interactions between the maternal and fetal environment in early gestation that lead to positive pregnancy outcomes. Although it is not clear how L-arginine enhances reproductive

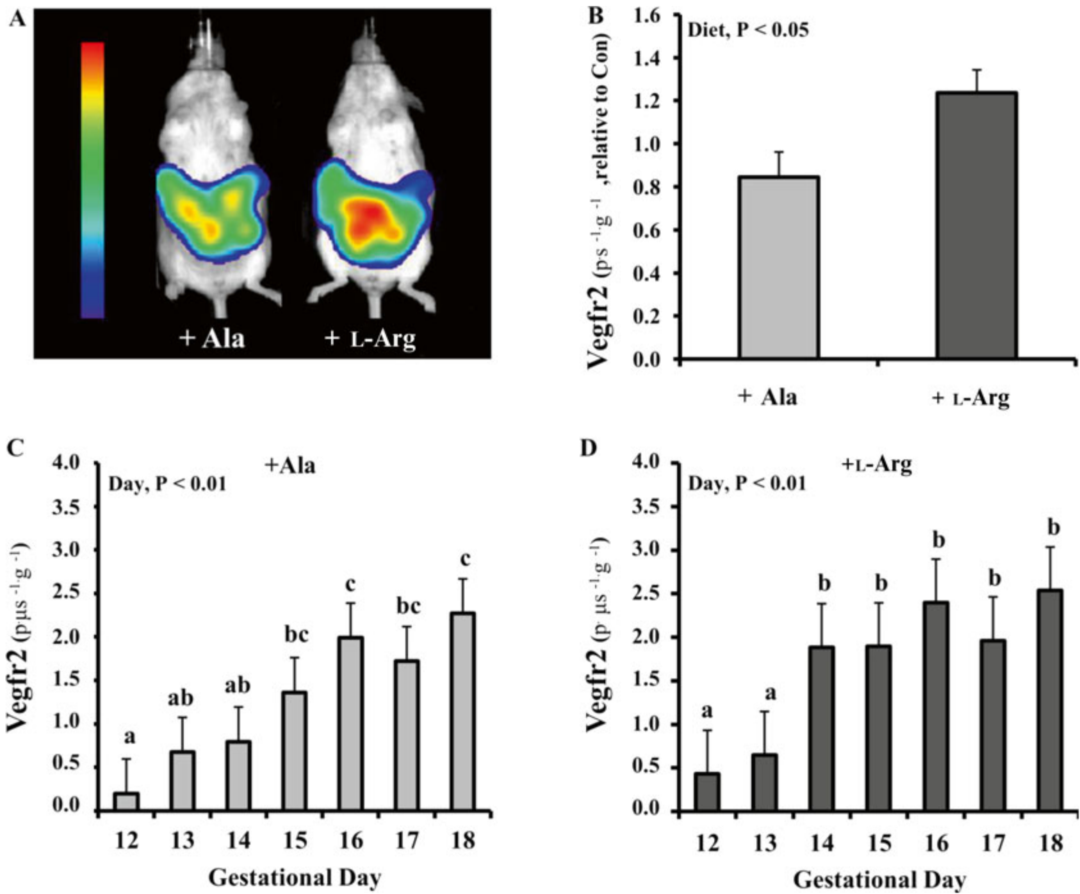

Fig. 22.6 Qualitative (**a**) and quantitative bioluminescent assessment of (**b**) mean and (**c** and **d**) daily fetoplacental VEGFR2 transcription activity from mice fed either the +Ala[1] ($n=5$) or +Arg[1] ($n=6$) diet during gestation. The color scheme represents a pseudo-color scale applied to the image, with the *red* colors indicating the greatest VEGFR2 transcription and the *purple* colors representing the least amount of VEGFR2 transcription. Means without a common letter differ $P \leq 0.05$. +Ala, L-alanine supplemented diet; +Arg, L-arginine supplemented diet. Data are presented as least square means ± the individual standard error of the mean. Reprinted from [81].

performance, it has been suggested that L-arginine may influence angiogenesis in the pregnant female via the VEGF and its cell surface receptor 2 (VEGFR2) system, two proteins that are critically important for angiogenesis to occur. To tackle this question, Greene and colleagues [81] employed an interesting mouse model where female mice bred to homozygous FVB/N-Tg vascular endothelial growth factor receptor 2-luc (Vegfr2-luc)-Xen male mice produced pregnancies where fetuses expressed the Vegfr2 gene whose transcription activity could be detected by bioluminescence imaging. Pregnant females (day 1 of gestation) were provided dietary supplementation of either L-arginine (2.0 % wt/wt) or alanine (4.1 % wt/wt) as an isonitrogenous control. As determined by bioluminescence imaging from days 12 to 18 of gestation, it was observed that L-arginine supplementation induced an earlier rise in Vegfr2 transcription activity as well as a significant increase in Vegfr2 transcription activity when corrected for fetal mass (Fig. 22.6). Moreover, L-arginine supplementation increased weight gain during the latter third of gestation, total litter size, number of pups born alive, number of placental attachment sites, litter birth weights, and litter weights of pups born alive (Table 22.1). The conclusion of these studies was that dietary L-arginine enhanced Vegfr2 transcription activity in the fetoplacental unit, thereby promoting placental and fetal growth and development. Dietary supplementation studies performed in the pregnant sow also demonstrated that L-arginine not only increased litter size, the

Table 22.1 Reproductive performance of mice fed either the +Ala or +Arg diet during gestation[a]

	Dietary treatment	
	+Ala	+Arg
Feed intake		
Total feed intake (g)	59.3 ± 2.16	59.9 ± 1.97
Total L-arginine intake (mg)	332 ± 49.6	1530 ± 45.3*
Reproductive performance parameters		
Weight gain (GD 12 to GD 18) (g)	5.95 ± 0.260	7.01 ± 0.230*
Total pups born per litter (*n*)	4.20 ± 0.730	8.50 ± 0.670*
Total pups born alive per litter (*n*)	4.20 ± 0.750	8.17 ± 0.690*
Number of placental attachment sites (*n*)	5.80 ± 1.10	9.67 ± 1.00**
Litter birth weight of all pups born (g)	5.40 ± 0.870	9.88 ± 0.790*
Litter birth weight of all pups born alive (g)	5.40 ± 0.920	9.62 ± 0.840*
Individual birth weight of pups born alive (g)	1.32 ± 0.0500	1.18 ± 0.0400**

[a]Data are presented as least square means ± the individual standard error of the mean. ANOVA *P*-values are *$P<0.01$; **$P<0.05$. +Ala, L-alanine supplemented diet ($n=5$); +Arg, L-arginine supplemented diet ($n=6$). Reprinted from [81]

average birth weight of piglets born alive, but also increased plasma vascular endothelial growth factor A (VEGFA) concentrations as well as VEGFA and endothelial nitric oxide synthase gene expression in allantochorionic tissue [79]. Thus, L-arginine appears to promote embryonic tissue development in the pig via similar mechanisms as observed in the mouse. Additional evidence to support this mechanism of action comes from both in vivo and in vitro studies where nitric oxide (NO) induces VEGF expression in placental tissue. Nitric oxide is produced from L-arginine and oxygen molecules by living cells, a process which is driven by the catalytic action of nitric oxide synthase (NOS).

L-Arginine is a component of the histotroph secretion which is the primary source of uterine nutrients in most mammalian species and is observed to increase in content as the preimplantation period of pregnancy advances where fetal and placental vascular tissues and membranes begin to proliferate [82]. Studies in rat placental tissues using nitric oxide synthase inhibitors, such as *N*-nitro-L-arginine-methyl ester, demonstrated a decline in VEGF expression, a potent stimulator of vascular development, as a direct result in the reduction of NO synthesis, while hypoxia-inducible factor (HIF)-1α and induced NOS expression increased [83]. These authors concluded that peak nitric oxide production is maintained by a reciprocal relationship between nitric oxide and VEGF through hypoxia-inducible factor (HIF)-1α. Further evidence in support of a direct role for L-arginine in the growth and development of the embryo and early conceptus was observed when ovine trophoblast cell proliferation was enhanced with L-arginine supplementation in vitro, and that an additive effect was observed with the addition of recombinant secreted phosphoprotein at physiological concentrations [82]. These authors hypothesized that L-arginine is the driving force for cell proliferation and that secreted phosphoprotein facilitates this cellular proliferative process in the developing conceptus in a cooperative manner.

Of additional interest here is the reported observation that dietary supplementation with L-arginine may be beneficial in lowering diastolic blood pressure and reducing the incidence of preeclampsia in pregnant women. Moreover, nitric oxide is a potent vasodilator and thought to play a role in regulation of blood pressure. During a normal pregnancy, women should experience a vascular adaptation that includes an increase in blood volume and cardiac output and a decrease in vascular resistance. The decrease in vascular resistance (hypotension) is attributed to nitric oxide activity in endothelial tissue, which is promoted by catalysis of L-arginine as described above. However, when nitric oxide activity is reduced or inhibited, hypertension or preeclampsia can be a negative outcome in the pregnancy. Studies in a preeclamptic rat model have shown that dietary L-arginine can reduce hypertension [84]. Since preeclampsia is a serious complicating factor in many human pregnancies (up to 8 % of pregnancies), meta-analysis of studies in the literature confirmed that dietary L-arginine supplementation may be beneficial in reducing the hypertensive state associated with preeclampsia [85]. Both cellular and animal

studies have shown that dietary supplementation of females during the pregnancy not only enhances preimplantation but also facilitates placental and fetal growth and development and affords a level of protection against pregnancy-associated hypertension experienced in some women.

Thus, it appears that L-arginine is nutritionally essential in ensuring that the appropriate amino acids are available in the uterine environment to facilitate normal placental and fetal angiogenesis during the early stages of placental and embryonic development. Lack of availability of this important amino acid may lead to inadequate synthesis of nitric oxide an important element in the induction of the VEGF system that is responsible for upregulating the angiogenic process in fetal and placental tissues during early gestation. In addition, L-arginine also is thought to enhance the fecundity of litter-bearing species and increase the incidence of live births at term.

Conclusion

Overall, even though L-arginine is not considered to be an essential amino acid in adult mammals, the preponderance of evidence suggests that this amino acid has a major role in the normal physiology of the uterus and placenta as well as a function during gestation. From its simple role as a building block in protein synthesis to serving as a component of the maternal histotroph and being an inducer of cell signaling cascades in uterine and placental tissues, the role of L-arginine in reproductive processes is now being further understood and is challenging the notion that amino acids should be classified solely on the role they have in growth. While the entire role of L-arginine in reproductive processes is not known, new investigations into this topic are increasing, and more data are becoming available.

References

1. Schulze E, Steiger E. Über das Arginin. Z Physiol Chem. 1886;11:43–65.
2. Hedin SG. Eine methode das lysin zu isolieren, nebst einigen Bemerkungen über das lysatinin. Z Physiol Chem. 1895;21:297–305.
3. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(Pt 1):1–17.
4. Scull CW, Rose WC. L-Arginine metabolism: I. The relation of the L-arginine content of the diet to the increments in tissue L-arginine during growth. J Biol Chem. 1930;89(1):109–23.
5. Rose WC, Haines WJ, Warner DT. The amino acid requirements of man: V. The role of lysine, L-arginine, and tryptophan. J Biol Chem. 1954;206(1):421–30.
6. Arnold A, Kline OL, Elvehjem CA, Hart EB. Further studies on the growth factor required by chicks: the essential nature of L-arginine. J Biol Chem. 1936;116(2):699–709.
7. Klose AA, Stokstad ELR, Almquist HJ. The essential nature of L-arginine in the diet of the chick. J Biol Chem. 1938;123(3):691–8.
8. Borman A, Wood TR, Black HC, et al. The role of L-arginine in the growth with some observations on the effects of arginic acid. J Biol Chem. 1946;166(2):585–94.
9. Visek WJ. L-Arginine needs, physiological state and usual diets. A reevaluation. J Nutr. 1986;116(1):36–46.
10. Bartol FF. Uterus, Nonhuman. In: Knobil E, Neill JD, editors. Encyclopedia of reproduction, vol. 4. San Diego, CA: Academic; 1998. p. 950–60.
11. Senger PL. Pathways to pregnancy and parturition. 2nd ed. Pullman, WA: Current Conceptions; 2003.
12. Constantinescu GM. Anatomy of reproductive organs. In: Schatten H, Constantinescu GM, editors. Comparative reproductive biology. Ames, IA: Blackwell; 2007. p. 5–59.
13. Grainger DA. Uterus, human. In: Knobil E, Neill JD, editors. Encyclopedia of reproduction, vol. 4. San Diego, CA: Academic; 1998. p. 942–50.
14. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. III. Cationic amino acid transporters in the ovine uterus and peri-implantation conceptuses. Biol Reprod. 2009;80(3):602–9.
15. Gao H, Wu G, Spencer TE, Johnson GA, Li X, Bazer FW. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biol Reprod. 2009;80(1):86–93.

16. Hugentobler SA, Diskin MG, Leese HJ, et al. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. Mol Reprod Dev. 2007;74(4):445–54.
17. Leese HJ, Hugentobler SA, Gray SM, et al. Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. Reprod Fertil Dev. 2007;20(1):1–8.
18. Casslen BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. J Reprod Med. 1987;32(3):181–4.
19. Greene J, Feugang J, Pfeiffer K, Stokes J, Bowers S, Ryan P. L-arginine enhances cell proliferation and reduces apoptosis in human endometrial RL95-2 cells. Reprod Biol Endocrinol. 2013;11(1):15.
20. Kim JY, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW. Select nutrients in the ovine uterine lumen. VIII. L-Arginine stimulates proliferation of ovine trophectoderm cells through MTOR-RPS6K-RPS6 signaling cascade and synthesis of nitric oxide and polyamines. Biol Reprod. 2011;84(1):70–8.
21. Kim J-Y, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW. Select nutrients in the ovine uterine lumen. VII. Effects of L-arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration. Biol Reprod. 2011;84(1):62–9.
22. Massmann GA, Zhang J, Figueroa JP. Functional and molecular characterization of nitric oxide synthase in the endometrium and myometrium of pregnant sheep during the last third of gestation. Am J Obstet Gynecol. 1999;181(1):116–25.
23. Kwon H, Wu G, Bazer FW, Spencer TE. Developmental changes in polyamine levels and synthesis in the ovine conceptus. Biol Reprod. 2003;69(5):1626–34.
24. Kwon H, Wu G, Meininger CJ, Bazer FW, Spencer TE. Developmental changes in nitric oxide synthesis in the ovine placenta. Biol Reprod. 2004;70(3):679–86.
25. Wu G, Pond WG, Flynn SP, Ott TL, Bazer FW. Maternal dietary protein deficiency decreases nitric oxide synthase and ornithine decarboxylase activities in placenta and endometrium of pigs during early gestation. J Nutr. 1998;128(12):2395–402.
26. Yu H, Yoo PK, Aguirre CC, et al. Widespread expression of arginase I in mouse tissues: biochemical and physiological implications. J Histochem Cytochem. 2003;51(9):1151–60.
27. Zeng X, Wang F, Fan X, et al. Dietary L-arginine supplementation during early pregnancy enhances embryonic survival in rats. J Nutr. 2008;138(8):1421–5.
28. Telfer JF, Irvine GA, Kohnen G, Campbell S, Cameron IT. Expression of endothelial and inducible nitric oxide synthase in non-pregnant and decidualized human endometrium. Mol Hum Reprod. 1997;3(1):69–75.
29. Cameron IT, Campbell S. Nitric oxide in the endometrium. Hum Reprod Update. 1998;4(5):565–9.
30. Purcell TL, Given R, Chwalisz K, Garfield RE. Nitric oxide synthase distribution during implantation in the mouse. Mol Hum Reprod. 1999;5(5):467–75.
31. Gouge RC, Marshburn P, Gordon BE, Nunley W, Huet-Hudson YM. Nitric oxide as a regulator of embryonic development. Biol Reprod. 1998;58(4):875–9.
32. Manser RC, Leese HJ, Houghton FD. Effect of inhibiting nitric oxide production on mouse preimplantation embryo development and metabolism. Biol Reprod. 2004;71(2):528–33.
33. Zhao Y-C, Chi Y-J, Yu Y-S, et al. Polyamines are essential in embryo implantation: expression and function of polyamine-related genes in mouse uterus during peri-implantation period. Endocrinology. 2008;149(5): 2325–32.
34. Rodriguez-Sallaberry C, Simmen FA, Simmen RCM. Polyamine- and insulin-like growth factor-i-mediated proliferation of porcine uterine endometrial cells: a potential role for spermidine/spermine N1-acetyltransferase during peri-implantation. Biol Reprod. 2001;65(2):587–94.
35. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. V. Nitric oxide synthase, GTP cyclohydrolase, and ornithine decarboxylase in ovine uteri and peri-implantation conceptuses. Biol Reprod. 2009;81(1):67–76.
36. Stewart MD, Johnson GA, Gray CA, et al. Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. Biol Reprod. 2000;62(6):1779–89.
37. Rosenfeld CS. Introduction to comparative placentation. In: Schatten H, Constantinescu GM, editors. Comparative reproductive biology. Ames, IA: Blackwell; 2007. p. 263–70.
38. Regnault TR, Friedman JE, Wilkening RB, Anthony RV, Hay Jr WW. Fetoplacental transport and utilization of amino acids in IUGR—a review. Placenta. 2005;26(Suppl A):S52–62.
39. Grillo M, Lanza A, Colombatto S. Transport of amino acids through the placenta and their role. Amino Acids. 2008;34(4):517–23.
40. Teasdale F, Jean-Jacques G. Morphometric evaluation of the microvillous surface enlargement factor in the human placenta from mid-gestation to term. Placenta. 1985;6(5):375–81.
41. Myatt L. Placental adaptive responses and fetal programming. J Physiol. 2006;572(1):25–30.
42. Ayuk PT-Y, Sibley CP, Donnai P, D'Souza S, Glazier JD. Development and polarization of cationic amino acid transporters and regulators in the human placenta. Am J Physiol: Cell Physiol. 2000;278(6):C1162–71.

43. Furesz TC, Smith CH. Identification of two leucine-sensitive lysine transport activities in human placental basal membrane. Placenta. 1997;18(8):649–55.
44. Ayuk PT-Y, Theophanous D, D'Souza SW, Sibley CP, Glazier JD. L-arginine transport by the microvillous plasma membrane of the syncytiotrophoblast from human placenta in relation to nitric oxide production: effects of gestation, preeclampsia, and intrauterine growth restriction. J Clin Endocrinol Metab. 2002;87(2):747–51.
45. Ishikawa T, Harada T, Koi H, Kubota T, Azuma H, Aso T. Identification of arginase in human placental villi. Placenta. 2007;28(2–3):133–8.
46. Myatt L, Brockman DE, Eis ALW, Pollock JS. Immunohistochemical localization of nitric oxide synthase in the human placenta. Placenta. 1993;14(5):487–95.
47. Swaisgood CM, Zu H-X, Perkins DJ, et al. Coordinate expression of inducible nitric oxide synthase and cyclooxygenase-2 genes in uterine tissues of endotoxin-treated pregnant mice. Am J Obstet Gynecol. 1997;177(5):1253–62.
48. Wu G, Bazer FW, Hu J, Johnson GA, Spencer TE. Polyamine synthesis from proline in the developing porcine placenta. Biol Reprod. 2005;72(4):842–50.
49. Buhimschi I, Yallampalli C, Dong Y-L, Garfield RE. Involvement of a nitric oxide-cyclic guanosine monophosphate pathway in control of human uterine contractility during pregnancy. Am J Obstet Gynecol. 1995;172(5):1577–84.
50. Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. Biochem Pharmacol. 1993;45(2):367–74.
51. Jovanović A, Grbović L, Tulić I. L-Arginine induces relaxation of human uterine artery with both intact and denuded endothelium. Eur J Pharmacol. 1994;256(1):103–7.
52. Taylor HS, Fei X. Emx2 regulates mammalian reproduction by altering endometrial cell proliferation. Mol Endocrinol. 2005;19(11):2839–46.
53. Zhang X, Chen CH, Confino E, Barnes R, Milad M, Kazer RR. Increased endometrial thickness is associated with improved treatment outcome for selected patients undergoing in vitro fertilization-embryo transfer. Fertil Steril. 2005;83(2):336–40.
54. Gonen Y, Casper RF. Prediction of implantation by the sonographic appearance of the endometrium during controlled ovarian stimulation for in vitro fertilization (IVF). J In Vitro Fert Embryo Transf. 1990;7(3):146–52.
55. Sher G, Herbert C, Maassarani G, Jacobs MH. Assessment of the late proliferative phase endometrium by ultrasonography in patients undergoing in-vitro fertilization and embryo transfer (IVF/ET). Hum Reprod. 1991;6(2):232–7.
56. Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. Dev Biol. 2001;240(1):182–93.
57. Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: insights from the sheep. Reproduction. 2004;128(6):657–68.
58. Kim J, Song G, Wu G, Gao H, Johnson GA, Bazer FW. L-Arginine, leucine, and glutamine stimulate proliferation of porcine trophectoderm cells through the MTOR-RPS6K-RPS6-EIF4EBP1 signal transduction pathway. Biol Reprod. 2013;88(5):113, 111–119.
59. Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G. Comparative aspects of implantation. Reproduction. 2009;138(2):195–209.
60. Wang X, Burghardt RC, Romero JJ, Hansen TR, Wu G, Bazer FW. Functional roles of L-arginine during the peri-implantation period of pregnancy. III. L-Arginine stimulates proliferation and interferon tau production by ovine trophectoderm cells via nitric oxide and polyamine-TSC2-MTOR signaling pathways. Biol Reprod. 2015;92(3):75, 71–17.
61. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod. 2001;64(4):1033–40.
62. Zheng J, Wen Y, Austin JL, Chen D-b. Exogenous nitric oxide stimulates cell proliferation via activation of a mitogen-activated protein kinase pathway in ovine fetoplacental artery endothelial cells. Biol Reprod. 2006;74(2):375–82.
63. Frank JW, Escobar J, Nguyen HV, et al. Oral N-carbamylglutamate supplementation increases protein synthesis in skeletal muscle of piglets. J Nutr. 2007;137(2):315–9.
64. Liu XD, Wu X, Yin YL, et al. Effects of dietary L-arginine or N-carbamylglutamate supplementation during late gestation of sows on the miR-15b/16, miR-221/222, VEGFA and eNOS expression in umbilical vein. Amino Acids. 2012;42(6):2111–9.
65. Lefevre PL, Palin MF, Murphy BD. Polyamines on the reproductive landscape. Endocr Rev. 2011;32(5):694–712.
66. Bazer FW, Kim J, Ka H, Johnson GA, Wu G, Song G. Select nutrients in the uterine lumen of sheep and pigs affect conceptus development. J Reprod Dev. 2012;58(2):180–8.
67. Wu G, Bazer FW, Satterfield MC, et al. Impacts of L-arginine nutrition on embryonic and fetal development in mammals. Amino Acids. 2013;45(2):241–56.

68. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37(1):153–68.
69. Krause BJ, Hanson MA, Casanello P. Role of nitric oxide in placental vascular development and function. Placenta. 2011;32(11):797–805.
70. Mateo RD, Wu G, Bazer FW, Park JC, Shinzato I, Kim SW. Dietary L-arginine supplementation enhances the reproductive performance of gilts. J Nutr. 2007;137(3):652–6.
71. Wu G, Bazer FW, Burghardt RC, et al. Impacts of amino acid nutrition on pregnancy outcomes in pigs: mechanisms and implications for swine production. J Anim Sci. 2010;88 Suppl 13:E195–204.
72. Gao K, Jiang Z, Lin Y, et al. Dietary L-arginine supplementation enhances placental growth and reproductive performance in sows. Amino Acids. 2012;42(6):2207–14.
73. de Boo HA, van Zijl PL, Smith DE, Kulik W, Lafeber HN, Harding JE. L-Arginine and mixed amino acids increase protein accretion in the growth-restricted and normal ovine fetus by different mechanisms. Pediatr Res. 2005;58(2):270–7.
74. Zhou W, Gosch G, Guerra T, et al. Amino acid profiles in first trimester amniotic fluids of healthy bovine cloned pregnancies are similar to those of IVF pregnancies, but not nonviable cloned pregnancies. Theriogenology. 2014;81(2):225–9.
75. Zeng X, Mao X, Huang Z, Wang F, Wu G, Qiao S. L-Arginine enhances embryo implantation in rats through PI3K/PKB/mTOR/NO signaling pathway during early pregnancy. Reproduction. 2013;145(1):1–7.
76. Vosatka RJ, Hassoun PM, Harvey-Wilkes KB. Dietary L-arginine prevents fetal growth restriction in rats. Am J Obstet Gynecol. 1998;178(2):242–6.
77. Gui S, Jia J, Niu X, et al. L-Arginine supplementation for improving maternal and neonatal outcomes in hypertensive disorder of pregnancy: a systematic review. J Renin Angiotensin Aldosterone Syst. 2014;15(1):88–96.
78. Zhu Q, Yue X, Tian QY, et al. Effect of L-arginine supplementation on blood pressure in pregnant women: a meta-analysis of placebo-controlled trials. Hypertens Pregnancy. 2013;32(1):32–41.
79. Wu X, Yin YL, Liu YQ, et al. Effect of dietary L-arginine and N-carbamylglutamate supplementation on reproduction and gene expression of eNOS, VEGFA and PlGF1 in placenta in late pregnancy of sows. Anim Reprod Sci. 2012;132(3–4):187–92.
80. Groebner AE, Zakhartchenko V, Bauersachs S, et al. Reduced amino acids in the bovine uterine lumen of cloned versus in vitro fertilized pregnancies prior to implantation. Cell Reprogram. 2011;13(5):403–10.
81. Greene JM, Dunaway CW, Bowers SD, Rude BJ, Feugang JM, Ryan PL. Dietary L-arginine supplementation during gestation in mice enhances reproductive performance and Vegfr2 transcription activity in the fetoplacental unit. J Nutr. 2012;142(3):456–60.
82. Wang X, Johnson GA, Burghardt RC, Wu G, Bazer FW. Uterine histotroph and conceptus development. I. cooperative effects of L-arginine and secreted phosphoprotein 1 on proliferation of ovine trophectoderm cells via activation of the PDK1-Akt/PKB-TSC2-MTORC1 signaling cascade. Biol Reprod. 2015;92(2):51.
83. Abe H, Ishikawa W, Kushima T, et al. Nitric oxide induces vascular endothelial growth factor expression in the rat placenta in vivo and in vitro. Biosci Biotechnol Biochem. 2013;77(5):971–6.
84. Altun ZS, Uysal S, Guner G, Yilmaz O, Posaci C. Effects of oral L-arginine supplementation on blood pressure and asymmetric dimethylarginine in stress-induced preeclamptic rats. Cell Biochem Funct. 2008;26(5):648–53.
85. Dorniak-Wall T, Grivell RM, Dekker GA, Hague W, Dodd JM. The role of L-arginine in the prevention and treatment of pre-eclampsia: a systematic review of randomised trials. J Hum Hypertens. 2014;28(4):230–5.

Chapter 23
Oral L-Arginine Supplementation in Young Males: Endocrinology, Metabolic, and Physiological Responses at Rest and During Exercise

Scott C. Forbes

Key Points

- L-arginine administered intravenously may alter hormones (i.e., growth hormone, insulin, IGF-1), metabolites (i.e., glucose and fat oxidation), and nitric oxide bioavailability.
- L-arginine ingested provides a lower physiological response compared to intravenous administration, which may be associated with absorption efficiency through the gut.
- L-arginine in theory may be beneficial for both aerobic and strength-trained athletes.
- The effect of L-arginine on performance is controversial.
- The hormonal, metabolic, physiological responses to acute L-arginine ingestion in athletes at rest and during exercise are limited, and future research is required.

Keywords L-Arginine • Exercise • Hormone • Growth hormone • Physiology • Nitric oxide • Metabolism

Abbreviations

GH	Growth hormone
IGF-1	Insulin-like growth factor-1
GHRH	Growth hormone–releasing hormone
GHIH	Growth hormone–inhibiting hormone
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Nitrate + nitrite
VO_2	Oxygen uptake

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Introduction

Athletic endeavors push the limits of human performance, and athletes often seek ergogenic aids to gain an edge. A nutritional ergogenic aid is defined as any nutrient capable of enhancing energy utilization, including energy production, control, and efficiency [1]. A nutritional ergogenic aid sought commonly by athletes is protein (or amino acid) supplementation [2–6]. L-arginine is an amino acid that has been purported to be ergogenic and, as such, has become very popular in the food supplement industry [2, 3, 5–8]. Recently, Maughan, Greenhaff, and Hespel [9] noted L-arginine as an emerging and growing trend among athletes.

L-arginine is one of the 20 most common amino acids; it has been shown to be in relatively high (e.g., as much as 16 % of the protein content) concentrations in foods such as watermelon juice, nuts, seeds, algae, meats, seafood, rice protein concentrate, and soy protein isolate [10–12]. A typical North American diet contains approximately 3–6 g day^{-1} of L-arginine [11]. L-arginine can also be synthesized endogenously in the kidney and liver [13] and therefore has traditionally been termed nonessential; however, during periods of rapid growth, in response to a traumatic incident, pathologic insult, or some other type of physiological stressor [14–18], the demand for L-arginine may not be fully met by de novo synthesis and normal dietary intake alone. Since exercise is a physiological stressor, athletes under heavy physical training regimes (catabolic stress) may benefit from dietary L-arginine supplementation [3, 5]. In older adults with cardiovascular diseases, such as heart failure, myocardial infarction, stable angina, and pulmonary hypertension, L-arginine has been shown to enhance exercise performance [8, 16, 19–21]. Despite the popularity of L-arginine as a nutritional supplement in physically active individuals, the underlying physiological mechanisms remain poorly understood. This chapter will (1) review briefly the influence of L-arginine on performance, (2) examine the potential ergogenic mechanisms for L-arginine for aerobic and strength-trained athletes, and (3) review the research on the hormonal, metabolic, and physiological responses to L-arginine supplementation at rest and during aerobic and resistance exercise in young healthy or athletic adults.

L-Arginine Supplementation and Performance

L-arginine supplementation has been effective in improving exercise performance (e.g., aerobic capacity or 6-min walk) in older adults with cardiovascular diseases such as stable angina, congestive heart failure, healed myocardial infarction, and pulmonary hypertension [16, 19–22]. However; L-arginine supplementation on performance in physically active healthy young subjects is limited [2, 23–27]. For example, Santos and colleagues [25] supplemented untrained men with 3 g of L-arginine for 15 days and underwent a test–retest protocol evaluating the resistance to muscular fatigue in the knee extensors using isokinetic dynamometry. This latter study was able to demonstrate a significant increase in the resistance to muscular fatigue, but they did not utilize a double-blinded protocol nor was there a placebo or control group. Conversely, Walberg–Rankin et al. [26] supplemented male weight lifters on a hypocaloric diet with 8 g of L-arginine daily and found no positive influences on muscle function (bicep/quadriceps isokinetic assessment) or body composition compared to a placebo condition. Recently, Alvares et al. [2] provided 6 g of L-arginine 60 min prior to an elbow extension protocol consisting of 3 sets of 10 repetitions and found no effect on peak torque, total work, and set total work. Liu and colleagues [27] also demonstrated no effect of short-term L-arginine supplementation (6 g day^{-1} for 3 days) on indirect measures of nitric oxide (NO) production [nitrate + nitrite (NOx), L-citrulline], metabolic markers, and repeated sprint performance in well-trained judo athletes. Buchman et al. [24] examined the effect of 10 g of L-arginine three times per day for 14 days on marathon performance and found a detrimental effect compared to a predicted time (+23 ± 21 min).

Table 23.1 Oral versus intravenous infusion on growth hormone response

References	Subject age (*y*)	Sex	Fitness/training status	Dosages	Oral/IV	Growth hormone response
Suminski et al. [28]	22.4 ± 0.8	Males and females	Resistance training, 2–3 days/week	1.5 g Arg + 1.5 g Lys	Oral	↑ 2.7-fold at 60 min
Isodori et al. [29]	15–20	Males	"Healthy"	1.2 g Arg + 1.2 g Lys	Oral	↑ Eightfold at 90 min
Lambert et al. [30]	22.6 ± 1.0	Males	Bodybuilders	2.4 g Arg + 2.4 g Lys	Oral	No effect
Merimee et al. [31]	17–35	Males and females	"Healthy"	183 mg Arg/kg	IV	Females ↑, males no change
				367 mg Arg/kg	IV	Females ↑, males ↑
Tanaka et al. [32]	17.2 ± 1.0	Males and females	BMI = 34.7	0.5 g Arg/kg	IV	↑ 13-fold
	25.3 ± 0.9	Males and females	BMI = 35.6	0.5 g Arg/kg	IV	↑ Sevenfold
	50.4 ± 3.4	Males and females	BMI = 35.5	0.5 g Arg/kg	IV	↑ Sixfold
Collier et al. [33]	24.8 ± 1.2	Male	"Healthy"	5 g Arg	Oral	↑
				9 g Arg	Oral	↑
				13 g Arg	Oral	No change
Forbes and Bell [34]	25 ± 5	Male	Recreationally active	0.075 Arg/kg	Oral	No change
				0.15 Arg/kg		No change

Thus, the ergogenic potential of L-arginine is difficult to evaluate because much of the literature is conflicting. Lastly, beyond the scope of this chapter is the potential interaction between other nutrients often contained in supplements on performance (see Tables 23.1 and 23.2).

Potential Ergogenic Mechanisms

The mechanisms by which L-arginine supplementation may play a role in enhancing performance are not fully understood. Intravenous administration of L-arginine stimulates growth hormone secretion from the anterior pituitary in humans [42, 43] primarily due to an inhibition of endogenous growth hormone–inhibiting hormone [44]. In addition, L-arginine is a necessary substrate involved in the detoxification of ammonia, produced during the catabolism of amino acids via the formation of urea [13]. L-arginine is also gluconeogenic because it has the potential to be converted to glucose in the liver. L-arginine may be catabolized to produce energy because it can be converted to alpha-ketoglutarate and enter the citric acid cycle [13]. Furthermore, L-arginine is utilized by a number of metabolic pathways that produce a variety of biologically active compounds such as nitric oxide and creatine [13]. Nitric oxide is an endogenously produced, cellular signaling molecule that is involved in a variety of endothelium-mediated effects in the vasculature. Nitric oxide serves as a second messenger to trigger blood vessel dilation and increase blood flow. L-arginine is the only endogenous nitrogen-containing amino acid substrate of nitric oxide synthase and thus is an important governor of the production of nitric oxide.

Potential ergogenic effects of L-arginine are unique due to the fact that L-arginine may be beneficial for both strength- and aerobically trained athletes (Fig. 23.1). Strength-trained athletes ingest L-arginine in an attempt to stimulate growth hormone secretion, believing that this practice will promote greater gains in muscle mass and strength compared to resistance training alone [4]. Growth hormone stimulates the production of hepatic insulin-like growth factor-1 through the JAK-STAT signaling pathway

Table 23.2 L-arginine containing compounds on performance

References	Subject number and age (*y*)	Fitness/ training status	Dosages	Oral/IV	Performance	Metabolic	Hormonal
Campbell et al. [35]	$n=35$, 38.9 ± 5.8	Resistance trained, ≥1year	6 g L-arginine + 6 g AAKG/day, 8 weeks	Oral	↑ 1RM bench press, Wingate peak power. No effects on body composition, muscular endurance, and aerobic capacity	No effect on lipid profiles, liver enzymes, renal function, electrolytes, calcium, total protein, nitrate/nitrite, agmatine, hematocrit	N/A
Elam et al. [36]	$n=22$	Healthy	1 g L-arginine + 1 g L-ornithine, 5 weeks	Oral	↑ Total strength, lean body mass.	↓ Urinary hydroxyproline	N/A
Colombani et al. [37]	$n=14$, 37 ± 2.5	Endurance trained	15 g L-arginine-L-aspartate, 14 days	Oral	No effect on marathon time	No effect on CHO and fat metabolites, ammonia, LDH, creatine kinase, RER. ↑ urea, L-arginine	No effect on cortisol, insulin. ↑ GH, glucagon
Abel et al. [38]	$n=30$, 38.6 ± 10.0	Endurance trained	High: 5.7 g L-Arg + 8.74 g aspartate. Low: 2.85 g L-Arg + 2.15 g aspartate, 4 weeks	Oral	No effect on time to exhaustion	No effect on VO_2, lactate, urea	No effect on GH, glucagon, cortisol, testosterone
Buford and Koch [39]	$n=10$, 21 ± 1	Physically active, ≥3 days/ week	11.2 g GAKIC	Oral	Less drop in mean power between sprints 1 and 2 compared to placebo	No effect on lactate	N/A
Stevens et al. [40]	$n=13$, 20.9 ± 1.9	Healthy	11.2 g GAKIC	Oral	↑ Muscle torque and work	N/A	N/A
Little et al. [41]	$n=35$, 22.8 ± 2.8	Physically active	0.075 g/kg AAKG + 0.1 g/kg creatine	Oral	↑ Muscular endurance, peak power. No effect on body composition	No effect on lactate	

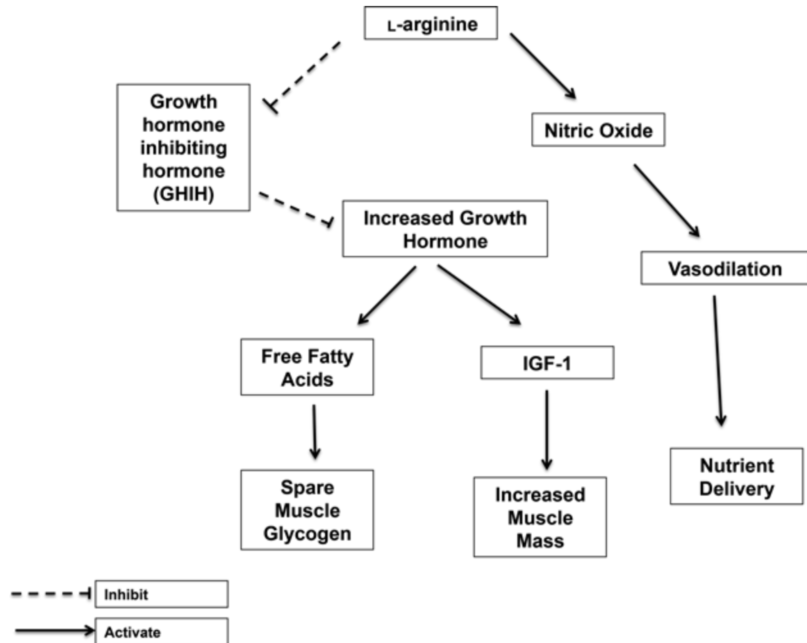

Fig. 23.1 The theory of L-arginine on exercise performance

which is known to enhance muscle protein synthesis. From an aerobic exercise perspective, enhanced L-arginine-induced growth hormone may influence endurance exercise performance by increasing lipolysis and fat oxidation [45]. During submaximal exercise, GH administration increases plasma glycerol and free fatty acids (FFA) in healthy and well-trained endurance athletes. These altered metabolic effects may in turn increase time to exhaustion during exercise by sparing skeletal muscle and/or liver glycogen. Secondly, L-arginine may enhance endurance performance through a nitric oxide-induced vasodilation. There is increasing evidence that interventions that influence nitric oxide bioavailability can alter the O_2 cost of exercise [46–48] and influence blood flow [49, 50], nutrient delivery [51–53], and aid in metabolic waste product removal [54].

Hormonal and Metabolic Responses of L-Arginine Supplementation at Rest

Although it is well known that intravenous infusion of L-arginine at rest stimulates a growth hormone response in clinical and some healthy populations [55, 56], oral supplementation is much more controversial. Oral L-arginine seems to result in a blunted response compared to intravenous infusion, which may be due to the low bioavailability of ingested L-arginine. Infusion with a high dose (e.g., 30 g) has been shown to be a potent secretagogue of both growth hormone and insulin [42]. In fact, intravenous infusion has been used clinically to determine the responsiveness of the growth hormone axis when growth hormone deficiency is suspected [8]. Oral L-arginine supplementation has also demonstrated increases in resting growth hormone in healthy individuals [33], while others have shown no effect [26]. One difficulty in interpreting the effectiveness of L-arginine taken orally has been due to the various dosages utilized, and it would be important to establish an effective dose of L-arginine to elicit a physiological effect [8]. Collier et al. [33] attempted to establish an effective dose for ingestion of oral L-arginine on the GH response. They used a randomized placebo-controlled repeated-measure design in which all the subjects received either a placebo or 5, 9, or 13 g of L-arginine in a double-blind fashion. An increase in the peak growth hormone and

area under the curve was observed with increasing doses of L-arginine up to 9 g. Furthermore, they demonstrated a peak growth hormone response 30 min post-ingestion. Forbes and Bell [34] performed a similar dose response study providing a placebo, low (0.075 g/kg of L-arginine) or a high dose (0.15 g/kg of L-arginine), and found no difference between groups for growth hormone, insulin-like growth factor-1, insulin, and markers of nitric oxide. Importantly, a side effect associated with a high dose of L-arginine is gastrointestinal distress which may not allow athletes to absorb a high enough dose required to achieve a large response. For example, Collier et al. [33] suggested that the highest absolute dose (13 g) used was not absorbed due to an osmotic imbalance that subsequently caused gastrointestinal distress.

Research examining nitric oxide production and blood flow at rest as a result of L-arginine supplementation is equivocal. Intravenous infusion of L-arginine enhanced blood flow in healthy young rats [57] and improved endothelial function in healthy older humans [58] and hypercholesterolemic patients [59]. Further, Vallance et al. [56] demonstrated that infusion of NG monomethyl-L-arginine, a specific inhibitor of nitric oxide synthesis, to healthy participants significantly impaired blood flow. In contrast, Kubota et al. [60] did not show any significant effect on blood flow in healthy young participants, when L-arginine (6 g) was ingested. Forbes and Bell [34], as mentioned earlier, found no effect of increasing oral doses on markers of nitric oxide. In summary, although L-arginine may promote a hormonal and metabolic effect at rest in some studies, the research is inconclusive; these contradictory findings may be related to individual differences and methods of administration.

Hormonal and Metabolic Responses of L-Arginine Supplementation and Strength Exercise

The potential mechanism(s) of L-arginine when combined with resistance exercise has been conflicting. Fahs and colleagues [61] examined the effects of acute L-arginine supplementation and resistance exercise on arterial function and found no significant difference between L-arginine supplementation and placebo for any hemodynamic or vascular response after a resistance training session. Collier and colleagues [62] examined the effects of 7 g of L-arginine ingestion combined with a whole body resistance exercise session, previously shown to stimulate GH secretion [63]. L-arginine alone resulted in a significant increase (twofold) in GH compared to the placebo, while exercise alone stimulated a fivefold increase; however, an attenuated response (threefold increase) was observed when L-arginine and resistance exercise were combined compared to resistance exercise alone. This attenuated effect has been supported in strength-trained athletes using a relative dose of 0.075 g/kg of body weight of L-arginine [44]. Marcell et al. [64] examined the effects of 5 g of L-arginine consumed orally that did not significantly change basal GH concentrations significantly nor did it enhance the GH response compared to resistance exercise alone. Contrary to Collier and colleagues [62], they found no significant difference between the resistance exercise trial alone and when resistance exercise was combined with L-arginine on GH. These studies suggest that L-arginine supplementation may in fact be detrimental to the positive GH response consequent to acute resistance exercise. Collier et al. [62] suggest two potential possibilities for the attenuation of GH. First, there may be a downregulation of GHRH-induced GH release, and secondly there may be an auto-negative feedback induced by elevated IGF-1 prior to the resistance exercise bout suppressing subsequent stimulation of GH (Fig. 23.2). Forbes and colleagues [44] found no statistical difference following resistance exercise for GHRH, GHIH, or ghrelin between the L-arginine and placebo conditions in strength-trained athletes; however, GHRH was blunted by ~28 % in the L-arginine condition.

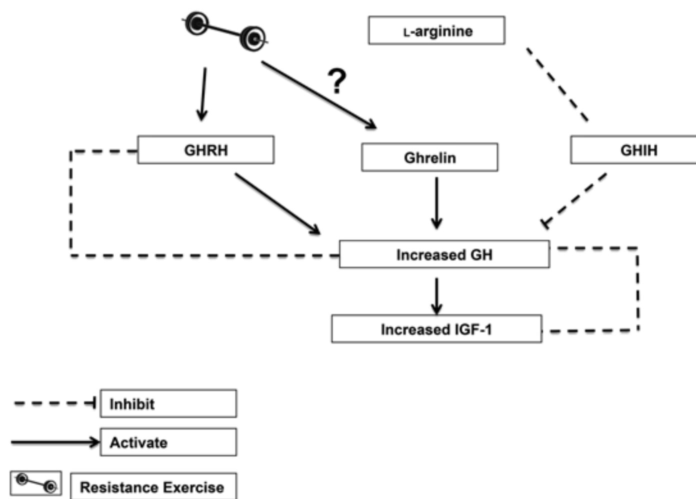

Fig. 23.2 L-arginine prior to exercise may increase IGF-1 and GH which may downregulate subsequent stimuli (i.e., resistance exercise) known to increase growth hormone

Hormonal and Metabolic Effects of L-Arginine Supplementation and Aerobic Exercise

Infusion of L-arginine influences blood pressure, heart rate, and blood flow at rest, while several other studies have shown that L-arginine has little effect on hemodynamics during exercise in human. Schaefer et al. [54] found that L-arginine administered intravenously reduced both lactate and ammonia and increased L-citrulline plasma concentrations during aerobic exercise, perhaps due to an enhanced NO production. In addition, Koppo et al. [60] demonstrated that oral L-arginine enhanced the speed of phase II pulmonary (slow component) VO_2 kinetics by 12 % at the onset of moderate-intensity aerobic exercise. Others have shown no effect on NO production during aerobic exercise following L-arginine ingestion [27, 65, 66]. Recently, Wideman et al. [55] found an enhanced postexercise GH secretion when L-arginine (30 g) was infused intravenously 30 min prior to a submaximal cycling protocol. Enhancing GH may increase gluconeogenesis and enhance lipolysis thereby sparing muscle glycogen. Forbes et al. [67] found no effect of L-arginine prior to submaximal exercise on carbohydrate, respiratory exchange ratio, VO_2, VCO_2, lactate, glucose, free fatty acids, and NOx. There was a small reduction in fat oxidation at the start of exercise, and others have shown an increase in carbohydrate oxidation [53, 66]. This may be associated with NO and/or a blunted GH response.

Conclusion

It is clear that interpreting the effectiveness of L-arginine as an ergogenic aid is difficult due to the different dosages utilized, method of delivery, and population investigated. Wideman et al. [55] have demonstrated elevated GH levels when L-arginine is infused, but since oral ingestion will be the predominate method of delivery for the potential ergogenic benefits in nonclinical populations such as athletes, establishing an effective oral dose of L-arginine is important [8]. Collier and colleagues [33]

examined the dose response effect of L-arginine on GH and observed increased GH levels in response to an absolute dose of L-arginine up to 9 g, but a reduced GH response was noted with a higher absolute dose (13 g). Furthermore, the ergogenic potential of L-arginine may only be realized when the individual is experiencing a particular stress [3]. L-arginine combined with resistance exercise seems to blunt the GH response, suggesting a potential detrimental effect of L-arginine supplementation [44]. With respect to aerobic exercise, Wideman et al. [55] found an enhanced postexercise GH secretion when L-arginine was infused prior to a submaximal cycling protocol. Schaefer et al. [54] infused L-arginine intravenously and demonstrated a reduced lactate and ammonia plasma concentration during an incremental cycling protocol. Others using an oral dose found no effect on the hormonal or metabolic profile [67]. Future research is required to examine the long-term adaptations and may have to examine the interaction between L-arginine and other nutrients.

References

1. Silver MD. Use of ergogenic aids by athletes. J Am Acad Orthop Surg. 2001;9(1):61–70.
2. Alvares TS, Conte CA, Paschoalin VM, et al. Acute L-arginine supplementation increases muscle blood volume but not strength performance. Appl Physiol Nutr Metab. 2012;37(1):115–26.
3. Campbell BI, La Bounty PM, Roberts M. The ergogenic potential of L-arginine. J Int Soc Sports Nutr. 2004;1(2):35–8.
4. Chromiak JA, Antonio J. Use of amino acids as growth hormone-releasing agents by athletes. Nutrition. 2002;18(7–8):657–61.
5. Paddon-Jones D, Borsheim E, Wolfe RR. Potential ergogenic effects of L-arginine and creatine supplementation. J Nutr. 2004;134 Suppl 10:2888S–94S. discussion 2895S.
6. Shao A, Hathcock JN. Risk assessment for the amino acids taurine, L-glutamine and L-arginine. Regul Toxicol Pharmacol. 2008;50(3):376–99.
7. McConell GK. Effects of L-arginine supplementation on exercise metabolism. Curr Opin Clin Nutr Metab Care. 2007;10(1):46–51.
8. Kanaley JA. Growth hormone, L-arginine and exercise. Curr Opin Clin Nutr Metab Care. 2008;11(1):50–4.
9. Maughan RJ, Greenhaff PL, Hespel P. Dietary supplements for athletes: emerging trends and recurring themes. J Sports Sci. 2011;29 Suppl 1:S57–66.
10. Hou Z, Peng H, Ayyanathan K, et al. The LIM protein AJUBA recruits protein L-arginine methyltransferase 5 to mediate SNAIL-dependent transcriptional repression. Mol Cell Biol. 2008;28(10):3198–207.
11. King DE, Mainous 3rd AG, Geesey ME. Variation in L-arginine intake follow demographics and lifestyle factors that may impact cardiovascular disease risk. Nutr Res. 2008;28(1):21–4.
12. Wu G, Bazer FW, Cudd TA, et al. Pharmacokinetics and safety of L-arginine supplementation in animals. J Nutr. 2007;137(6 Suppl 2):1673S–80.
13. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336(Pt 1):1–17.
14. Daly JM, Reynolds J, Thom A, et al. Immune and metabolic effects of L-arginine in the surgical patient. Ann Surg. 1988;208(4):512–23.
15. Saito H, Trocki O, Wang SL, Gonce SJ, Joffe SN, Alexander JW. Metabolic and immune effects of dietary L-arginine supplementation after burn. Arch Surg. 1987;122(7):784–9.
16. Ceremuzynski L, Chamiec T, Herbaczynska-Cedro K. Effect of supplemental oral L-arginine on exercise capacity in patients with stable angina pectoris. Am J Cardiol. 1997;80(3):331–3.
17. Luiking YC, Poeze M, Dejong CH, Ramsay G, Deutz NE. Sepsis: an L-arginine deficiency state? Crit Care Med. 2004;32(10):2135–45.
18. Witte MB, Barbul A. L-Arginine physiology and its implication for wound healing. Wound Repair Regen. 2003;11(6):419–23.
19. Bednarz B, Jaxa-Chamiec T, Gebalska J, Herbaczynska-Cedro K, Ceremuzynski L. L-arginine supplementation prolongs exercise capacity in congestive heart failure. Kardiol Pol. 2004;60(4):348–53.
20. Rector TS, Bank AJ, Mullen KA, et al. Randomized, double-blind, placebo-controlled study of supplemental oral L-arginine in patients with heart failure. Circulation. 1996;93(12):2135–41.
21. Nagaya N, Uematsu M, Oya H, et al. Short-term oral administration of L-arginine improves hemodynamics and exercise capacity in patients with precapillary pulmonary hypertension. Am J Respir Crit Care Med. 2001;163(4):887–91.

22. Tangphao O, Chalon S, Moreno Jr H, Hoffman BB, Blaschke TF. Pharmacokinetics of L-arginine during chronic administration to patients with hypercholesterolaemia. Clin Sci (Lond). 1999;96(2):199–207.
23. Alvares TS, Conte-Junior CA, Silva JT, Paschoalin VM. L-arginine does not improve biochemical and hormonal response in trained runners after 4 weeks of supplementation. Nutr Res. 2014;34(1):31–9.
24. Buchman AL, O'Brien W, Ou CN, et al. The effect of L-arginine or glycine supplementation on gastrointestinal function, muscle injury, serum amino acid concentrations and performance during a marathon run. Int J Sports Med. 1999;20(5):315–21.
25. Santos RS, Pacheco MTT, Martins RABL, Villaverde AB, Giana HE, Baptista F, et al. Study of the effect of oral administration of L-arginine on muscular performance in healthy volunteers: an isokinetic study. Isokinet Exerc Sci. 2002;10:153–8.
26. Walberg-Rankin J, Hawkins CE, Fild DS, Sebolt DR. The effect of oral L-arginine during energy restriction in male weight trainers. J Strength Cond Res. 1994;8:170–7.
27. Liu TH, Wu CL, Chiang CW, Lo YW, Tseng HF, Chang CK. No effect of short-term L-arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. J Nutr Biochem. 2009;20(6):462–8.
28. Suminski RR, Robertson RJ, Goss FL, et al. Acute effect of amino acid ingestion and resistance exercise on plasma growth hormone concentration in young men. Int J Sport Nutr. 1997;7(1):48–60.
29. Isidori A, Lo Monaco A, Cappa M. A study of growth hormone release in man after oral administration of amino acids. Curr Med Res Opin. 1981;7(7):475–81.
30. Lambert LE, French JF, Whitten JP, Baron BM, McDonald IA. Characterization of cell selectivity of two novel inhibitors of nitric oxide synthesis. Eur J Pharmacol. 1992;216(1):131–4.
31. Merimee TJ, Fineberg SE, Tyson JE. Fluctuations of human growth hormone secretion during menstrual cycle: response to L-arginine. Metabolism. 1969;18(7):606–8.
32. Tanaka K, Inoue S, Shiraki J, et al. Age-related decrease in plasma growth hormone: response to growth hormone-releasing hormone, L-arginine, and L-dopa in obesity. Metabolism. 1991;40(12):1257–62.
33. Collier SR, Casey DP, Kanaley JA. Growth hormone responses to varying doses of oral L-arginine. Growth Horm IGF Res. 2005;15(2):136–9.
34. Forbes SC, Bell GJ. The acute effects of a low and high dose of oral L-arginine supplementation in young active males at rest. Appl Physiol Nutr Metab. 2011;36(3):405–11.
35. Campbell B, Roberts M, Kerksick C, et al. Pharmacokinetics, safety, and effects on exercise performance of L-arginine alpha-ketoglutarate in trained adult men. Nutrition. 2006;22(9):872–81.
36. Elam RP, Hardin DH, Sutton RA, Hagen L. Effects of L-arginine and ornithine on strength, lean body mass and urinary hydroxyproline in adult males. J Sports Med Phys Fitness. 1989;29(1):52–6.
37. Colombani PC, Bitzi R, Frey-Rindova P, et al. Chronic L-arginine aspartate supplementation in runners reduces total plasma amino acid level at rest and during a marathon run. Eur J Nutr. 1999;38(6):263–70.
38. Abel T, Knechtle B, Perret C, Eser P, von Arx P, Knecht H. Influence of chronic supplementation of L-arginine aspartate in endurance athletes on performance and substrate metabolism – a randomized, double-blind, placebo-controlled study. Int J Sports Med. 2005;26(5):344–9.
39. Buford BN, Koch AJ. Glycine-arginine-alpha-ketoisocaproic acid improves performance of repeated cycling sprints. Med Sci Sports Exerc. 2004;36(4):583–7.
40. Stevens BR, Godfrey MD, Kaminski TW, Braith RW. High-intensity dynamic human muscle performance enhanced by a metabolic intervention. Med Sci Sports Exerc. 2000;32(12):2102–8.
41. Little JP, Forbes SC, Candow DG, Cornish SM, Chilibeck PD. Creatine, L-arginine alpha-ketoglutarate, amino acids, and medium-chain triglycerides and endurance and performance. Int J Sport Nutr Exerc Metab. 2008;18(5):493–508.
42. Merimee TJ, Rabinowitz D, Riggs L, Burgess JA, Rimoin DL, McKusick VA. Plasma growth hormone after L-arginine infusion. Clinical experiences. N Engl J Med. 1967;276(8):434–9.
43. Hembree WC, Ross GT. L-Arginine infusion and growth-hormone secretion. Lancet. 1969;1(7584):52.
44. Forbes SC, Harber V, Bell GJ. Oral L-arginine prior to resistance exercise blunts growth hormone in strength trained males. Int J Sport Nutr Exerc Metab. 2013;24(2):236–44.
45. Gravholt CH, Schmitz O, Simonsen L, Bulow J, Christiansen JS, Moller N. Effects of a physiological GH pulse on interstitial glycerol in abdominal and femoral adipose tissue. Am J Physiol. 1999;277(5 Pt 1):E848–54.
46. Bailey SJ, Winyard PG, Vanhatalo A, et al. Acute L-arginine supplementation reduces the O_2 cost of moderate-intensity exercise and enhances high-intensity exercise tolerance. J Appl Physiol (Bethesda, Md: 1985). 2010;109(5):1394–403.
47. Koppo K, Taes YE, Pottier A, Boone J, Bouckaert J, Derave W. Dietary L-arginine supplementation speeds pulmonary VO_2 kinetics during cycle exercise. Med Sci Sports Exerc. 2009;41(8):1626–32.
48. Bailey SJ, Winyard PG, Vanhatalo A, et al. Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J Appl Physiol. 2009;107:1144–55.

49. Shen W, Lundborg M, Wang J, et al. Role of EDRF in the regulation of regional blood flow and vascular resistance at rest and during exercise in conscious dogs. J Appl Physiol (Bethesda, Md: 1985). 1994;77(1):165–72.
50. Bode-Boger SM, Boger RH, Creutzig A, et al. L-arginine infusion decreases peripheral arterial resistance and inhibits platelet aggregation in healthy subjects. Clin Sci (Lond). 1994;87(3):303–10.
51. Apostol AT, Tayek JA. A decrease in glucose production is associated with an increase in plasma citrulline response to oral L-arginine in normal volunteers. Metabolism. 2003;52(11):1512–6.
52. Schellong SM, Boger RH, Burchert W, et al. Dose-related effect of intravenous L-arginine on muscular blood flow of the calf in patients with peripheral vascular disease: a $H_2{}^{15}O$ positron emission tomography study. Clin Sci (Lond). 1997;93(2):159–65.
53. McConell GK, Huynh NN, Lee-Young RS, Canny BJ, Wadley GD. L-Arginine infusion increases glucose clearance during prolonged exercise in humans. Am J Physiol Endocrinol Metab. 2006;290(1):E60–6.
54. Schaefer A, Piquard F, Geny B, et al. L-arginine reduces exercise-induced increase in plasma lactate and ammonia. Int J Sports Med. 2002;23(6):403–7.
55. Wideman L, Weltman JY, Patrie JT, et al. Synergy of L-arginine and GHRP-2 stimulation of growth hormone in men and women: modulation by exercise. Am J Physiol Regul Integr Comp Physiol. 2000;279(4):R1467–77.
56. Vallance P, Collier J, Moncada S. Nitric oxide synthesised from L-arginine mediates endothelium dependent dilatation in human veins in vivo. Cardiovasc Res. 1989;23(12):1053–7.
57. Ohta F, Takagi T, Sato H, Ignarro LJ. Low-dose L-arginine administration increases microperfusion of hindlimb muscle without affecting blood pressure in rats. Proc Natl Acad Sci USA. 2007;104(4):1407–11.
58. Bode-Boger SM, Muke J, Surdacki A, Brabant G, Boger RH, Frolich JC. Oral L-arginine improves endothelial function in healthy individuals older than 70 years. Vasc Med. 2003;8(2):77–81.
59. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest. 1992;90(4):1248–53.
60. Kubota T, Imaizumi T, Oyama J, Ando S, Takeshita A. L-arginine increases exercise-induced vasodilation of the forearm in patients with heart failure. Jpn Circ J. 1997;61(6):471–80.
61. Fahs CA, Heffernan KS, Fernhall B. Hemodynamic and vascular response to resistance exercise with L-arginine. Med Sci Sports Exerc. 2009;41(4):773–9.
62. Collier SR, Collins E, Kanaley JA. Oral L-arginine attenuates the growth hormone response to resistance exercise. J Appl Physiol (Bethesda, Md: 1985). 2006;101(3):848–52.
63. Kraemer WJ, Marchitelli L, Gordon SE, et al. Hormonal and growth factor responses to heavy resistance exercise protocols. J Appl Physiol (Bethesda, Md: 1985). 1990;69(4):1442–50.
64. Marcell TJ, Taaffe DR, Hawkins SA, et al. Oral L-arginine does not stimulate basal or augment exercise-induced GH secretion in either young or old adults. J Gerontol A Biol Sci Med Sci. 1999;54(8):M395–9.
65. Bescos R, Gonzalez-Haro C, Pujol P, et al. Effects of dietary L-arginine intake on cardiorespiratory and metabolic adaptation in athletes. Int J Sport Nutr Exerc Metab. 2009;19(4):355–65.
66. Linden KC, Wadley GD, Garnham AP, McConell GK. Effect of L-arginine infusion on glucose disposal during exercise in humans. Med Sci Sports Exerc. 2011;43(9):1626–34.
67. Forbes SC, Harber V, Bell GJ. The acute effects of L-arginine on hormonal and metabolic responses during submaximal exercise in trained cyclists. Int J Sport Nutr Exerc Metab. 2013;23(4):369–77.

Chapter 24
Metabolic Precursors of L-Arginine Supplementation in Sports: A Focus on L-Citrulline and L-Ornithine

Antoni Pons, Raúl Bescós, Antoni Sureda, and Josep A. Tur

Key Points

- Supplementation of L-citrulline is more effective in increasing plasma levels of L-arginine and nitric oxide availability than L-arginine supplementation.
- There is a lack of studies showing that dietary supplementation of L-citrulline improves exercise performance of trained subjects.
- L-Citrulline supplementation has been shown to preserve splanchnic perfusion and to attenuate intestinal injury of subjects performing physical exercise under stressing environmental conditions.
- In addition, there is some evidence indicating that the combination of dietary L-citrulline and malate is effective at increasing nitric oxide metabolites and exercise capacity in healthy humans.
- No strong evidence regarding oral supplementation with L-ornithine is reported in augmenting the exercise-induced growth hormone increase or in altering body composition in athletes. However, oral supplementation with L-ornithine regulates blood ammonia level after an incremental exhaustive cycle ergometer exercise in trained subjects.
- There is evidence that a combination of L-arginine and L-ornithine supplementation increases total strength and lean body mass during a progressive strength-training programme.

Keywords Amino acids • L-Arginine • Exercise • Nitric oxide • Performance • Supplementation

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Abbreviations

ADMA	Asymmetric dimethylarginine
ATP	Adenosine triphosphate
cGMP	Cyclic guanosine monophosphate
GH	Growth hormone
IMP	Inosine monophosphate
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor-binding protein-3
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
NOx	Nitrate and nitrite
NO	Nitric oxide
NOS	Nitric oxide synthases

Introduction

Exercise induces rapid adenosine triphosphate (ATP) consumption in muscles, resulting in energy deficiency, an important factor in fatigue [1]. Moreover, during intense exercise, there is an augmented production of ammonia and inosine monophosphate (IMP) in exercised muscle. The accumulation of ammonia is very toxic for the organism and has to be eliminated in the form of urea through the urea cycle [2]. Thus, the effect of supplementation by L-citrulline and L-ornithine, which are intermediates of the urea cycle and metabolic precursors of L-arginine, on L-arginine availability, ammonia detoxification and nitric oxide (NO) synthesis from L-arginine, a substrate for nitric oxide synthases (NOS), has been investigated (Fig. 24.1). L-Arg is a conditionally essential amino acid during periods of rapid growth, in response to a traumatic or pathologic insult [3, 4], or during exercise [5, 6], and the demand for L-arginine may not be fully met by de novo synthesis and normal dietary intake alone. The demands for L-arginine in physically active subjects are increased with respect to healthy individuals in order to satisfy the increased protein and creatine turnover and urea as well as NO production associated to acute exercise, but the dietary and de novo synthesis of L-arginine also increases L-arginine availability to meet L-arginine demands in active people (Fig. 24.2). The balance of these processes could be modified in function of the intensity, duration, frequency and type of exercise. Plasma levels of L-arginine are used as a marker of the balance between availability and demand of L-arginine in different pathological and physiological situations [7]. There is evidence that acute exercise decreases the plasma levels of L-arginine [8], but there is also evidence that the plasma levels of L-arginine are maintained after acute exercise [7, 9]. Diet supplementation with L-arginine or its metabolic precursors such as L-ornithine and L-citrulline has recently received much interest in sports science.

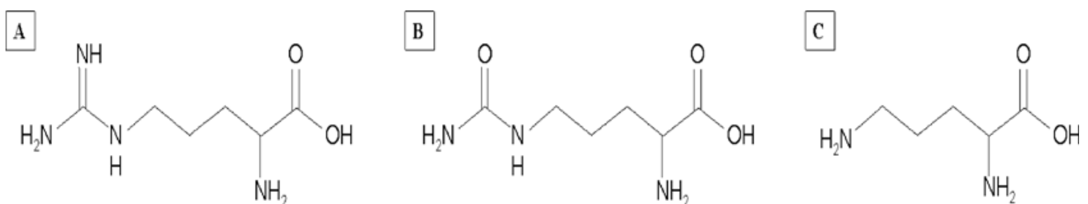

Fig. 24.1 Chemical structure of L-arginine and precursors. Chemical structure of the semi-essential amino acid L-arginine (**a**) and its metabolic precursors L-citrulline (**b**) and L-ornithine (**c**)

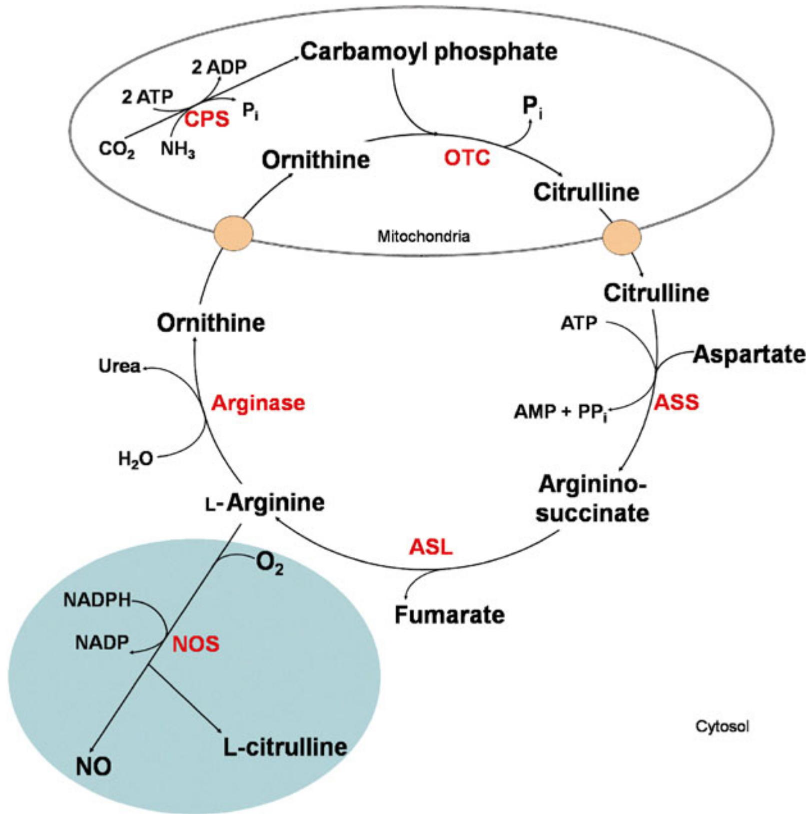

Fig. 24.2 Urea cycle and nitric oxide synthesis. The urea cycle produces urea from ammonia and consists of five reactions, two mitochondrial and three cytosolic. L-Arginine is also a substrate for nitric oxide synthases (NOS) to generate nitric oxide (NO). *CPS* carbamoyl phosphate synthetase, *OTC* ornithine transcarbamylase, *ASS* argininosuccinate synthase, *ASL* argininosuccinate lyase

L-Ornithine Metabolism and Supplementation

L-Ornithine is a free amino acid that is not incorporated into proteins. L-Ornithine is synthesised from L-arginine and metabolised by ornithine aminotransferase to generate α-ketoglutarate, by ornithine decarboxylase to generate polyamines and by ornithine transcarbamylase to form L-citrulline in the urea cycle [10]. As L-ornithine is a central part of the urea cycle, this amino acid is considered to inhibit the increase in blood ammonia level caused by exercise. In addition, L-ornithine promotes growth hormone (GH) release by stimulating the pituitary gland and, consequently, promoting the metabolism of carbohydrates, proteins and lipids [11]. L-Ornithine is contained in significant quantity in *Corbicula japonica* or dried *Lentinula edodes*. However, it is difficult to obtain sufficient amounts of L-ornithine from ordinary meals because it is a free amino acid and not a protein amino acid.

To date there have only been few studies evaluating the effects of L-ornithine as ergogenic aid. The ingestion or intravenous infusion of L-ornithine results in increased levels in serum of this amino acid and in the acceleration of the ammonia metabolism by activation of the urea cycle [12, 13]. In a study performed by Bucci et al. [14], L-ornithine (40, 100 and 170 mg/kg) was administered to bodybuilders reporting a significant increase in amino acid levels in serum (330, 400 and 570 mmol/L, respectively) 45 min after ingestion. Mean serum GH levels tended to rise with L-ornithine ingestion but only rose significantly at 90 min post-administration at the highest dosage. In another study conducted by the same group [15] with a similar experimental procedure, no changes were evidenced in serum insulin

levels at any dose of L-ornithine indicating that L-ornithine is not an insulin secretagogue. In addition, in a placebo-controlled double-blind study during 3-week heavy-resistance training, significant increases were observed in GH and insulin-like growth factor 1 (IGF-1) serum levels after L-ornithine supplementation immediately after the cessation of the exercise protocol and after 1 h of recovery, whereas a significant decrease in insulin-like growth factor-binding protein-3 (IGFBP-3) was reported during the recovery period [16]. No differences were reported in testosterone, cortisol or insulin levels [16]. The authors concluded that the GH/IGF-1/IGFBP-3 complex may be the major player in muscle tissue response to short-term resistance training after L-ornithine supplementation [16].

Demura et al. [17] reported that the blood ammonia level was lower immediately and 15 min after incremental exhaustive ergometer bicycle exercise in 14 healthy young trained male adults ingesting L-ornithine (0.1 g/kg body mass) than in those ingesting a placebo. These results suggest that the metabolism of ammonia produced in skeletal muscle during intense exercise is increased by the ingestion of L-ornithine. Demura and colleagues [18] also evaluated the effect of L-ornithine ingestion on ammonia metabolism and performance after intermittent maximal anaerobic cycle ergometer exercise. In this study, ten healthy young adults with regular training experience ingested L-ornithine hydrochloride (0.1 g/kg body mass) and reported that peak pedalling revolution (rpm) was significantly greater in the supplemented group with respect to the placebo group and serum L-ornithine was also significantly greater with L-ornithine ingestion. However, the improvement reported seemed not to depend on an increase in ammonia metabolism with L-ornithine hydrochloride ingestion. Finally, Elam et al. [19] orally administered combined dosages between 1 and 2 g of each, L-arginine and L-ornithine, to adult men who participated in a 5-week progressive strength-training programme and found that the supplements increased total strength and lean body mass.

L-Citrulline Metabolism and Supplementation

L-Citrulline is a non-essential amino acid under normal physiological conditions, but it is considered semi-essential in situations where intestinal function is compromised. This amino acid is present at low amounts in some food mainly watermelon. However, the main source of L-citrulline is endogenous production. L-Citrulline is synthesised endogenously from glutamine, glutamate and proline in the mitochondria of enterocytes [20]. All the enzymes involved in L-citrulline synthesis are located in the mitochondria of enterocytes. Interestingly, the activity of the two main enzymes that catabolise L-citrulline [argininosuccinate synthase (ASS); argininosuccinate lyase (ASL)] is very low in the intestine, and, consequently, L-citrulline cannot be catabolised in enterocytes. L-Citrulline is released by enterocytes into the portal circulation, bypasses metabolism by periportal hepatocytes and is transported to the kidneys where around 80 % is catabolised to L-arginine by enzymes ASS and ASL. This metabolic pathway generates 5–15 % of the endogenous L-arginine production. In fact, L-citrulline acts as a precursor for the synthesis of L-arginine which is the substrate for NOS. These enzymes catalyse the synthesis of NO from L-arginine and molecular oxygen [21]. In sports science, NO has received much interest because of its function as modulator of blood flow and mitochondrial respiration during physical exercise [22]. Accordingly, L-citrulline has been indicated to be a secondary NO donor in the NOS-dependent pathway.

Therefore, L-citrulline and L-arginine metabolism are closely linked. However, L-citrulline has better absorption and systemic bioavailability than L-arginine. Oral supplementation of L-citrulline is able to increase plasma levels of L-arginine even more than supplementation of the same dose of L-arginine. Consequently, dietary L-citrulline supplementation has been investigated in order to increase nitric oxide (NO) availability. It was reported that single oral doses of 10 g of L-arginine or L-citrulline resulted in maximum plasma concentrations of 300 μmol/L for L-arginine and 2800 μmol/L

for L-citrulline [23]. In patients with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome, L-citrulline supplementation has been shown to be more effective to increase NO production than L-arginine [24]. This fact has increased the interest in L-citrulline which may have a better therapeutic effect than L-arginine.

As regards to the key role played by L-citrulline in NO availability, it has been suggested that a dietary supplementation with this amino acid can help to increase exercise performance. However, there is a lack of studies showing that L-citrulline increases NO markers in healthy subjects.

As for exercise capacity, an initial study in mice found that L-citrulline supplementation resulted effective for reducing blood ammonia and lactate during exhaustive exercise [25]. This fact was related with a significant increase in exercise capacity. However, such results have not been corroborated in humans. To the best of our knowledge, only one study has investigated the effect of L-citrulline on exercise performance in healthy humans. This study did not find any change on plasma levels of NO metabolites, and, in contrast to the hypothesis, exercise performance capacity was impaired when subjects ingested L-citrulline compared with placebo [26].

Other studies have investigated the link between strenuous exercise and intestinal function in regard of L-citrulline supplementation [27]. In regard of this issue, recent experiments in humans have found that L-citrulline supplementation can be effective to preventing an increase in permeability and bacterial translocation during exercise under hot environmental conditions [27]. Prolonged dynamic exercise, particularly when performed in hot environments, shifts the blood flow from the splanchnic and renal vascular beds to the muscular and skin vascular beds. Depending on the amount of the environmental heat stress and the exercise intensity, splanchnic vasoconstriction can result in local ischemia, which can enhance oxidative stress. This fact has been suggested that may disrupt the integrity of the intestinal barrier, allowing the passage of bacteria from the intestine to systemic circulation. Thus, in accordance with this new evidence, L-citrulline supplementation can be an essential compound to preserve intestinal function during exercise in humans.

On the other hand, some experiments investigating the ergogenic effect of L-citrulline have combined this amino acid with other components such as malate which is an intermediate component of the tricarboxylic acid cycle (Table 24.1). An initial study using this combination found a significant increase in the rate of oxidative ATP production during exercise and an increase in the rate of phosphocreatine recovery [28]. However, this study had several weaknesses such as the lack of a placebo group or a blind condition. On the other hand, two other studies conducted by our research group showed an increase in plasma NO metabolites in professional cyclists when they were supplemented with L-citrulline plus malate before a real competition [29, 30]. However, the protocol of our previous studies made not possible to assess exercise performance. Many factors, such as strategy, environmental conditions, nutrition, drafting and breakdown of material, can affect the results during field sport events, limiting the use of these data to make conclusions between dietary interventions and performance. Regarding exercise performance, another experiment in humans found that an acute dose of L-citrulline with malate increased muscle work capacity in healthy humans [31]. However, this experiment had also some weaknesses such as the lack of the analysis of NO metabolites. A recent study reported that L-citrulline with malate (8 g) supplementation improved exercise performance during lower-body multiple-bout resistance exercise in advanced resistance trained men [32]. However, no differences between groups were reported in blood lactate, blood pressure and heart rate. In addition, L-citrulline participates in other metabolic pathways independent of NO that may enhance exercise capacity. For example, L-citrulline can enhance the synthesis of intramuscular creatine. Malate can also be involved in the beneficial effects on energy production because it is an intermediate of tricarboxylic acid cycle [33]. Finally, it is noticeable in a study evidencing that L-citrulline (10 g) co-ingestion with a low quantity of protein was ineffective in augmenting the anabolic properties of protein compared with non-essential amino acids in elderly males (65–80 year) after acute resistance exercise [34].

Table 24.1 Studies investigating the effect of L-citrulline supplementation alone and in combination with other components in healthy humans

Dose/day (g)	Other components	Duration (days)	Sample size	Training status	Performance	Other effects	NO metabolites	References
9		1	17	M	↓ Endurance	↓ Citrulline levels after exercise	?	[26]
10		1	10	M	?	↓ Splanchnic hypoperfusion	?	[27]
6	Malate	1	17	H	?	↓ Plasma amino acids	↑ Nitrite	[29]
6	Malate	1	17	H	?	↑ No effects on oxidative stress	↑ Nitrite	[30]
6	Malate	15	18	U	?	ATP synthesis	?	[33]
6	Malate	1	41	M	↑	Stomach discomfort	?	[31]
8	Malate	1	12	H		↑ Repetitions in leg press, hack squat and leg extension machines	?	[32]
10	Whey protein	1	21	U	?	↑ L-Arginine levels after exercise	?	[34]

Conclusions

Many athletes use specific amino acids to stimulate GH secretion and to promote gains in muscle mass. Exercise per se is a potent stimulus for GH release, and no strong evidence is reported regarding oral supplementation with L-ornithine in augmenting exercise-induced GH increase or in altering body composition in athletes. Moreover, an important point to consider is the fact that consumption of high amounts of amino acids can result in stomach cramping and diarrhoea. At this moment, more studies about the potential effects of L-ornithine in stimulating GH release to promote greater gains in muscle mass and strength and to alter body composition are needed.

Currently, there is no evidence supporting that dietary L-citrulline supplementation enhances exercise performance in healthy humans. However, recent evidence has shown that oral ingestion of L-citrulline can be important in order to preserve the integrity of the intestinal barrier of athletes performing exercise under hot environmental conditions. On the other hand, the addition of malate to L-citrulline has been shown to increase significantly NO markers in well-trained athletes as well as to improve work capacity of skeletal muscle. While this response is totally related with NO synthesis, other metabolic pathways are currently unknown. New and more mechanistic studies are needed to investigate how the addition of certain compounds to L-arginine and L-citrulline can be effective in enhancing exercise performance and health of healthy subjects.

Acknowledgements This work was supported by a grant from the Spanish government CIBEROBN CB12/03/30038 and Balearic Island government (35/2011 and 23/2012) and FEDER funds.

References

1. Sahlin K, Tonkonogi M, Söderlund K. Energy supply and muscle fatigue in humans. Acta Physiol Scand. 1998;162:261–6.
2. Meneguello MO, Mendonça JR, Lancha Jr AH, et al. Effect of L-arginine, ornithine and citrulline supplementation upon performance and metabolism of trained rats. Cell Biochem Funct. 2003;21:85–91.

3. Paddon-Jones D, Børsheim E, Wolfe RR. Potential ergogenic effects of L-arginine and creatine supplementation. J Nutr. 2004;134 Suppl 10:2888S–94. discussion 2895S.
4. Witte MB, Barbul A. L-Arginine physiology and its implication for wound healing. Wound Repair Regen. 2003;11:419–23.
5. Alvares TS, Conte-Junior CA, Silva JT, et al. L-arginine does not improve biochemical and hormonal response in trained runners after 4 weeks of supplementation. Nutr Res. 2014;34(1):31–9.
6. Linden KC1, Wadley GD, Garnham AP, et al. Effect of L-arginine infusion on glucose disposal during exercise in humans. Med Sci Sports Exerc. 2011;43(9):1626–34.
7. Luiking YC, Ten Have GA, Wolfe RR, et al. L-Arginine de novo and nitric oxide production in disease states. Am J Physiol Endocrinol Metab. 2012;303:E1177–89.
8. Reid J, Skelton G, Clark M, et al. Effects of 7 days of L-arginine-alpha-ketoglutarate supplementation using NO2 Platinum on brachial artery blood flow and the levels of plasma L-arginine, nitric oxide, and eNOS after resistance exercise. J Int Soc Sports Nutr. 2010;7 Suppl 1:22.
9. Tang JE, Lysecki PJ, Manolakos JJ, et al. Bolus L-arginine supplementation affects neither muscle blood flow nor muscle protein synthesis in young men at rest or after resistance exercise. J Nutr. 2011;141:195–200.
10. Sugino T, Shirai T, Kajimoto Y, et al. L-ornithine supplementation attenuates physical fatigue in healthy volunteers by modulating lipid and amino acid metabolism. Nutr Res. 2008;28:738–43.
11. Evain-Brion D, Donnadieu M, Roger M, et al. Simultaneous study of somatotrophic and corticotrophic pituitary secretions during ornithine infusion test. Clin Endocrinol. 1982;17:119–22.
12. Tsujino S, Miyamoto T, Kanazawa N. Mitochondrial ornithine transporter deficiency. Nippon Rinsho. 2002;60(4):779–82.
13. Tsujino S, Suzuki T, Azuma T, et al. Hyperornithinemia, hyperammonemia and homocitrullinuria—a case report and study of ornithine metabolism using in vivo deuterium labelling. Clin Chim Acta. 1991;14(201):129–33.
14. Bucci L, Hickson Jr JF, Pivarnik JM, et al. Ornithine ingestion and growth hormone release in bodybuilders. Nutr Res. 1990;10:239–45.
15. Bucci LR, Hickson Jr JF, Wolinsky I, et al. Ornithine supplementation and insulin release in bodybuilders. Int J Sport Nutr. 1992;2(3):287–91.
16. Zajac A, Poprzecki S, Zebrowska A, et al. L-Arginine and ornithine supplementation increases growth hormone and insulin-like growth factor-1 serum levels after heavy-resistance exercise in strength-trained athletes. J Strength Cond Res. 2010;24(4):1082–90.
17. Demura S, Yamada T, Yamaji S, et al. The effect of L-ornithine hydrochloride ingestion on performance during incremental exhaustive ergometer bicycle exercise and ammonia metabolism during and after exercise. Eur J Clin Nutr. 2010;64:1166–71.
18. Demura S, Morishita K, Yamada T, et al. Effect of L-ornithine hydrochloride ingestion on intermittent maximal anaerobic cycle ergometer performance and fatigue recovery after exercise. Eur J Appl Physiol. 2011;111:2837–43.
19. Elam RP, Hardin DH, Sutton RA, et al. Effects of L-arginine and ornithine on strength, lean body mass and urinary hydroxyproline in adult males. J Sports Med. 1989;29:52–6.
20. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
21. Schmidt HH, Nau H, Wittfoht W, et al. L-Arginine is a physiological precursor of endothelium-derived nitric oxide. Eur J Pharmacol. 1988;154:213–6.
22. Schrage WG, Wilkins BW, Johnson CP, et al. Roles of nitric oxide synthase and cyclooxygenase in leg vasodilation and oxygen consumption during prolonged low-intensity exercise in untrained humans. J Appl Physiol (1985). 2010;109(3):768–77.
23. Cynober L. Pharmacokinetics of L-arginine and related amino acids. J Nutr. 2007;37(6 Suppl 2):1646S–9.
24. El-Hattab AW, Hsu JW, Emrick LT, et al. Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and L-arginine supplementation. Mol Genet Metab. 2012;105:607–14.
25. Takeda K, Machida M, Kohara A, et al. Effects of citrulline supplementation on fatigue and exercise performance in mice. J Nutr Sci Vitaminol (Tokyo). 2011;57:246–50.
26. Hickner RC, Tanner CJ, Evans CA, et al. L-citrulline reduces time to exhaustion and insulin response to a graded exercise test. Med Sci Sports Exerc. 2006;38:660–6.
27. van Wijck K, Wijnands KA, Meesters DM, et al. L-citrulline improves splanchnic perfusion and reduces gut injury during exercise. Med Sci Sports Exerc. 2014;46(11):2039–46.
28. Stevens BR, Godfrey MD, Kaminski TW, et al. High-intensity dynamic human muscle performance enhanced by a metabolic intervention. Med Sci Sports Exerc. 2000;32:2102–8.
29. Sureda A, Cordova A, Ferrer MD, et al. L-citrulline-malate influence over branched chain amino acid utilization during exercise. Eur J Appl Physiol. 2010;110:341–51.
30. Sureda A, Cordova A, Ferrer MD, et al. Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise. Free Radic Res. 2009;6:1–8.

31. Perez-Guisado J, Jakeman PM. Citrulline malate enhances athletic anaerobic performance and relieves muscle soreness. J Strength Cond Res. 2010;24:1215–22.
32. Wax B, Kavazis AN, Weldon K, et al. Effects of supplemental citrulline malate ingestion during repeated bouts of lower-body exercise in advanced weight lifters. J Strength Cond Res. 2014; Sep 15 2015;29(3):786-92.
33. Bendahan D, Mattei JP, Ghattas B, et al. Citrulline/malate promotes aerobic energy production in human exercising muscle. Br J Sports Med. 2002;36:282–9.
34. Churchward-Venne TA, Cotie LM, MacDonald MJ, et al. Citrulline does not enhance blood flow, microvascular circulation, or myofibrillar protein synthesis in elderly men at rest or following exercise. Am J Physiol Endocrinol Metab. 2014;307(1):E71–83.

Part V
L-*Arginine* and Diseases of the Gastrointestinal Tract

Chapter 25
L-Arginine and Its Use in Ameliorating *Cryptosporidium parvum* Infection in Undernourished Children

Reinaldo Barreto Oriá, Orleâncio Gomes Ripardo de Azevedo, Theídes Batista Carneiro, Aldo Ângelo Lima, and Richard L. Guerrant

Key Points

- Diarrheal and enteric diseases may have profound effects on children's development.
- *Cryptosporidium parvum* commonly infects undernourished children in endemic areas leading to a prolonged vicious cycle of malnutrition–infection.
- L-arginine ameliorates intestinal barrier function and bacterial translocation in models of intestinal injury in vivo and in vitro.
- Compelling evidence suggests that L-arginine supplementation benefits *C. parvum* infection in undernourished children with NOS and arginase pathway involvement.
- Caution is needed to balance therapy efficacy of citrulline and L-arginine supplementation to children living in endemic areas of *C. parvum* infection and malnutrition.

Keywords *Cryptosporidium* • Diarrhea • L-Arginine • Arginase • Inflammation • Environmental enteropathy

Abbreviations

ADC L-Arginine decarboxylase
AGAT L-Arginine/glycine amidinotransferase
ARG Arginase

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ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
C. parvum	*Cryptosporidium parvum*
NOS	Nitric oxide synthase
L-NAME	*N*-nitro-L-arginine methyl ester
NO	Nitric oxide
NTZ	Nitazoxanide
ORT	Oral rehydration therapy
TLR	Toll-like receptor

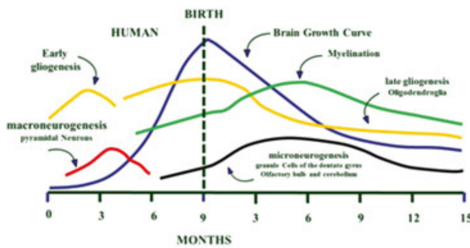
Introduction

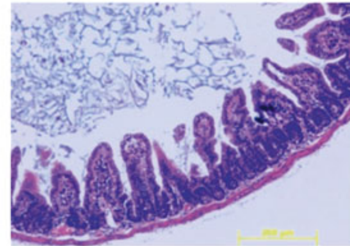
In a recent survey published in *The Lancet*, diarrheal illnesses were among the five leading causes of death, killing 1.3 million people worldwide annually, especially young children [1]. However, overall case numbers have declined considerably compared to earlier surveys. Although the diarrheal disease burden worldwide is declining due to widespread, although still needed oral rehydration therapy (ORT) in endemic areas, its morbidity remains a serious health threat, especially in developing areas among children below 5 years old, where it may lead to a lasting and often neglected disability [2, 3]. These deleterious effects include growth shortfalls, poor educational performance, and various levels of cognitive impairment, overall reducing human capital [4, 5]. Several infections such as cryptosporidiosis in children may cause lasting growth and cognitive impairments even without overt diarrhea [4, 6, 7] and may even favor metabolic diseases over time [8, 9].

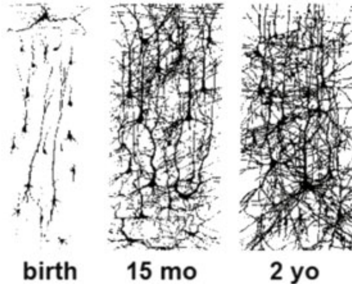
Cryptosporidium parvum is one of the most common waterborne enteric pathogens that can lead to a devastating disease, especially in the immunocompromised host, such as in the undernourished children living in unsanitary and disenfranchised environments. In undernourished children, cryptosporidiosis may cause a severe diarrheal illness accompanied by life-threatening dehydration and weight loss, or it may cause a more smoldering persisting or recurring infection, leading to a vicious cycle of infection and malnutrition [10, 11].

C. parvum is an intracellular protozoan from the extracytoplasmic group of Coccidia, which develops its life cycle in only one host (9). Although various species of *Cryptosporidium* have been identified in humans, *C. parvum* and *C. hominis* represent over 90 % of cases of human cryptosporidiosis [12].

C. parvum is a protozoan parasite which replicates inside intestinal epithelial cells, compromising the intestinal barrier function, triggering great amount of enterocyte's apoptosis in the villus tip [13], with progressive villus atrophy, nutrient malabsorption, and severe diarrhea. Chronic and recurrent infections in children may lead to a state of environmental enteropathy with intestinal inflammation and epithelial disruption, distressing the intestinal barrier function and halting the adequate nutrient supply to the brain in a critical time window of development (Fig. 25.1). Currently, there is no effective treatment or vaccination for cryptosporidiosis, and likely novel and safe therapeutics are warranted to protect afflicted children and safeguard their full cognitive and physical potential. In this chapter, we review the etiology, epidemiology, and disease burden of cryptosporidiosis in undernourished children and the potential therapeutical benefits of L-arginine supplementation against this devastating disease.







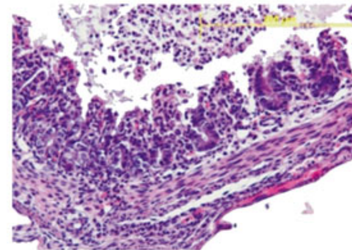


Fig. 25.1 Critical time window of brain development (and only one opportunity) that overlaps with common early enteric infections by *C. parvum*. Early weaning predisposes children to *C. parvum* infections that may disrupt the intestinal barrier function and jeopardize the nutrient supply to the developing brain. *Left panel*: Pre- and postnatal human brain development milestones. Adapted from Morgane et al. [14]. *Right panel*: Representative histology of normal and *C. parvum*-infected mouse ileum

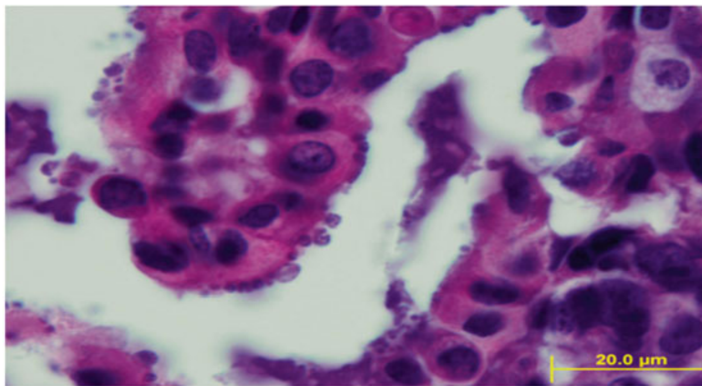
Cryptosporidium parvum Etiology and Infection Cycle

Cryptosporidium is an intracellular protozoon, with zoonotic potential, first described in the early twentieth century by Tyzzer [15]. In 1907, it was described initially in the digestive tract of the laboratory mouse and later in 1955 in turkeys and in 1971 in calves and recognized as a causative agent of gastroenteritis in humans in 1976 [16]. Amidst the main species of *Cryptosporidium* with public health importance, *C. parvum* is the one that commonly infects humans and cattle [17]. *C. parvum*'s taxonomy is as follows: phylum Apicomplexa, subclass Coccidia, order Eucoccidiida, suborder Eimeriina, family Cryptosporidiidae, and genus *Cryptosporidium*. There are different *C. parvum* genotypes (genotype 1, genotype 2, genotype guinea pig, genotype monkey, and Koala); however, genotype 1 is of great importance, since it is responsible for the majority of human infections, while genotype 2 probably is the most widespread in the environment and can also infect animals and humans [18].

C. parvum oocysts are spherical to ovoid and may be detected by immunofluorescence techniques, a feature that aids laboratory diagnostics (Fig. 25.2). Each sporulated oocyst contains four sporozoites. The oocysts are very resistant to chloride and can remain viable for many months in a wet

A.

B.



C.

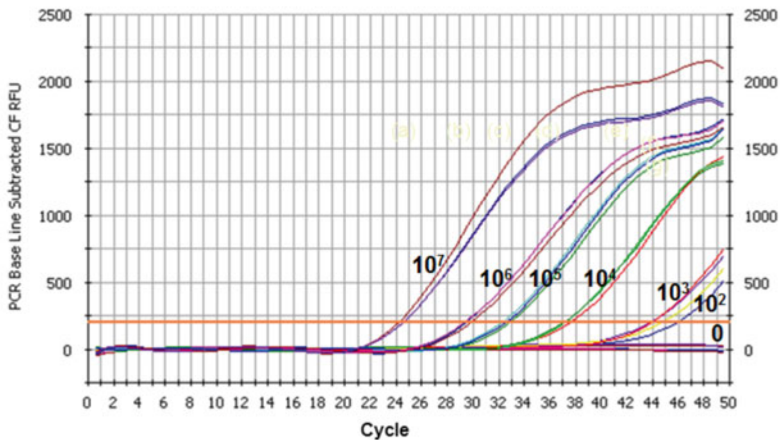


Fig. 25.2 (**a**) Immunofluorescence detection and counting of *C. parvum* oocysts using Merifluor® in stools. (**b**) Hematoxylin–eosin staining of *C. parvum* oocysts in the villus tip. (**c**) Highly sensitive q-RT-PCR for detection of the 18S rRNA of *C. parvum* in the stools [19]

environment; therefore, cryptosporidiosis may be found in swimming waters causing recreational-borne diarrhea [10].

The oocysts (4–5 μm in diameter) are ingested from contaminated food and water. In the intestine, the infecting sporozoites can penetrate the enterocytes by schizogony (asexual cycle), generating merozoites (5–7 μm in diameter). They can undergo schizogonic cycles or switch to gametogony (sexual cycle) leading to the production of oocysts, which are then eliminated in the feces, contaminating the environment (Fig. 25.3). After a series of asexual replications, a small proportion of the parasite differentiates into sexual stages, resulting in the production of micro- and macrogametes that eventually merge to form new zygotes and oocysts.

Cryptosporidiosis Burden in Undernourished Children

Cryptosporidium is one of the most common enteric parasites afflicting humans and domestic animals. Outbreaks of cryptosporidiosis occur frequently around the world, both in developing and developed world, where most of the incidents have mainly been attributed to infection by consumption of contaminated water or infected water of bathers. Outbreaks of cryptosporidiosis in developed countries also occur. One of the largest documented outbreaks occurred in Milwaukee, Wisconsin, in

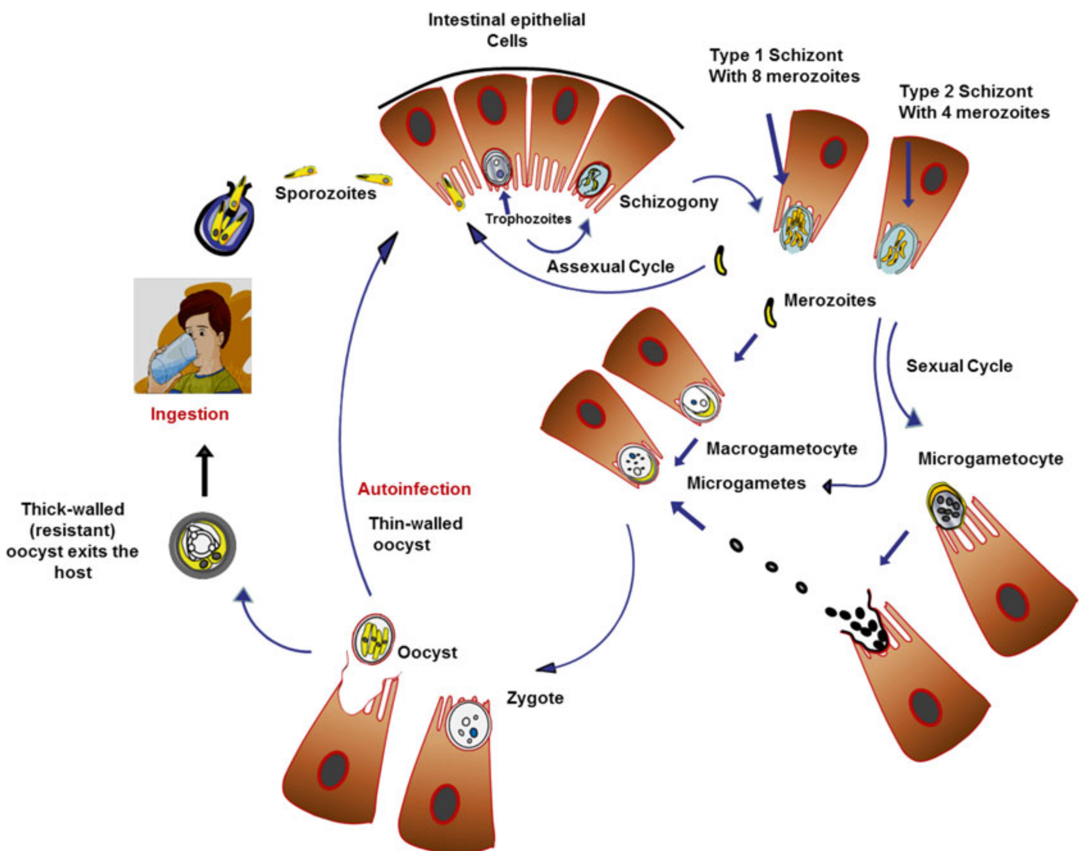


Fig. 25.3 Life cycle of *Cryptosporidium parvum* since acquiring the parasite from contaminated water to intestinal colonization and shedding. Adapted from Smith et al. [20]

1993, due to city water contamination; as a result, 403,000 people suffered ill gastrointestinal-related symptoms and over 100 people died [21].

In the developing world, recurrent *Cryptosporidium* infections are facilitated by crowded households, impoverished living conditions, and unsanitary environments, especially affecting weanling undernourished children exposed to contaminated water. Cryptosporidiosis has been identified as a major cause of recurrent diarrhea in children below 5 years. Ongoing malnutrition in children may aggravate weight loss and dehydration due to *C. parvum* infection, worsening diarrheal illnesses and the intestinal barrier dysfunction. Conversely, cryptosporidiosis aggravates the malnutrition state, debilitating the host and the ability of its immune system to resolve the infection.

In one recent study, when weaned mice were subjected to low-protein diet and then inoculated with a high dose of excysted *C. parvum* oocysts, undernourished mice shed more oocysts than their nourished counterparts and developed a poorer TLR signaling and Th1-mediated immune response. More importantly nitazoxanide (NTZ), ~100–150 mg/kg (one of the few drugs approved for *C. parvum* treatment in humans) was not effective in reducing parasite burden [22]. Data in neonatal mice also demonstrates that early postnatal malnutrition worsens *C. parvum* intestinal infection in C57BL6J mice model of maternal–offspring separation [23]. If this scenario holds in humans, novel and effective interventions are needed to look at the problem to break this self-amplifying cycle of malnutrition–infection [23, 24].

Cryptosporidiosis is likely more severe and prolonged in the undernourished children, increasing the chances to amplify the parasite spread in the environment and further disseminate the disease.

L-Arginine Nutritional and Immunological Effects in Cryptosporidiosis

L-Arginine is considered a conditionally essential amino acid in catabolic states and is critical for growing children due to ever increasing anabolic requirements and protein construction. L-Arginine is a substrate for the synthesis of L-citrulline, which in turn through argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) may back generate L-arginine and nitric oxide (NO) by nitric oxide synthase (NOS) activity. Moreover, L-arginine may form ornithine and polyamines by entering the arginase pathway (Fig. 25.4). Therefore, it is a key constituent of complex immunologic, inflammatory, and wound-healing responses. L-Arginine is also required for the detoxification of ammonia, which is potentially toxic to the central nervous system.

This amino acid is found in approximately 57 % of the amino acid content of a normal adult healthy diet. Nonetheless, early in life, the human breast-milk may not support the high L-arginine need for protein accretion during the burst of growth. In addition, the small intestine poorly produces arginase before weaning. Likewise, the proximal tubule of the kidney where most of the citrulline is converted to L-arginine in the adult has low activity of ASS and ASL in the neonatal period. It has been recognized that most of required L-arginine at this time is produced from proline, and this production relies on a healthy intestinal function [25].

L-Arginine in the body is produced by dietary protein degradation, endogenous synthesis, and protein turnover. L-Arginine may be metabolized via various pathways, including the activity of NOS, arginase (ARG), L-arginine/glycine amidinotransferase (AGAT), and L-arginine decarboxylase (ADC), generating NO, polyamines, proline, glutamate, creatine, and agmatine, all of them with potential biological activities. It is noteworthy by entering the arginase pathway (there are two arginase isoforms: arginase 1, "liver arginase," and arginase 2) that L-arginine can form polyamines (e.g. spermine, spermidine, and putrescine) through the activity of ornithine decarboxylase from the ornithine substrate (Fig. 25.4). Polyamines are recognized to be anti-inflammatory, to promote cell proliferation and to protect the epithelial intestinal barrier [26, 27].

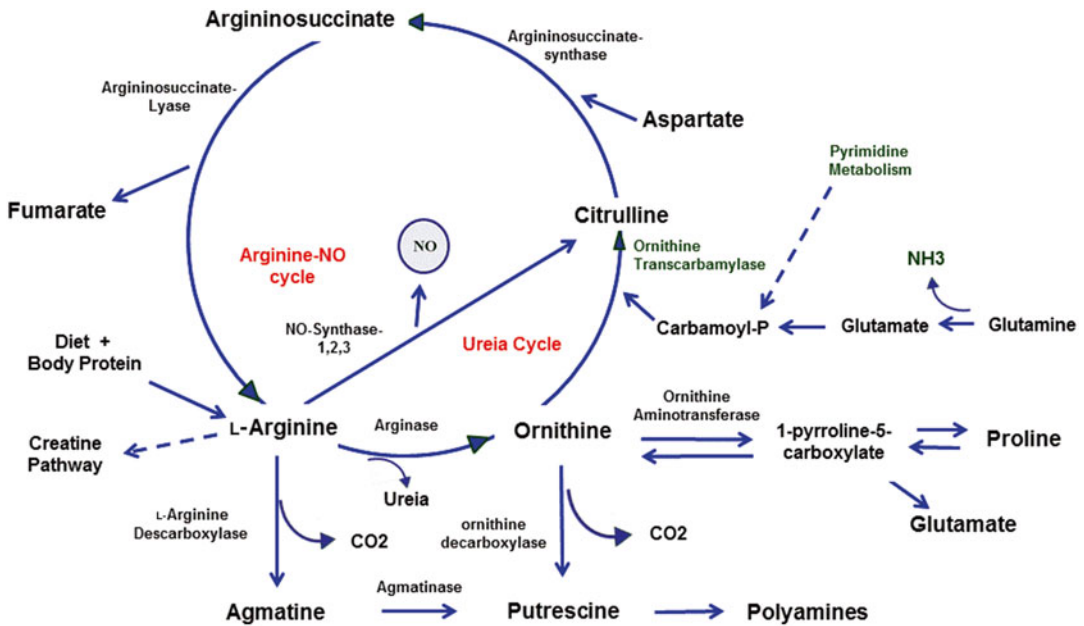


Fig. 25.4 The L-arginine metabolic pathway showing the relationship of L-arginine with the urea cycle and related amino acid biosynthesis and the polyamine biosynthesis pathways (some enzymes and metabolites were omitted)

L-Arginine also plays a role in immune function, since L-arginine is a substrate for NO synthesis by inducible NOS (iNOS or NOS2), which is important to increase thymic weight and to improve lymphocyte proliferation and T-cell receptor expression and to booster NK cell function. In addition, it is also important to B-cell function and development [28]. Thus, L-arginine plays an important role in both innate and acquired immunity. Data show that inadequate intake of dietary L-arginine impairs NO synthesis by both constitutive and inducible NOS in mammals [29], all effects that may compromise *C. parvum* infection control, which is highly dependent on a robust Th1-T-cell-derived immune response [30].

C. parvum infection results in epithelial induction of NOS2 (23); knocking out or pharmacologically inhibiting iNOS activity results in significant increases in epithelial *Cryptosporidium* parasitism and oocyst excretion [31], mainly mediated by lymphocyte induction of iNOS activation by the intestinal epithelium [32], suggesting that nitric oxide has a role against the parasite.

The amino acid L-arginine has been demonstrated in several models of intestinal injury to promote epithelial defense and repair [33]. L-arginine is recognized to improve epithelial tight junction's proteins [34] and to reduce luminal intestinal bacterial translocation following intestinal barrier breakdown [35, 36]. More recently, it has been shown that dietary L-arginine supplementation activates the immune system with increased TLR signaling, secretory IgA, mucins, and defensins in the small intestine of mice with the involvement of the intestinal microbiome [37]. These factors could render the host with a better intestinal adaptation to cope with *C. parvum* infections in endemic areas, therefore reducing (or preventing) the disease burden and its deleterious short- and long-term effects. In addition, dietary L-arginine may be converted by intestinal bacteria to polyamines, which may further improve intestinal barrier function [38].

Cryptosporidiosis compounded with malnutrition is associated with ileal crypt hyperplasia, villus blunting, and mucosal inflammation, altering the small intestine architecture and overall reducing the absorptive surface. L-arginine was able to improve mucosal histology after the infection. *N*-Nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, abrogated these L-arginine-induced

improvements. The infected control mice showed an intense arginase 1 expression, which was even greater with L-NAME treatment. L-arginine decreased the parasite burden, an effect that was reversed by L-NAME. These findings show a protective role of L-arginine during *C. parvum* infection in undernourished mice, with involvement of arginase I and NOS enzymatic actions [39]. Additionally, experimental cryptosporidiosis may induce significant reduction in amino acid absorption even after the parasite clearance with compensatory upregulation of peptide transporters [40]. L-Arginine also has been found to protect against mucosal damage in a murine model of colitis [41].

C. parvum may induce a higher enterocyte expression of arginase I to limit the availability of L-arginine to the iNOS pathway as a survival mechanism. Moreover, arginase plasma levels may increase after tissue injury aggravating even more L-arginine deficiency [29] in undernourished individuals. In clinical settings, in endemic areas of early childhood malnutrition and *C. parvum* infection, caution is needed to define the best L-arginine supplementation strategy, since L-arginine may elicit a myriad of effects due to activation of distinct signaling pathways. There is evidence that L-arginine-derived NO may elicit prostaglandin-dependent secretory diarrhea without improving cryptosporidiosis [42]. The metabolism of arginase and L-arginine-related enzymes in the intestinal milieu (and within the intestinal microbiome), liver, and kidney is a key factor regulating the benefit or not of L-arginine supplementation against cryptosporidiosis, something that deserves further investigation to define the best therapeutic strategy.

L-Arginine given by oral supplementation may be converted to ornithine and urea by the liver. In addition, higher doses of L-arginine may induce increased circulating levels of arginase and therefore further L-arginine breakdown [43, 44]. One way to overcome this problem is by oral citrulline supplementation, since citrulline per se can generate L-arginine (see Fig. 25.4) and oral citrulline is not subjected to liver metabolism nor induces too much arginase activity. Citrulline supplementation has been beneficial in improving intestinal barrier function in models of 5-FU-induced intestinal mucositis and intestinal obstruction [45, 46].

Conclusion

Our experimental findings using a model of malnutrition–infection in suckling mice support the importance of NO-mediated responses against cryptosporidial infections and document the benefit of L-arginine treatment with the involvement of arginase in mucosal healing. More studies are warranted to understand the role of the intestinal microbiome on the biological effects of L-arginine in undernourished children, especially when L-arginine deficiency occurs, as orally given L-arginine may induce polyamine synthesis by the colonic microbiome.

Finally, comparative studies in weanling mice are needed to balance L-arginine versus citrulline supplementation to ameliorate the intestinal barrier function following the compound effect of cryptosporidiosis and malnutrition. Since the malnutrition and infection cycle may jeopardize NTZ efficacy to treat cryptosporidiosis due to impaired immune responses, effective and safe nutritional interventions are needed to boost the innate immune system (without overt inflammation) against the parasite with ameliorated mucosal recovery.

These interventions could have a key role in protecting children in most need by reducing the overall short- and long-term impact of *C. parvum* infection in the developing world where malnutrition is endemic, redirecting their development toward the full genetic potential.

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References

1. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet2015;385:117–171.
2. Lima AA, Guerrant RL. Persistent diarrhea in children: epidemiology, risk factors, pathophysiology, nutritional impact, and management. Epidemiol Rev. 1992;14:222–42.
3. Moore SR, Lima NL, Soares AM, et al. Prolonged episodes of acute diarrhea reduce growth and increase risk of persistent diarrhea in children. Gastroenterology. 2010;139(4):1156–64.
4. Guerrant RL, Oria RB, Moore SR, Oria MO, Lima AA. Malnutrition as an enteric infectious disease with long-term effects on child development. Nutr Rev. 2008;66(9):487–505.
5. Kotloff KL, Nataro JP, Blackwelder WC, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet. 2013;382(9888):209–22.
6. Checkley W, White Jr AC, Jaganath D, et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. Lancet Infect Dis. 2014.
7. Guerrant DI, Moore SR, Lima AA, Patrick PD, Schorling JB, Guerrant RL. Association of early childhood diarrhea and cryptosporidiosis with impaired physical fitness and cognitive function four-seven years later in a poor urban community in northeast Brazil. Am J Trop Med Hyg. 1999;61(5):707–13.
8. Anatskaya OV, Sidorenko NV, Vinogradov AE, Beyer TV. Impact of neonatal cryptosporidial gastroenteritis on epigenetic programming of rat hepatocytes. Cell Biol Int. 2007;31(4):420–7.
9. DeBoer MD, Lima AA, Oria RB, et al. Early childhood growth failure and the developmental origins of adult disease: do enteric infections and malnutrition increase risk for the metabolic syndrome? Nutr Rev. 2012;70(11): 642–53.
10. Dillingham RA, Lima AA, Guerrant RL. Cryptosporidiosis: epidemiology and impact. Microbes Infect. 2002;4(10):1059–66.
11. Agnew DG, Lima AA, Newman RD, et al. Cryptosporidiosis in northeastern Brazilian children: association with increased diarrhea morbidity. J Infect Dis. 1998;177(3):754–60.
12. Bushen OY, Kohli A, Pinkerton RC, et al. Heavy cryptosporidial infections in children in northeast Brazil: comparison of *Cryptosporidium hominis* and *Cryptosporidium parvum*. Trans R Soc Trop Med Hyg. 2007;101(4):378–84.
13. Foster DM, Stauffer SH, Stone MR, Gookin JL. Proteasome inhibition of pathologic shedding of enterocytes to defend barrier function requires X-linked inhibitor of apoptosis protein and nuclear factor kappaB. Gastroenterology. 2012;143(1):133–44.
14. Morgane PJ, Mokler DJ, Galler JR. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci Biobehav Rev. 2002;26(4):471–83.
15. Tyzzer EE. An extracellular coccidium, *Cryptosporidium Muris* (Gen. Et Sp. Nov.), of the gastric glands of the common mouse. J Med Res. 1910;23(3):487–510.
16. Tzipori S, Widmer G. A hundred-year retrospective on cryptosporidiosis. Trends Parasitol. 2008;24(4):184–9.
17. Robinson G, Elwin K, Chalmers RM. Unusual cryptosporidium genotypes in human cases of diarrhea. Emerg Infect Dis. 2008;14(11):1800–2.
18. Caccio SM. Molecular epidemiology of human cryptosporidiosis. Parassitologia. 2005;47(2):185–92.
19. Parr JB, Sevilleja JE, Samie A, et al. Detection and quantification of cryptosporidium in HCT-8 cells and human fecal specimens using real-time polymerase chain reaction. Am J Trop Med Hyg. 2007;76(5):938–42.
20. Smith HV, Nichols RA, Grimason AM. Cryptosporidium excystation and invasion: getting to the guts of the matter. Trends Parasitol. 2005;21(3):133–42.
21. Mac Kenzie WR, Hoxie NJ, Proctor ME, et al. A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. N Engl J Med. 1994;331(3):161–7.
22. Costa LB, JohnBull EA, Reeves JT, et al. Cryptosporidium-malnutrition interactions: mucosal disruption, cytokines, and TLR signaling in a weaned murine model. J Parasitol. 2011;97(6):1113–20.
23. Coutinho BP, Oria RB, Vieira CM, et al. Cryptosporidium infection causes undernutrition, and conversely, weanling undernutrition intensifies infection. J Parasitol. 2008;94(6):1225–32.
24. Costa LB, Noronha FJ, Roche JK, et al. Novel in vitro and in vivo models and potential new therapeutics to break the vicious cycle of cryptosporidium infection and malnutrition. J Infect Dis. 2012;205(9):1464–71.
25. Tomlinson C, Rafii M, Sgro M, Ball RO, Pencharz P. L-Arginine is synthesized from proline, not glutamate, in enterally fed human preterm neonates. Pediatr Res. 2011;69(1):46–50.
26. Gao JH, Guo LJ, Huang ZY, Rao JN, Tang CW. Roles of cellular polyamines in mucosal healing in the gastrointestinal tract. J Physiol Pharmacol. 2013;64(6):681–93.
27. Timmons J, Chang ET, Wang JY, Rao JN. Polyamines and gut mucosal homeostasis. J Gastrointest Dig Syst. 2012;2(Suppl 7).

28. de Jonge WJ, Kwikkers KL, te Velde AA, et al. L-Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice. J Clin Invest. 2002;110(10):1539–48.
29. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37(1):153–68.
30. Chen XM, Keithly JS, Paya CV, LaRusso NF. Cryptosporidiosis. N Engl J Med. 2002;346(22):1723–31.
31. Gookin JL, Chiang S, Allen J, et al. NF-kappaB-mediated expression of iNOS promotes epithelial defense against infection by *Cryptosporidium parvum* in neonatal piglets. Am J Physiol Gastrointest Liver Physiol. 2006;290(1):G164–74.
32. Nordone SK, Gookin JL. Lymphocytes and not IFN-gamma mediate expression of iNOS by intestinal epithelium in murine cryptosporidiosis. Parasitol Res. 2010;106(6):1507–11.
33. Ziegler TR, Evans ME, Fernandez-Estivariz C, Jones DP. Trophic and cytoprotective nutrition for intestinal adaptation, mucosal repair, and barrier function. Annu Rev Nutr. 2003;23:229–61.
34. Beutheu S, Ghouzali I, Galas L, Dechelotte P, Coeffier M. Glutamine and L-arginine improve permeability and tight junction protein expression in methotrexate-treated Caco-2 cells. Clin Nutr. 2013;32(5):863–9.
35. Quirino IE, Cardoso VN, Santos R, et al. The role of L-arginine and inducible nitric oxide synthase in intestinal permeability and bacterial translocation. JPEN J Parenter Enteral Nutr. 2013;37(3):392–400.
36. Costa KA, Soares AD, Wanner SP, et al. L-arginine supplementation prevents increases in intestinal permeability and bacterial translocation in male Swiss mice subjected to physical exercise under environmental heat stress. J Nutr. 2014;144(2):218–23.
37. Ren W, Chen S, Yin J, et al. Dietary L-arginine supplementation of mice alters the microbial population and activates intestinal innate immunity. J Nutr. 2014;144(6):988–95.
38. Kibe R, Kurihara S, Sakai Y, et al. Upregulation of colonic luminal polyamines produced by intestinal microbiota delays senescence in mice. Sci Rep. 2014;4:4548.
39. Castro IC, Oliveira BB, Slowikowski JJ, et al. L-Arginine decreases *Cryptosporidium parvum* infection in undernourished suckling mice involving nitric oxide synthase and arginase. Nutrition. 2012;28(6):678–85.
40. Barbot L, Windsor E, Rome S, et al. Intestinal peptide transporter PepT1 is over-expressed during acute cryptosporidiosis in suckling rats as a result of both malnutrition and experimental parasite infection. Parasitol Res. 2003;89(5):364–70.
41. Gobert AP, Cheng Y, Akhtar M, et al. Protective role of arginase in a mouse model of colitis. J Immunol. 2004;173(3):2109–17.
42. Gookin JL, Foster DM, Coccaro MR, Stauffer SH. Oral delivery of L-arginine stimulates prostaglandin-dependent secretory diarrhea in *Cryptosporidium parvum*-infected neonatal piglets. J Pediatr Gastroenterol Nutr. 2008;46(2):139–46.
43. Curis E, Nicolis I, Moinard C, et al. Almost all about citrulline in mammals. Amino Acids. 2005;29(3):177–205.
44. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and apoptotic cell clearance during pregnancy. Am J Reprod Immunol. 2004;51(4):275–82.
45. Antunes MM, Leocadio PC, Teixeira LG, et al. Pretreatment with L-citrulline positively affects the mucosal architecture and permeability of the small intestine in a murine mucositis model. JPEN J Parenter Enteral Nutr. 2015.
46. Batista MA, Nicoli JR, Martins FS, et al. Pretreatment with citrulline improves gut barrier after intestinal obstruction in mice. JPEN J Parenter Enteral Nutr. 2012;36(1):69–76.

Chapter 26
L-Arginine and Inflammatory Bowel Diseases (IBD)

Wenkai Ren, Gang Liu, Shuai Chen, and Yulong Yin

Key Points

- Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract.
- The pathogenesis of IBD is associated with the individual's genetic susceptibility, external environment, intestinal microbial flora, and immune responses.
- L-Arginine could potentially be useful as therapy for IBD.
- Mechanistically, L-arginine regulates the progression of IBD through oxidative system, intestinal immunity, intestinal microbiota, tight junction proteins, and metabolic products, like nitric oxide and polyamines.

Keywords Inflammatory bowel diseases • L-Arginine • Innate immunity • Nitric oxide • Polyamines

Abbreviations

AAI	L-Arginine availability index
Akt	Protein kinase B
AOPPs	Advanced oxidation protein products
CD	Crohn's disease
DSS	Dextran sulfate sodium
ERK	Extracellular signal-regulated kinase
IBD	Inflammatory bowel diseases
IgA	Immunoglobulin A
IL	Interleukin
JNK	c-Jun *N*-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

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MDA Malondialdehyde
MLCK Myosin light-chain kinase
MLC20 20-kDa myosin regulatory light chain
MPO Myeloperoxidase
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NO Nitric oxide
ODC Ornithine decarboxylase
PI3K Phosphoinositide-3-kinases
SIgA Secretory immunoglobulin A
TJ Tight junction
TLRs Toll-like receptors
TNBS Trinitrobenzene sulfonic acid
TNF-α Tumor necrosis factor alpha
T-SOD Total superoxide dismutase
UC Ulcerative colitis
ZO-1 Zonula occludens protein 1

Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases of the gastrointestinal tract and include Crohn's disease (CD) and ulcerative colitis (UC). Although both are chronically relapsing illnesses, each is distinct in its presentation and course. CD causes transmural inflammation and affects all parts of the gastrointestinal tract, while UC is characterized by mucosal inflammation and is limited to the colon [1]. While the pathogenesis of IBD remains unclear, increasing evidence indicates that alterations in an individual's genetic susceptibility, external environment, intestinal microbial flora, and immune response may all play a role [2–4]. Since IBD is a global healthcare problem that is consistently becoming more prevalent [5], effective therapeutic strategies continue to be investigated. Since an imbalance between pro-inflammatory mediators, i.e., reactive oxygen mediators and cytokines, and anti-inflammatory responses is considered to be a key factor in the development and perpetuation of IBD [6], current treatments usually include anti-inflammatory agents such as immunomodulators and biologic agents [7]. Nutrients, which can control the pro-inflammatory response, have been shown to be beneficial in models of spontaneous and induced colitis [8, 9]. Notably, compelling evidence has indicated that L-arginine might be a good candidate for the low-risk treatment of IBD [9, 10].

This chapter will review recent evidence on the beneficial impact of L-arginine on IBD and will propose some possible mechanisms by which L-arginine regulates the progression of IBD.

L-Arginine and IBD

An interesting study in 2001 reported that while a normal amount of L-arginine in the diet is not harmful for trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats, both the absence of L-arginine and supplementation with high doses of L-arginine may be deleterious for colitis [11]. The author thought that the absence of L-arginine could reduce the nitrogen retention in injured rats, whereas high doses of L-arginine could cause nitric oxide (NO)-mediated tissue damage and collagen deposition [11]. In further studies, serum levels of L-arginine were correlated with the severity of UC, which suggests that a defect in the uptake of L-arginine by cells in the inflamed colon may contribute to the pathogenesis of UC [12, 13]. Subsequent research has demonstrated that L-arginine supplementation could be a potential

therapy for IBD [14]. In this well-designed study, the authors found that L-arginine supplementation enhances various clinical parameters, such as survival rate, body weight, and colon weight, and concurrently decreases indexes of disease severity, including colonic permeability, the number of myeloperoxidase-positive neutrophils, and the expression of pro-inflammatory cytokines and chemokines in dextran sulfate sodium (DSS)-induced colitis [14]. Importantly, a genomic analysis by microarray demonstrated that DSS-treated mice supplemented with L-arginine cluster more closely with mice that have not been exposed to DSS than to those that received DSS alone, indicating L-arginine supplementation normalizes of expression of multiple genes in DSS colitis [14]. The difference between this study and the report in 2001 may be related to the animal model used (DSS in mice vs. TNBS in rats), the duration of supplementation (4 days after 6 days of DSS vs. 2 h after the induction of colitis up to 7 days after TNBS), or the route of supplementation (drinking water vs. gavage) [11, 14]. In a recent publication, the authors found that L-arginine could be a potential therapy for intestinal inflammatory diseases [15]. In this study, authors measured the serum profile of amino acids at days 3, 7, 10, and 12 (5 days after treatment with DSS) in DSS-induced colitis and found that the L-arginine availability index (AAI) after DSS treatment is significantly lower than that in control group at day 12 (Fig. 26.1) [15]. This decrease is associated with indexes of disease activity after DSS treatment, such as neutrophilic infiltration and colon length [15].

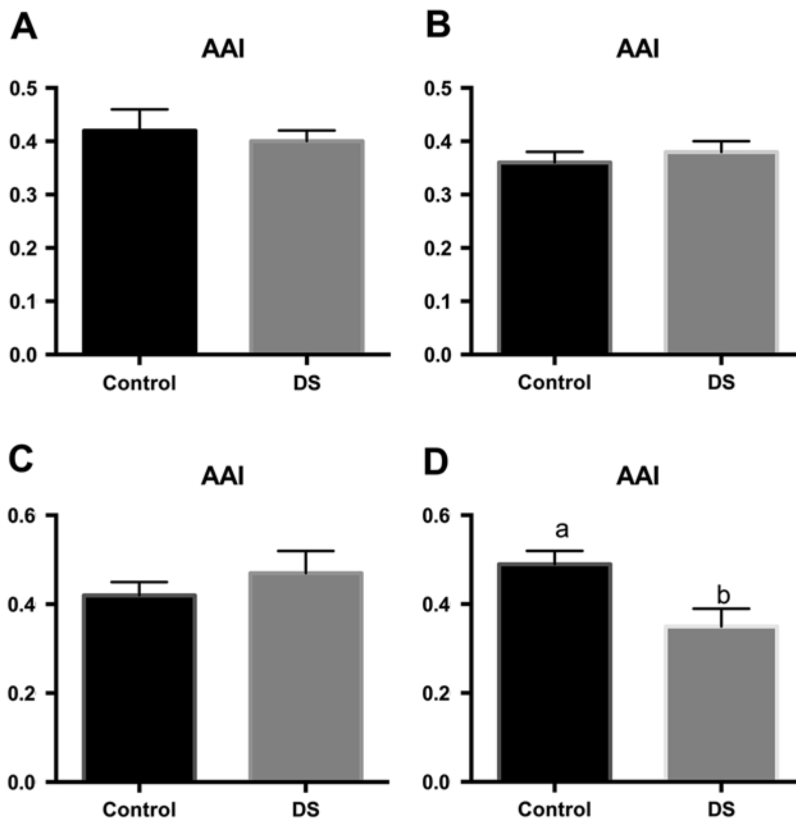

Fig. 26.1 L-Arginine availability index (AAI) after DSS treatment (modified from [15]). (**a**) AAI at 3 days post DSS treatment. (**b**) AAI at 5 days post DSS treatment. (**c**) AAI at 7 days post DSS treatment. (**d**) AAI at 12 days post DSS treatment. Mice are treated with DSS (DS) or normal drinking (control). Data are presented as mean ± SEM, $n=6$, with $a-b$ used to indicate a statistically significant difference ($P<0.05$, Student's t-test). AAI: L-arginine availability index = serum [L-Arg]/([L-Orn] + [L-Lys]). *DSS* dextran sulfate sodium

Consequently, dietary L-arginine supplementation significantly increases the AAI at day 7 and 12 (Fig. 26.2), which led to a longer colon length and less neutrophilic infiltration at day 7 and 12 [15]. Likewise, L-arginine supplementation reverses the decrease in activity of total superoxide dismutase (T-SOD) and expression of interleukin (IL)-17 and tumor necrosis factor alpha (TNF-α) in the colon at day 12 [15]. Mechanically, the beneficial function of L-arginine in DSS-induced colitis is associated with the phosphoinositide-3-kinases (PI3K)/PI3K-protein kinase B (Akt) and the myosin light-chain kinase (MLCK)-myosin light chain (MLC20) pathway in the colon [15].

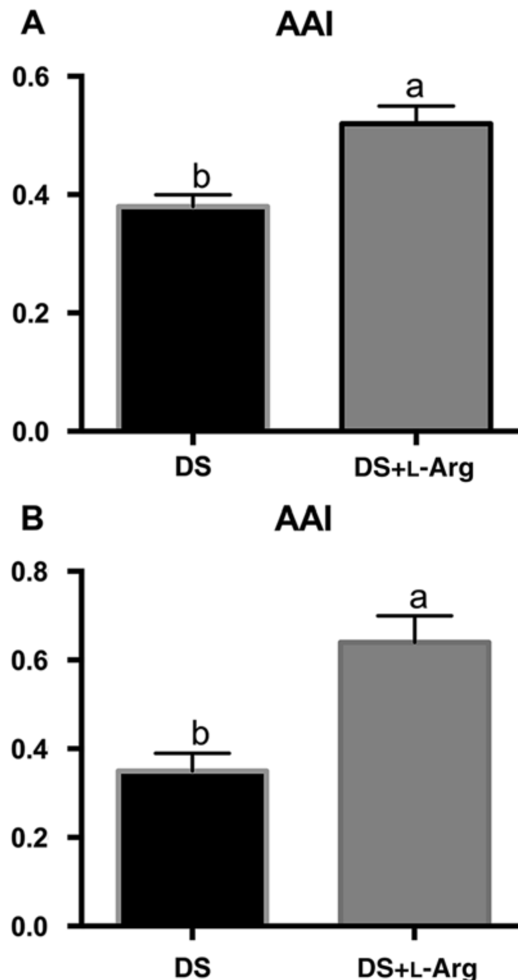

Fig. 26.2 L-Arginine availability index after L-arginine supplementation (modified from [15]). (**a**) AAI at 7 days post DSS treatment. (**b**) AAI at 12 days post DSS treatment. At day 7, serum amino acid profile in mice treated with DSS feeding basal diet (DS) or 1.5 % L-arginine supplementation (DS + Arg) is detected. At day 12, serum amino acid profile in mice treated with DSS feeding basal diet (DS) or 0.4 % L-arginine supplementation (DS + Arg) is detected. Data are presented as mean ± SEM, $n = 6$, with $a - b$ used to indicate a statistically significant difference from control ($P < 0.05$, Student's *t*-test). AAI: L-arginine availability index = serum [L-Arg]/([L-Orn] + [L-Lys]). *DSS* dextran sulfate sodium, *Arg* L-arginine

Mechanisms by Which L-Arginine Regulates IBD

Since the pathogenesis of IBD includes various factors such as intestinal microbial flora and immune responses [2–4], L-arginine could regulate IBD through any of these factors (Fig. 26.3).

L-Arginine May Regulate IBD Through the Oxidative System

Oxidative stress has been suggested to be necessary for the initiation and progression of IBD [2–4]. For example, IBD is associated with a higher content of advanced oxidation protein products (AOPPs), a novel protein marker of oxidative damage [16], with a higher level of malondialdehyde (MDA), an indicator of lipid peroxidation [17], and with a lower level of SOD [15]. It has been widely documented that L-arginine can enhances the anti-oxidative capacity in the body [18, 19]. For instance, dietary L-arginine supplementation significantly increases the SOD activity and total antioxidant capacity in pregnant porcine circovirus-infected mice [18]. Thus, the oxidative system could be a potential target by which L-arginine regulates the pathogenesis of IBD. Indeed, L-arginine supplementation significantly reverses the decrease in T-SOD activity caused by DSS treatment in mice (Fig. 26.4), but has little effect on the catalase or glutathione peroxidase in mice treated with DSS [15].

L-Arginine May Regulate IBD Through Intestinal Immunity

Intestinal immunity, especially innate immunity, is an important factor in the initiation and progression of IBD [2–4]. Innate immunity is initiated by the recognition of microbial antigens through pattern-recognition receptors, including toll-like receptors (TLRs) and NOD-like receptors [20]. Recent studies have found that individuals with IBD clearly show a reduced function of

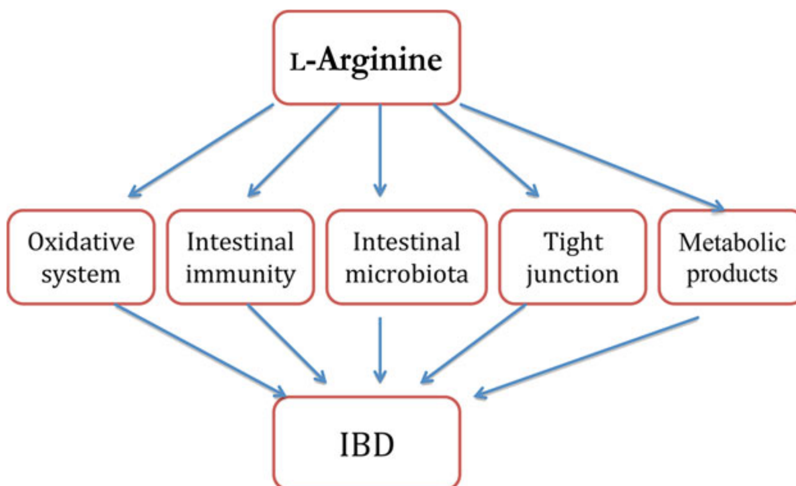

Fig. 26.3 The possible mechanism by which L-arginine regulates the pathogenesis of IBD. L-Arginine affects the pathogenesis of IBD through oxidative system, intestinal immunity, intestinal microbiota, tight junction protein, and even its metabolic products, like NO and polyamines. *IBD* inflammatory bowel diseases; *NO* nitric oxide

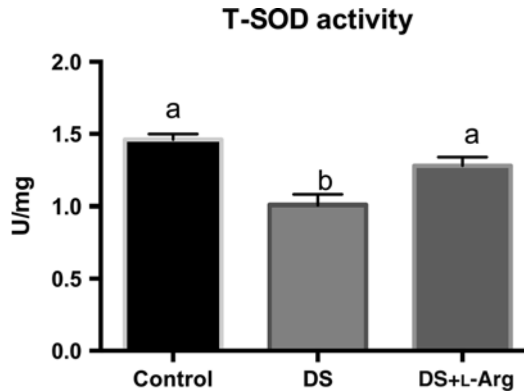

Fig. 26.4 T-SOD activities in each group (modified from [15]). Mice are treated with normal drinking (control), or distilled water containing 5 % (wt/vol) dextran sulfate sodium (DS), or dextran sulfate sodium water with dietary 0.4 % L-arginine supplementation (DS+Arg). Data are presented as mean±SEM, $n=6$, with $a-b$ used to indicate a statistically significant difference ($P<0.05$, one way ANOVA method). *T-SOD* total superoxide dismutase

innate immunity, including altered expression and function of both TLRs and NOD proteins, and defective expression of the mucosal layer and antimicrobial peptides [4]. Dietary L-arginine supplementation has been shown to enhance the intestinal immunity, such as by increasing the numbers of immunoglobulin A (IgA)-secreting cells and decreasing the apoptosis of lymphocytes in Peyer's patches, in pigs challenged by lipopolysaccharide (LPS) [21]. Dietary L-arginine supplementation promotes innate immune activation in mice through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), and PI3K-pAkt signaling pathways [22]. In this study, mice supplemented with 0.4 % L-arginine show a higher expression of TLR signaling, as well as the expression of secretory immunoglobulin A (SIgA), mucins, and Paneth antimicrobials in the jejunum and ileum, compared to controls [22]. Thus, it is not yet known whether L-arginine supplementation regulates the progression of IBD by affecting intestinal innate immunity. Compellingly, L-arginine has been shown to reduce the expression of IL-17 and TNF-α in mice with DSS-induced colitis (Fig. 26.5) [15].

CD was known to be characterized by a Th1 immune response which is induced by IL-12 to produce a high level of IFN-gamma, while UC had been considered to be a Th2-mediated disease which is associated with the release of IL-4, IL-5, and IL-13 [4, 23]. However, recent findings led us to reconsider the Th1/Th2 paradigm in CD and UC, although it is clear that these are adaptive immunity-mediated diseases [23]. The effect of L-arginine on adaptive immunity has been well documented. For example, dietary L-arginine supplementation has an immunostimulatory effect in mice that were immunized with the inactivated *Pasteurella multocida* vaccine [19]. In this study, mice fed diets supplemented with L-arginine have higher levels of antibody titers against the pathogen, resulting in higher protection against the challenge, compared to mice without L-arginine supplementation [19]. Thus, as with the oxidative system, adaptive immunity could also be a potential target by which L-arginine regulates the pathogenesis of IBD. However, there is as yet no direct evidence to support this hypothesis.

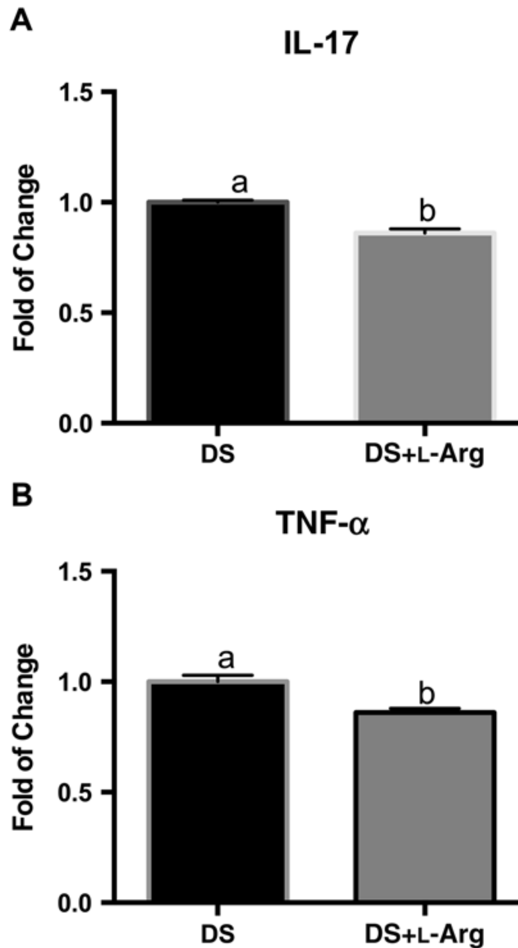

Fig. 26.5 Expression of pro-inflammatory cytokines (modified from [15]). (**a**) mRNA expression of IL-17 at 12 days post treatment with dextran sulfate sodium. (**b**) mRNA expression of TNF-α at 12 days post treatment with dextran sulfate sodium. Mice are treated with distilled water containing 5 % (wt/vol) dextran sulfate sodium (DS) or dextran sulfate sodium water with dietary 0.4 % L-arginine supplementation (DS+Arg). Data are presented as mean ± SEM, $n=6$, with $a-b$ used to indicate a statistically significant difference ($P<0.05$, Student's *t*-test). *IL* interleukin, *TNF* tumor necrosis factor

L-Arginine May Regulate IBD Through the Intestinal Microbiota

The pathogenesis of IBD has been shown to be related to dysbiosis of the gut microbiota including reduced biodiversity, unstable composition, and an altered abundance of components [24–27]. L-arginine supplementation affects the intestinal microenvironment, which leads to a change in the intestinal microbiota composition. For example, L-arginine supplementation regulates the metabolism and utilization of amino acids by small-intestinal bacteria, such as *Escherichia coli* [28], which in turn affects the composition and activity of other microorganisms. Meanwhile, L-arginine supplementation affects feed intake, which results in a change in the intestinal microbiota composition because increased

feed intake augments the abundance of Firmicutes in the gut, while food deprivation reverses the bacterial balance to favor Bacteroides [29, 30]. Likewise, L-arginine supplementation changes the abundance of Bacteroidetes and the abundance of Firmicutes in the mouse jejunum and ileum [22]. Thus, it would be interesting to validate whether L-arginine affects the progression of IBD by manipulating the intestinal microbiota. Interestingly, one study found that the coadministration of NO during exposure to DSS inhibits colonic myeloperoxidase (MPO) activity, colonic permeability, and histological inflammation, indicating that the production of NO from L-arginine by microorganisms in the gut lumen is a possible mechanism by which L-arginine can play a beneficial role in colitis [13].

L-Arginine May Regulate IBD Through Tight Junction (TJ) Proteins

A defective epithelial barrier and increased intestinal permeability have long been observed in IBD patients [31]. Consequently numerous studies have been conducted to identify and characterize the factors that underlie barrier disruption in IBD, and these have highlighted the alterations in epithelial TJ in IBD, such as a reduced number of horizontal TJ strands and an altered TJ protein expression and subcellular distribution [32]. L-arginine has been recognized to have a beneficial effect on TJ. For example, pretreatment of methotrexate-treated Caco-2 cells with L-arginine reverses the decrease in the expression of zonula occludens protein 1 (ZO-1) and occludin and changes in their cellular distribution, through c-Jun *N*-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and NF-κB pathways [33]. Thus, TJ function could be a target by which L-arginine regulates the progression of IBD. Indeed, L-arginine has been shown to enhance the abundance of claudin-1 in a DSS-induced colitis model (Fig. 26.6) [15].

L-Arginine May Regulate IBD Through Metabolic Products

L-Arginine is metabolized by nitric oxide synthase (NOS) to generate NO and citrulline and by arginase to produce ornithine, which is catabolized by ornithine decarboxylase (ODC) for polyamine biosynthesis [34]. Thus, it is possible that L-arginine may regulate the progression of IBD through these metabolic products.

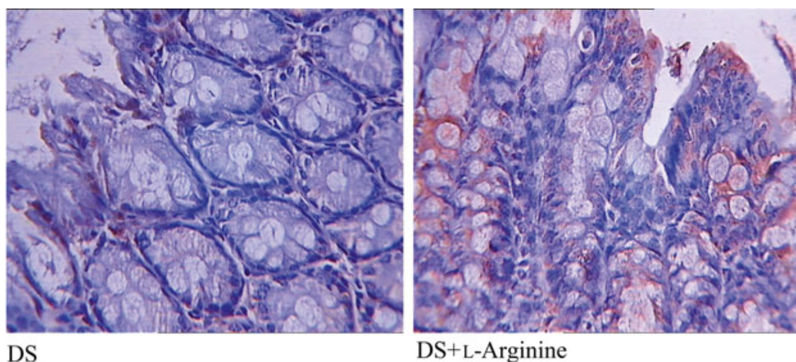

Fig. 26.6 Expression of claudin1 in the colon (modified from [15]). Claudin-1 is analyzed with immunohistochemistry analyses in DSS-induced colitis model at day 7. Mice are treated with distilled water containing dextran sulfate sodium (DS) or dextran sulfate sodium water with dietary 1.5 % L-arginine supplementation (DS + L-Arginine). *DSS* dextran sulfate sodium

Nitric Oxide (NO) and IBD

The regulatory role of this metabolic pathway in IBD is the first and most widely studied. Thus there are several good reviews on this topic [35–37]. In 1993, an important study in Lancet showed that citrulline concentrations are significantly higher in rectal biopsy specimens from patients with active ulcerative colitis than in those from patients with quiescent disease or with normal histology, which suggests that mucosal NO biosynthesis is increased in active colitis and that NO plays a pathogenic role in ulcerative colitis [38]. Subsequently, various investigations have demonstrated that both the expression and activity of inducible NO synthase increase in ulcerative colitis patients [39–43]. Unsurprisingly, NOx generation is increased in the inflamed colonic mucosa of patients with active ulcerative colitis [42]. Interestingly, there is no correlation between this activity and the severity of bowel inflammation in patients with Crohn's disease [39, 41]. Consequently, treatment with a selective inhibitor of iNOS, like aminoguanidine, *N*-[3-(aminomethyl)benzyl]acetamidine, is beneficial for the treatment of experimental colitis in rats [44–48]. Meanwhile, many investigations have also found that treatment with nonselective [1-nitroso-L-arginine methyl ester (L-NAME)] inhibitors alleviates various disease indicators in colitis induced by TNBS [47, 49–51], sulfhydryl alkylator [52]. However, others have found that nonselective NOS inhibition with L-NAME provides no benefit in colitis [45, 53] or even aggravates the course of experimental colitis [54], suggesting that NO has both protective and deleterious effects in colitis [48, 55–57]. Further studies have suggested that pretreatment with L-NAME, which can inhibit constitutive NO synthase, exacerbates the colitis, whereas the delayed administration of L-NAME at the time of inducible NO synthase expression has a beneficial effect in colitis [48, 56, 58].

Polyamines and IBD

Polyamines, including putrescine, spermidine, and spermine, are prerequisites for cellular metabolism and are essential for the proliferation and differentiation of the rapidly renewing intestinal mucosa, indicating its regulatory function in IBD. Indeed, a lack of spermine may aggravate severe ulcerative colitis and chronic DSS colitis [59]. Through the competitive inhibition of iNOS, arginase plays a protective role in a *Citrobacter rodentium* model of colitis by enhancing the generation of polyamines, indicating that the modulation of the arginase-ODC metabolic pathway may represent a new strategy for regulating IBD [60]. However, both the activity and expression of arginase are increased in UC and CD submucosal tissues [61], and this enhanced arginase could lead to the decreased production of NO from NOS, contributing to the pathogenesis of the colonic inflammation in DSS-induced colitis [62]. Thus, the regulatory function of arginase and polyamine in IBD merits further investigation.

Conclusion

Based on current evidence, L-arginine could potentially be useful as therapy for intestinal inflammatory diseases. However, the optimal dosage, route, and duration of L-arginine supplementation in IBD merit further investigation. The metabolic pathway for L-arginine in IBD should also be considered. Other mechanisms by which L-arginine regulates the initiation and progression of IBD continue to be explored.

References

1. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med. 2009;361:2066–78.
2. Danese S, Fiocchi C. Etiopathogenesis of inflammatory bowel diseases. World J Gastroenterol. 2006;12:4807–12.
3. Kugathasan S, Fiocchi C. Progress in basic inflammatory bowel disease research. Semin Pediatr Surg. 2007;16:146–53.
4. Zhang YZ, Li YY. Inflammatory bowel disease: pathogenesis. World J Gastroenterol. 2014;20:91–9.
5. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 2007;448:427–34.
6. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol. 2008;8:458–66.
7. Yamamoto T. Nutrition and diet in inflammatory bowel disease. Curr Opin Gastroenterol. 2013;29:216–21.
8. Rutgeerts P, Vermeire S, Van Assche G. Biological therapies for inflammatory bowel diseases. Gastroenterology. 2009;136:1182–97.
9. Ren WK, Yin J, Zhu XP, Liu G, Li NZ, et al. Glutamine on intestinal inflammation: a mechanistic perspective. Eur J Inflam. 2013;11:315–26.
10. Coeffier M, Marion-Letellier R, Dechelotte P. Potential for amino acids supplementation during inflammatory bowel diseases. Inflamm Bowel Dis. 2010;16:518–24.
11. Mane J, Fernandez-Banares F, Ojanguren I, Castella E, Bertran X, et al. Effect of L-arginine on the course of experimental colitis. Clin Nutr. 2001;20:415–22.
12. Singh K, Coburn LA, Barry DP, Asim M, Scull BP, et al. Deletion of cationic amino acid transporter 2 exacerbates dextran sulfate sodium colitis and leads to an IL-17-predominant T cell response. Am J Physiol Gastrointest Liver Physiol. 2013;305:G225–40.
13. Hong SK, Maltz BE, Coburn LA, Slaughter JC, Chaturvedi R, et al. Increased serum levels of L-arginine in ulcerative colitis and correlation with disease severity. Inflamm Bowel Dis. 2010;16:105–11.
14. Coburn LA, Gong X, Singh K, Asim M, Scull BP, et al. L-arginine supplementation improves responses to injury and inflammation in dextran sulfate sodium colitis. PLoS One. 2012;7:e33546.
15. Ren W, Yin J, Wu M, Liu G, Yang G, et al. Serum amino acids profile and the beneficial effects of L-arginine or L-glutamine supplementation in dextran sulfate sodium colitis. PLoS One. 2014;9:e88335.
16. Xie F, Sun S, Xu A, Zheng S, Xue M, et al. Advanced oxidation protein products induce intestine epithelial cell death through a redox-dependent, c-jun N-terminal kinase and poly (ADP-ribose) polymerase-1-mediated pathway. Cell Death Dis. 2014;5:e1006.
17. Gul M, Kayhan B, Elbe H, Dogan Z, Otlu A. Histological and biochemical effects of dexmedetomidine on Liver during an inflammatory bowel disease. Ultrastruct Pathol. 2013. doi:10.3109/01913123.2013.829150
18. Ren WK, Yin YL, Liu G, Yu XL, Li YH, et al. Effect of dietary L-arginine supplementation on reproductive performance of mice with porcine circovirus type 2 infection. Amino Acids. 2012;42:2089–94.
19. Ren WK, Zou LX, Li NZ, Wang Y, Liu G, et al. Dietary L-arginine supplementation enhances immune responses to inactivated *Pasteurella multocida* vaccination in mice. Br J Nutr. 2013;109:867–72.
20. Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. J Immunol. 2005;174:4453–60.
21. Zhu HL, Liu YL, Xie XL, Huang JJ, Hou YQ. Effect of L-arginine on intestinal mucosal immune barrier function in weaned pigs after *Escherichia coli* LPS challenge. Innate Immun. 2013;19:242–52.
22. Ren W, Chen S, Yin J, Duan J, Li T, et al. Dietary L-arginine supplementation of mice alters the microbial population and activates intestinal innate immunity. J Nutr. 2014;144(6):988–95.
23. Di Sabatino A, Biancheri P, Rovedatti L, MacDonald TT, Corazza GR. New pathogenic paradigms in inflammatory bowel disease. Inflamm Bowel Dis. 2012;18:368–71.
24. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut. 2011;60:631–7.
25. Andoh A, Imaeda H, Aomatsu T, Inatomi O, Bamba S, et al. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. J Gastroenterol. 2011;46:479–86.
26. Martinez C, Antolin M, Santos J, Torrejon A, Casellas F, et al. Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission. Am J Gastroenterol. 2008;103:643–8.
27. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut. 2004;53:685–93.
28. Dai ZL, Li XL, Xi PB, Zhang J, Wu G, et al. Regulatory role for L-arginine in the utilization of amino acids by pig small-intestinal bacteria. Amino Acids. 2012;43:233–44.
29. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, et al. Obesity alters gut microbial ecology. Proc Natl Acad Sci USA. 2005;102:11070–5.

30. Crawford PA, Crowley JR, Sambandam N, Muegge BD, Costello EK, et al. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. Proc Natl Acad Sci USA. 2009;106:11276–81.
31. Salim SY, Soderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. Inflamm Bowel Dis. 2011;17:362–81.
32. Hering NA, Fromm M, Schulzke JD. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. J Physiol. 2012;590:1035–44.
33. Beutheu S, Ghouzali I, Galas L, Dechelotte P, Coeffier M. Glutamine and L-arginine improve permeability and tight junction protein expression in methotrexate-treated Caco-2 cells. Clin Nutr. 2013;32:863–9.
34. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37:1–17.
35. Perner A, Rask-Madsen J. Review article: the potential role of nitric oxide in chronic inflammatory bowel disorders. Aliment Pharmacol Ther. 1999;13:135–44.
36. Kubes P. Inducible nitric oxide synthase: a little bit of good in all of us. Gut. 2000;47:6–9.
37. Cross RK, Wilson KT. Nitric oxide in inflammatory bowel disease. Inflamm Bowel Dis. 2003;9:179–89.
38. Middleton SJ, Shorthouse M, Hunter JO. Increased nitric oxide synthesis in ulcerative colitis. Lancet. 1993;341:465–6.
39. Boughton-Smith NK, Evans SM, Hawkey CJ, Cole AT, Balsitis M, et al. Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. Lancet. 1993;342:338–40.
40. Kimura H, Hokari R, Miura S, Shigematsu T, Hirokawa M, et al. Increased expression of an inducible isoform of nitric oxide synthase and the formation of peroxynitrite in colonic mucosa of patients with active ulcerative colitis. Gut. 1998;42:180–7.
41. Guihot G, Guimbaud R, Bertrand V, Narcy-Lambare B, Couturier D, et al. Inducible nitric oxide synthase activity in colon biopsies from inflammatory areas: correlation with inflammation intensity in patients with ulcerative colitis but not with Crohn's disease. Amino Acids. 2000;18:229–37.
42. Rachmilewitz D, Stamler JS, Bachwich D, Karmeli F, Ackerman Z, et al. Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. Gut. 1995;36:718–23.
43. Godkin AJ, De Belder AJ, Villa L, Wong A, Beesley JE, et al. Expression of nitric oxide synthase in ulcerative colitis. Eur J Clin Invest. 1996;26:867–72.
44. Yamaguchi T, Yoshida N, Ichiishi E, Sugimoto N, Naito Y, et al. Differing effects of two nitric oxide synthase inhibitors on experimental colitis. Hepatogastroenterology. 2001;48:118–22.
45. Kankuri E, Vaali K, Knowles RG, Lahde M, Korpela R, et al. Suppression of acute experimental colitis by a highly selective inducible nitric-oxide synthase inhibitor, N-[3-(aminomethyl)benzyl]acetamidine. J Pharmacol Exp Ther. 2001;298:1128–32.
46. Kankuri E, Hamalainen M, Hukkanen M, Salmenpera P, Kivilaakso E, et al. Suppression of pro-inflammatory cytokine release by selective inhibition of inducible nitric oxide synthase in mucosal explants from patients with ulcerative colitis. Scand J Gastroenterol. 2003;38:186–92.
47. Pilichos CJ, Kouerinis IA, Zografos GC, Korkolis DP, Preza AA, et al. The effect of nitric oxide synthases inhibitors on inflammatory bowel disease in a rat model. In Vivo. 2004;18:513–6.
48. Rumi G, Tsubouchi R, Nishio H, Kato S, Mozsik G, et al. Dual role of endogenous nitric oxide in development of dextran sodium sulfate-induced colitis in rats. J Physiol Pharmacol. 2004;55:823–36.
49. Hogaboam CM, Jacobson K, Collins SM, Blennerhassett MG. The selective beneficial effects of nitric oxide inhibition in experimental colitis. Am J Physiol. 1995;268:G673–84.
50. Rachmilewitz D, Karmeli F, Okon E, Bursztyn M. Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity. Gut. 1995;37:247–55.
51. Neilly PJ, Kirk SJ, Gardiner KR, Anderson NH, Rowlands BJ. Manipulation of the L-arginine-nitric oxide pathway in experimental colitis. Br J Surg. 1995;82:1188–91.
52. Rachmilewitz D, Karmeli F, Okon E. Sulfhydryl blocker-induced rat colonic inflammation is ameliorated by inhibition of nitric oxide synthase. Gastroenterology. 1995;109:98–106.
53. Armstrong AM, Campbell GR, Gannon C, Kirk SJ, Gardiner KR. Oral administration of inducible nitric oxide synthase inhibitors reduces nitric oxide synthesis but has no effect on the severity of experimental colitis. Scand J Gastroenterol. 2000;35:832–8.
54. Dobosz M, Mionskowska L, Dobrowolski S, Dymecki D, Makarewicz W, et al. Is nitric oxide and heparin treatment justified in inflammatory bowel disease? An experimental study. Scand J Clin Lab Invest. 1996;56:657–63.
55. Pfeiffer CJ, Qiu BS. Effects of chronic nitric oxide synthase inhibition on TNB-induced colitis in rats. J Pharm Pharmacol. 1995;47:827–32.
56. Yoshida Y, Iwai A, Itoh K, Tanaka M, Kato S, et al. Role of inducible nitric oxide synthase in dextran sulphate sodium-induced colitis. Aliment Pharmacol Ther. 2000;14 Suppl 1:26–32.
57. Videla S, Vilaseca J, Medina C, Mourelle M, Guarner F, et al. Modulatory effect of nitric oxide on mast cells during induction of dextran sulfate sodium colitis. Dig Dis Sci. 2007;52:45–51.

58. Kiss J, Lamarque D, Delchier JC, Whittle BJ. Time-dependent actions of nitric oxide synthase inhibition on colonic inflammation induced by trinitrobenzene sulphonic acid in rats. Eur J Pharmacol. 1997;336:219–24.
59. Weiss TS, Herfarth H, Obermeier F, Ouart J, Vogl D, et al. Intracellular polyamine levels of intestinal epithelial cells in inflammatory bowel disease. Inflamm Bowel Dis. 2004;10:529–35.
60. Gobert AP, Cheng Y, Akhtar M, Mersey BD, Blumberg DR, et al. Protective role of arginase in a mouse model of colitis. J Immunol. 2004;173:2109–17.
61. Horowitz S, Binion DG, Nelson VM, Kanaa Y, Javadi P, et al. Increased arginase activity and endothelial dysfunction in human inflammatory bowel disease. Am J Physiol Gastrointest Liver Physiol. 2007;292:G1323–36.
62. Akazawa Y, Kubo M, Zhang R, Matsumoto K, Yan F, et al. Inhibition of arginase ameliorates experimental ulcerative colitis in mice. Free Radic Res. 2013;47:137–45.

Chapter 27
Dietary L-Arginine and Intestinal Recovery

Igor Sukhotnik

Key Points

- Arginine and nitric oxide are critical to the normal physiology of the gastrointestinal tract and maintain the mucosal integrity of the intestine in various intestinal disorders.
- Endogenous formation of nitric oxide maintains the mucosal integrity of the intestine and protects the gut from injuries from blood-borne toxins and tissue-destructive mediators.
- Exposure to oral L-arginine has a protective effect in the intestinal mucosa from damage caused by lipopolysaccharide endotoxemia in a rodent model.
- Dietary L-arginine did not protect the intestinal mucosa from damage caused by ischemia-reperfusion (IR); however exposure to oral L-arginine significantly enhanced intestinal recovery following an IR event.
- Dietary L-arginine protects the intestinal mucosa from damage caused by methotrexate.

Keywords L-Arginine • Intestine • Inflammatory processes • Intestinal ischemia-reperfusion • Short bowel syndrome • Chemotherapy-induced mucositis • Intestinal cell turnover • Cell proliferation • Cell apoptosis • Intestinal regrowth • Intestinal recovery • Intestinal mucosa • Nitric oxide

Abbreviations

NO	Nitric oxide
NOS	Nitric oxide synthase
LPS	Lipopolysaccharide
IR	Ischemia-reperfusion
TNF	Tumor necrosis factor
IL	Interleukin
MTX	Methotrexate
ARG	L-Arginine

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Introduction

L-Arginine: (2-amino-5-guanidinovaleric acid) is a nonessential amino acid which is metabolically processed by the urea cycle and is one of the most versatile amino acids in animal cells [1, 2]. It was first isolated from lupin seedlings in 1886 and subsequently was found to be a major amino acid in the basic proteins of many mammals' cells and tissues. Physiological and nutritional studies during the last 70 years started a new area of L-arginine research. It has been shown that L-arginine is required for the synthesis of nitric oxide, polyamines, proline, glutamate, and creatinine [1]. L-Arginine was classified as a dispensable (nonessential) amino acid for healthy adult humans and as an essential amino acid for young, growing mammals (Fig. 27.1) [3].

L-Arginine was shown to influence metabolism in mammalian cells directly or through stimulation of the secretion of hormones such as insulin, growth hormone, glucagon, and prolactin. In the last two decades, L-arginine has attracted major interest since it has been identified as the natural substrate of nitric oxide and is now recognized to play a major role in many regulation processes. L-Arginine is converted to nitric oxide and citrulline by the enzyme nitric oxide synthase (NOS) [4]. Nitric oxide (NO) is an important molecule involved in neurotransmission, vascular homeostasis, immune regulation, and host defense [5]. There is growing interest in the potential roles of the L-arginine and NO as regulators of cell proliferation and apoptosis in general and in the gastrointestinal tract in particular. NO has been shown to promote apoptosis in some cells, whereas it inhibits apoptosis in other cells depending on the amount, duration, and the site of NO production, and kind of target cells [6].

L-Arginine and Intestine

The intestinal epithelium is a highly dynamic tissue in which cellular turnover can be completed in a matter of a few days [7]. The dynamic process of epithelial cell turnover is a function of the rates of crypt cell proliferation, migration along the small bowel crypt-villus axis, differentiation, and cell

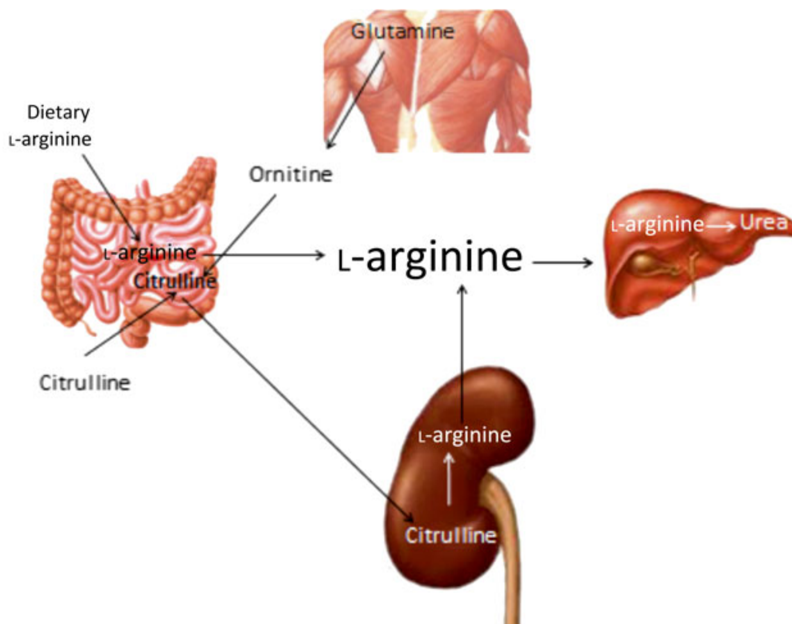

Fig. 27.1 Metabolic pathways of L-arginine

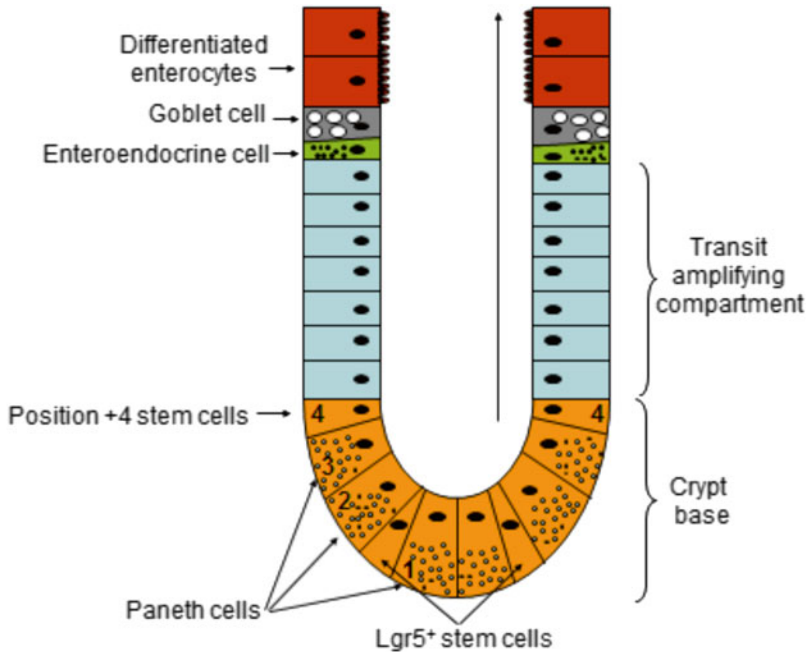

Fig. 27.2 Proliferation and differentiation of intestinal stem cells

death via apoptosis. This process may be affected by nutritional status, the route of feeding, and the adequacy of specific nutrients in the diet. Under normal circumstance, newly formed immature intestinal cells divide in the crypts of Lieberkuhn (fingerlike invaginations of the epithelium into the underlying connective tissue) and migrate up to the villus tip, differentiate, mature, and become functional, with an important role in absorption of nutrients. The renewal process operates continually, with cells taking two to seven days to make the journey from the site of their final division cycle in the crypt to the point of their exfoliation from the villus tip [8]. Intestinal stem cells reside near the base of the crypt, providing immature progeny, which continue to divide as they migrate up the crypt until they differentiate into either intestinal absorptive cells (enterocytes) or secretory lineages (Fig. 27.2) (goblet, Paneth, and enteroendocrine cells).

Absorptive enterocytes, the primary cell type of the epithelial layer, comprise about 90 % of the cells in the entire epithelium and are responsible for the terminal digestion and absorption of luminal nutrients. The goblet cells, located throughout the epithelial layer, secrete mucus that helps protect the epithelial layer from digestion, as well as trefoil factor-3, which is ultimately involved in post-injury repair of the small bowel. The enteroendocrine cells comprise only 1 % of the intestinal epithelium and are a part of the enteric endocrine system sense the luminal environment and secrete hormones such as cholecystokinin and gastrin into blood. The Paneth cells reside at the base of the crypt and are classically believed to have an antimicrobial function in the small intestine (production of lysozyme, an enzyme that destroys bacteria. The adjacent location of these cells to the intestinal stem cell zone and recent research demonstrating their secretion of cytokines, growth factors, and other products, including soluble Wnt proteins known to regulate proliferation, raises the possibility of a potential role for Paneth cells initiating and maintaining the adaptive response [9].

L-Arginine and NO are critical to normal physiology of the gastrointestinal tract. The importance of L-arginine signaling in the intestine, as applied to all species and developmental ages, was reviewed in detail by Rhoads and Wu [10]. However, supplemental L-arginine in neonatal piglets has long been known to be beneficial in intestinal integrity and function because it is an essential substrate for

synthesis of nitric oxide, a major nonadrenergic, noncholinergic vasodilator. More recently, supplemental L-arginine before a razor wound injury in neonatal piglet intestinal IPEC-J2 cells was shown to increase cell migration in a dose-dependent biphasic manner via NO and focal adhesion kinase-dependent mechanisms [11, 12]. Additionally, it was shown that L-arginine-dependent migration required synthesis of polyamines and that L-arginine is a major amino acid precursor of polyamines essential in gastrointestinal repair [11, 12]. The increased NO production and increased enterocyte migration are important mechanisms after mucosal injury for rapid restoration of epithelial continuity over the denuded villus surface area and may be mechanistically related to previous work demonstrating attenuation of necrotizing enterocolitis in newborn pigs pretreated with intravenous L-arginine [11, 12].

Effects of L-Arginine on Gut Mucosal Injury Caused by Lipopolysaccharide Endotoxemia

Bacterial translocation, which is defined as a migration of bacteria and endotoxin across the intestinal mucosa, has been postulated as an important factor in development of multiple organ failure [13, 14]. Mechanisms responsible for bacterial translocation depend on factors related to the microorganisms and the host defenses. A considerable number of animal experiments have been performed to elucidate the pathogenesis of impaired gut barrier function in order to find a rationale for new therapeutic options. A complex of several immunologic and nonimmunologic factors maintains the barrier function of the gastrointestinal tract, which includes salivary secretions, gastric acid, bile, pancreatic secretions, mucus production, intact intestinal mucosa, and a normally functioning local immune system [14]. The integrity of the gastrointestinal mucosa is a key element in maintaining gut barrier function. Mucosal injury has been considered key in the translocation process. Therefore, identification of those factors that maintain mucosal integrity of the gastrointestinal tract will suggest new therapeutic strategies for improvement of gut barrier function.

The mechanism by which L-arginine activates the immune system is poorly understood. Its importance for macrophage and T-lymphocytes has been reported recently by Bansal et al. [15]. In the last two decades, L-arginine has attracted major interest since it has been identified as the natural substrate of nitric oxide and is now recognized to have a major role in many regulation processes (Fig. 27.3).

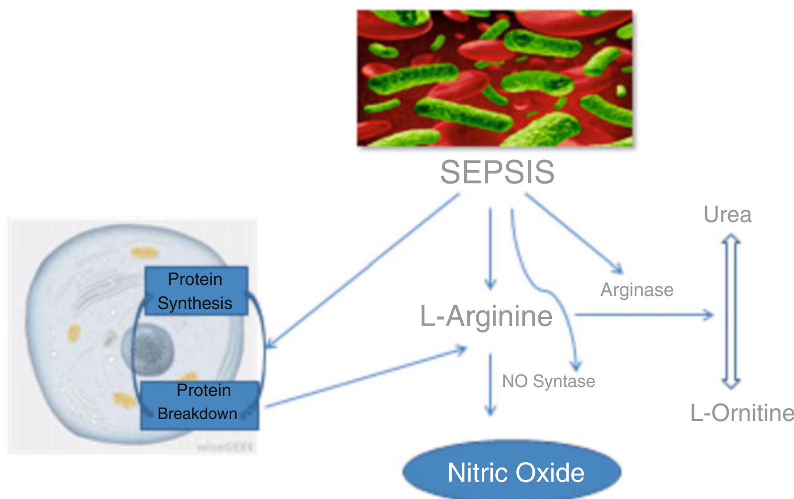

Fig. 27.3 Metabolism of L-arginine during sepsis

L-Arginine and NO are critical to the normal physiology of the gastrointestinal tract. Several studies have suggested that endogenous formation of nitric oxide maintains the mucosal integrity of the intestine and protects the gut from injuries from blood-borne toxins and tissue-destructive mediators [16, 18]. In weanling piglets, supplemental L-arginine decreases the mucosal injury caused by LPS endotoxemia by decreasing intestinal lesions and increasing cell proliferation [18]. L-Arginine maintains the mucosal integrity of the intestine. However, not all investigators support this concept. It is apparent that in chronic injury, NO is detrimental by combining with reactive oxygen species to form potent free radicals. In contrast, inhibition of NO synthesis after acute injury may exacerbate damage and inflammation. In a recent study, Gookin et al. reported that iNOS-derived NO is a key mediator of early villous reepithelialization following acute injury caused by deoxycholate in porcine ileal mucosa [19]. The mechanisms of this positive effect are still unclear; however, a suppressive effect of appropriate amounts of NO on apoptotic cell death in the gastrointestinal tract may be considered as one of them. In the recent study, the effect of lipopolysaccharide endotoxemia and oral L-arginine supplementation on small intestine morphology and cell turnover was evaluated [20]. This study has shown that lipopolysaccharide endotoxemia causes a marked intestinal mucosal injury. Decreased enterocyte proliferation and increased cell apoptosis were responsible for this negative effect. Exposure to oral L-arginine has protective effect in the intestinal mucosa from damage caused by LPS endotoxemia. Increased enterocyte proliferation rather than apoptosis was responsible for this beneficial effect. The results of the present study add to the body of evidence that suggests that maintaining mucosal integrity of the gastrointestinal tract with concomitant improvement in gut barrier function may be the reason for the positive effect of oral L-arginine in septic patients.

Effects of L-Arginine on Intestinal Recovery Following Intestinal Ischemia-Reperfusion Injury

Intestinal ischemia-reperfusion (IR) is a significant problem in a variety of clinical settings that commonly occurs in critically ill patients and may lead to systemic inflammation and multiple organ failure and is associated with a high morbidity and mortality [21]. Although the mechanisms involved in the pathogenesis of gut IR injury have not been fully elucidated, it is generally believed that oxidative stress with subsequent inflammatory injury plays an important role. The introduction of oxygen during reperfusion of ischemic tissues exacerbates this tissue damage via the formation of reactive oxygen species and reactive nitrogen species and changes in lipid mediator synthesis [22]. There is also a dramatic increase in lamina propria and intraepithelial lymphocytes in the mucosa and accumulation of inflammatory cells, which release several cytokines (TNF-α, IL-1, IL-8), platelet-activating factor, eicosanoids, leukotrienes, and other mediators that can promote a systemic inflammatory response, multiple organ failure, and death [23]. Injury to the intestinal barrier during ischemia-reperfusion leads to translocation of bacteria and bacterial products such as endotoxin, with the systemic dissemination linked to the development of the systemic inflammatory response syndrome. Although necrosis is responsible for the intestinal cell death during ischemic phase, recent evidence suggests that enterocyte apoptosis plays an important role in cell loss following intestinal IR. Reactive oxygen species and cytokines releasing during IR event and intracellular regulatory proteins (e.g., Bcl-2, Bax, Fas, p53) were identified to be involved in apoptosis induction or reduced expression.

Therapy of IR injury should include the pharmacological agents that can prevent ischemic injury and can prevent production of reactive oxygen species or agents in order to improve intestinal rehabilitation following the IR event. Several studies have suggested that endogenous formation of nitric oxide maintains the mucosal integrity of the intestine and protects the gut from injuries from blood-borne toxins and tissue-destructive mediators [17]. It has been reported previously that at low concentration NO may have a protective physiologic function, while high NO production may cause intestinal injury.

The positive effect of inhaled NO in preventing pulmonary damage induced by intestinal IR has been reported by several investigators [24]. There are few and controversial studies concerning the effects of NO and its precursor L-arginine on intestinal recovery following IR. In a recent study Khanna and coworkers have demonstrated that intraluminal nitroglycerin, which is an exogenous NO donor, produces several beneficial local and systemic effects in a rat model of intestinal IR [22]. Luo et al. have shown that pretreatment with L-NAME, a specific inhibitor of NO production, exacerbates intestinal mucosal injury and increases intestinal permeability following bowel IR in the rat [25]. Ward et al. have shown that L-arginine given intravenously prior to ischemia as well as intraluminal L-arginine given during the reperfusion period inhibited mucosal injury caused by IR. Cellular mechanisms of enterocyte turnover (enterocyte proliferation and apoptosis) were not studied in this experiment [27]. The mechanisms by which oral L-arginine preserved intestinal mucosa from IR injury are unclear; however, overproduction of NO from L-arginine may be considered as one of them. NO might attenuate various aspects of IR injury through scavenging the oxygen-derived free radical superoxide anion [28], inactivation of xanthine oxidase, and decrease in xanthine oxidase/xanthine dehydrogenase ratio [5] or by a direct inhibitory effect on neutrophil activation [29]. In a recent study, the effects of oral L-arginine on intestinal recovery following IR injury in rat were investigated, and the mechanisms by which L-arginine affects enterocyte turnover including its effect on cell proliferation and death via apoptosis were determined [30]. Results of the present study show that dietary L-arginine did not protect the intestinal mucosa from damage caused by IR. However, exposure to oral L-arginine significantly enhanced intestinal recovery following an IR event. The present data also suggest that L-arginine increased mucosal proliferation and decreased cell apoptosis rate in functioning intestine that represent an additional mechanisms that maintain mucosal structure following IR (Fig. 27.4).

Effects of L-Arginine on Intestinal Recovery Following Chemotherapy-Induced Mucositis

Oral and gastrointestinal mucositis is a debilitating, dose-limiting, and costly side effect of cancer therapy. Mucositis occurs in 40 % of cancer patients after standard doses of treatment and in almost 100 % of patients treated with high doses of chemotherapy, in particular with drugs affecting DNA synthesis (such as fluorouracil, methotrexate, and cytarabine) [31, 32]. Mucositis can affect the entire gastrointestinal tract causing discomfort, nausea, vomiting, bloating, diarrhea, ulceration, and bleeding and in some cases result in septicemia, contributing not only to the morbidity of treatment but its cost as well. Mucositis limits the patient's ability to tolerate chemotherapy or radiation therapy, prolongs hospital stay, increases readmission rates, compromises the patient's nutritional status, affects the patient's quality of life, and is occasionally fatal. Severe inflammation of the intestinal mucosa plays a significant role in the development of chemotherapy-induced mucositis and is a major characteristic of the condition [33]. Different inflammatory mediators and cytokines, such as leukotriene B4 and prostaglandin E2, act to amplify signaling cascades, induce apoptosis, and cause further tissue damage. The pathogenesis of chemotherapy-induced gastrointestinal mucositis has been described by Sonis et al. and includes five phases: initiation by chemotherapy, upregulation and generation of messenger signals, signaling by pro-inflammatory cytokines and amplification of mucosal injury, ulceration of the mucosa, and, finally, healing. The initial stages of inflammation in mucositis include increased pro-inflammatory cytokine levels, which act as a homing marker for inflammatory immune cells in the submucosa [32].

The role of L-arginine in prevention of chemotherapy-induced intestinal damage is unclear. Dietary supplementation with L-arginine stimulates small intestinal mucosal recovery following experimental radiation enteritis [17]. The mechanisms of these positive effects are still unclear;

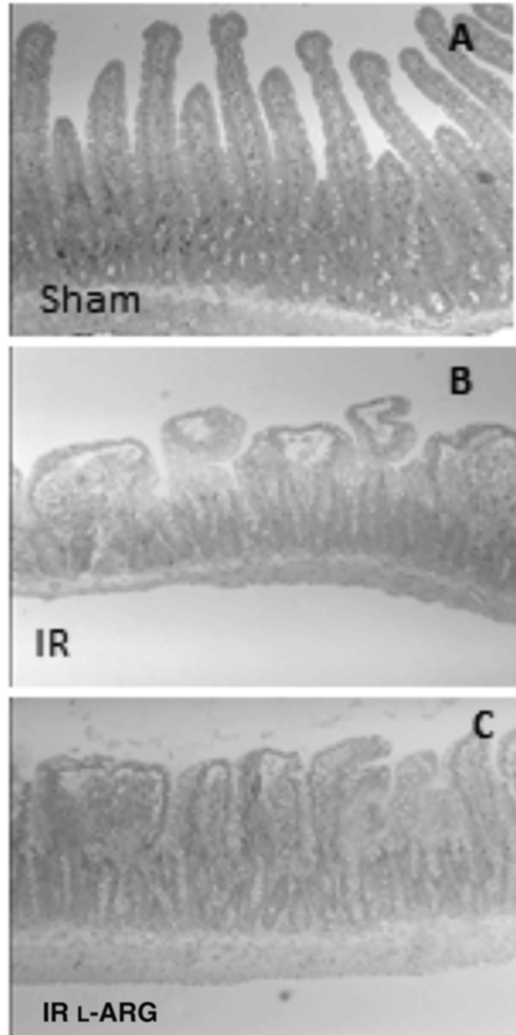

Fig. 27.4 Low-power photomicrographs of the full-thickness H&E-stained sections of distal ileum in sham (**a**), ischemia-reperfusion (**b**), and ischemia-reperfusion rats treated with oral L-arginine (**c**). Distal ileum section from sham rats shows the normal architecture of the intestinal epithelium. Distal ileum from IR rats demonstrates subepithelial space at villus tip, inflammatory cells infiltration extending through the wall, shortening and loss of villi, and reduction of crypt depth. Distal ileum of IR-ARG rats shows similar to IR rat signs of intestinal injury but increasing villus height and crypt depth

however, a stimulating effect of appropriate amounts of NO on enterocyte proliferation and a suppressive effect on enterocyte death via apoptosis may be considered as one of them. Hämäläinen et al. have shown recently that chemotherapy inhibits iNOS expression and subsequent NO production, in a dose-dependent manner at therapeutically achievable drug concentrations in a human colon epithelial cell line [33]. In a recent experiment, Gulgun et al. have demonstrated that proanthocyanidin, L-arginine, and glutamine supplementation had a positive effect in the protection of the small intestine from methotrexate-induced injury [34]. The mechanisms of this effect were not investigated. In a recent clinical trial, Izaola et al. have shown that L-arginine- and glutamine-enhanced formula decreases the rate of radiotherapy-induced oral mucositis in patients with head

and neck cancer [35]. In a recent study, the effect of oral L-arginine on intestinal recovery following methotrexate-induced intestinal damage was investigated in a rat model [36]. Results of the present study show that dietary L-arginine protects the intestinal mucosa from damage caused by MTX. While MTX rats showed severe villous atrophy, epithelial flattening, extensive crypt loss and signs of crypt remodeling, marked cellularity, and an increased number of blood vessels in stroma, L-arginine-treated rats showed more preserved architecture as well as the presence of newly formed crypts and regeneration. While the proliferative zone in MTX rats moved progressively upward in the crypts toward the crypt-villus junction, the proliferative zone of MTX-ARG rats was only mildly affected, showing a slight shift upward within the crypts.

In addition, exposure to oral L-arginine significantly enhanced intestinal recovery following methotrexate-induced damage. This was evident from the significant increase in bowel and mucosal

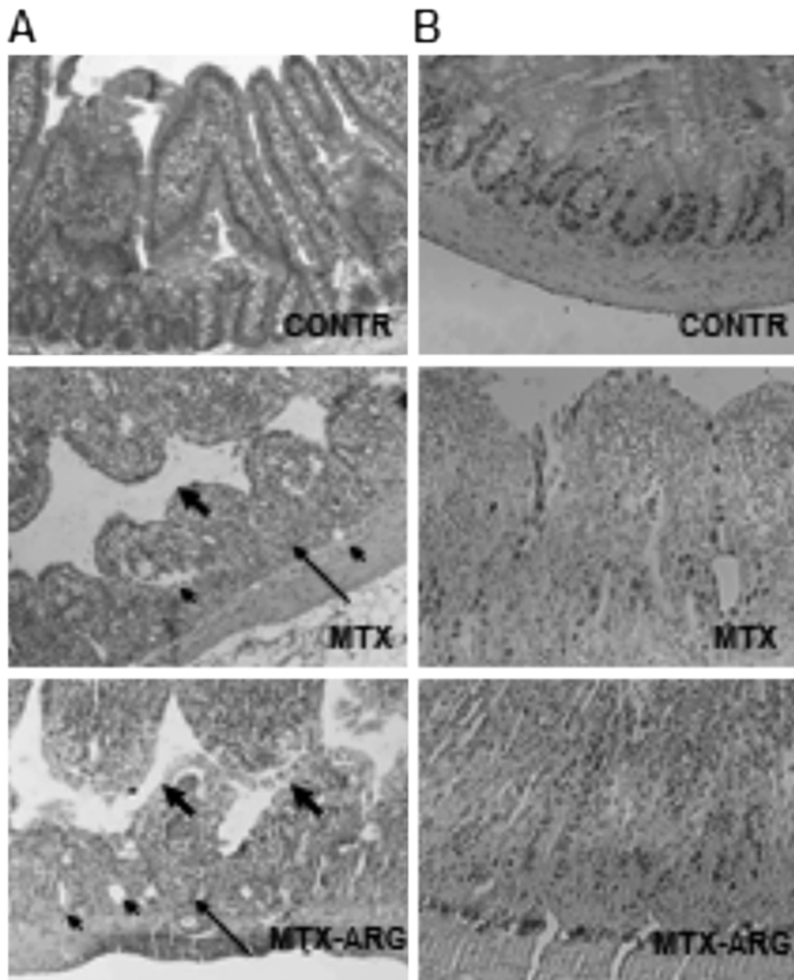

Fig. 27.5 (**a**) Staining with H&E. Intestinal injury caused by MTX. MTX rats demonstrated a significant epithelial atrophy (*thick arrows*) and signs of crypt remodeling (*thin arrows*) which were accompanied by marked cellularity and an increased number of blood vessels (*arrowheads*) in the stroma. Following ARG administration rats showed less significant epithelial atrophy and crypt remodeling compared to MTX rats. (**b**) BrdU staining (cell proliferation). Control rats show normal crypt compartment. The proliferative zone in MTX rats moved progressively upward in the crypts toward the crypt-villus junction. The proliferative zone of MTX-ARG rat was only mildly affected and showed the presence of newly formed crypts and signs of regeneration

DNA and protein content. Histologically, marked increases in villus height in both jejunum and ileum suggested increased absorptive surface area and closely correlated with increased cell mass. The present data suggested that L-arginine did not change significantly mucosal proliferation in functioning intestine, but decreased significantly cell apoptosis rate, which may represent the main mechanism that maintains mucosal structure following MTX-induced damage. The presented results showed that the intrinsic pathway, with its regulation by the bcl-2 family of proteins, was altered by L-arginine in accordance with changes in cell apoptosis: the mRNA and protein levels of the pro-apoptotic bax decreased, while those of the antiapoptotic bcl-2 protein levels increased. Correspondingly, bax/bcl-2 ratio decreased in L-arginine-treated rats compared to methotrexate animals, suggesting increased enterocyte survival (Fig. 27.5).

Conclusions

In the last two decades, L-arginine has attracted major interest since it has been identified as the natural substrate of nitric oxide and is now recognized to play a major role in many regulation processes. Nitric oxide is a multifunctional intercellular messenger molecule that plays an important role in a variety of physiological processes. L-Arginine and nitric oxide are critical to the normal physiology of the gastrointestinal tract and maintain the mucosal integrity of the intestine in various intestinal disorders. The rationale for supplementation with L-arginine to promote the health of the gastrointestinal tract lies in the anti-inflammatory effects of this amino acid, although there are controversial results that may reside in the different study designs used as well as in the various formulations and dosages used.

References

1. Cynober L, Le Boucher J, Vasson MP. L-Arginine metabolism in mammals. Nutr Biochem. 1995;6:402–13.
2. Kirk SJ, Barbul A. Role of L-arginine in trauma, sepsis and immunity. JPEN. 1990;14(5 Suppl):226S–9.
3. Scull CW, Rose WC. L-Arginine metabolism: the relation of the L-arginine content of the diet to the increments in tissue L-arginine during growth. J Biol Chem. 1930;39:109–14.
4. Anggard E. Nitric oxide: mediator, murderer, and medicine. Lancet. 1994;14:1199–206.
5. Cote CG, Yu FS, Zulueta JJ, Vosatka RJ, Hassoun PM. Regulation of intracellular xanthine oxidase by endothelial-derived nitric oxide. Am J Physiol. 1996;271:L869–74.
6. Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. Biochem Biophys Res Commun. 2001;282:1075–9.
7. Potten CS, Nellet M, Roberts SA, Revi RA, Wilson GD. Measurement of in vivo proliferation in human colorectal mucosa using bromodeoxyuridine. Gut. 1992;33:71–8.
8. Wright N, Alison M. The biology of epithelial cell populations. Oxford: Clarendon; 1984.
9. Bjerknes M, Cheng H. The stem-cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse. Am J Anat. 1981;160:51–63.
10. Rhoads J, Wu GY. Glutamine, L-arginine, and leucine signaling in the intestine. Amino Acids. 2009;37:111–22.
11. Rhoads JM, Chen W, Gookin J, Wu GY, Fu Q, Blikslager AT, Rippe RA, Argenzio RA, Cance WG, Weaver EM. L-Arginine stimulates intestinal cell migration through a focal adhesion kinase dependent mechanism. Gut. 2004;53:514–22.
12. Rhoads M, Fu Q, Rippe R, Odle J, Graves LM. Focal adhesion kinase (FAK) and p70 s6 kinase are critical for L-arginine stimulated intestinal cell migration. J Investig Med. 2004;52:S291–2.
13. Deitch EA. Multiple organ failure. Ann Surg. 1992;216:117–25.
14. Lemaire LCJM, van Lanschot JJB, Stoutenbeek CP, van Deventer SJH, Wells CL, Gouma DJ. Bacterial translocation in multiple organ failure: cause or epiphenomenon still unproven. Br J Surg. 1997;84:1340–5.
15. Bansal V, Ochoa JB. L-Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care. 2003;6:223–9.

16. Gurbuz AT, Kunzelman J, Ratzer EE. Supplemental dietary L-arginine accelerates intestinal mucosal regeneration and enhances bacterial clearance following radiation enteritis in rats. J Surg Res. 1996;74:149–56.
17. Schleiffer R, Raul F. Prophylactic administration of L-arginine improves the intestinal barrier function after mesenteric ischemia. Gut. 1996;39:194–9.
18. Liu Y, Huang JJ, Hou YQ, Zhu HL, Zhao SJ, Ding BY, Yin YL, Yi GF, Shi JX, Fan W. Dietary L-arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. Br J Nutr. 2008;100:552–60.
19. Gookin JL, Rhoads JM, Argenzio RA. Inducible nitric oxide synthase mediates early epithelial repair of porcine ileum. Am J Physiol Gastrointest Liver Physiol. 2002;283:G157–63.
20. Sukhotnik I, Mogilner J, Krausz MM, Lurie M, Hirsh M, Coran AG, Shiloni E. Oral L-arginine reduces gut mucosal injury caused by lipopolysaccharide endotoxemia in rat. J Surg Res. 2004;122:256–62.
21. Haglund U, Bergqvist D. Intestinal ischemia—the basics. Langenbecks Arch Surg. 1999;384:233–8.
22. Khanna A, Rossman J, Caty MG, Fung HL. Beneficial effects of intraluminal nitroglycerin in intestinal ischemia-reperfusion injury in rats. J Surg Res. 2003;114:15–24.
23. Granger DN, Hollwarth ME, Park DA. Ischemia reperfusion injury: role of oxygen-derived free radicals. Acta Physiol Scand Suppl. 1986;548:47–56.
24. Terada LS, Mahr NN, Jacobson ED. Nitric oxide decreases lung injury after intestinal ischemia. J Appl Physiol. 1996;81:2456–60.
25. Luo CC, Chen HM, Chiu CH, Lin JN, Chen JC. Effect of N-nitro-L-arginine methyl ester on intestinal permeability following intestinal ischemia-reperfusion injury in a rat model. Biol Neonate. 2001;80:60–3.
26. Ward DT, Lawson SA, Gallagher CM, Conner WC, Shea-Donohue T. Sustained nitric oxide production via L-arginine administration ameliorates effects of intestinal ischemia-reperfusion. J Surg Res. 2000;89:13–9.
27. Rubanyi GM, Ho EH, Cantor EH, Lumma WC, Botelho LH. Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. Biochem Biophys Res Commun. 1991;181:1392–7.
28. Kubes P, Suzuki M, Granger DN. Nitric oxide. An endogenous modulator of leukocyte adhesions. Proc Natl Acad Sci USA. 1991;88:4651–7.
29. Sukhotnik I, Helou H, Mogilner J, Lurie M, Bernsteyn A, Coran AG, Shiloni E. Oral L-arginine improves intestinal recovery following ischemia-reperfusion injury in rat. Pediatr Surg Int. 2005;21:191–6.
30. Naidu MUR, Ramana GV, Rani PU. Chemotherapy-induced and/or radiation therapy-induced oral mucositis? Complicating the treatment of cancer. Neoplasia. 2004;6:423–31.
31. Sonis ST. Complications of cancer and their treatment: oral complications. In: Holland JF, Frei E, Bast RC, editors. Cancer medicine. 3rd ed. Philadelphia: Lea and Febiger; 1993. p. 2381–8.
32. Sonis ST, Elting LS, Keefe DMK, Peterson DE, Schubert M, Hauer-Jensen M, Bekele BN, Raber-Durlacher J, Donnelly JP, Rubenstein EB. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer. 2004;100:1995–2025.
33. Hämäläinen M, Lahti A, Moilanen E. Calcineurin inhibitors, cyclosporin A and tacrolimus inhibit expression of inducible nitric oxide synthase in colon epithelial and macrophage cell lines. Eur J Pharmacol. 2002;448:239–44.
34. Gulgun M, Karaoglu A, Kesik V. Effect of proanthocyanidin, L-arginine and glutamine supplementation on methotrexate-induced gastrointestinal toxicity in rats. Methods Find Exp Clin Pharmacol. 2010;32:657–61.
35. Izaola O, de Luis DA, Cuellar L. Influence of an immuno-enhanced formula in postsurgical ambulatory patients with head and neck cancer. Nutr Hosp. 2010;25:793–6.
36. Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran AG, Sukhotnik I. Dietary L-arginine supplementation reduces methotrexate-induced intestinal mucosal injury in rat. BMC Gastroenterol. 2012;12:41–7.

Chapter 28
Enteral and Parenteral L-Arginine Supplementation in Intestinal Ischaemia and Reperfusion Injury

Chun-Hong Lai and Hui-Chen Lo

Key Points

- Intestinal I–R is a life-threatening abdominal emergency that requires rapid restoration of mesenteric blood flow; however, reperfusion may result in mucosal injury and multiple organ damage, including damage to the liver, lung, kidneys, heart and brain.
- In prolonged ischaemia, the depletion of ATP provokes xanthine oxidase activity to increase the production of reactive oxygen species (ROS), which further mediates reperfusion-induced injury.
- In intestinal I–R injury, polymorphonuclear neutrophils (PMNs) are believed to be the major sources of ROS, and their interaction with endothelial cells allow PMNs to infiltrate into the tissues and organs, leading to multiple organ failure (MOF).
- The inflammatory mediators, such as pro-inflammatory cytokines, nitric oxide (NO), and transcriptional factor nuclear factor (NF)-κB, play major roles in the histological changes and apoptosis in intestinal I–R injury.
- The controversial results of L-arginine administration in intestinal I–R might be associated with the therapeutic time, dosing, route and disease severity.
- The pretreatment of enteral L-arginine may have beneficial effects on ameliorating survival and impaired contractile response in the intestine, thereby improving mucosal barrier function, enhancing cell proliferation and decreasing ROS and lipid peroxidation in the circulation and intestine. These advantages are associated with the increased serum NO and inactivated inducible nitric oxide synthase (iNOS).
- Intravenous L-arginine administration prior to reperfusion may be used to sustain the constitutive nitric oxide synthase (cNOS)-derived production of NO to alleviate capillary leak via a neutrophil-dependent mechanism in intestinal I–R-induced mucosal injury. However, pretreatment with large doses of parenteral L-arginine may result in uncontrolled immune cell responses, which leads to deleterious outcomes.

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Abbreviations

AP-1	Activator protein-1
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
cNOS	Constitutive nitric oxide synthase
DAMPs	Damage-associated molecular proteins
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signalling-regulated kinase
HO	Haem oxygenase
I–R	Ischaemia and reperfusion
ICAMs	Intercellular adhesion molecules
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
L-NAME	L-N^G-nitroarginine methyl ester
LT	Leukotrienes
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
MPO	Myeloperoxidase
NF-κB	Nuclear factor-kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NT	Nitrotyrosine
$ONOO^-$	Peroxynitrite
PAF	Platelet activation factor
PMNs	Polymorphonuclear neutrophils
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SECs	Sinusoidal endothelial cells
SMA	Superior mesenteric artery
SOD	Superoxide dismutase
TdT	Terminal deoxynucleotidyl transferase
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
TUNEL	TdT-mediated dUTP nick end labelling
VCAM	Vascular cellular adhesion molecule
XO	Xanthine oxidase

Introduction

Among the internal organs, the intestine, especially the small intestine, is the most sensitive organ to ischaemia and reperfusion (I–R) damages. Intestinal ischaemia, a common and serious clinical condition resulting from numerous clinical diseases, including haemorrhagic shock, necrotising enterocolitis, sepsis, burn, vascular surgery, small bowel transplantation, cardiopulmonary bypass and abdominal aortic surgery, is a life-threatening abdominal emergency. When intestinal ischaemia occurs, rapid restoration of mesenteric blood flow is crucial; however, reperfusion may exacerbate vascular and tissue damage and result in local mucosal injury, systemic inflammation and multiple organ failure (MOF) [1]. In the late 1990s, the mortality rate of intestinal I–R was 60–80 % in the USA. In recent years, the in-hospital mortality rates of intestinal I–R remain as high as 55.1 %, mainly due to MOF, even with advanced clinical techniques [2].

Intestinal I–R injury involved in nutritive perfusion failure, inflammatory cell response, mediator surge and the pathogenesis of intestinal barrier dysfunction, which are associated with the concomitant translocation of enteric bacteria and toxins and the potential development of MOF [1]. Numerous strategies have been proposed to ameliorate intestinal I–R injury. L-Arginine, the sole source of nitric oxide (NO) in our body, has been indicated to have a potential to prevent the development of MOF in the management of intestinal I–R [3]. L-Arginine has a variety of physiological functions, including cell division, wound healing, urea synthesis, immune function and hormone secretion. In this chapter, we reviewed the pathophysiology and mechanisms of intestinal I–R and the effects of enteral and parenteral L-arginine supplementation on intestinal I–R.

Intestinal Ischaemia and Reperfusion

Pathophysiology of Intestinal I–R Injury

Intestinal Ischaemia

The intestines require consistent oxygen and nutrition supplies via three arteries to maintain normal functions. Under normal physiological conditions, the intestinal mucosa uses approximately 20 % of the total resting oxygen consumption in the body, receives up to 70–80 % of the mesenteric artery blood flow and provides 60 % of its blood flow directly to the epithelial cells at the villi [4]. Alterations of the blood supply exceeding 50 % may result in intestinal ischaemia and progressive damages [5]. The first detectable sign of intestinal mucosal injury in ischaemia is the increased capillary permeability. The ischaemic damages are mainly caused by the blockage of oxygen and nutrient supply that leads to the depletion of adenosine triphosphate (ATP) and homeostatic imbalance in the enterocytes [6].

When ischaemia is continuous in the intestine, mucosal permeability is further increased with subsequent epithelial cell injury and irreversible necrosis. The intestines accumulate metabolites and toxic mediators with the consequent compromise of oxidative phosphorylation in the mitochondria, which causes cellular injury and a series of events in multiple organ dysfunction and death [7]. If the intestinal ischaemia is corrected before irreversible injury by returning oxygen to remove toxic mediators and by providing nutrients to restore energetic metabolism, the functions of intestinal cells may be partially or fully recovered [8].

Reperfusion

Paradoxically, blood restoration to the ischaemic intestine may initiate a series of events that leads to more prominent injury than ischaemia [9]. It has been demonstrated that the mucosal injury observed after 3 h of ischaemia and 1 h of reperfusion was more severe than that produced by 4 h of ischaemia without reperfusion in felines [7]. Coopersmith et al. [10] indicated that after ischaemia, apoptotic cells appeared in the intestinal epithelium within 4 h after reperfusion, with a maximum response occurring at 24 h after reperfusion. At the time of reperfusion, oxygen is added suddenly and in excess. The burst of generated superoxide triggers a free radical chain reaction most likely via the hypoxanthine–xanthine oxidase (XO) system. The increased oxygen-derived free radicals may directly cause mucosal damages and the inflammatory response [8].

The intestinal mucosa is a major site for the production of acute phase proteins, gut hormones and inflammatory mediators, such as cytokines, NO and transcription factors [5]. Intestinal I–R decreases ATP production from the cellular mitochondria and increases mucosal and vascular permeability, the activation and adhesion of polymorphonuclear neutrophils (PMNs), the release of pro-inflammatory substances and the formation of both nitrogen-derived and oxygen-derived free radicals. These alterations are closely related to the pathophysiological changes in the mucosa and submucosa that cause endothelial destruction [5].

In animal studies, intestinal I–R significantly decreased mucosa weight, DNA and protein contents, villus height and crypt depth [11, 12] and significantly altered intestinal morphology, including mucosal cell desquamation, mucosal cell and crypt necrosis and muscularis layer thinning in the small intestine [9]. The destruction of the mucosal barrier in intestinal I–R may result in the translocation of enteric bacterial products, the release of PMNs and the activation of systemic inflammatory responses. All of these events lead to pathophysiological effects on distant organs, including the lungs, liver, kidney, heart and brain, as well as high rates of morbidity and mortality.

Mechanisms of Intestinal I–R Injury

Reactive Oxygen Species

In prolonged ischaemia, hypoxanthine is derived from the catabolism of cellular ATP. The depletion of ATP results in a loss of ATP-dependent ion channel regulation, which produces a condition in which sodium ions, calcium ions and water diffuse in the cytoplasm, followed by cell swelling. The increased cellular calcium ions and the hypoxic condition provoke xanthine dehydrogenase to convert to the oxygen radical-producing XO. When blood flow supply of the ischemic tissue is restored, the introduced oxygen and excess hypoxanthine are catalysed by XO to form xanthine, toxic superoxide and hydroxyl radicals, the so-called ROS (Fig. 28.1) [13]. Subsequently, toxic superoxide is catalysed by superoxide dismutase (SOD) or spontaneously reacts with protons to produce H_2O_2, a highly reactive and cytotoxic material. These XO-derived ROS play an important role in increasing lipid peroxidation and protein carbonyl formation, recruiting and activating granulocytes and further mediating reperfusion-induced microvascular injury [7].

Polymorphonuclear Neutrophils

Endothelial dysfunction is the first phenomena of intestinal I–R, which is regarded as the “trigger” of reperfusion injury by inducing a catastrophic cascade of events. I–R injury may cause significant infiltration of PMNs, macrophages and T cells into wounded tissue within 24 h [14]. PMNs are believed to be the major sources of ROS via their activated myeloperoxidase (MPO), which results in

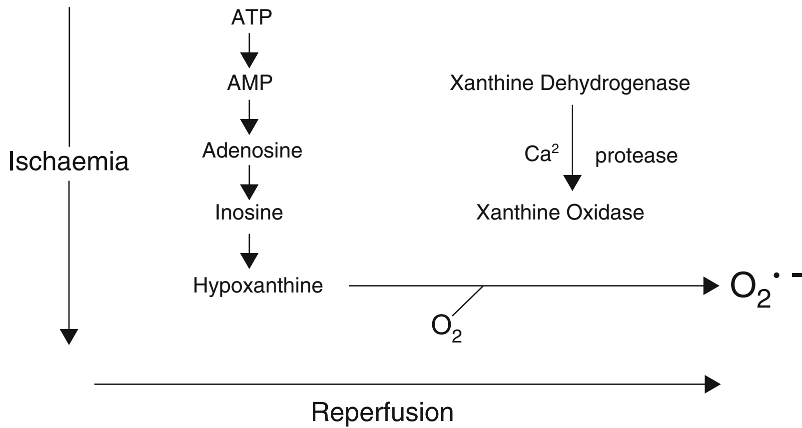

Fig. 28.1 Mechanism of xanthine oxidase-mediated free radical injury in ischaemia and reperfusion. Reprinted with permission from Mallick et al. [13]

mucosal injury during reperfusion [15]. PMNs-secreted proteolytic enzymes, such as elastase from cytoplasmic granules, play a crucial role in mucosal destruction. It is proposed that the intestinal I–R--induced endothelium dysfunction is mediated by ROS during the early stages and by the activation of PMNs during the late stages [6]. This late-stage injury involves an interaction between PMNs and endothelial cells accompanied with a complex process of adhesion molecules, including PMN rolling, leucocyte adherence and trans-endothelial migration [13].

The migration of PMNs to areas of inflammation is a significant indication in intestinal I–R injury. Initially, the upregulated adhesion molecules, such as P-selectin and E-selectin, on the surface of endothelium interact with L-selectin on the PMNs. This interaction attracts chemokines, which bind PMNs and promote β2 integrin binding to adhesion molecules, such as intercellular adhesion molecules (ICAMs)-1 and vascular cellular adhesion molecule (VCAM)-1, expressed on endothelial cells. Subsequently, the strengthened adhesion between PMNs and endothelial cells allows PMNs to infiltrate into the interstitium from the circulation and to lead to cellular damage in the tissues and organs (Fig. 28.2) [16].

Inflammatory Mediators

Reperfusion of an ischaemic bed may lead to inflammation locally and systemically. The inflammatory mediators, such as pro-inflammatory cytokines, NO and transcriptional factor nuclear factor (NF)-κB, play major roles in the histological changes and apoptosis in intestinal I–R injury. The damaged mucosa barrier allows bacteria, especially the gram-negative bacteria, to translocate to the mesenteric lymphatic nodes and travel to the other organs or tissues, such as the lung and liver, via the circulatory system [9]. These invading bacteria may induce uncontrolled inflammatory responses and further lead to local and remote organ damages.

NO, a double-edged sword, acts as both a cytotoxic and a cytoprotective molecule in intestinal I–R injury. The constitutive forms of neuronal and endothelial NO synthase, i.e. nNOS and eNOS, are essential to maintain normal physiology, and the inducible form of NO synthase (iNOS) produces large amounts of NO and peroxynitrite to lead to tissue injury. During the early phase of reperfusion, the inactivated nNOS and eNOS and accumulated superoxide limit the availability of NO to perform its beneficial effects in preventing the adhesion between PMNs and endothelial cells and in avoiding the aggregation of platelets. The absence of NO and the excess of superoxide result in the formation of H_2O_2 which triggers the inflammatory responses, including the accumulation of platelet activation

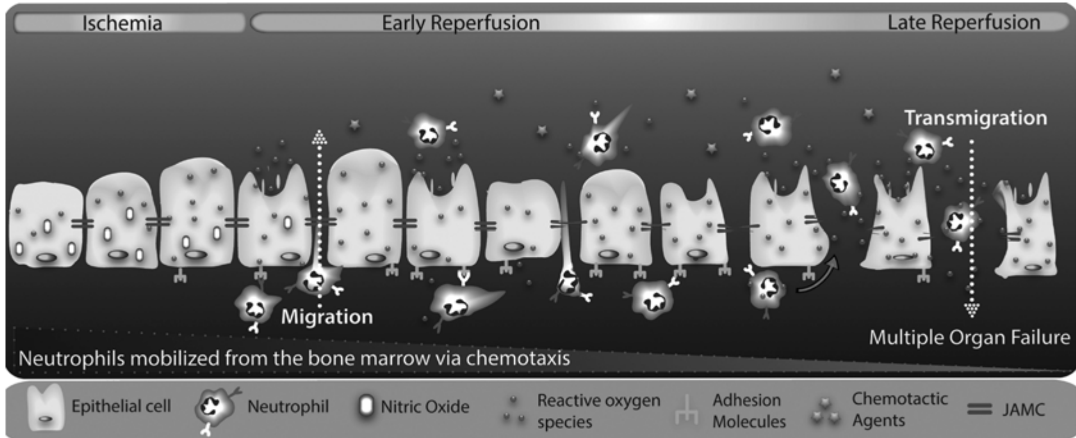

Fig. 28.2 Neutrophil migration is initiated by various chemotactic agents produced at the site of I–R. Neutrophils produce ROS and inadvertently destroy local endothelial or epithelial cells that were unaffected by the initial I–R insult. Junctional adhesion molecule-C (JAM-C) is disrupted by neutrophil proteases and cell disruption, enabling neutrophils to transmigrate out of the tissue (reverse migration). Neutrophils become more resilient to apoptosis and gain enhanced ROS production, where they can subsequently migrate to other organs and destroy tissues through ROS production, leading to ARDS, systemic inflammatory response syndrome or MOF. Reprinted with permission from Schofield et al. [16]

factor (PAF) and leukotrienes (LTs), the activation of NF-κB transcription and the increased expression of cytokines and adhesion molecules [13]. However, during the late phase of reperfusion, elevated expression of iNOS produces extensive NO. The excess NO exerts its cytotoxic effects by reacting with superoxide to further produce potent oxidant peroxynitrite ($ONOO^-$), which causes accentuated lipid peroxidation, protein and DNA modifications and cellular damage [17].

The small intestinal mucosa contains large numbers of immune cells, including T and B lymphocytes, macrophages, mast cells, eosinophils and neutrophils [18]. In animals suffering from intestinal I–R, the immune cells in the intestine are activated to extensively release pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, IL-1 and IL-6, via the activation of transcription factor NF-κB [19]. The activated pro-inflammatory cytokines may be released into the circulation and induce a systemic inflammatory response. Therefore, intestinal I–R-induced NF-κB overexpression may result in injuries in the intestine and remote organs including the liver, lung and kidneys [20, 21].

Apoptotic Signalling Pathways

Apoptosis, a form of programmed cell death, has been known to be the major mode of cell death in the destruction of small intestinal epithelial cells in intestinal I–R injury [22]. Several factors mediate this injury and apoptosis, including ROS, inflammatory leucocytes, mitochondrial dysfunction and the release of cytochrome c from mitochondria into the cytosol. The morphological characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation, cytoskeletal reorganisation, nuclear envelop injury and systemic internucleosomal DNA fragmentation. The results of in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) methods for apoptosis detection confirm that apoptosis is an important form of cell death in rat small intestine after I–R injury that occurs at 30 min, maximises at 12 h and returns to baseline levels by 24 h of reperfusion [22].

Oxidative stress is known to induce apoptosis by activating mitogen-activated protein kinase (MAPK) family proteins, including extracellular signalling-regulated kinase (ERK), c-Jun N-terminal

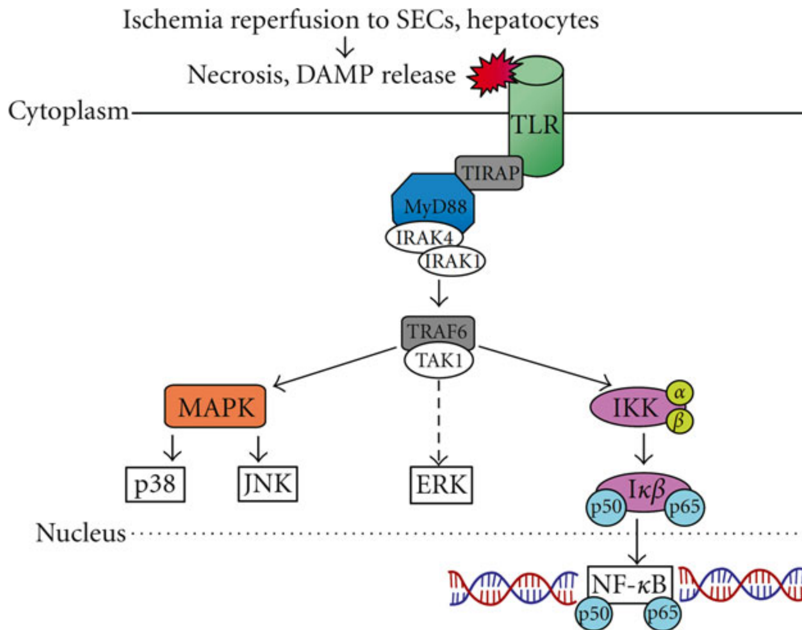

Fig. 28.3 TLR-mediated pathway during I–R injury. Ligation of the TLR leads to activation of upstream MyD88 and activation of the downstream NF-κB, p38 and JNK pathways, resulting in cellular injury. *SECs* Sinusoidal endothelial cells, *DAMPs* Damage-associated molecular proteins. Reprinted with unrestricted permission from Siriussawakul et al. [26]

kinase (JNK) and p38 MAPKs. Rats pretreated with JNK and/or p38 inhibitors exhibit significantly attenuated histological damage in the small intestine after suffering from I–R [23]. Accumulating evidence reveals that JNK is upregulated by various stimuli during intestinal I–R, including ROS, pro-inflammatory cytokines, osmotic and mechanical stress and toll-like receptor activation [24].

Toll-like receptors (TLRs), a family of signalling receptors, play a critical role in the regulation of inflammatory and innate immune responses. Using TLR4 mutant mice, Ben et al. [25] demonstrated that TLR4 plays an important role in the pathogenesis of intestinal I–R-induced acute lung injury and inflammation. The authors found that when TLR4 was recognised by bacterial lipopolysaccharides that activate multiple intracellular signalling systems, including ERK, JNK, and p38 MAPK and NF-κB pathways, to trigger the inflammatory immune responses and apoptosis in the lung (Fig. 28.3) [25, 26].

Enteral L-Arginine Supplementation in Intestinal I–R Injury

In the past few decades, the therapeutic effects of nutrition support, especially amino acids and antioxidants, have been tested in attenuating intestinal I–R-induced local and systemic injuries. For example, immune-enhancing nutrients, including glutamine, L-arginine, omega-3-fatty acids and nucleotides, may reduce septic morbidity and MOF in trauma patients without segregating the individual effects of these nutrients. L-Arginine is considered to have both nutritional and therapeutic roles in I–R injury because I–R injury is associated with deficiencies in L-arginine and/or reduced bioavailability of NO.

It is conceivable that L-arginine-derived NO is an important mediator in the restitution of intestinal mucosa by minimising cell injury during reperfusion. NO, a free radical and volatile gas with a half-life in the body, is important in sustaining mucosal integrity; however, it also results in the

pathogenesis of I–R-induced tissue injury. The role of NO in the I–R syndrome remains a matter of controversy. In rats undergoing bowel transplantation, exogenous NO administration resulted in less necrosis but greater apoptosis. In addition, the NO donor molsidomine prevented intestinal I–R injury by depressing tissue malondialdehyde (MDA) levels, a marker of lipid peroxidation, reducing mucosal injury scores and restoring the intestinal microcirculatory blood flow [27]. In animals with intestinal I–R injury, L-arginine treatment may enhance survival rates, improve intestinal integrity and function [11], decrease intestinal lipid peroxidation [28], suppress the release of pro-inflammatory cytokines [29] and inhibit lung leakage and neutrophil infiltration [30]. These results imply that L-arginine may be beneficial in improving intestinal I–R injury.

In contrast, the adverse effects of enteral L-arginine supplementation have been reported in recent years. Several studies demonstrated that L-arginine administration at an early time point after the intestinal I–R appears to have no beneficial effects on survival and may worsen the outcomes via excessive priming and activation of circulating myeloid cells and iNOS expression [31, 32]. The inconsistent results may be due to dose-dependent effects of L-arginine because a recent study revealed that dietary supplementation with a lower level of L-arginine (0.7 %) had beneficial effects on the microvascular development of early-weaned pigs, but a higher level of L-arginine supplementation (1.2 %) caused adverse effects [33]. Therefore, the controversial results of L-arginine administration in intestinal I–R might be associated with the therapeutic time, dosing, route and disease severity [12].

The roles of different NOS have been discussed in affecting the development of intestinal I–R injury. In rats with intestinal transplantation, the decreased nNOS and increased iNOS activities contribute to intestinal I–R injury and acute rejection, and nNOS activity was closely related to graft structure and function [34]. In ovariectomised female rats with 45-min superior mesenteric artery (SMA) occlusion followed by 2 h of reperfusion, lung vascular permeability was significantly reduced by the selective iNOS inhibitor aminoguanidine, and the protective effects of estradiol were abolished by the nonselective NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) [35]. These results suggest that the beneficial and adverse effects of L-arginine administration may be closely associated with the profiles of NOS activities.

Pretreatment of Enteral L-Arginine

The effects of L-arginine administration may vary according to the therapeutic time and route. Pretreatment with L-arginine may ameliorate survival after intestinal I–R and improve mucosal barrier function in rats with 90-min SMA occlusion [36]. Sukhotnik et al. [11] reported that pretreatment with L-arginine in the drinking water (2 %) for 3 days significantly increased the rate of enterocyte proliferation in the jejunum and ileum and improved mucosal recovery, as evidenced by the alleviated decreases in duodenal and jejunal bowel weight; duodenal, jejunal and ileal mucosal weight; ileal mucosal DNA; and jejunal and ileal mucosal protein. In addition, pretreatment with L-arginine significantly decreased the apoptotic index in the ileum.

It has been proposed that the beneficial effects of L-arginine pretreatment may be related to its metabolite polyamines. Polyamines, the small basic molecules synthesised from ornithine via arginase, are involved in repair processes in the synthesis of DNA, RNA and protein after intestinal ischaemia [11]. Intragastric administration of male Wistar rats with 0.5 g/kg L-arginine at 17 and 2 h before ischaemia (90 min) did not prevent ischaemic damage but accelerated morphological repair and enhanced cell proliferation after 4 h of reperfusion. These effects were accompanied by increases in polyamines and cyclic GMP in the intestine. However, when animals were administered L-NAME to block the activity of NOS, the effects of L-arginine pretreatment on mucosal repair were abolished. These results imply that L-arginine-derived NO instead of polyamines is the major mediator in minimising cell injury and repairing the mucosa during reperfusion [37].

The beneficial effects of L-arginine pretreatment on mucosal injury are proposed to be related to the decrease in endothelin, a protein produced primarily in the endothelium to constrict blood vessels and raise blood pressure. In rats with burn injury, oral L-arginine (0.14 g/day) significantly lowered endothelin content, elevated NO content and alleviated mucosal injury in the intestine. These results suggest that oral L-arginine may be able to ameliorate intestinal I–R injury and protect the barrier function of the intestinal mucosa via the maintenance of a stable ratio of endothelin to NO by an increase in mucosal NO [38].

Moreover, L-arginine treatment may ameliorate intestinal I–R injury by decreasing neutrophil–endothelial interactions, stimulating free radical scavenging and reducing lipid peroxidation. In rats with intestinal I–R, L-arginine pretreatment significantly decreased serum MDA levels, attenuated histopathological damage and promoted the healing of the intestinal mucosa [27]. In addition, oral administration of L-arginine (500 mg/day) for 5 days prior to I–R injury significantly reduced leucocyte adherence, decreased ROS and lipid peroxidation in the intestine and inhibited the release of endotoxins into the blood [39]. These changes were associated with increased serum NO in L-arginine-treated rats, as these beneficial effects were abolished by NOS inhibitor.

In rats subjected to 30-min mesenteric ischaemia and 3-h reperfusion, L-arginine pretreatment reversed I–R-induced decreases in acetylcholine-stimulated contractile response and attenuated I–R--induced increases in lipid peroxidation, decreases in glutathione and mucosal injury severity. These results suggest that L-arginine pretreatment has a protective effect on ameliorating impaired contractile response and mucosal injury in intestinal I–R injury, which may be mediated in part by alleviating lipid peroxidation and improving endogenous antioxidants [28].

However, more recent reports suggest that enteral diets enriched with L-arginine may be harmful by enhancing inflammation. In a rat study with jejunal sacs filled with 10 mmol/L L-arginine prior to intestinal I–R, L-arginine resulted in worsened mucosal injury, disrupted actin cytoskeleton, decreased tissue ATP and enhanced permeability [40]. These adverse effects of L-arginine may be associated with enhanced activator protein (AP)-1 expression (c-Jun but not c-Fos), not NF-kappa B [24]. AP-1, a stress-activated DNA-binding protein, is regulated by MAPK, especially the inflammatory mediator JNK. Ban et al. [24] confirmed that early enteral L-arginine may augment neutrophil infiltration and AP-1 DNA-binding activity as well as increase iNOS expression, which worsens the gut injury in rats with mesenteric I–R. In addition, intestinal I–R-induced gut injury was alleviated by the JNK inhibitor SP600125 but not the iNOS inhibitor 1400W [24]. In intestinal I–R, the induction of iNOS is harmful, whereas the induction of haem oxygenase 1 (HO)-1 and peroxisome proliferator-activated receptor-gamma (PPAR-gamma) is protective. Lumen L-arginine significantly increased iNOS and was associated with increased MPO activity, mucosal injury and heat shock proteins as well as decreased PPAR-gamma in rats with 60-min SMA occlusion and 6 h of reperfusion [32]. These adverse effects of L-arginine were attenuated by the iNOS inhibitor 1400W. These results reveal that L-arginine pretreatment may augment the intestinal I–R-induced gut injury via AP-1 activation and iNOS expression.

The xanthine oxidase system is a major source of ROS, and NO plays a crucial but controversial role in intestinal I–R. Male Wistar rats subjected to occlusion of the SMA for 60 min and reperfusion for 0, 1, 8 or 24 h were orally administered L-arginine (800 mg/kg body weight), an NOS inhibitor L-NAME (50 mg/kg), or a xanthine oxidase inhibitor allopurinol (100 mg/kg) at 24, 12 and 1 h before the surgical operation. After reperfusion, the allopurinol-treated rats exhibited decreased MPO, iNOS and nitrotyrosine (NT), the indirect index of reactive nitrogen species (RNS), in the intestine and had the mildest histological lesions compared with the L-arginine- or L-NAME-treated rats. The L-arginine-treated rats exhibited fewer intestine lesions and increased iNOS and NT levels in the intestine, whereas the L-NAME-treated rats exhibited increased intestinal lesions, serum nitrite and catalase activity (Table 28.1) [41]. These findings suggest that preventing the production of ROS and RNS is a useful approach to alleviate intestinal I–R injury.

Table 28.1 Tissue nitrotyrosine and iNOS levels during reperfusion

Group	Time of reperfusion (h) 0	1	8	24
Nitrotyrosine (*score*)				
Control	0.63 ± 0.18[a]	1.88 ± 0.23[b]	2.00 ± 0.19[b]	1.50 ± 0.33
L-Arg	2.00 ± 0.27	2.00 ± 0.27	1.38 ± 0.26	2.13 ± 0.30
L-NAME	1.25 ± 0.25	0.88 ± 0.23[a]	1.38 ± 0.18	1.17 ± 0.31
Allo	0.88 ± 0.23[a]	1.00 ± 0.33	1.88 ± 0.23	0.75 ± 0.25[a, c]
iNOS (*score*)				
Control	1.38 ± 0.26	1.13 ± 0.30	1.50 ± 0.19	1.25 ± 0.16[a]
L-Arg	1.75 ± 0.16	2.00 ± 0.19	2.25 ± 0.31	2.38 ± 0.18
L-NAME	1.00 ± 0.27	0.88 ± 0.23[a]	1.57 ± 0.27	1.33 ± 0.49
Allo	1.00 ± 0.27	0.75 ± 0.31[a]	0.75 ± 0.25[a]	1.13 ± 0.23[a]

Tissue nitrotyrosine and iNOS levels in rats with 60-min SMA occlusion and 0, 1, 8 or 24 h of reperfusion. Rats were orally administered with L-arginine (800 mg/kg body weight; L-Arg group), L-NAME (50 mg/kg; L-NAME group) or allopurinol (100 mg/kg; Allo group) in 3 equal doses 24, 12 and 1 h before the surgical operation. Reprinted with permission from Margaritis et al. [41]

Values are expressed as mean ± SEM

[a] $P < 0.05$ versus L-Arg group

[b] $P < 0.05$ versus ischaemia

[c] $P < 0.05$ versus 8 h reperfusion

Post-treatment of Enteral L-Arginine

In fact, nutrition management before the development of intestinal I–R may be difficult in most cases. The establishment of new nutritional strategies after the insult, that is, during the intestinal I–R period, would have great advantages in clinical settings. Enteral L-arginine is a specific stimulus for neonatal intestinal blood flow and mucosal growth. In newborn mice suffering from hypoxia and reoxygenation, L-arginine administration twice a day for 3 days significantly improves structural preservation rates and NO content in the ileum [42]. In pigs with partial enteral nutrition, acute 3-h enteral L-arginine supplementation increased plasma L-arginine dose dependently and 4-day enteral L-arginine supplementation induced mucosal growth in the intestine, neither of which were affected by L-NAME. These results suggest that post-treatment of L-arginine in partial enteral feeding modestly increases intestinal mucosal growth, and this effect was NO independent [43].

Lee and colleagues [12] tested the effects of long-term enteral L-arginine administration on intestinal morphology and inflammatory response in rats with 3 h of ileal ischaemia and continuous infusion of 2 % L-arginine via duodenal cannulation for 7 days. The authors reported that long-term intra-duodenal L-arginine administration may not have observable benefits on intestinal morphology or systemic/jejunal inflammatory responses in rats with intestinal I–R injury. The necessity of long-term L-arginine supplementation for patients with intestinal I–R injury remains questionable and requires further investigation.

Parenteral L-Arginine Supplementation in Intestinal I–R Injury

Parenteral L-arginine supplementation may be a potential adjunct for attenuating intestinal I–R injury to prevent the development of ARDS and MODS. However, the results of parenteral L-arginine supplementation on intestinal I–R injury are also controversial. Whether these contradictory results were due to the therapeutic time and dose are still not concluded.

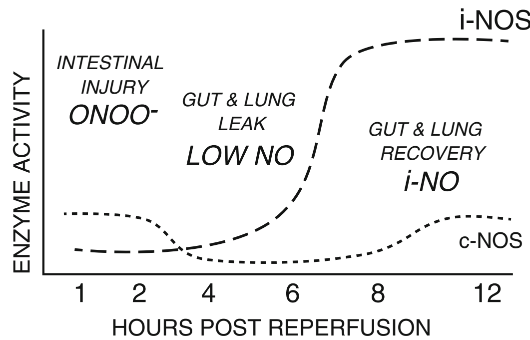

Fig. 28.4 Hypothetical schematic demonstrating the involvement of the cNOS (*dotted line*) and iNOS (*dashed line*) enzymes in the ischaemia and reperfusion model. Reprinted with permission from Ward et al. [30]

Pretreatment of Parenteral L-Arginine

In the past two decades, intravenous L-arginine has been investigated by numerous studies testing its ability to prevent intestinal I–R damage. Ward et al. [30] proposed that there is a time course relationship between the recovery and injury of intestinal I–R with the activity of cNOS and iNOS (Fig. 28.4).

As shown in Fig. 28.4, intestinal I–R-induced injury is presumably due to peroxynitrite ($ONOO^-$) formed from NO within 2 h of reperfusion. Diminished cNOS activity occurs at 4–6 h of reperfusion, which correlates with the onset of gut and lung capillary leak. With over 6 h of reperfusion, increased iNOS-derived NO is correlated with resolution of mucosal injury.

By quantitating neutrophil numbers in the intestinal mucosa and sustaining NOS activity, Ward and colleagues [30] demonstrated that intravenous L-arginine administration (4 mg/kg/min) 60 min prior to ischaemia significantly attenuated intestinal I–R-induced mucosal injury, PMN infiltration, lung leak and systemic capillary permeability and significantly increased serum NO in rats undergoing 30 min of SMA occlusion followed by 4 h of reperfusion. To compare the time effects of L-arginine administration, the authors further post-treated with intravenous L-arginine during the reperfusion period and found that intestinal I–R-induced lung leak and neutrophil infiltration were reduced, but the mucosal injury was not improved. Using a concentration approximately equal to the dose infused intravenously, intraluminal L-arginine administration significantly reduced mucosa injury but had no effect on capillary leak in the intestine and lung.

These results suggest that early production of NO in intestinal I–R injury, i.e. initially following ischaemia, may lead to mucosal injury. However, continued production of NO is essential to reduce the systemic inflammatory response and the consequence of organ failure [30]. Because diminished NO production during the reperfusion period may lead to endothelial dysfunction, intravenous L-arginine administration prior to reperfusion may be used to sustain the cNOS-derived production of NO to alleviate capillary leak via a neutrophil-dependent mechanism (Fig. 28.5).

Intestinal I–R injury is initiated by the up-regulation and interaction of adhesion molecules on the endothelium and PMNs. Reperfusion of ischemic intestine is associated with P-selectin-dependent adhesion of leucocytes in the liver microcirculation. In mice with 15 min of SMA occlusion and 30 min of reperfusion, intravenous L-arginine (2.5 mg/kg/h) administered at 15 min before ischaemia effectively attenuated the gut I–R-induced increases in the number of rolling leucocytes in the terminal hepatic venules and P-selectin expression in the liver, intestine and lung. The gut I–R-induced increases in both rolling leucocytes and P-selectin expression in the liver and intestine were exaggerated in rats with a NOS inhibitor N^G-monomethyl-L-arginine and were attenuated in rats with the co-administration of L-arginine and NOS inhibitor. These findings indicated that NO modulates gut I–R-induced recruitment of rolling leucocytes by acting on P-selectin expression [44].

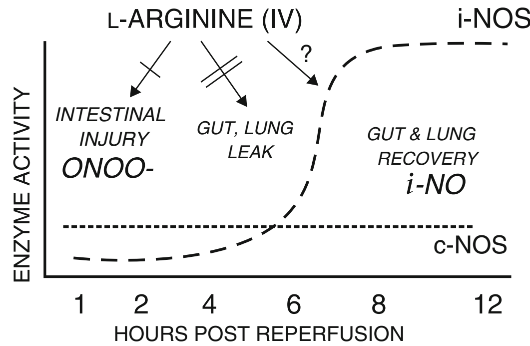

Fig. 28.5 Schematic demonstrating the effects of intravenous L-arginine administration on intestinal ischaemia reperfusion. Note the sustained levels of cNOS compared with Fig. 28.4. Reprinted with permission from Ward et al. [30]

Gut hypoperfusion is considered a mechanism for early MOF after severe surgical insults. L-Arginine may enhance immune cell responses; however, uncontrolled excess immune responses may cause deleterious effects. For example, mice with intestinal I–R receiving L-arginine infusion (1 % L-arginine hydrochloride solution, 1 mL/h) during 60 min of ischaemia exhibit significantly increased production of reactive oxygen intermediates and myeloid cells at 4 h and exhibit decreased survival rates at 12 h of reperfusion. Although L-arginine infusion significantly improved intestinal blood perfusion during ischaemia, the primed, activated and excessive circulating myeloid cells resulted in the reduction in survival rates [31]. In contrast, in pigs with intestinal I–R, administration of L-arginine (5 mg/kg/min) during ischaemia and continuing throughout reperfusion significantly increased SMA blood flow during reperfusion, reduced mucosal injury, alleviated intestinal I–R-induced denudation of villous tips and destruction of glandular architecture and suppressed the release of TNF-α, IL-1 and IL-6 [29]. These results reveal that parenteral L-arginine infusion during ischaemia may improve the blood flow during reperfusion; however, large doses of parenteral L-arginine may result in uncontrolled immune cell responses, which lead to deleterious outcomes.

Evidence indicates that L-arginine may stimulate free radical scavenging and reduce lipid peroxidation. In immature rats with 60-min SMA occlusion followed by 90-min reperfusion, intravenous administration of L-arginine (250 mg/kg) 45 min after ischaemia, i.e. 15 min prior to reperfusion, significantly increased serum NO and decreased serum MDA and lung endothelin, MDA and PMNs, which resulted in the improvement of systemic endothelial function. However, white blood cell counts in bronchoalveolar lavage and serum and lung TNF-alpha, MDA and NO content in lung tissues were not significantly altered [3]. In rats with small bowel transplantation, intravenous L-arginine (50 mg/kg) injection 90 min before harvesting resulted in lower MDA and MPO levels and better histological structures and graft survival rates after the grafts were preserved at 4 °C in Ringer's solution for 8 h [45]. These results suggest that pretreatment with L-arginine may act as a useful adjunct to preserve the grafted intestine from ischaemia injury, at least partially, by improving antioxidant activities.

The effects of L-arginine on intestinal I–R-reduced gut function have been studied in rats subjected to 30-min mesenteric ischaemia followed by 180-min reperfusion. Intravenous L-arginine infusion at a dose of 10 mg/kg at 5 min before reperfusion ameliorated intestinal I–R-induced impairment in spontaneous basal activity and acetylcholine-induced contractile response in the ileum [46]. In addition, Taha et al. [47] indicated that pretreatment with intravenous L-arginine (100 mg/kg body weight) may attenuate intestinal dysfunction in jejunal contractions and enteric nerves due to ischaemia. Gomez et al. [48] treated rats with the same amount of L-arginine intravenously 60 min before SMA occlusion and/or during 120 min of reperfusion. The authors reported that jejunal contractions were not affected by pretreatment of L-arginine, i.e. before ischaemia, but were decreased by peri-treatment of L-arginine,

i.e. during reperfusion. Jejunal enteric nerves were damaged in rats with peri-treatment of L-arginine but not in those with pretreatment of L-arginine. Based on these results, the authors suggested that parenteral L-arginine may attenuate intestinal dysfunction induced by ischaemia but not by reperfusion [48].

Peri-treatment of Parenteral L-Arginine

Few studies have investigated the effects of peri-treatment of parenteral L-arginine, and most studies have reported the deleterious effects of the late use of parenteral L-arginine, i.e. during the reperfusion period, in intestinal I–R injury. The study of Ward et al. [30] revealed that treatment of rats with intravenous L-arginine during the reperfusion period may reduce intestinal I–R-induced lung leak and neutrophil infiltration, but the intestinal leak and mucosal injury were not prevented. The authors concluded that peri-treatment of intravenous L-arginine may be too late to prevent the formation of peroxynitrite from superoxide. Therefore, the intestinal I–R-induced local injury may still occur. Taha et al. [47] also reported that initiated intravenous L-arginine infusion (100 mg/kg body weight) during reperfusion did not attenuate intestinal dysfunction in jejunal contractions and enteric nerves in rabbits with occlusion of the SMA. These studies noted that the timing of intravenous L-arginine infusion may result in different outcomes, which may be associated with the homeostasis of NO.

Conclusions

Intestinal I–R injury is a complex process that involves oxygen and energy depletion and restoration, the hypoxanthine–xanthine oxidase system, ROS and RNS production, the inflammatory response and apoptosis. Under uncontrolled conditions, significant infiltration of PMNs is accompanied by mucosal damage and multiple organ dysfunction and leads to death. Evidence indicates that enteral L-arginine administration has beneficial effects on survival, intestine structure and function and NO homeostasis, and recovery is observed only when L-arginine is provided before the initiation of ischaemia and/or reperfusion. Similar, intravenous L-arginine administration prior to ischaemia may alleviate intestinal I–R-induced adverse effects. However, it has been noted that pretreatment with large doses of parenteral L-arginine may result in deleterious outcomes. Thus far, almost all of these results were obtained from animal studies with inconsistent outcomes. These controversial results of L-arginine administration in intestinal I–R may be associated with the therapeutic time, dosing, route and disease severity. Therefore, the application of L-arginine supplementation for patient with intestinal I–R injury remains questionable and needs to be further investigated.

References

1. Vollmar B, Menger MD. Intestinal ischemia/reperfusion: microcirculatory pathology and functional consequences. Langenbeck's Arch Surg. 2011;396(1):13–29.
2. Alhan E, Usta A, Cekic A, Saglam K, Turkyilmaz S, Cinel A. A study on 107 patients with acute mesenteric ischemia over 30 years. Int J Surg. 2012;10(9):510–3.
3. Fu TL, Zhang WT, Zhang L, Wang F, Gao Y, Xu M. L-arginine administration ameliorates serum and pulmonary cytokine response after gut ischemia-reperfusion in immature rats. World J Gastroenterol. 2005;11(7):1070–2.
4. de Aguilar-Nascimento JE, Dock-Nascimento DB, Bragagnolo R. Role of enteral nutrition and pharmaconutrients in conditions of splanchnic hypoperfusion. Nutrition. 2010;26(4):354–8.
5. Cerqueira NF, Hussni CA, Yoshida WB. Pathophysiology of mesenteric ischemia/reperfusion: a review. Acta Cir Bras. 2005;20(4):336–43.

6. Thomson AB, Keelan M, Thiesen A, Clandinin MT, Ropeleski M, Wild GE. Small bowel review: diseases of the small intestine. Dig Dis Sci. 2001;46(12):2555–66.
7. Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. Anesthesiology. 2001;94(6):1133–8.
8. Haglund U. Gut ischaemia. Gut. 1994;35 Suppl 1:S73–6.
9. Ozkan OV, Yuzbasioglu MF, Ciralik H, et al. Resveratrol, a natural antioxidant, attenuates intestinal ischemia/reperfusion injury in rats. Tohoku J Exp Med. 2009;218(3):251–8.
10. Coopersmith CM, O'Donnell D, Gordon JI. Bcl-2 inhibits ischemia-reperfusion-induced apoptosis in the intestinal epithelium of transgenic mice. Am J Physiol. 1999;276(3 Pt 1):G677–86.
11. Sukhotnik I, Helou H, Mogilner J, et al. Oral L-arginine improves intestinal recovery following ischemia-reperfusion injury in rat. Pediatr Surg Int. 2005;21(3):191–6.
12. Lee CH, Hsiao CC, Hung CY, Chang YJ, Lo HC. Long-term enteral L-arginine supplementation in rats with intestinal ischemia and reperfusion. J Surg Res. 2012;175(1):67–75.
13. Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischemia-reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci. 2004;49(9):1359–77.
14. Watson MJ, Ke B, Shen XD, et al. Intestinal ischemia/reperfusion injury triggers activation of innate toll-like receptor 4 and adaptive chemokine programs. Transplant Proc. 2008;40(10):3339–41.
15. Sasaki M, Joh T. Oxidative stress and ischemia-reperfusion injury in gastrointestinal tract and antioxidant, protective agents. J Clin Biochem Nutr. 2007;40(1):1–12.
16. Schofield ZV, Woodruff TM, Halai R, Wu MC, Cooper MA. Neutrophils—a key component of ischemia-reperfusion injury. Shock. 2013;40(6):463–70.
17. Sureda A, Cordova A, Ferrer MD, et al. Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise. Free Radic Res. 2009;43(9):828–35.
18. Perdue MH, McKay DM. Integrative immunophysiology in the intestinal mucosa. Am J Physiol. 1994;267(2 Pt 1):G151–65.
19. Cavriani G, Oliveira-Filho RM, Trezena AG, et al. Lung microvascular permeability and neutrophil recruitment are differently regulated by nitric oxide in a rat model of intestinal ischemia-reperfusion. Eur J Pharmacol. 2004;494(2–3):241–9.
20. Yao JH, Zhang XS, Zheng SS, et al. Prophylaxis with carnosol attenuates liver injury induced by intestinal ischemia/reperfusion. World J Gastroenterol. 2009;15(26):3240–5.
21. Tian XF, Yao JH, Zhang XS, et al. Protective effect of carnosol on lung injury induced by intestinal ischemia/reperfusion. Surg Today. 2010;40(9):858–65.
22. Tóth Š, Pomfy M, Wohlfahrt P, et al. Detection of early stages of apoptosis in experimental intestinal ischemia-reperfusion injury. Biologia. 2007;62(4):491–7.
23. Murayama T, Tanabe M, Matsuda S, et al. JNK (c-Jun NH2 terminal kinase) and p38 during ischemia reperfusion injury in the small intestine. Transplantation. 2006;81(9):1325–30.
24. Ban K, Santora R, Kozar RA. Enteral L-arginine modulates inhibition of AP-1/c-Jun by SP600125 in the postischemic gut. Mol Cell Biochem. 2011;347(1–2):191–9.
25. Ben DF, Yu XY, Ji GY, et al. TLR4 mediates lung injury and inflammation in intestinal ischemia-reperfusion. J Surg Res. 2012;174(2):326–33.
26. Siriussawakul A, Chen LI, Lang JD. Medical gases: a novel strategy for attenuating ischemia-reperfusion injury in organ transplantation? J Transplant. 2012;2012:819382.
27. Ozturk H, Aldemir M, Dokucu AI, Yagmur Y, Kilinc N, Sahin AH. The nitric oxide donor molsidomine prevents ischemia/reperfusion injury of the adult rat small intestine. Pediatr Surg Int. 2003;19(4):305–8.
28. Sayan H, Ozacmak VH, Altaner S, Aktas RG, Arslan SO. Protective effects of L-arginine on rat terminal ileum subjected to ischemia/reperfusion. J Pediatr Gastroenterol Nutr. 2008;46(1):29–35.
29. Spanos CP, Papaconstantinou P, Spanos P, Karamouzis M, Lekkas G, Papaconstantinou C. The effect of L-arginine and aprotinin on intestinal ischemia-reperfusion injury. J Gastrointest Surg. 2007;11(3):247–55.
30. Ward DT, Lawson SA, Gallagher CM, Conner WC, Shea-Donohue T. Sustained nitric oxide production via L-arginine administration ameliorates effects of intestinal ischemia-reperfusion. J Surg Res. 2000;89(1):13–9.
31. Fukatsu K, Ueno C, Maeshima Y, et al. Effects of L-arginine infusion during ischemia on gut blood perfusion, oxygen tension, and circulating myeloid cell activation in a murine gut ischemia/reperfusion model. J Parenter Enteral Nutr. 2004;28(4):224–30. discussion 230–221.
32. Sato N, Moore FA, Kone BC, et al. Differential induction of PPAR-gamma by luminal glutamine and iNOS by luminal L-arginine in the rodent postischemic small bowel. Am J Physiol Gastrointest Liver Physiol. 2006;290(4):G616–23.
33. Zhan Z, Ou D, Piao X, Kim SW, Liu Y, Wang J. Dietary L-arginine supplementation affects microvascular development in the small intestine of early-weaned pigs. J Nutr. 2008;138(7):1304–9.
34. Li XL, Zou XM, Nie G, Song ML, Li G. Roles of neuronal nitric oxide synthase and inducible nitric oxide synthase in intestinal transplantation of rats. Transplant Proc. 2013;45(6):2497–501.

35. Breithaupt-Faloppa AC, Fantozzi ET, Assis-Ramos MM, et al. Protective effect of estradiol on acute lung inflammation induced by an intestinal ischemic insult is dependent on nitric oxide. Shock. 2013;40(3):203–9.
36. Schleiffer R, Raul F. Prophylactic administration of L-arginine improves the intestinal barrier function after mesenteric ischaemia. Gut. 1996;39(2):194–8.
37. Raul F, Galluser M, Schleiffer R, Gosse F, Hasselmann M, Seiler N. Beneficial effects of L-arginine on intestinal epithelial restitution after ischemic damage in rats. Digestion. 1995;56(5):400–5.
38. Chen B, Fu JF, Yuan WH, et al. Study on the mechanism of protective effect of oral L-arginine on intestine after scald injury in rats. Zhonghua Shao Shang Za Zhi. 2005;21(4):259–61.
39. Krauss H, Sosnowski P, Biczysko M, et al. Effects of L-arginine and NG-nitro L-arginine methyl ester (L-NAME) on ischemia/reperfusion injury of skeletal muscle, small and large intestines. Chin J Physiol. 2011;54(1):7–18.
40. Kozar RA, Verner-Cole E, Schultz SG, et al. The immune-enhancing enteral agents L-arginine and glutamine differentially modulate gut barrier function following mesenteric ischemia/reperfusion. J Trauma. 2004;57(6):1150–6.
41. Margaritis EV, Yanni AE, Agrogiannis G, et al. Effects of oral administration of (L)-arginine, (L)-NAME and allopurinol on intestinal ischemia/reperfusion injury in rats. Life Sci. 2011;88(23–24):1070–6.
42. Cintra AE, Martins JL, Patricio FR, Higa EM, Montero EF. Nitric oxide levels in the intestines of mice submitted to ischemia and reperfusion: L-arginine effects. Transplant Proc. 2008;40(3):830–5.
43. Puiman PJ, Stoll B, van Goudoever JB, Burrin DG. Enteral L-arginine does not increase superior mesenteric arterial blood flow but induces mucosal growth in neonatal pigs. J Nutr. 2011;141(1):63–70.
44. Horie Y, Wolf R, Anderson DC, Granger DN. Nitric oxide modulates gut ischemia-reperfusion-induced P-selectin expression in murine liver. Am J Physiol. 1998;275(2 Pt 2):H520–6.
45. Cao B, Li N, Wang Y, Li JS. Protective effect of L-arginine preconditioning on ischemia and reperfusion injury associated with rat small bowel transplantation. World J Gastroenterol. 2005;11(19):2994–7.
46. Arslan SO, Gelir E, Sayan H, Ozacmak VH. L-arginine and melatonin interaction in rat intestinal ischemia–reperfusion. Fundam Clin Pharmacol. 2005;19(5):533–5.
47. Taha MO, Miranda-Ferreira R, Paez RP, et al. Role of L-arginine, a substrate of nitric oxide biosynthesis, on intestinal ischemia-reperfusion in rabbits. Transplant Proc. 2010;42(2):448–50.
48. Gomez JS, Miranda-Ferreira R, Taha NS, et al. Study of L-arginine in intestinal lesions caused by ischemia-reperfusion in rats. Transplant Proc. 2012;44(8):2309–12.

Chapter 29
Mucosal Protection by L-Arginine in the Upper Gastrointestinal Tract

Koji Takeuchi

Key Points

- The intragastric administration of L-arginine prevented the occurrence of acid-reflux esophagitis, which was induced in rats by ligation of both the pylorus and forestomach.
- This effect was mimicked by L-arginine, but was not affected by the pretreatment with indomethacin or N^G-nitro-L-arginine methyl ester.
- The oral administration of L-arginine prevented 0.6 N HCl-induced gastric lesions in rats. This effect was also observed with L-arginine, but not with an equimolar dose of mannitol, and was attenuated by the prior administration of indomethacin, but not N^G-nitro-L-arginine methyl ester.
- The mucosal application of L-arginine produced a reduction in the potential difference, inhibited gastric motility, and increased mucosal blood flow, and the blood flow and motility responses were mitigated by the pretreatment with indomethacin, but not N^G-nitro-L-arginine methyl ester.
- Protective effects against esophagitis may have occurred via the amelioration of defensive mechanisms mediated by factors other than prostaglandins and nitric oxide, while protective effects against gastric lesions may be afforded by it acting as a mild irritant and are mainly mediated by endogenous prostaglandins, but not the nitric oxide-dependent pathway.

Keywords L-Arginine • Acid-reflux esophagitis • HCl-induced gastric lesion • Mucosal protection • Nitric oxide • Prostaglandin

Abbreviations

i.g.	Intragastric
s.c.	Subcutaneous
L-NAME	N^G-nitro-L-arginine methyl ester
PGs	Prostaglandins

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NO Nitric oxide
PD Potential difference
MBF Mucosal blood flow

Introduction

L-Arginine, a conditionally nonessential amino acid, is synthesized from citrulline by the sequential action of the cytosolic enzymes argininosuccinate synthetase and argininosuccinate lyase in the body and does not need to be obtained directly through the diet. However, since this biosynthetic pathway does not produce sufficient amounts of L-arginine, some needs to be consumed through the diet. This amino acid plays an important role in cell division, the healing of wounds, removal of ammonia from the body, immune function, and the regulation of cardiovascular aspects [1–3]. In addition, L-arginine is the precursor of nitric oxide (NO), which is synthesized from this amino acid by NO synthase, an enzyme involved in the modulation of various biological actions mainly through the activation of guanylyl cyclase under physiological conditions [4]. Several studies [5–10] demonstrated that L-arginine exhibited protective effects against various lesion models in the gastrointestinal tract, including the esophagus. Although the mechanisms responsible have yet to be elucidated, the protective effects were reportedly related to processes dependent on or independent of NO [7–10].

We herein reviewed the protective effects of L-arginine against acid-reflux esophagitis and HCl-induced gastric lesions, mainly based on the findings of our previously published studies [6, 9, 10], and discussed the pathogenesis of these lesions as well as the factors involved in the protective effects of L-arginine.

Esophageal Protection

Reflux esophagitis, an endoscopically positive gastroesophageal reflux disease, is mainly caused by excessive exposure to gastric contents due to impairments in various protective mechanisms that prevent reflux into the esophagus and resist the refluxate [11, 12]. Since gastric acid plays a key role in the pathogenesis of reflux esophagitis, luminal pH control is considered to be important in the management of this disease [12]. Antisecretory drugs, such as histamine H_2 receptor antagonists and proton pump inhibitors, were previously shown to be effective against acid-reflux esophagitis in humans and animals [13–15]. We also showed the unique influences of L-arginine on — this esophageal injury [10].

Induction of Acid-Reflux Esophagitis and Its Pathogenesis

Reflux esophagitis is a chronic disease caused by repeated contact between the gastric contents and the esophageal epithelium. We used a rat esophagitis model to investigate the pathogenesis of this disease and examine the effects of antisecretory drugs on esophagitis. In brief, rats were kept in individual cages with raised mesh bottoms and deprived of food, but were allowed free access to tap water for 18 h prior to the experiments. Under ether anesthesia, the abdomen was incised along the middle and both the pylorus and junction between the forestomach and corpus were ligated [15, 16] (Fig. 29.1a). Severe hemorrhagic damage then developed in the proximal 3 cm of the esophagus in a time-dependent manner (Fig. 29.1b). The severity of acid-reflux esophagitis induced by double ligation of the pylorus and forestomach for 4 h was prevented by the prior intraduodenal administration of antisecretory drugs (Fig. 29.1c). In addition, the intragastric (i.g.) administration of antipeptic

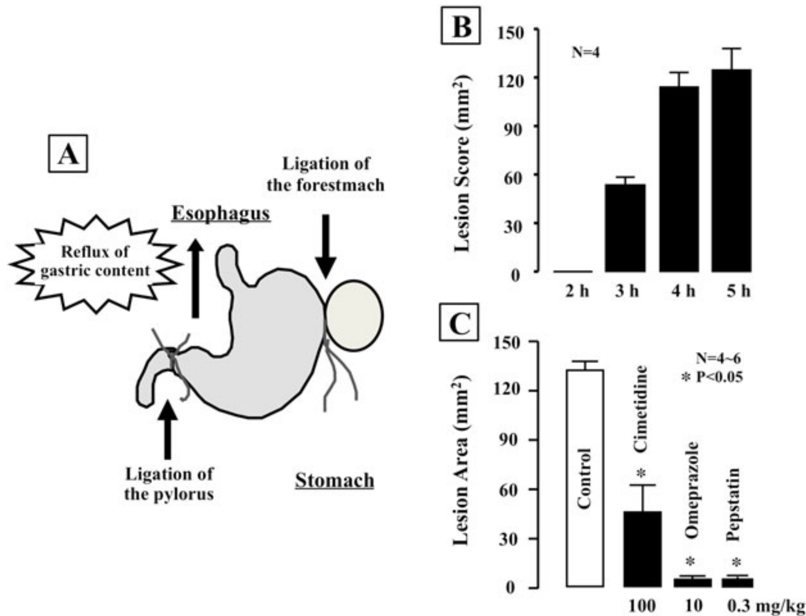


Fig. 29.1 (**a**) Induction of acid-reflux esophagitis in rats. Under ether anesthesia, the abdomen was incised, and both the pylorus and junction between the corpus and forestomach were ligated. Three or four hours later, animals were killed by an overdose of ether, and the esophagus was removed, opened, and examined for hemorrhagic lesions. (**b**) Time-course changes in the development of acid-reflux esophagitis in rats. Under ether anesthesia, both the pylorus and forestomach were ligated, and the esophageal mucosa was examined 2–5 h later. Data were presented as the mean ± SE for four rats. (**c**) Effects of cimetidine, omeprazole, and pepstatin on acid-reflux esophagitis in rats. Cimetidine (100 mg/kg) and omeprazole (10 mg/kg) were given i.d. immediately after the dual ligation, while pepstatin (0.3 mg/kg) was given i.g. immediately after the ligation. Data were presented as the mean ± SE for 4–6 rats. *Significantly different from the control, at $P < 0.05$ (from Ref. [10] after modifications)

drugs also mitigated the occurrence of these esophageal lesions, whereas porcine pepsin worsened the severity of esophageal damage induced by double ligation for 4 h.

We found that antisecretory drugs, such as omeprazole and cimetidine, significantly prevented the development of esophageal lesions, and the findings suggested a key role of gastric acid in the pathogenesis of esophageal lesions [15, 17]. Pepstatin also potently prevented the occurrence of acid-reflux esophagitis [9, 17]. This drug was previously shown to exhibit potent and specific inhibitory effects against the proteolytic activity of pepsin [18, 19]. We also observed that exogenously administered pepsin significantly worsened the severity of esophageal lesions in the present model. These findings strongly suggested that pepsin may play a major role in the pathogenesis of acid-reflux esophagitis. In addition to gastric acid and pepsin, bile acids and pancreatic enzymes were also included in the refluxate into the esophagus. However, because this model was induced in pylorus-ligated stomachs in which no regurgitation occurred from the duodenal contents into the stomach, it is unlikely that bile acids and pancreatic enzymes participated in the pathogenesis observed in this model of rat esophagitis.

Effects of L-Arginine on Esophagitis

When L-arginine was administered i.g. 10 min before the ligation of the pylorus and forestomach, this amino acid dose-dependently reduced the severity of esophageal lesions, with complete inhibition

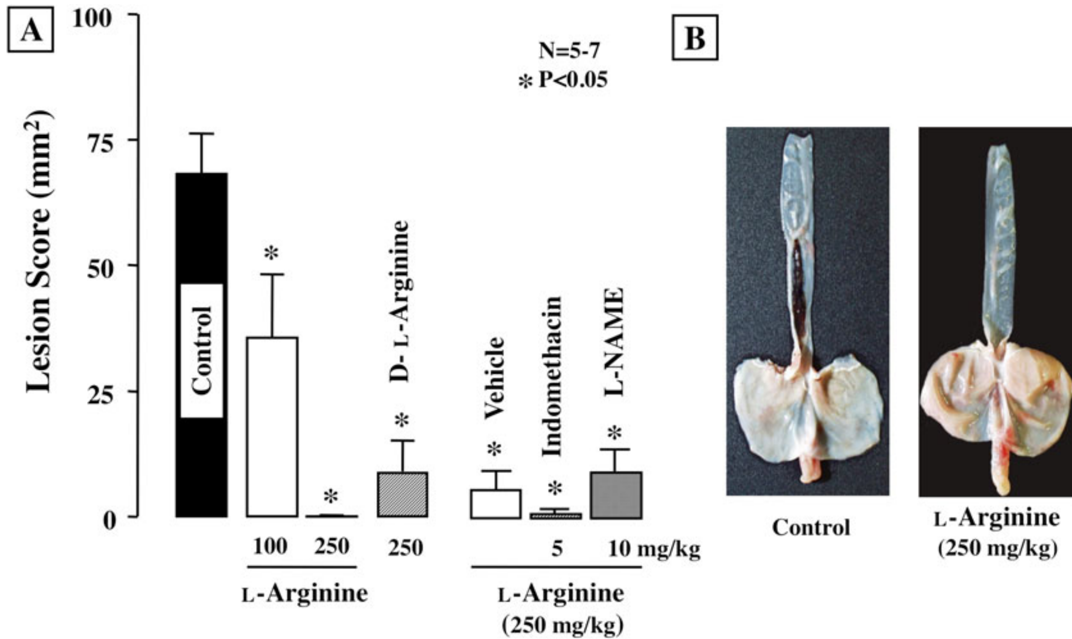


Fig. 29.2 Effects of L- and D-arginine on acid-reflux esophagitis in rats in the absence or presence of indomethacin or L-NAME. Both the pylorus and forestomach were ligated under ether anesthesia, and the esophageal mucosa was examined 4 h later. L-Arginine (100 and 250 mg/kg) or D-arginine (250 mg/kg) was given i.g. 10 min after the ligation, while indomethacin (5 mg/kg) or L-NAME (10 mg/kg) was given s.c. 30 min before the ligation. Data are presented as the mean ± SE for 5–7 rats. *Significantly different from the control, at $P < 0.05$. (**b**) shows the macroscopic appearance of esophageal lesions induced by the dual ligation for 3 h. Note that L-arginine potently inhibited the development of hemorrhagic esophageal lesions (from Ref. [10] after modifications)

being observed at 250 mg/kg (Fig. 29.2a, b). We also examined the effects of D-arginine in order to determine whether the protective effects of L-arginine were due to its physicochemical properties or were mediated by its biological actions; therefore, the same experiments were repeated using D-arginine. As shown in Fig. 29.2a, D-arginine also provided good protection against acid-reflux esophagitis, inhibition at 250 mg/kg being 89.0 %. To further investigate the involvement of endogenous PGs and NO in these protective actions, the effects of L-arginine were examined in the presence of indomethacin and N^G-nitro-L-arginine methyl ester (L-NAME). The prior subcutaneous (s.c.) administration of indomethacin or L-NAME did not significantly affect the protective effects of L-arginine, with the degree of protection in the presence of these agents being equivalent to that in the vehicle-treated group (Fig. 29.2a). On the other hand, the severity of acid-reflux esophagitis was not significantly affected by L-arginine given i.v. or mannitol given i.g. at a concentration equimolar to that of L-arginine (250 mg/kg).

Reflux esophagitis has been attributed to impairments in epithelial defense against acid-pepsin contact [20, 22]. The mechanisms underlying these phenomena remain unclear; however, they may be mediated, at least partly, by endogenous PGs and NO [23–25]. Since L-arginine is a substrate for the production of NO, it is possible that the protective effects of this amino acid may be partly mediated by NO. However, the role of NO in the pathogenesis of esophagitis remains controversial [25–27]. Lanas et al. [25] reported that rabbit esophageal mucosa showed mucosal adaptation to acid and pepsin, and this phenomenon was, at least in part, dependent on NO-mediated mechanisms. Konturek et al. [26] also reported that melatonin protected against acid-reflux esophagitis mediated by NO and

calcitonin gene-related peptide released from sensory nerves and the suppression of inflammatory cytokine production. In contrast, Ishiyama et al. [27] showed that exogenous NO exacerbated tissue damage in a rat model of reflux esophagitis. We reported that NO increased the secretion of pepsin via the stimulation of guanylyl cyclase [28]. However, since we found that the protective effects of L-arginine were not significantly antagonized by L-NAME, a NO synthase inhibitor, it was unlikely that L-arginine afforded protection against acid-reflux esophagitis by a NO-dependent mechanism. This is supported by the finding that L-arginine had a similar protective effect to L-arginine at the same dose because L-arginine cannot be used as a substrate for NO production. Further studies are necessary to elucidate the mechanism underlying the protective effects of L-arginine.

Effect of L-Arginine on Acid Secretion and Acid-Buffering Action

Since the severity of esophagitis is known to be affected by antisecretory drugs and influenced by the pH of the gastric contents, we examined the effects of L-arginine on gastric secretion, including the pH of the gastric contents in pylorus-ligated rats. Ligation of the pylorus for 3 h resulted in accumulation of approximately 6 ml of gastric juice in the stomach, with the pH of the gastric contents being 1.30 ± 0.05 (Table 29.1). L-Arginine at 250 mg/kg did not significantly affect the volume of the gastric contents, but significantly elevated the pH to 1.82 ± 0.05. Because this model was produced by the corrosive actions of acid and pepsin, it is possible that the mechanism underlying the protection against these lesions may be associated with changes in peptic activity. The proteolytic activity of pepsin has been shown to be dependent on pH and was maximal at a pH of approximately 2.0 [9, 10]. Pepsin also plays an important role in the pathogenesis of acid-reflux esophagitis. It is possible that the effects of L-arginine on acid-reflux esophagitis may be attributable to its acid-buffering capability; it may modify the optimal pH for the proteolytic action of pepsin. To examine this possibility, we titrated a solution of L-arginine and varied the pH with the addition of HCl in vitro. The L-arginine solution was a strong base, pH 10.6, and when the solution was titrated with the addition of 150 mM HCl, this amino acid exhibited a potent buffering action against HCl at pH of approximately 2.0 (Fig. 29.3).

We found that the pH of the gastric contents was increased by L-arginine (250 mg/kg) from 1.30 ± 0.05 to 1.82 ± 0.05. Since L-arginine increased the pH of the gastric contents due to its acid-buffering capability to around 2.0, it is assumed that this amino acid may increase pepsin activity. Since L-arginine prevented, but not aggravated, esophagitis, it is unlikely that the acid-buffering capability of this amino acid contributed to its protective effects. In addition, we demonstrated that other amino acids such as L-alanine, glycine, and L-glutamine also had potent buffering effects and increased the pH of the gastric contents to approximately 2.0; however, protection against the esophagitis model was only observed with glycine, but not L-alanine or L-glutamine [10]. Thus, L-arginine may exert a protective effect against acid-reflux esophagitis due to as yet unknown mechanisms, in spite of the increase in gastric pH and possibly pepsin activity.

Table 29.1 Effects of L-arginine on the volume and pH of the gastric contents in pylorus-ligated rats

Drugs	Doses (mg/kg)	No. of rats	Volume (ml)	Gastric pH
Control	–	5	6.38 ± 0.87	1.30 ± 0.05
L-Arginine	250	4	6.30 ± 0.41	1.82 ± 0.05*

L-Arginine (250 mg/kg) was given orally immediately after the pylorus was ligated. The gastric contents were collected 3 h later. Following centrifugation for 10 min at 3000 rpm, the volume of each sample was measured, and the pH was determined using a pH meter. Values are presented as the mean ± SE for 4–5 rats

*Significantly different from the control, at $P < 0.05$

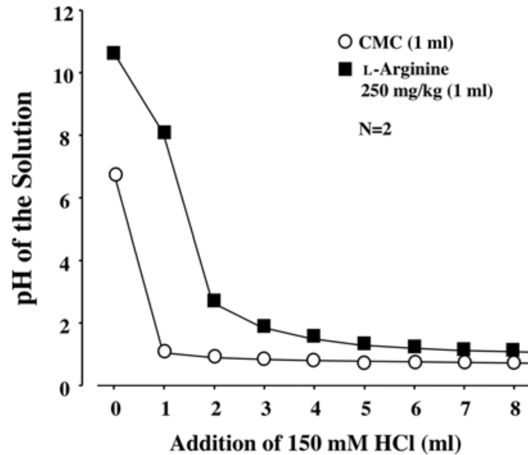


Fig. 29.3 Buffering capability of L-arginine against HCl in vitro. L-Arginine (250 mg/kg) was suspended or dissolved in a 0.5 % CMC solution, and 1 ml of the solution was titrated by the addition of 150 mM HCl. Changes in the pH of the solution were determined by a pH meter, and data are presented as the mean for two rats

Gastric Protection

Several studies [5, 29, 30] reported that amino acids, including L-arginine, exhibited protective effects against the gastric lesions induced by aspirin, indomethacin, and necrotizing agents. On the other hand, endogenous NO was shown to be involved in certain types of gastroprotection induced by capsaicin [31], lafutidine [32], and mild irritants [33]. However, the effects of L-arginine, a precursor of NO, on gastric mucosal integrity have not been studied in detail. We introduced the protective effects of L-arginine on gastric lesions induced by 0.6 N HCl in rats, including the possible mechanisms involved in this action [6].

Effects of L-Arginine on HCl-Induced Gastric Damage

The oral administration of 0.6 N HCl (1 ml) induced hemorrhagic lesions in the glandular stomach, with a lesion score of 118.3 ± 10.2 mm^2. L-Arginine, given p.o. 30 min before HCl, prevented the development of gastric lesions caused by HCl in a dose-related manner; the lesion score at 300 mg/kg was 40.2 ± 17.3 mm^2 (Fig. 29.4). D-Arginine also dose-dependently protected against HCl-induced gastric lesions, and the degree of protection at 300 mg/kg, p.o. was 62.3 %, which was equivalent to that obtained by L-arginine at 300 mg/kg. On the other hand, L- and D-arginine given i.v. had no effect on the severity of these lesions. Mannitol given p.o. at an equimolar concentration to L-arginine (300 mg/kg, p.o.) was also ineffective in reducing the severity of these lesions. The prior s.c. administration of L-NAME 30 min before L-arginine did not affect the protective activity of L-arginine against HCl-induced gastric lesions (Fig. 29.5a, b). In contrast, the effects of L-arginine were significantly mitigated by the prior administration of indomethacin; the degree of protection afforded by L-arginine was only 12.3 % in the presence of indomethacin, which was significantly lower than that (68.3 %) observed in the absence of this agent. Neither L-NAME nor indomethacin alone had any effect on the severity of HCl-induced gastric lesions.

L-Arginine potently protected against HCl-induced gastric mucosal lesions when it was administered p.o. However, when this amino acid was given i.v., it did not confer any protection against damage at the dose (200 mg/kg) that antagonized the elevated blood pressure response induced by the NO biosynthesis

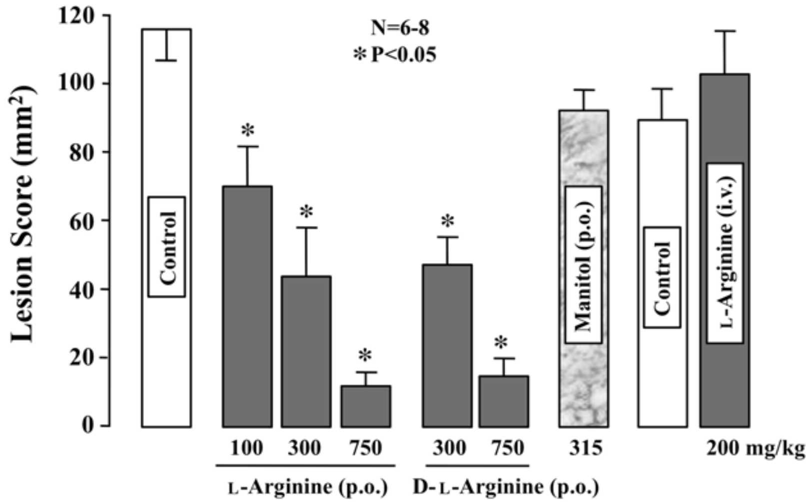


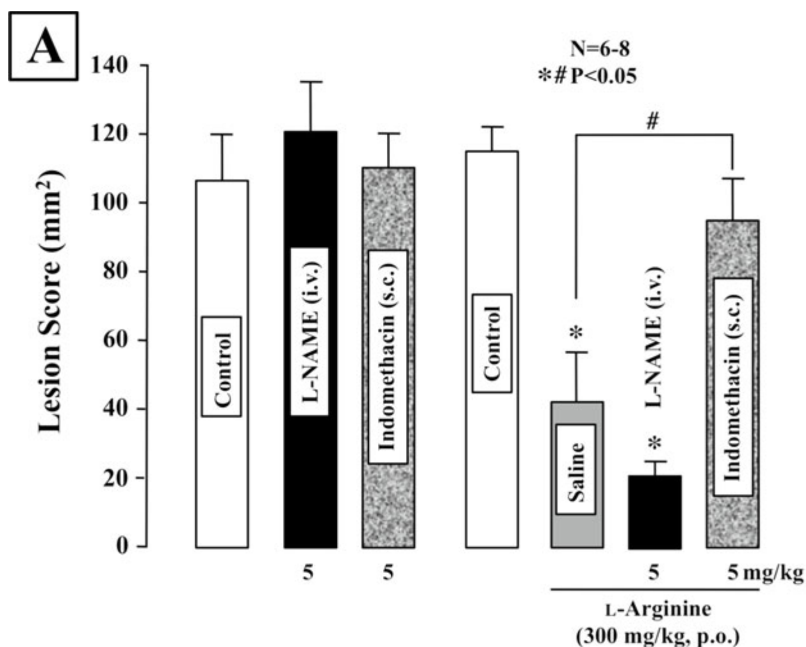
Fig. 29.4 Effects of L- or D-arginine on gastric lesions induced by 0.6 N HCl in rats. Animals were given 1 ml of 0.6 N HCl p.o. and killed 1 h later. L-Arginine (100, 300, and 750 mg/kg), D-arginine (300 and 750 mg/kg), or mannitol (315 mg/kg) was given p.o. 30 min or i.v. 10 min, respectively, before the HCl treatment. Data are presented as the mean ± SE for 6–8 rats. *Significantly different from the control, at $P < 0.05$ (from Ref. [6] after modifications)

inhibitor L-NAME [6]. In addition, the protective effects of L-arginine were mimicked by the enantiomer D-arginine and were unaffected by the prior administration of L-NAME and almost totally disappeared in the presence of indomethacin. These findings suggested that the mucosal protective effects afforded by L-arginine (p.o.) may involve endogenous PGs but are unrelated to the NO-mediated pathway.

Effects of L-Arginine on Gastric PD, MBF, and Motility

The mucosal application of L-arginine (300 mg/kg) in the stomach caused a decrease in the potential difference (PD) and increase in mucosal blood flow (MBF) (Figs. 29.6 and 29.7). Similar results were obtained with D-arginine but not with an equimolar concentration of D-mannitol applied to the mucosa (data not shown). In addition, L-arginine given i.v. did not produce any change in either PD or MBF. The changes in MBF induced by the mucosal application of L-arginine were significantly mitigated by the prior administration of indomethacin, although the degree of the reduction in PD remained unchanged. The pretreatment with L-NAME did not significantly affect either the PD or MBF response induced by L-arginine. On the other hand, gastric motility was inhibited by L-arginine given i.g. at 300 mg/kg, but not i.v. at 200 mg/kg. The motility inhibition caused by L-arginine was mitigated by the prior s.c. administration of indomethacin, but not L-NAME (Fig. 29.8). Gastric motility was also inhibited by the i.g. administration of D-arginine but not D-mannitol at an equimolar concentration (data not shown).

The mucosal application of L-arginine reduced PD, followed by an increase in MBF. These responses were mimicked by D-arginine, and the increased MBF response was mitigated by pretreatment with indomethacin. These responses have frequently been reported in the stomach after exposure to mild irritants such as hypertonic NaCl [34]. A histological examination revealed damage in the surface epithelium of the mucosa immediately after exposure to L-arginine at 300 mg/kg or greater, which suggested that L-arginine acted on the stomach in a similar manner to mild irritants. The mechanism by which the mucosal application of L-arginine caused a reduction in PD remains unknown. These effects of L-arginine were not reproduced by the mucosal application of mannitol at an



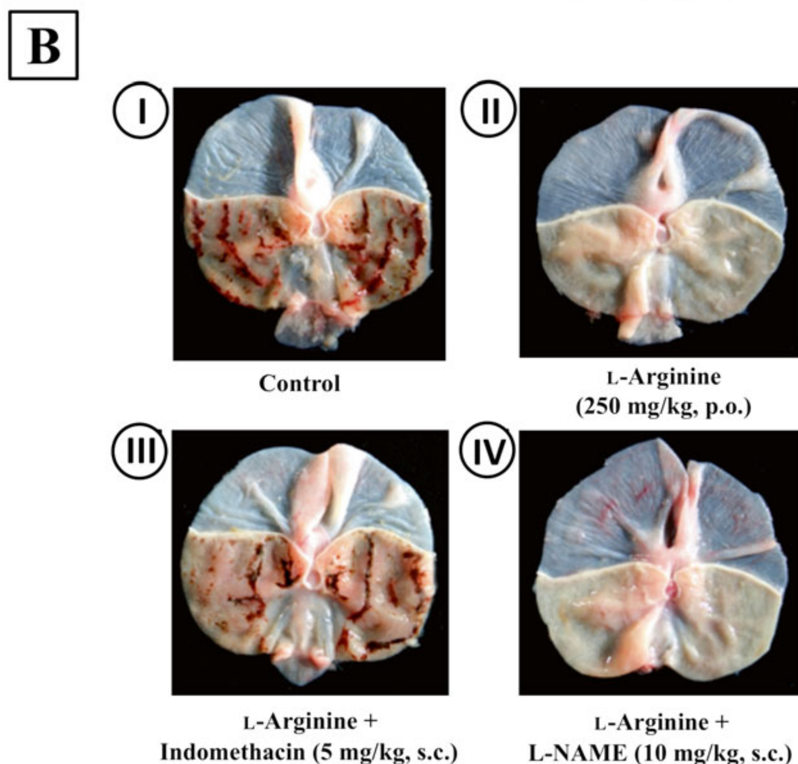


Fig. 29.5 (**a**) Effects of L-arginine on the gastric lesions induced by 0.6 N HCl in rats, in the absence or presence of indomethacin or L-NAME. Animals were given 1 ml of 0.6 N HCl p.o. and killed 1 h later. L-Arginine (300 mg/kg) was given p.o. 30 min before the HCl treatment, while indomethacin (5 mg/kg, s.c.) or L-NAME (5 mg/kg, i.v.) was given 30 min or 10 min, respectively, before L-arginine. Data are presented as the mean ± SE for 6–8 rats. *Significant difference at *P*<0.05; * from the control; # from the saline. (**b**) Macroscopic observations of gastric lesions induced by 0.6 N HCl in the absence or presence of L-arginine (300 mg/kg) with or without indomethacin or L-NAME. Figures show I, control; II, L-arginine (300 mg/kg); III, indomethacin+ L-arginine; and IV, L-NAME + L-arginine. Note that L-arginine prevented the development of hemorrhagic gastric lesions, but this effect was attenuated by the pretreatment with indomethacin, but not L-NAME (from Ref. [6] after modifications)

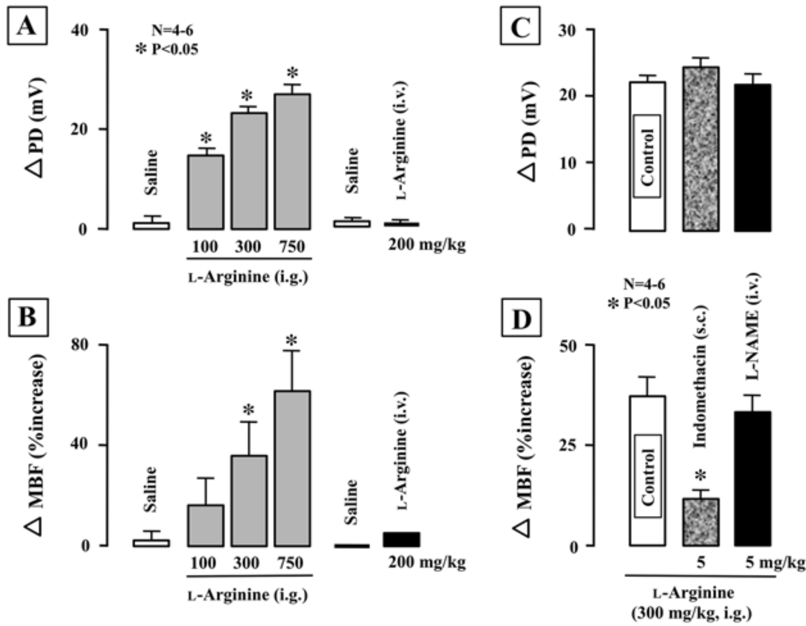


Fig. 29.6 Effects of L-arginine on PD (**a**) and MBF (**b**) responses in the chambered stomach of anesthetized rats. L-Arginine was applied topically to the chamber at a dose of 300 mg/kg for 30 min or given i.v. at a dose of 200 mg/kg. On the *right*, effects of indomethacin and L-NAME on changes in PD (**C**) and MBF (**D**) caused by L-arginine applied to the stomach for 30 min. Values indicate the maximal changes after the treatment with L-arginine and represent the mean ± SE from 4 to 6 rats. *Significantly different from the control (saline), at $P<0.05$ (from Ref. [6] after modifications)

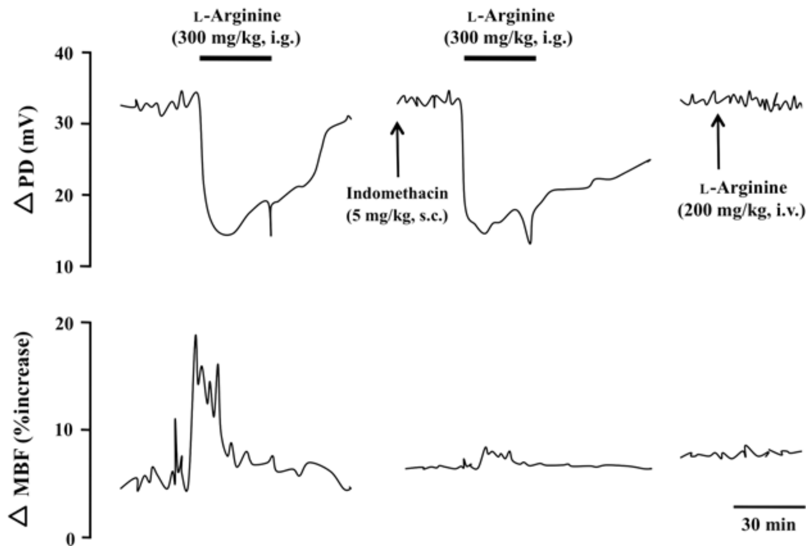


Fig. 29.7 Representative recordings showing changes in PD and MBF after the mucosal application of L-arginine (300 mg/kg for 30 min) in the absence or presence of indomethacin (5 mg/kg, s.c.) or after the i.v. administration of L-arginine (200 mg/kg) to anesthetized rats (from Ref. [6] after modifications)

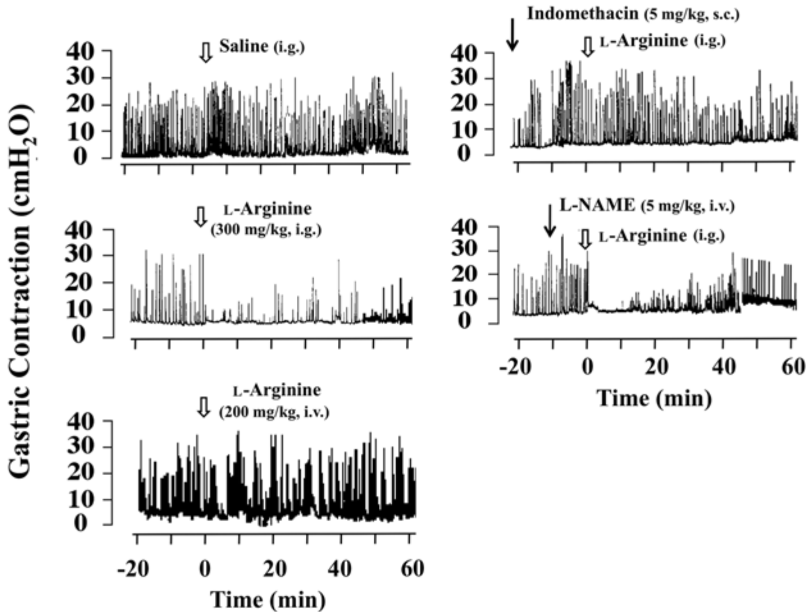


Fig. 29.8 Representative recordings of gastric motility changes after the i.g. administration of L-arginine (300 mg/kg) in the absence or presence of L-NAME and indomethacin in rats. L-NAME (5 mg/kg, i.v.) or indomethacin (5 mg/kg, s.c.) was given 10 min or 30 min, respectively, before the i.g. administration of L-arginine (from Ref. [6] after modifications)

equimolar concentration. Thus, these effects of L-arginine may be attributed to its chemical properties, but not to its hyperosmolarity.

The involvement of endogenous PGs in the mucosal protection induced in the stomach by mild irritants has been demonstrated in previous studies [34–36]. The increased MBF response induced by L-arginine was significantly attenuated by indomethacin, but not by L-NAME. If the irritation of the stomach by L-arginine was associated with the activation of constitutive NO synthase or expression of inducible NO synthase, the MBF response would also be inhibited by L-NAME. Since the mucosal application of L-arginine increased the MBF response even in animals pretreated with L-NAME, it is clear that the MBF response to L-arginine is mainly mediated by endogenous PGs, irrespective of whether NO synthase was inhibited by L-NAME. On the other hand, the inhibition of gastric motility may be functionally associated with gastric protection [37]. As expected, the i.g. administration of L-arginine inhibited gastric motility at a dose that also protected against HCl-induced lesions. This effect was attenuated by indomethacin, but not L-NAME, which is consistent with the previous finding that mild irritants inhibited gastric motility mediated by endogenous PGs [38]. Thus, the mechanism responsible for the protection afforded by L-arginine may mainly be mediated by endogenous PGs and functionally associated with the inhibition of motility and the increase in MBF.

Summary and Future Prospects

This review introduced the mucosal protective effects of L-arginine in the gastrointestinal tract against acid-reflux esophagitis and HCl-induced gastric damage. L-Arginine (i.g.) was found to be highly protective against esophagitis and was mimicked by D-arginine but was unaffected by the pretreatment

with indomethacin or L-NAME. Although the mechanisms responsible have yet to be confirmed, the protective effects of L-arginine in the esophagus may occur via the amelioration of defensive mechanisms that are mediated by factors other than PGs and NO. The oral administration of L-arginine also prevented 0.6 N HCl-induced gastric lesions, and this effect was observed with D-arginine but not an equimolar dose of mannitol and was attenuated by the prior administration of indomethacin, but not L-NAME. The mucosal application of L-arginine produced a reduction in PD, inhibited gastric motility, and increased MBF, and these responses, except for the PD response, were mitigated by the pretreatment with indomethacin, but not L-NAME. The protection afforded by L-arginine in the stomach may occur by it acting as a mild irritant and is mainly mediated by endogenous PGs, but not the NO-dependent pathway. Although further studies are needed to elucidate the mechanisms responsible for these actions, the findings presented here may contribute to the development of a novel therapeutic approach for the treatment of various diseases in the gastrointestinal tract.

Conclusion

L-Arginine given orally has protective effects against the acid-reflux esophagitis and the HCl-induced gastric damage. Protective effects against esophagitis may have occurred via the amelioration of defensive mechanisms mediated by factors other than PGs and NO, while those against gastric lesions may be afforded by it acting as a mild irritant and are mainly mediated by endogenous PGs, but not the NO-dependent pathway.

Statement of Conflict of Interest The authors declare that there is no conflict of interests regarding the publication of this manuscript.

References

1. Masumoto K, Nagata K, Oka Y, Kai H, et al. Successful treatment of an infected wound in infants by a combination of negative pressure wound therapy and L-arginine supplementation. Nutrition. 2011;27:1141–5.
2. Zhu HL, Liu YL, Xie XL, et al. Effect of L-arginine on intestinal mucosal immune barrier function in weaned pigs after Escherichia coli LPS challenge. Innate Immun. 2013;19:242–52.
3. Lorin J, Zeller M, Guilland JC, et al. L-Arginine and nitric oxide synthase: regulatory mechanisms and cardiovascular aspects. Mol Nutr Food Res. 2014;58:101–16.
4. Whittle BJR. Nitric oxide in gastrointestinal physiology and pathology. In: Johnson LR, editor. Physiology of the gastrointestinal tract. 3rd ed. New York: Raven; 1994. p. 267–94.
5. Okabe S, Takeuchi K, Honda K, et al. Effects of various amino acids on gastric lesions induced by acetylsalicylic acid and gastric secretion in pylorus-ligated rats. Arzneimittelforschung. 1997;26:534–7.
6. Takeuchi K, Ohuchi T, Kato S, et al. Cytoprotective action of L-arginine against HCl-induced gastric injury in rats: involvement of nitric oxide? Jpn J Pharmacol. 1993;61:13–21.
7. Tanaka A, Kunikata T, Konaka A, et al. Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. J Physiol Pharmacol. 1999;50:405–17.
8. Ohno R, Yokota A, Tanaka A, et al. Induction of small intestinal damage in rats following combined treatment with cyclooxygenase-2 and nitric oxide synthase inhibitors. J Pharmacol Exp Ther. 2004;310:821–7.
9. Nagahama K, Yamato M, Nishio H, et al. Essential role of pepsin in pathogenesis of acid reflux esophagitis in rats. Dig Dis Sci. 2006;51:303–9.
10. Nagahama K, Nishio H, Yamato M, et al. Orally administered L-arginine and glycine are highly effective against acid reflux esophagitis in rats. Med Sci Monit. 2012;18:BR9–15.
11. Orlando RC. Pathogenesis of reflux esophagitis and Barrett's esophagus. Med Clin North Am. 2005;89:219–41.
12. Hunt RH. Importance of pH control in the management of GERD. Arch Intern Med. 1999;159:649–57.
13. Bell NJ, Burget D, Howden CW, et al. Appropriate acid suppression for the management of gastro-oesophageal reflux disease. Digestion. 1992;51 Suppl 1:59–67.

14. Hamamoto N, Hashimoto T, Adachi K, et al. Comparative study of nizatidine and famotidine for maintenance therapy of erosive esophagitis. J Gastroenterol Hepatol. 2005;20:281–6.
15. Nagahama K, Yamato M, Kato S, et al. Protective effect of lafutidine, a novel H_2 receptor antagonist on acid reflux esophagitis in rats through capsaicin-sensitive afferent neurons. J Pharmacol Sci. 2003;93:55–61.
16. Inatomi N, Nagaya H, Takami K, et al. Effects of a proton pump inhibitor, AG-1749 (lansoprazole), on reflux esophagitis and experimental ulcers in rats. Jpn J Pharmacol. 1991;55:437–51.
17. Takeuchi K, Nagahama K. Animal model of acid-reflux esophagitis: pathogenic roles of acid/pepsin, prostaglandins, and amino acids. BioMed Res Int. 2014;2014:1–10.
18. Umezawa H, Aoyagi T, Morishima H, et al. Pepstatin, a new pepsin inhibitor produced by actinomycetes. J Antibiot. 1970;23:259–62.
19. Kunimoto S, Aoyagi T, Morishima H, et al. Mechanism of inhibition of pepsin by pepstatin. J Antibiot. 1972;25:251–5.
20. Souza RF, Huo X, Mittal V, et al. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. Gastroenterology 2009; 137: 1776–84.
21. De Backer A, Haentjens P, Willems G. Hydrochloric acid: a trigger of cell proliferation in the esophagus of dogs. Dig Dis Sci. 1994;9:884–90.
22. Hollwarth ME, Smith ME, Kvietys RP, et al. Esophageal blood flow in the cat: normal distribution and effects of acid perfusion. Gastroenterology. 1986;90:622–8.
23. Yamato M, Nagahama K, Kotani T, et al. Biphasic effect of prostaglandin E_2 on a rat model of esophagitis mediated by EP1 receptors: relation to pepsin secretion. Digestion. 2005;72:109–18.
24. Takeuchi K, Kato S, Amagase K. Prostaglandin EP receptors involved in modulating gastrointestinal mucosal integrity. J Pharmacol Sci. 2010;114:248–61.
25. Lanas AI, Blas JM, Ortego J, et al. Adaptation of esophageal mucosa to acid- and pepsin-induced damage: role of nitric oxide and epidermal growth factor. Dig Dis Sci. 1997;42:1003–12.
26. Konturek PC, Brozowska I, Targosz A, et al. Esophagoprotection mediated by esogenous and endogenous melatonin in an experimental model of reflux esophagitis. J Pineal Res 2013; 55: 46-57..
27. Ishiyama F, Iijima K, Asanuma K, et al. Exogenous luminal nitric oxide exacerbates esophagus tissue damage in a reflux esophagitis model of rats. Scand J Gastroenterol. 2009;44:527–37.
28. Ito Y, Okuda S, Ohkawa F, et al. Dual role of nitric oxide in gastric hypersecretion in distended stomach: inhibition of acid secretion and stimulation of pepsin secretion. Life Sci. 2008;83:886–92.
29. Urushidani T, Okabe S, Takeuchi K, et al. Effects of various amino acids on indomethacin-induced gastric ulcers in rats. Jpn J Pharmacol. 1977;27:316–9.
30. Kitagawa H, Ikejiri T, Nishiwaki H, et al. Endothelium-dependent adaptive gastric protection. Jpn J Pharmacol. 1992;58(Supp 1):131P.
31. Szolcsányi J, Barthó L. Capsaicin-sensitive afferents and their role in gastroprotection: an update. J Physiol Paris. 2001;95:181–8.
32. Onodera S, Nishida K, Takeuchi K. Unique profile of lafutidine, a novel histamine H_2-receptor antagonist: mucosal protection throughout gastrointestinal tract mediated by capsaicin-sensitive afferent neurons. Drug Des Rev Online. 2004;1:133–44.
33. Yamamoto H, Hirata T, Araki H, et al. Inducible types of cyclooxygenase and nitric oxide synthase in adaptive cytoprotection in rat stomachs. J Physiol Paris. 1999;93:405–12.
34. Matsumoto J, Takeuchi K, Ueshima K, et al. Role of capsaicin-sensitive afferent neurons in mucosal blood flow response of rat stomach induced by mild irritants. Dig Dis Sci. 1992;37:1336–44.
35. Robert A, Nezamis JE, Lancaster C, et al. Mild irritants prevent gastric necrosis through “adaptive cytoprotection” mediated by prostaglandins. Am J Physiol. 1983;245:G113–21.
36. Nobuhara Y, Takeuchi K, Okabe S. Vinegar is a dietary mild irritant to the rat gastric mucosa. Jpn J Pharmacol. 1986;41:101–8.
37. Takeuchi K, Nishiwaki H, Ueshima K, et al. Gastric motility as a possible mechanism of gastric cytoprotection in the rat. In: Kitajima M, editor. Cytoprotection and Cyto-biology. Tokyo: Excerpta Medica; 1992. p. 15–21.
38. Takeuchi K, Nishiwaki H, Okada M, et al. Mucosal protective action of histamine against gastric lesions induced by HCl in rats: importance of antigastric motor activity mediated by H_2-receptors. J Pharmacol Exp Ther. 1988;245:632–8.

Chapter 30
Enteral L-Arginine and Necrotizing Enterocolitis

Sophia Zachaki, Stavroula Gavrili, Elena Polycarpou, and Vasiliki I. Hatzi

Key Points

- Necrotizing enterocolitis is an acute inflammatory bowel disease that affects preterm neonates and is associated with high mortality, morbidity, and hospitalization cost.
- Given that diagnostic and treatment strategies still remain difficult, preventive modalities are critical for reducing the incidence and/or severity of this devastating disease.
- Hypoargininemia is frequently observed in prematurely born neonates.
- L-Arginine supplementation could be proven to be an effective preventive measure by promoting nitric oxide synthesis.
- It is very important to find the right dose, route, and duration of L-arginine administration.

Keywords Necrotizing enterocolitis (NEC) • Amino acid administration • Hypoargininemia • L-Arginine • Nitric oxide (NO)

Abbreviations

NEC	Necrotizing enterocolitis
NO	Nitric oxide
NOS	Nitric oxide synthase
LBW	Low birth weight
FHN	Focal hemorrhagic necrosis
ELBW	Extremely low birth weight
TPN	Total parenteral nutrition

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VLBW	Very low birth weight
RDS	Respiratory distress syndrome
ADMA	Asymmetric dimethylarginine
EDRF	Endothelium-derived relaxing factor
SOD	Superoxide dismutase
GSH-Px	Glutathione peroxidase
NOx	Nitrate plus nitrite
IVH	Intraventricular hemorrhage

Introduction

Necrotizing enterocolitis (NEC) is the most common acquired gastrointestinal disease in premature infants. The molecular basis of this multifactorial disease is poorly understood, and NEC diagnosis and treatment strategies still remain difficult and challenging. In current research preventive approaches are of the utmost priority. Providing human milk to premature infants has been shown to be an effective intervention that decreases the incidence of NEC. Interestingly, recent reports reveal that L-arginine supplementation may be a promising prevention modality of NEC by promoting nitric oxide (NO) synthesis. Deficient or inadequate NO levels due to L-arginine deficiency or immaturity of NO synthase (NOS) activity in premature infants may lead to vasoconstriction and ischemia–reperfusion injury and may predispose the neonates to develop NEC. The role of L-arginine supplementation in NEC has been highlighted in numerous studies on experimental animal models and human neonates. These researches reveal that the prophylactic administration of L-arginine in low doses is safe with no adverse effects for the premature neonates and also that L-arginine supplementation appears to be effective in reducing the overall incidence of NEC in preterm infants and especially the incidence of Bell's stage III NEC. The recent evidence on the L-arginine role in NEC pathophysiology opens up a new area in the field of NEC prevention and further supports the essential role of amino acids in nutrition and disease prevention.

Necrotizing Enterocolitis: Clinical Concepts and Pathogenetic Mechanisms

NEC is an acute inflammatory disease that affects the intestine of neonates resulting in intestinal necrosis, systemic sepsis, and multisystem organ failure. According to Blakely et al., NEC has been characterized as the leading cause of death and long-term disability from gastrointestinal disease in preterm infants [1]. In NEC the small (most often distal) and/or large bowel becomes injured and develops intramural air, and this may progress to frank necrosis with perforation [2]. The mortality of NEC ranges between 20 and 30 %, with the greatest mortality among neonates requiring surgery [3]. Despite the great progress in perinatal care, the mortality and morbidity from NEC have not been significantly improved during the last 40 years, underlying the necessity for improved treatment and prevention.

In NEC there is an excessive inflammatory response initiated in the highly immunoreactive intestine spreading systemically and affecting distant organs such as the brain and the retina. Neonates with NEC have been shown to have an increased risk for neurodevelopmental delays and retinopathy of prematurity [3, 4]. Despite the extensive research the pathophysiology of "classic NEC" is incompletely understood. This form of NEC is seen most commonly in preterm infant with incidence and mortality increasing in inverse proportion to birth weight and gestational age. The low-birth-weight (LBW) and the most premature infants are at greatest risk [5].

Based on the pathology of ischemic necrosis in NEC, earlier studies hypothesized that perinatal hypoxic–ischemic events including low Apgar scores, episodes of apnea or bradycardia, umbilical arterial catheterization, and indomethacin administration increase the risk of NEC in preterm infants [6]. Such concerns, however, are not supported by several prospective and properly controlled studies [7]. Prematurity and LBW are the most important risk factors (Fig. 30.1). Typically, the "classic" form of NEC in a preterm infant presents at about 29–32 weeks of postmenstrual age, with initial subtle signs such as abdominal distension, feeding intolerance, episodes of apnea, and bloody stools [8–10]. Symptoms may progress rapidly, often within hours, to abdominal discoloration, intestinal perforation, and peritonitis leading to deteriorating vital signs/shock requiring intensive medical and/or surgical support.

Unlike the classic form of NEC in premature infants in late preterm or term infants, NEC is often associated with underlying disorders or predisposing disorders such as perinatal asphyxia, chorioamnionitis, exchange transfusion, polycythemia, prolonged rupture of membranes, congenital heart disease, and neural tube defects [11]. Therefore, there is a heterogeneity with different initiating events and possibly pathophysiology in what is termed NEC in premature compared with mature infants.

Another entity which must be differentiated from NEC is spontaneous isolated or focal intestinal perforation. Isolated intestinal perforation is a distinct clinical entity different from NEC not generally accompanied by an inflammatory component or by diffuse necrosis. Focal hemorrhagic necrosis (FHN) with well-defined margins is seen in contrast to ischemic coagulative necrosis in NEC, and the bowel proximal and distal to perforation is normal. FHN occurs earlier than NEC and is strongly associated with the combined use of glucocorticoids and early postnatal use of indomethacin [12, 13].

The categorization of NEC into three different stages (stages I–III NEC), originally developed by Bell et al. firstly published in 1978 [14] and subsequently refined by Walsh [15], is a commonly used instrument that has been helpful in the diagnosis and management of NEC (Table 30.1). However, stage I NEC (suspected NEC) is characterized by nonspecific clinical signs which are not pathognomonic of intestinal pathology.

Given that the diagnosis and treatment of NEC still remain difficult and challenging and that the molecular basis of this multifactorial disease is poorly understood, prevention, rather than treatment,

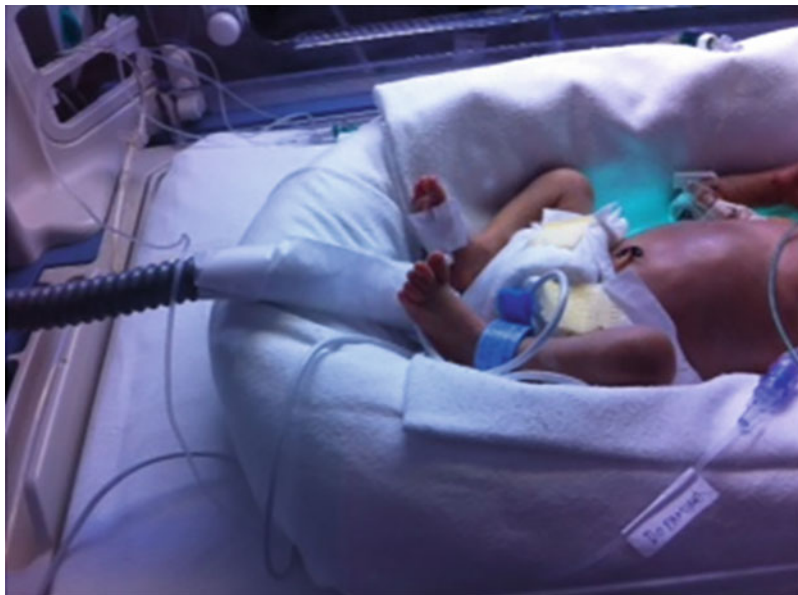

Fig. 30.1 Premature neonate with NEC presenting with abdominal distension

Table 30.1 Modified Bell's staging criteria for necrotizing enterocolitis (NEC) [14–16]

NEC stage	Systemic signs	Intestinal signs	Radiologic signs	Treatment
I. Suspected				
A	Temperature instability, apnea	Elevated pregavage residuals, mild abdominal distension, occult blood in stool	Normal or mild ileus	NPO, antibiotics × 3 days
B	Same as IA	Same as IA, plus gross blood in stool	Same as IA	Same as IA
II. Definite				
A: Mildly ill	Same as IA	Same as I, plus absent bowel sounds, abdominal tenderness	Ileus, pneumatosis intestinalis	NPO, antibiotics × 7–10 days
B: Moderately ill	Same as I, plus mild metabolic acidosis, mild thrombocytopenia	Same as I, plus absent bowel sounds, definite abdominal tenderness, abdominal cellulites, right lower quadrant mass	Same as IIA, plus portal vein gas, with or without ascites	NPO, antibiotics × 14 days
III. Advanced				
A: Severely ill, bowel intact	Same as IIB, plus hypotension, bradycardia, respiratory and metabolic acidosis, disseminated intravascular coagulation, neutropenia	Same as I and II, plus signs of generalized peritonitis, marked tenderness, and distension of abdomen	Same as IIB, plus definite ascites	NPO, antibiotics × 14 days, fluid resuscitation, inotropic support, ventilator therapy, paracentesis
B: Severely ill, bowel perforated	Same as IIIA	Same as IIIA	Same as IIB, plus pneumoperitoneum	Same as IIA, plus surgery

is of the utmost priority. Several preventive modalities have been studied, including the use of probiotics [17], prebiotics [18], and synbiotics [19]. Feeding with human milk is the most widely studied and the only intervention that is known to be effective in the prevention on NEC until now [20]. Interestingly, several recent reports reveal the role of L-arginine for the prevention of the disease. Specifically, as it will be analyzed in the following sections, L-arginine supplementation is a promising method for the prevention of NEC by promoting nitric oxide (NO) synthesis, and its potential use in NEC treatment opens up a new era in the fight of the disease.

The Dual Role of L-Arginine and Its Role in Nutrition and Pathophysiology of NEC

L-Arginine, the essential amino acid which is a precursor for proteins [21], plays a crucial role in the formation of nitric oxide (NO) by the nitric oxide synthase (NOS) family of enzymes [22] (Fig. 30.1). Interestingly, L-arginine has been shown to possess a dual profile and has been characterized as a "conditional" amino acid. This characterization is based on the evidence that endogenous L-arginine production covers metabolic requirements in healthy, unstressed adults but, under conditions of increased need, e.g., growth or tissue repair, or in catabolic states such as sepsis and starvation levels of endogenous production, may not suffice metabolic demands, thus becoming an essential amino acid [21].

Several studies have been carried out on the plasma L-arginine levels [23, 24]. Nowadays it is well known that hypoargininemia is frequently observed in prematurely born human [25] and animal neonates [26] (Fig. 30.2). L-Arginine deficiency is an important metabolic problem for the premature infant [27–30]. It has been found that human milk and mothers' milk of pigs and other species are deficient in L-arginine [31]. In addition, recently there are indications that L-arginine concentration remains low in current formulas for parenteral nutrition [28, 32]. Specifically, plasma L-arginine levels were found to be 95 ± 25 μmol/l in full-term breast milk-fed (n = 16) infants [30]. In extremely low-birth-weight infants (ELBW, birth weight <1000 g) who were on formula feeds (n = 2), the mean L-arginine level was found to be 37 μmol/l (range 13–60 μmol/l), while in ELBW breast milk-fed (n = 9) infants, the mean L-arginine level was 53 μmol/l (range 3–116 μmol/l) [33].

Snyderman et al. [27] first reported that the concentration of plasma L-arginine (34 μmol/l) is extremely low in preterm infants receiving enterally 2 g protein/kg/day (calculated protein requirements for infants). Heird et al. [34] and other researcher groups [25, 35] discovered that life-threatening hyperammonemia occurred in preterm infants who remained in total parenteral nutrition (TPN) and could be effectively treated by intravenous L-arginine administration. These findings suggest that neonatal hyperammonemia is due to hypoargininemia rather than deficiency of the enzymes involved in the urea cycle. Furthermore, Batshaw and Bursilow [35] and Batshaw et al. [25] also showed that life-threatening hypoargininemia (plasma L-arginine = 32 μmol/l) and hyperammonemia syndrome appeared in more than 50 % of preterm neonates. Recent studies have shown that hypoargininemia still occurs in premature infants. For example, in very low-birth-weight (VLBW) infants (<29 weeks' gestational age) entirely maintained in FreAmine III parenteral feeding (Kendall McGaw Laboratories, Irvine, CA), the mean plasma L-arginine concentration remained extremely low (19 μmol/l) at the third day of life [36]. Preterm neonates of <30 weeks' gestational age receiving intravenously glucose solution 10 % had also low plasma L-arginine levels (μmol = 28/l) [29]. Zamora et al. [28] recently studied premature infants of <32 weeks' gestational age that remained primarily in TrophAmine parenteral feeding (Kendall McGaw Laboratories, Irvine, CA), which contains the highest L-arginine amount between all currently used parenteral solutions. The authors reported that the mean plasma L-arginine concentration was only 40 μmol/l on the third day of life. This concentration is less than 50 % of normal L-arginine levels of healthy breast milk-fed infants (95.3 μmol/l) [30] and approaches the critical value of 32 μmol/l in which it often occurs as the hyperammonemia syndrome in premature infants [25, 34, 35, 37]. Moreover, hypoargininemia in preterm infants has been associated with increased severity of respiratory distress syndrome (RDS) and decreased systemic oxygenation [38]. Becker et al. [32] and Zamora et al. [22] also found that L-arginine deficiency is associated with

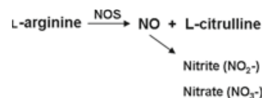

Fig. 30.2 L-Arginine, the precursor for nitric oxide (NO) synthesis

increased incidence of NEC in preterm infants. The recent findings that in preterm infants, hypoarginінemia during the first days of life is associated with cardiovascular, pulmonary, and intestinal dysfunction are very important. It is also important that increased receiving of exogenous L-arginine prevents hyperammonemia and NEC in preterm neonates [39], persistent pulmonary hypertension (a potentially fatal condition) in infants with mean L-arginine plasma levels of 12.5 μmol/l [40], and hyperammonemia and death in parenterally fed neonates [41]. Additionally, decreased synthesis of intestinal L-arginine contributes significantly to hypoargininemia in preterm infants [42]. Thus, L-arginine is considered to be an essential amino acid in infants, especially under conditions of increased metabolic demands such as infection and prematurity [41, 42]. Plasma L-arginine concentrations based on the above published studies are presented diagrammatically in Figs. 30.3 and 30.4.

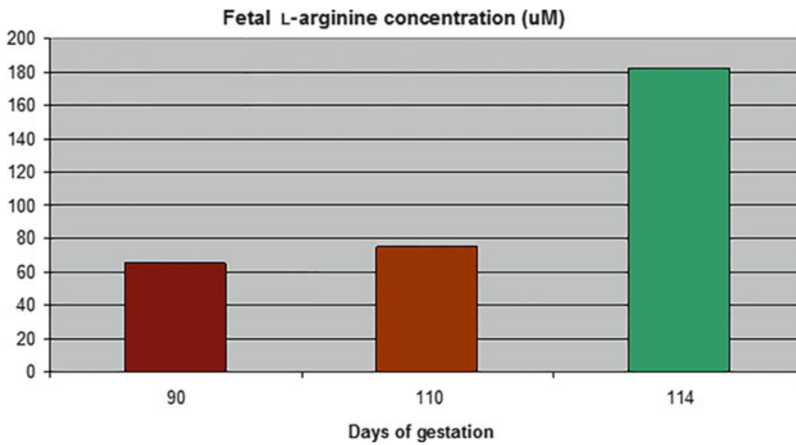

Fig. 30.3 Plasma arginine concentrations in preterm and term piglets obtained by hysterectomy based on the study of Wu et al. [26]

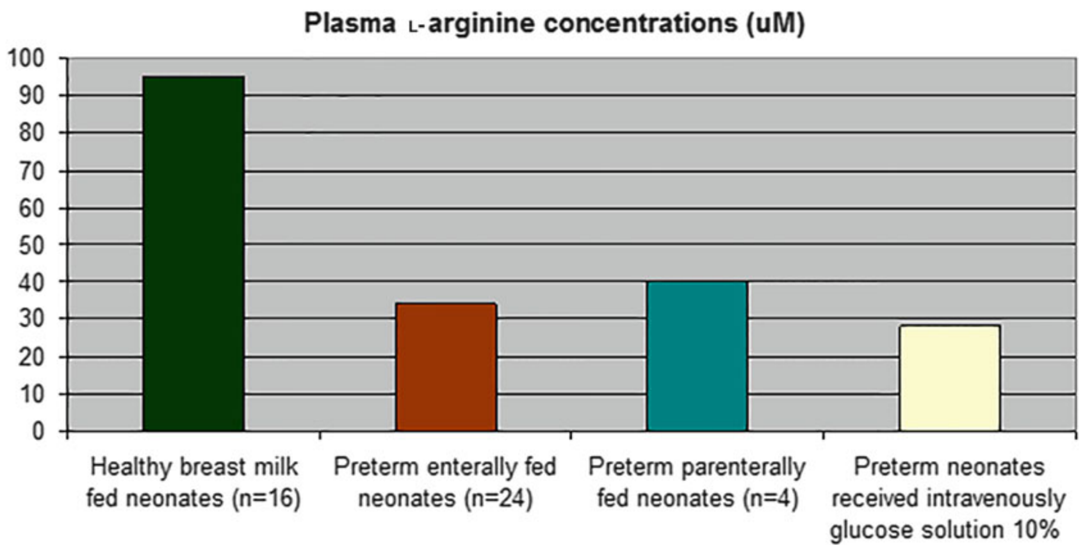

Fig. 30.4 Plasma arginine concentration found in healthy breast milk-fed neonates [30], preterm enterally fed neonates [27], preterm parenterally fed neonates [28], and preterm neonates who received intravenously glucose solution 10 % [29]

The role of L-arginine in NEC is supported by several recent reports. Becker et al. in a case–control study found that premature infants who developed NEC had similar L-arginine and glutamine levels on the third day but significantly lower on days 7 and 14 comparing with premature infants of comparable gestation, birth weight, and Apgar scores who did not develop NEC [32]. Zamora et al. found a statistically significant decreased in plasma L-arginine concentration in preterm infants who developed NEC compared to control infants even after adjusting for the intake of L-arginine and day of life [28]. Richir et al. suggested that diminished NO production may be involved in the pathophysiology of NEC, after they found that in premature infants with NEC not only the NO substrate L-arginine but also the endogenous NOS inhibitors (asymmetric dimethylarginine, ADMA) and the L-arginine/ADMA ratio were lower than in infants without NEC [43]. In addition, in their study low L-arginine and L-arginine/ADMA ratio were associated with mortality in infants with NEC. Conclusively VLBW infants who develop NEC present lower L-arginine plasma levels comparing to gestation comparable neonates who do not develop NEC and additionally have higher L-arginine requirements due to higher utilization and therefore higher metabolic needs.

Although extensive research has investigated the pathophysiology of NEC and L-arginine metabolism, the exact role of L-arginine in the pathophysiology of the disease still remains to be elucidated. However, it is widely recognized that the pathology of NEC frequently resembles intestinal ischemia–reperfusion injury. Nitric oxide (NO) is also known as the endothelium-derived relaxing factor or EDRF and is a weak radical biosynthesized endogenously from L-arginine, oxygen, and NADPH by various NOS enzymes. It has a major role on the regulation of vascular perfusion being a powerful vasodilator important in the protection of organs from ischemic damage and is also an important cellular signaling molecule in gut microcirculation involved in the maintenance of mucosal integrity, intestinal barrier function, and regulation of mucosal flow in the face of inflammation or injury. Inhibition of NO synthesis in a variety of animal models, in which bowel injury is induced, increases the area of intestinal damage [44, 45]. Lack or inadequacy of NO synthesized from the amino acid L-arginine can alter intestinal motility, predisposing premature neonates in NEC development. Therefore, it is hypothesized that a relative L-arginine deficiency or immaturity of NOS activity in premature infants may lead to deficient or inadequate tissue NO levels, vasoconstriction, and ischemia–reperfusion injury and may predispose the neonates to develop NEC [24, 46].

Studies About the Role of L-Arginine Supplementation in NEC

The role of L-arginine supplementation in NEC has been highlighted in numerous studies on experimental animal models and human neonates. In an animal experiment using young rats as a model, repeated stress conditions have been shown to significantly decrease plasma L-arginine levels [47]. Lorenzo et al., based on the intraluminal model of NEC in the developing neonatal piglet of Clark et al. [48], showed that continuous intravenous infusion with the NO synthase substrate L-arginine markedly attenuates intestinal injury in this neonatal piglet model of NEC [49]. More recently, experimental work on young mouse NEC model has shown that dietary supplementation with L-arginine and L-carnitine for 7 days significantly improves the histological evidence of hypoxia–reoxygenation-induced intestinal injury and decreases lipid peroxidation in hypoxia–reoxygenation-induced bowel injury [50]. Furthermore, the authors based on these findings suggested that the beneficial effects of L-arginine and L-carnitine in the animal model that was used may be mediated via mechanisms preventing free radical damage. Moreover, experiments on newborn rats showed that enteral L-arginine supplementation has favorable effects on oxidative stress in both experimental models of hypoxia–reoxygenation injury and healthy newborn rats [51]. In a study by Cintra et al., supply of L-arginine increased tissue levels of nitric oxide and reduced morphologic intestinal injury among mice undergoing ischemia–reperfusion (Cintra 2008). In 2012 the research group of Çekmez et al. attempted to evaluate the effectiveness of inhaled NO administration compared to L-arginine usage in a NEC

model of newborn rats [52]. The results of their study showed that superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and nitrate plus nitrite (NOx) were significantly higher in the group of NEC newborn rats (subjected to NEC) who received L-arginine supplementation compared to the group of NEC newborn rats who did not receive L-arginine supplementation, indicating a significantly lower intestinal injury and apoptosis index scoring in the NEC supplemented with L-arginine group comparing to NEC group without L-arginine supplementation. Moreover, the experimental results have also showed that SOD, GSH-Px, and NOx levels were significantly higher in the group of NEC pups that inhaled NO compared to the group of NEC pups that received L-arginine supplementation. The authors suggested that the inhaled NO administration could be served as a novel therapeutic agent like L-arginine in NEC, but more studies are needed to confirm this finding.

The effect of L-arginine supplementation in premature human infants on the reduction of NEC incidence in premature neonates has been firstly shown by Amin et al. [39]. Specifically Amin et al. showed that there was a statistically significant reduction in the risk of developing NEC of any stage in the L-arginine group when compared with that in the placebo group, suggesting that L-arginine supplementation in premature infants can reduce the incidence of NEC (all stages).

In the study of Amin et al. [39], 1.5 mmol/kg/day of L-arginine or placebo was added to parenteral nutrition, and when enteral feeds were >40 % of total daily fluid intake, L-arginine or placebo was administered orally over the first 28 days of life. The selected L-arginine supplementation dose of 1.5 mmol/kg/day (equal to 261 mg/kg/day) was based on a previous study of the same group [28] to achieve plasma L-arginine concentrations similar to normal term newborns. They observed no increase in hypotension, hypoglycemia, or intraventricular hemorrhage (IVH). Polycarpou et al. [53] also suggested that L-arginine supplementation in premature infants can reduce the incidence and severity of NEC. In the second study of Polycarpou et al., 1.5 mmol/kg/day of L-arginine from the 3rd to the 28th day of life was administered exclusively orally considering that L-arginine could have beneficial effect locally on the intestinal mucosa itself increasing proliferation and differentiation due to the production of other amino acids and polyamines [21, 54]. The incidence of NEC stage III was significantly lower in the L-arginine supplemented group. The authors concluded that enteral L-arginine supplementation of 1.5 mmol/kg/day bid can be safely administered in VLBW neonates from the 3rd to the 28th day of life and that enteral L-arginine supplementation appears to reduce the incidence of stage III NEC in VLBW infants. Both studies excluded neonates with severe congenital anomalies or inborn errors in metabolism, but Amin et al. additionally excluded neonates with evidence of IVH grade ≥ II on cranial ultrasound scan by day 3 of life, conjugated hyperbilirubinemia, and exchange transfusion during the study period or with preexisting kidney failure (<0.5 ml/kg/h urine for >8 h). Both groups suggest that no adverse effects from enteral L-arginine supplementation of 1.5 mmol/kg/day were noted and consequently L-arginine supplementation can be safely administered in the dose used in VLBW neonates. The safety even of higher doses of L-arginine can be proposed based on the higher therapeutic doses of L-arginine that are used without adverse effects in the management of urea cycle disorders (loading 600 mg/kg followed by an infusion of 250 mg/kg/day) [25, 55]. Moreover, neonatal pigs as well as adult rats can tolerate large amounts of enteral supplemental L-arginine without the appearance of any adverse effect [24, 56]. In conclusion, prophylactic administration of L-arginine in low doses is safe and appears to be effective in reducing the overall incidence of NEC in preterm infants and especially the incidence of Bell's stage III NEC.

Conclusions

The recent evidence on the L-arginine role in NEC pathophysiology opens up a new area in the field of NEC prevention and further supports the essential role of amino acids in nutrition and disease prevention. Despite the extensive research, the pathophysiology of this multifactorial disease is

incompletely understood, and its treatment is a challenging issue. Therefore, nutritional preventive approaches rather than treatment seem to be of high priority. Based on recent evidence, L-arginine supplementation in premature infants is safe and reduces the incidence of all NEC stages. Thus, it seems to be a promising prophylaxis for improving health besides human milk.

Clear diagnostic criteria including development of highly sensitive and specific predictive and diagnostic biomarkers that would detect predispositions to NEC need to be developed. In this direction, epidemiological genetic studies would further contribute in the elucidation of the "high risk" genetic background for NEC development. Consistent diagnostic criteria will also be helpful for evaluating the effects of different clinical practices among neonatal intensive care units and then applying strategies that are found in the most successful units. Future research should focus on the identification of the upper safe dose of L-arginine, the right route, and duration of administration in order to achieve the maximum benefits for the premature neonates.

References

1. Blakely ML, Lally KP, McDonald S, et al. Postoperative outcomes of extremely low birth-weight infants with necrotizing enterocolitis or isolated intestinal perforation: a prospective cohort study by the NICHD neonatal research network. Ann Surg. 2005;241(6):984–9. discussion 989–994.
2. Srinivasan PS, Brandler MD, D'Souza A. Necrotizing enterocolitis. Clin Perinatol. 2008;35(1):251–72.
3. Lodha A, Asztalos E, Moore AM. Cytokine levels in neonatal necrotizing enterocolitis and long-term growth and neurodevelopment. Acta Paediatr. 2010;99:338–43.
4. Martin CR, Dammann O, Allred EN, et al. Neurodevelopment of extremely preterm infants who had necrotizing enterocolitis with or without late bacteremia. J Pediatr. 2010;157:751–6.
5. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet. 2006;368:1271–83.
6. Davey AM, Wagner CL, Cox C. Feeding premature infants while low umbilical artery catheters are in place: a prospective, randomized trial. J Pediatr. 1994;124:795–9.
7. Moss RL, Kalish LA, Duggan C, et al. Clinical parameters do not adequately predict outcome in necrotizing enterocolitis: a multiinstitutional study. J Perinatol. 2008;28:665–74.
8. Neu J. Neonatal necrotizing enterocolitis: an update. Acta Paediatr. 2005;94(Suppl):100–5.
9. Blau J, Calo JM, Dozor D, et al. Transfusion-related acute gut injury: necrotizing enterocolitis in very low birth weight neonates after packed red blood cell transfusion. J Pediatr. 2011;158:403–9.
10. Llanos AR, Moss ME, Pinzòn MC, et al. Epidemiology of neonatal necrotising enterocolitis: a population based study. Paediatr Perinat Epidemiol. 2002;16:342–9.
11. Martinez-Tallo E, Claure N, Bancalari E. Necrotizing enterocolitis in full-term or near-term infants: risk factors. Biol Neonate. 1997;71:292–8.
12. Stark AR, Carlo WA, Tyson JE, et al. Adverse effects of early dexamethasone treatment in extremely-low-birth-weight infants. N Engl J Med. 2001;344:95–101.
13. Attridge JT, Clark R, Walker MW, et al. New insights into spontaneous intestinal perforation using a national data set: (1) SIP is associated with early indomethacin exposure. J Perinatol. 2006;26:93–9.
14. Bell MJ, Ternberg JL, Feigin RD, et al. Neonatal necrotizing enterocolitis: therapeutic decisions based upon clinical staging. Ann Surg. 1978;187:1–7.
15. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. Pediatr Clin North Am. 1986;33:179–201.
16. Neu J. Necrotizing enterocolitis: the search for a unifying pathogenic theory leading to prevention. Pediatr Clin North Am. 1996;43(2):409–32.
17. Alfaleh K, Anabrees J, Bassler D, et al. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev. 2011;3:CD005496.
18. Modi N, Uthaya S, Fell J, et al. A randomized, double blind, controlled trial of the effect of prebiotic oligosaccharides on enteral tolerance in preterm infants (ISRCTN77444690). Pediatr Res. 2010;68:440–5.
19. Underwood MA, Salzman NH, Bennett SH, et al. A randomized placebo-controlled comparison of 2 prebiotic/probiotic combinations in preterm infants: impact on weight gain, intestinal microbiota, and fecal short-chain fatty acids. J Pediatr Gastroenterol Nutr. 2009;48:216–25.
20. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet. 1990;336:1519–23.
21. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.

22. Zamora SA, Amin HJ, McMillan DD, et al. Plasma L-arginine concentration, oxygenation index, and systemic blood pressure in premature infants. Crit Care Med. 1998;26:1271–6.
23. Kohler ES, Sankaranarayanan S, van Ginneken CJ, et al. The human neonatal small intestine has the potential for L-arginine synthesis; developmental changes in the expression of L-arginine-synthesizing and -catabolizing enzymes. BMC Dev Biol. 2008;8:107.
24. Wu G, Jaeger LA, Bazer FW, et al. L-Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. J Nutr Biochem. 2004;15(8):442–51.
25. Batshaw ML. Long term treatment of inborn errors of urea synthesis. In: Kleinberger G, editor. Advances in hepatic encephalopathy and urea cycle diseases. Basel: Karger; 1984. p. 187–95.
26. Wu G, Bazer FW, Tou W. Developmental changes of free amino acid concentrations in fetal fluids of pigs. J Nutr. 1995;125:2859–68.
27. Snyderman SEHL, Norton PM, et al. Protein requirements of the premature infant. II. Influence of protein intake on free amino acid content of plasma and red blood cells. Am J Clin Nutr. 1970;23:890–5.
28. Zamora SA, Amin HJ, McMillan DD, et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. J Pediatr Gastroenterol Nutr. 1997;131:226–32.
29. Van Goudoever JB, Colen T, Wattimenta JLD, et al. Immediate commencement of amino acid supplementation in preterm infants: effect on serum amino acid concentrations and protein kinetics on the first day of life. J Pediatr. 1995;127:458–65.
30. Wu PY, Edwards N, Storm MC. Plasma amino acid patterns in normal term breast-fed infants. J Pediatr. 1986;109:347–9.
31. Wu G, Knabe DA. Free and protein-bound amino acids in sow's colostrum and milk. J Nutr. 1994;124:415–24.
32. Becker RM, Wu G, Galanko JA, et al. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. J Pediatr. 2000;137:785–93.
33. Ventura V, Brooke OG. Plasma amino acids in small preterm infants fed on human milk or formula. Arch Dis Child. 1987;62:1257–64.
34. Heird WC, Driscoll JM, Schullinger JN, et al. Hyperammonemia resulting from intravenous alimentation using a mixture of synthetic L-amino acids: a preliminary report. J Pediatr. 1972;81:162–5.
35. Batshaw ML, Bursilow SW. Asymptomatic hyperammonemia in low-birth weight infants. Pediatr Res. 1978;12:221–4.
36. Srinivasan G, Amin A, Pildes RS, et al. Plasma amino acid patterns in very low birth weight infants during parenteral nutrition. Indian J Pediatr. 1990;57:93–7.
37. Thomas DW, Sinatra FR, Hack SL, et al. Hyperammonemia in neonates receiving intravenous nutrition. J Parenter Enteral Nutr. 1982;6:503–6.
38. Ball RH, Wykes LL, Pencharz PB, et al. A piglet model for neonatal amino acid metabolism during total parenteral nutrition. New York: Plenum Press; 1996.
39. Amin HJ, Zamora SA, McMillan DD, et al. L-Arginine supplementation prevents necrotizing enterocolitis in the premature infant. J Pediatr Gastroenterol Nutr. 2002;140:425–31.
40. McCaffrey MJ, Bose CL, Reiter PD, et al. Effects of L-arginine infusion on infants with persistent pulmonary hypertension of the newborn. Biol Neonate. 1995;67:240–3.
41. Brunton JA, Bertolo RF, Pencharz PB, et al. Proline ameliorates L-arginine deficiency during enteral but not parenteral feeding in neonatal piglets. Am J Physiol Endocrinol Metab. 1999;277:223–31.
42. Wu G, Meininger CJ, Knabe DA, et al. L-Arginine nutrition in development, health and disease. Curr Opin Clin Nutr Metab Care. 2000;3:59–66.
43. Richir MC, Siroen MP, van Elburg RM, et al. Low plasma concentrations of L-arginine and asymmetric dimethylarginine in premature infants with necrotizing enterocolitis. Br J Nutr. 2007;97:906–11.
44. Cintra AE, Martins JL, Patrício FR, et al. Nitric oxide levels in the intestines of mice submitted to ischemia and reperfusion: L-arginine effects. Transplant Proc. 2008;40:830–5.
45. Fukatsu K, Ueno C, Maeshima Y, et al. Effects of L-arginine infusion during ischemia on gut blood perfusion, oxygen tension, and circulating myeloid cell activation in a murine gut ischemia/reperfusion model. J Parenter Enteral Nutr. 2004;4:224–30.
46. Nankervis CA, Giannone PJ, Reber KM. The neonatal intestinal vasculature: contributing factors to necrotizing enterocolitis. Semin Perinatol. 2008;32:83–91.
47. Milakofsky L, Harris N, Vogel WH. Effects of repeated stress on plasma L-arginine levels in young and old rats. Physiol Behav. 1993;54:725–8.
48. Clark DA, Thompson JE, Weiner LB, et al. Necrotizing enterocolitis: intraluminal biochemistry in human neonate and a rabbit model. Pediatr Res. 1985;19:919–21.
49. Di Lorenzo M, Bass J, Krantis A. Use of L-arginine in the treatment of experimental necrotizing enterocolitis. J Pediatr Surg. 1995;30(2):235–40.
50. Akisu M, Ozmen D, Baka M, et al. Protective effect of dietary supplementation with L-arginine and L-carnitine on hypoxia/reoxygenation-induced necrotizing enterocolitis in young mice. Biol Neonate. 2002;81:260–5.

51. Kul M, Vurucu S, Demirkaya E, et al. Enteral glutamine and/or L-arginine supplementation have favorable effects on oxidative stress parameters in neonatal rat intestine. J Pediatr Gastroenterol Nutr. 2009;49:85–9.
52. Cekmez F, Purtuloğlu T, Aydemir G, et al. Comparing beneficial effects of inhaled nitric oxide to L-arginine in necrotizing enterocolitis model in neonatal rats. Pediatr Surg Int. 2012;28:1219–24.
53. Polycarpou E, Zachaki S, Tsolia M, et al. Enteral L-arginine supplementation for prevention of necrotizing enterocolitis in very low birth weight neonates: a double-blind randomized pilot study of efficacy and safety. J Parenter Enteral Nutr. 2013;37(5):617–22.
54. Neu J. L-Arginine supplementation for neonatal necrotizing enterocolitis: are we ready? Br J Nutr. 2007;97:814–5.
55. Group UCDC. Consensus statement from a conference for the management of patients with urea cycle disorders. J Pediatr Gastroenterol Nutr. 2001;138(Suppl):S1–5.
56. Wu G, Bazer FW, Cudd TA, et al. Pharmacokinetics and safety of L-arginine supplementation in animals. J Nutr Biochem. 2007;137(6 Suppl 2):1673S–80.

Part VI
Therapeutic Uses of L-*Arginine*: Diabetes, Obesity and Cardiovascular Diseases

Chapter 31
L-Arginine Usage in Type I Diabetes: From the Autoimmune Event to Human Dietary Supplementation

Mauricio Krause, Ana Paula Trussardi Fayh, and Alvaro Reischak-Oliveira

Key Points

- L-Arginine and its precursor L-citrulline come up as new potential therapeutic tools for the treatment and prevention of type 1 diabetes (T1DM).
- L-Arginine availability in the pancreatic islets is crucial for beta-cell function and survival during the autoimmune event installation.
- L-Arginine supplementation elicits significant improvements in blood flow in type 1 diabetic subjects.
- The positive effects of L-arginine supplementation observed in T1DM subjects may affect endothelial cells, increasing nitric oxide availability, lowering oxidative stress, and improving mitochondrial function.
- The effects of L-arginine and L-citrulline are beneficial for T1DM; however, the effects may be different for different stages of the disease.

Keywords L-Arginine • Type 1 diabetes • Inflammation • Oxidative stress • Nutrition

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Abbreviations

ACE	Angiotensin-converting enzyme
Ach	Acetylcholine
AGE	Advanced glycation end products
AMPK	5′ adenosine monophosphate-activated protein
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
DN	Diabetic nephropathy
FMD	Flow-mediated dilation
GPX	Glutathione peroxidase
GSH	Glutathione
GTP	Guanosine triphosphate
HbA1c	Glycated hemoglobin
HbNO	Nitrosyl hemoglobin
HO-3	Heme oxygenase-3
HSP70	Heat shock protein 70 kDa
iNOS	Inducible isoform of NO synthase
iNOS	Inducible nitric oxide synthase
NF-κB	Nuclear factor-κB
NO	Nitric oxide
O_2•	Superoxide radical
$ONOO^-$	Peroxynitrite
PGC-1α	PPARγ co-activator-1α
PKC	Protein kinase C
RDA	Recommended dietary allowance
RSNOs	*S*-nitrosothiols
sGC	Soluble guanylate cyclase
sGC	Soluble guanylyl cyclase
SOD	Superoxide dismutase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCR	T cell receptor
tNOx	Nitric oxide metabolites

Introduction

L-Arginine is an amino acid synthesized from glutamine, glutamate, and proline via the intestinal-renal axis in humans and most other mammal glutamate. The γ-carboxyl group of glutamate is first phosphorylated, producing phosphoryl glutamate. This compound is then reduced to yield glutamate γ-semialdehyde plus phosphate. This step is followed by transamination to form ornithine, which is then converted to L-arginine via the enzymes of the urea cycle. Although L-arginine can be produced by the adult human body (synthesized) [1], this amino acid is considered as a "conditionally essential" amino acid since its availability may be decreased under special conditions such as diabetes, and the additional ingestion may be required to normalize the plasma levels [2]. Briefly, proline, glutamine, and glutamate (from L-glutamine metabolism) are the major precursors for L-arginine synthesis, being the liver the major supplier of glutamate to the circulation [3]. Additionally, glutamine from the diet is

metabolized to citrulline, which is released in the circulation and uptake by the kidney [4]. The kidney is the physiological producer of L-arginine being the only organ known to take up L-citrulline released from the metabolism of glutamine in the gut and release L-arginine into the blood, although other tissues strongly express argininosuccinate synthetase and lyase but without any net delivery to the circulation [5]. In fasted humans, the contribution of glutamine via L-citrulline to the de novo synthesis of L-arginine is about 65 % in neonates, where the gut is the major source of systemic L-arginine, even though some residual production in the adult gut could account for by L-arginine release as well [5].

The average L-arginine consumption in the Western diet is around 5.4 g/day, mainly from animal protein sources (beef, chicken, and fish), seafood, soy, chocolate, dairy products, and seeds (sunflower and pumpkin) [6]. Until now, the National Research Council's Food and Nutrition Board did not have an official recommendation on the L-arginine recommended dietary allowance (RDA) [2]. The amount of L-arginine used on studies with L-arginine supplementation may reach five times the normal ingestion; however, the average range is between 6 and 8 g/day in addition to the dietary intake [7, 8]. L-Arginine is well absorbed in the gut, reaching a peak plasma concentration after 2 h of ingestion [9]. Parenteral administration of L-arginine is well tolerated when used up to 500 mg/kg/day [9]. The amino acid clearance occurs by kidney where it is almost fully absorbed by the kidney tubules [9]. L-Arginine deficiency is known to induce asthenia symptoms, reduce insulin secretion, and alter glucose and lipid metabolism in the liver [10]. Most studies using L-arginine supplementation did not report any collateral effects when the dosage used ranges between 3 and 20 g. However, few subjects have reported gastrointestinal discomfort when the dosage is higher than 8 g/day [2] (Table 31.1).

The importance of L-arginine is clearly observed in diseases with abnormalities in the vascular tone control. The normal vascular endothelium plays an important role in maintaining vessel wall homeostasis, through synthesizing substances such as prostacyclin and the free radical nitric oxide (NO), which modulate vascular tone, prevent thrombosis, and stimulate smooth muscle growth [5]. Additionally, NO plays an important role in many other functions in the body, not only regulating vasodilatation and blood flow but also inflammation, immune system activation, insulin secretion and sensitivity [11, 12], mitochondrial function, and neurotransmission. L-Arginine is the main precursor of NO via nitric oxide synthase (NOS) activity which diffuses to underlying smooth muscle cells to activate soluble guanylyl cyclase (sGC) resulting in smooth muscle relaxation, and then, the availability of this amino acid may modulate NO production [13–15] (Fig. 31.1).

Vascular disease is the principal cause of death and disability in patients with type 1 diabetes (T1DM) [16], being endothelial dysfunction one of the major causes in its pathogenesis [4]. Strategies aiming to maintain/restore a normal vascular function are important for vascular disease prevention in diabetes. Rather than the use of therapeutic drugs for the control of cholesterol, hyperglycemia, and other risk factors related to atherogenesis process, blood flow restoration seems to be essential. Endothelial dysfunction, which is defined by decreased endothelium-dependent vasodilation, is the hallmark of cardiovascular complications in diabetes [8]. Not surprisingly, NO is found reduced in diabetic subjects, compromising their vascular tonus control [11, 17]. Reductions in the NO availability may occur when the levels of its precursor L-arginine are decreased [18, 19]. Indeed, L-arginine has been used as a strategy to improve endothelial function [20], insulin secretion, and pancreatic beta-cell protection [12], besides adiposity control in obesity and diabetes [21].

Specifically, in animal models of T1DM, L-arginine administration in vivo resulted in protection against the effects of many diabetic agents, such as alloxan and streptozotocin [22–26]. Interestingly, a single administration of watermelon, rich in L-citrulline (L-arginine precursor), was able to reduce the serum concentrations of cardiovascular risk factors such as homocysteine, improve glycemic control, and ameliorate vascular dysfunction in obese insulin-resistant animals [27]. In addition, several studies have identified L-arginine supplementation in humans as a tool for the treatment for cardiovascular complications and diabetes, improving endothelial function, reducing oxidative stress markers, and increasing NO availability [8, 28–32]. Thus, dietary L-arginine and L-citrulline supplements may increase levels of NO metabolites, and their supplementation may induce several benefits to patients with T1DM.

Table 31.1 Foods highest in L-arginine

Foods highest in L-arginine	L-Arginine (based on levels per 200-calorie serving) (mg)
Seeds, sesame flour, low fat	4465
Gelatins, dry powder, unsweetened	3951
Soy protein isolate	3947
Peanut flour, defatted	3818
Crustaceans, crab, Alaska king, raw	3805
Crustaceans, shrimp, mixed species, cooked	3689
Spinach, frozen, chopped or leaf, unprepared	3317
Turkey, fryer roasters, breast, meat only, cooked, roasted	3107
Spinach, frozen, chopped or leaf, cooked, boiled, drained, without salt	2877
Watercress, raw	2727
Chicken breast, oven roasted, fat-free, sliced	2724
Egg, white, raw, fresh	2700
Fish, tilapia, raw	2660
Fish, tuna, light, canned in water, drained solids	2632
Fish, cod, Pacific, raw	2612
Tofu, silken, lite, firm	2540
Pork, fresh, enhanced, loin, tenderloin, separable lean only, cooked, roasted	2479
Beef, round, top round, separable lean only, trimmed to 0″ fat, select, cooked, braised	2403
Pork, cured, ham – water added, rump, bone-in, separable lean only, heated, roasted	2265
Seeds, pumpkin and squash seed kernels, roasted, with salt added	2076
Fish, salmon, pink, raw	2057
Fish, trout, rainbow, wild, cooked, dry heat	1829
Mustard greens, frozen, unprepared	1810
Beans, mung, mature seeds, sprouted, canned, drained solids	1683

Data retrieved from http://nutritiondata.self.com/foods-000089000000000000000.html

The Importance of L-Arginine Availability to Pancreatic β-Cell Function and Viability in T1DM

According to Krause et al. [12], destruction of β cells during islet inflammation can be mediated by direct contact with activated macrophages and by exposure to soluble mediators secreted by dendritic cells, macrophages, and T lymphocytes, including cytokines, oxygen free radicals, and nitric oxide. Indeed, overproduction of NO by β cells themselves is one of the most significant mechanisms leading to β-cell dysfunction and death [33]. The production of NO is promoted by inflammatory cytokines which activate the transcription of β-cell inducible nitric oxide synthase (iNOS, encoded by the NOS-2 gene), an enzyme whose expression is nuclear factor-κB (NF-κB) driven and which uses L-arginine as substrate [33]. Curiously, macrophages can release, into the inflammatory islet microenvironment, the enzyme arginase [34], which splits L-arginine into urea and L-ornithine, thus avoiding its conversion into NO and favoring resolution of inflammation [35]. Interestingly, the depletion of L-arginine itself is sufficient to inhibit T cell proliferation by the downregulation of the ζ chain, the main signal transduction component of the T cell receptor (TCR) complex [36]. It has also been demonstrated that β cells possess a cytokine-inducible arginase activity [37], which may account for reduction in NO synthesis under appropriate conditions. Although the release of arginase by infiltrating macrophages may promote the resolution of inflammation, restriction of L-arginine availability in

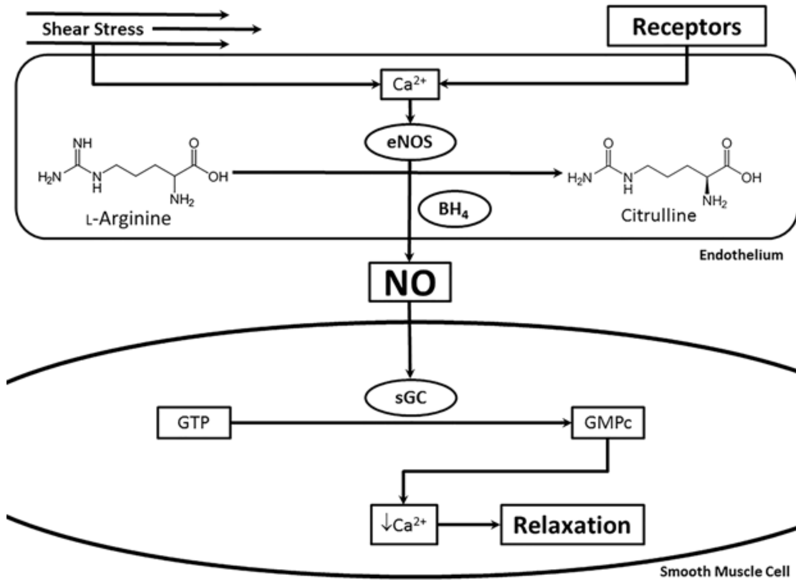

Fig. 31.1 Agonists bind receptors and/or shear stress stimulates endothelial NO synthase (eNOS) through an increase in Ca^{2+}. Endothelial NO synthase (eNOS) converts L-arginine to citrulline in the presence of optimal concentration of the cofactor tetrahydrobiopterin (BH4), producing NO, which diffuses to smooth muscle cell in order to activate soluble guanylate cyclase (sGC). sGC then converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), reducing Ca^{2+} and, consequently, resulting in relaxation of vascular tone (based on [14, 15])

the islet microenvironment may be detrimental for β-cell metabolism, antioxidant defenses, and insulin secretion [13]. Interestingly, decreased plasma and intracellular concentrations of L-arginine have been reported in patients with T1DM and type 2 diabetes [19]. L-Arginine also increased β-cell neogenesis and antioxidant defenses in rats treated with alloxan, a model of T1DM, and aided recovery of endothelium-dependent relaxation in patients with type 2 diabetes [19, 22]. Administration of L-arginine has also been reported to reduce adiposity in obese-diabetic humans, diet-induced and genetically obese rats, as well as finishing pigs [38].

In order to further investigate the importance of L-arginine for β-cell function, we recently tested if changes in the availability of L-arginine to clonal β cell and pancreatic islets placed in a pro-inflammatory environment (simulating the islet inflammation in diabetes) would result in changes in β-cell function and insulin secretion [12]. The main finding in this study was that L-arginine is essential for β-cell survival during the onset of inflammation due to the fact that this amino acid promotes glutathione (GSH) synthesis and heat shock protein 70 kDa (HSP70) expression to an ideal level. The activation of the iHSP70 is sine qua non for the promotion of tissue repair, since the expression of this chaperone confers cytoprotection and also exerts anti-inflammatory effects [39]. The increase in the levels of HSP72 induced by L-arginine in β cells could be one of the key mechanisms mediating the observed protection against stress and inflammation. Thus, the higher level of HSP72 together with the increment in GSH synthesis induced by L-arginine may provide effective β-cell protection against inflammatory insult caused by the autoimmune event (Fig. 31.2).

Summarizing, L-arginine exerts a broad spectrum of beneficial effects on clonal β cells and isolated islets in addition to simple membrane depolarization and triggering of insulin secretion. These novel findings suggest an important role of L-arginine in the promotion of GSH synthesis and antioxidant defense that may encourage the development of novel strategies for the protection of β cells against chemical/immune insult and both type 1 (T1DM) and type 2 diabetes (T2DM).

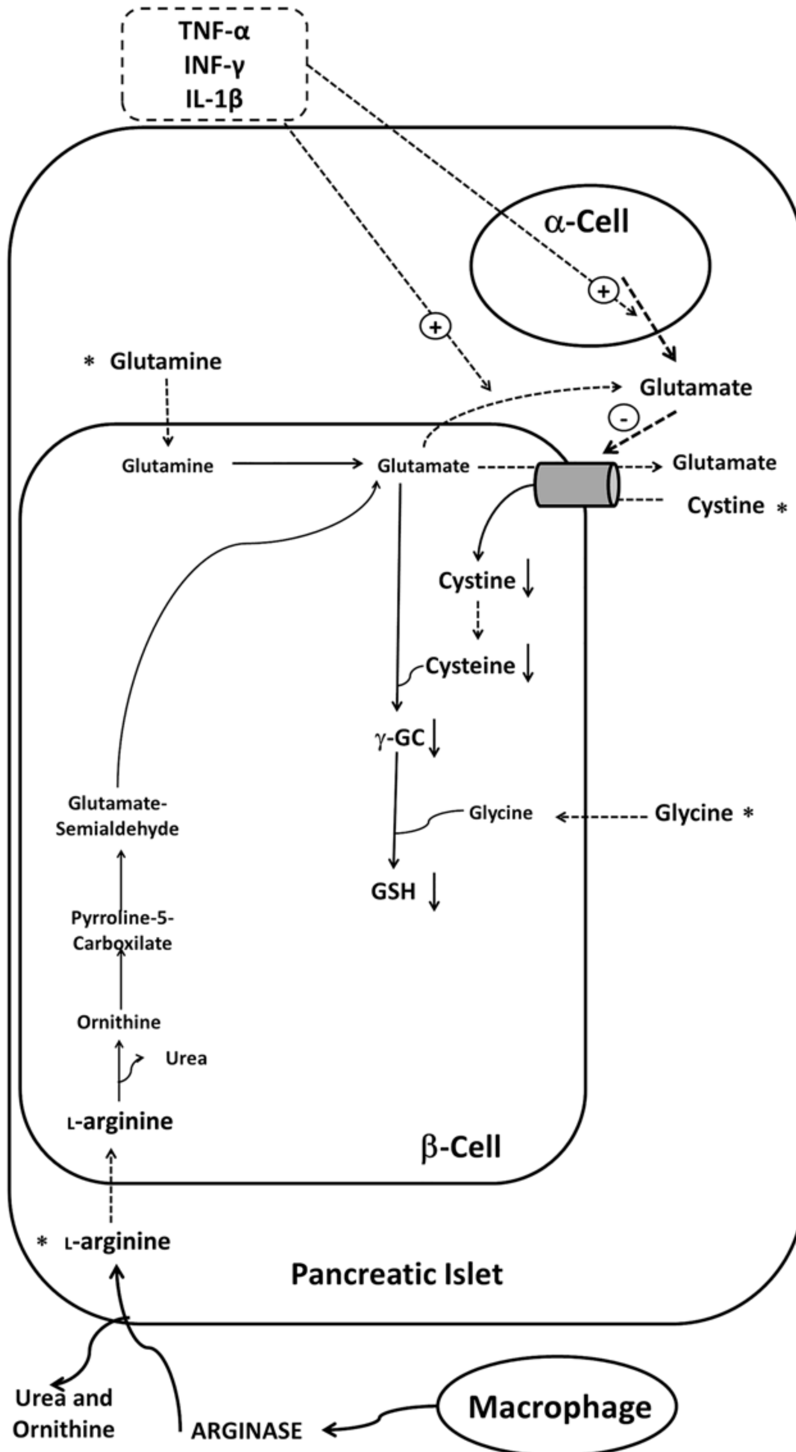

Fig. 31.2 Failure of beta-cell antioxidant production induced by inflammation and macrophage arginase secretion. Failure in glutathione (GSH) synthesis can result in changes in redox state, loss of beta-cell function, and cell death. Glutathione synthesis involves three key amino acids: glutamate, glycine and cysteine. Changes on the availability of these amino acids may lead to decreased levels of GSH and cell death. L-glutamine and L-arginine are also important for intracellular glutamate synthesis. Extracellular glutamate plays an essential role on the beta-cell function/survival.

L-Arginine Supplementation in Type 1 Diabetes

Several clinical studies and meta-analysis have shown the positive impact of L-arginine supplementation. For example, Dong and collaborators [41] conducted a systematic review on the effects of L-arginine supplementation (from 4 to 24 g/day for at least 4 weeks) over the blood pressure in human patients. The meta-analysis results show that, compared to the placebo groups, L-arginine supplementation significantly reduced the values of systolic blood pressure in 5.39 mmHg (IC 95 % −8.54 and −2.25, $P=0.001$) and diastolic in 2.66 mmHg (IC 95 % −3.77 and −1.54, $P<0.001$). In another meta-analysis, Bai and collaborators have found that in healthy humans and in people with cardiovascular diseases, L-arginine supplementation improved the blood flow only when this was already compromised by the disease [flow- mediated dilation (FMD) < 7 %], showing no effects in patients without endothelial dysfunction [42].

Studies on L-arginine supplementation and diabetes are mainly focused in type 2 diabetes mellitus (T2DM) and normally for short-term periods. In humans, long-term L-arginine ingestion has shown positive effects in several parameters such as the glucose metabolism, insulin resistance, and endothelial dysfunction in patients with T2DM and insulin resistant [43]. When supplemented for 18 months, L-arginine (6.8 g/day) reduced the incidence of diabetes in patients with glucose intolerance [44].

Although many studies have shown positive effects of L-arginine supplementation in T2DM, little is known about the effects of L-arginine supplementation in humans with T1DM. To our knowledge, most of the studies have investigated the effects of L-arginine in animal models of type 1 diabetes, specially looking at its potential to restore NO in conditions when this gas is likely to be reduced [12, 21–23, 25–27]. Results have consistently shown that L-arginine ameliorates several metabolic parameters such as lipid oxidation [21], reduction of the polyol pathway [25], eNOS activation [45], and ameliorated vascular dysfunction [27].

In humans with T1DM and in animal models of T1DM, such as the alloxan-induced experimental diabetes, oxidative stress is a known player of the disease complications. Importantly, L-arginine administration in vivo resulted in protection against the effects of many diabetic agents, such as alloxan and streptozotocin [22–26]. β-cell neogenesis seems to be promoted, as well as increased GPX (glutathione peroxidase) [22], SOD (superoxide dismutase) and CAT (catalase) activities, plus augmented GSH (glutathione) content [23]; while polyol and protein kinase C (PKC) pathway activation are reduced though [25]. Interestingly, a single administration of watermelon, rich in L-citrulline (L-arginine precursor), was able to reduce serum concentrations of cardiovascular risk factors such as homocysteine, improve glycemic control, and ameliorate vascular dysfunction in obese insulin-resistant animals [27]. L-Arginine supplementation in Zucker diabetic fatty rats resulted in powerful activation of heme oxygenase-3 (HO-3), 5′ adenosine monophosphate-activated protein (AMPK), and PPARγ co-activator-1α (PGC-1a), which would be expected to increase mitochondrial biogenesis and increase oxidative metabolism in the skeletal and cardiac muscle, brain, liver, and adipose tissue [21]. In addition, this amino acid seems to act as antiatherogenic agent by reducing oxidative stress [20].

An additional complication in T1DM is the diabetic nephropathy (DN). DN is a major cause of end-stage renal disease, and it is associated with endothelial dysfunction. L-Arginine supplementation was suggested to improve the vascular function since it is a direct precursor for NO synthesis [13]; however, in many cases, it failed to improve vascular function [4]. This may be caused by the higher levels of the enzyme arginase in the plasma that occurs in many conditions such as in diabetes [18,

Fig. 31.2 (continued) This amino acid is an inhibitor of cysteine transport. Increased levels of glutamate (from beta and/or alpha cells) can be induced by pro-inflammatory cytokines and metabolic overload, leading to a blockage of cysteine uptake. When the level of cysteine/cysteine falls, glutathione levels decreases, leading to oxidative stress and cell death. Arginase secretion from macrophages leads to the reduction of L-arginine in the microenvironment causing stress and beta-cell dysfunction and death (Newsholme et al. [40])

19]. We believe that arginase, actively released by macrophages, or by damaged cells, is the reason why L-arginine may not be efficient in these conditions: the amino acid would be metabolized in the plasma before reaching the target cells. For this reason, L-citrulline, which is the precursor of L-arginine, may be effective to restore endothelial function by increasing intracellular levels of L-arginine. This is possible because most cells have high activity of argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) being capable of synthesizing L-arginine from L-citrulline [4]. Indeed, recent evidences have shown that L-citrulline supplementation increases L-arginine availability on the vasculature [46]. The authors also found that L-citrulline induced anti-inflammatory cytokine release and restored nitric oxide balance in the kidney proximal tubules cells [46]. L-citrulline has been shown to prevent coronary vascular dysfunction in diabetic rats [47], with concomitant reduction of endothelial arginase I activity, which was also recently shown to contribute to coronary endothelial dysfunction in patients with diabetes mellitus [48] and in diabetic mice [47, 49]. Thus, L-citrulline is now considered a new strategy to improve endothelial function, nitric oxide, and L-arginine levels and to reduce oxidative damage and pancreatic beta-cell function in T1DM; however, the efficacy of this supplementation in humans with T1DM remains to be verified.

A recent report has tested the effects of L-arginine supplementation (7 g/day for 1 week) over the endothelial function and oxidative stress markers in young male adults with uncomplicated type 1 diabetes. To our knowledge, this was the first work that shown evidences of the benefits of L-arginine supplementation for this population [8]. In this work, L-arginine supplementation consisted in oral ingestion of identical pills containing either amide compound (as placebo) or 7 g of L-arginine hydrochloride. The main finding of this study is that L-arginine supplementation (7 g/day) could elicit significant improvements in the lower limb vascular function (i.e., blood flow) in type 1 diabetic subjects (Fig. 31.3). Improvements in blood flow and cardiovascular function are related to a diminution in oxidative stress [50]. In this report the authors found that, in accordance with other studies [51], diabetic subjects' present higher levels of ROS damage as indicated by the results of TBARS and carbonyls. In this regard, supplementation with L-arginine has failed to reverse these parameters, then it is unlikely that the improvements in blood flow caused by L-arginine administration is related to any concurrent upregulation of antioxidant mechanisms as was previously suggested [20, 23].

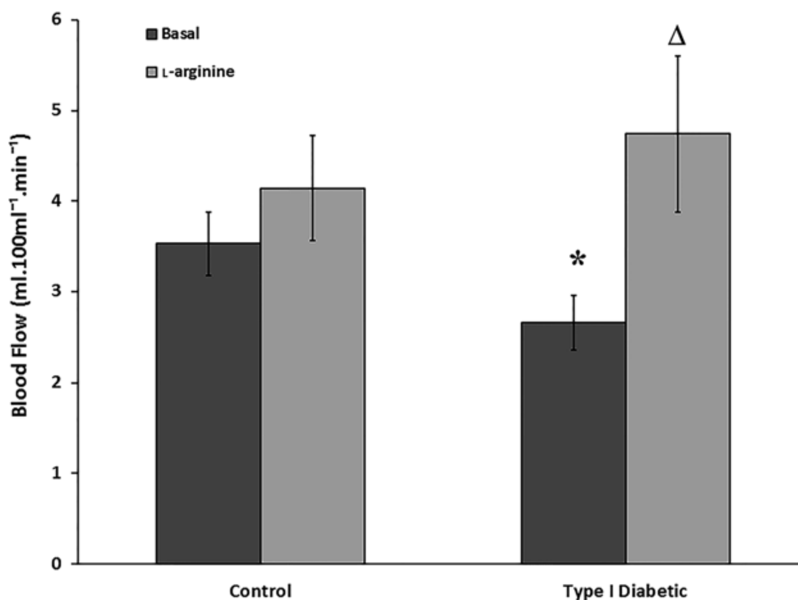

Fig. 31.3 Baseline comparison of lower limb blood flow (mL 100 mL^{-1} min^{-1}), measured by a venous occlusion plethysmography. $^{*}P<0.05$ when compared diabetic with control group; $^{\Delta}P<0.05$ when compared before and after L-arginine supplementation (Fayh et al. [8])

The possible beneficial effects of L-arginine could be attributed to changes in the viability of nitric oxide; however, the L-arginine supplementation used did not change the plasma levels of nitric oxide metabolites (nitrite and nitrate, tNOx) in any circumstance or group studied. Levels of tNOx may change during the time course of type 1 diabetes, with higher levels during the first stage of the disease and decrements on the production in later stages [52]. The first stage seems to be related to the autoimmune process itself, while the latter one looks to be associated to decreased levels of L-arginine [39], caused by altered endothelial signal transduction or by increased formation of O_2• (superoxide radical), which reacts with NO to produce $ONOO^-$ (peroxynitrite). In addition, hyperglycemia accelerates the formation of advanced glycation end products (AGE), including glycated hemoglobin (HbA1c), albumin, and other plasma proteins, changing their binding affinity for NO, thereby altering the metabolic fate of NO in type 1 diabetic patients [53]. Thus, protein glycosylation can alter nitric oxide binding affinity of hemoglobin and plasma proteins, hence reducing nitric oxide availability and causing NO metabolism alterations [53]. This process seems to be enhanced when the level of HbA1c rises dramatically above 8 % [53], condition found in our type 1 diabetes subjects. Hence, the fact that we did not found increments in nitric oxide levels even with L-arginine supplementation could be explained by the rapidly binding of nitric oxide to blood proteins, preventing the appearance of its metabolite tNOx in the plasma [8].

The formation of relatively stable nitric oxide metabolites seems to provide a protected transport mechanism, allowing nitric oxide to be transported through regions of high production, such as arteries, to be delivered in the microcirculation, and thus changing the microvascular tone [53]. Nitric oxide release within the systemic microcirculation is suggested by an arteriovenous gradient of nitrite and HbNO (nitrosyl hemoglobin) after nitric oxide inhalation in normal subjects [53], of nitrite and high -molecular weight RSNOs (*S*-nitrosothiols) following exercise in normal subjects [53], and of nitrate and tNOx during exercise training in hypercholesterolemic patients [53]. Nitric oxide can also be transferred from high-molecular weight to low-molecular weight thiols [53], possibly allowing nitric oxide to be delivered to the vascular bed to elicit vasodilation. In basal conditions, we found that patients with diabetes present lower blood flow than their controls [8]. Since L-arginine supplementation was able to partially recover this cardiovascular deficit, we suggest that this amino acid administration could result into a higher nitric oxide formation, which was immediately converted into one of its stable metabolites (other than nitrate/nitrite), causing subsequent changes in vascular tone. Furthermore, the lower VO_{2max} founded in the diabetic group could be attributed, at least in part, to the lower nitric oxide metabolites formation, leading to a decreased vasodilation and finally disrupting the gas exchange for the active skeletal muscle cells.

Although most of the L-arginine effects are mediated primarily by NO production, some effects of L-arginine are known to be independent of NO production [54]. Among them, this amino acid can act as an antioxidant, scavenging superoxide and reducing copper-induced lipid peroxidation [55]. Even more relevant, L-arginine is an inhibitor of angiotensin-converting enzyme (ACE) [56], reducing plasma angiotensin II levels and thus amplifying its hypotensive effect. Thus, our finding that L-arginine increases blood flow in basal conditions could also be attributed to this function of L-arginine.

Even though L-arginine supplementation increases blood flow in basal conditions, the amino acid did not change this variable after exercise in T1DM patients [8]. This could indicate that during exercise, other mechanisms of vasodilation in the microcirculation of active muscles may be involved, rather than NO production. For instance, prostaglandin activity could fill this vasoactive function. As previously mentioned, the normal vascular endothelium plays an important role in maintaining vessel wall homeostasis, synthesizing substances such as prostacyclin and the free radical NO, which modulate vascular tone, prevent thrombosis, and influence smooth muscle growth [57]. Once the inhibition of NO synthesis does not completely abolish vasodilation, an alternative pathway may be involved [57]. There is evidence that vasodilatory prostanoids may be important in determining responses to acetylcholine (Ach) in both diabetic [58] and nondiabetic subjects [59], being their effects mediated through an increase in cyclic AMP. Recent findings [57] suggest that

vasodilatory prostanoids are important in determining endothelial response to Ach in diabetic and nondiabetic subjects. Increased prostaglandin-mediated vasodilation may compensate for attenuated responses to NO previously reported in diabetic subjects. These reports partly explain the conflicting reports of endothelial dysfunction in patients with type 1 diabetes. Finally, L-arginine was able to restore the reduced rest blood flow in type 1 diabetic subjects, without any influence in relation to exercise. Therefore, we suggest that in rest, blood flow may be controlled/dependent on nitric oxide synthesis (in the form of stable metabolites) and by a possible reduction in vasoconstrictive molecules, such as endothelin and ACE activation. Furthermore, during an acute bout of exercise, a lack of NO production can be compensated by other vasoactive molecules such as prostaglandins; despite NO is still an essential molecule.

Conclusions and Perspectives

L-Arginine and its precursor L-citrulline come up as new potential therapeutic tools for the treatment and perhaps prevention of T1DM. More studies are needed to provide information on the effects of L-arginine and L-citrulline in the population of people with T1DM. Our hypothesis is that the effects of both amino acids are beneficial for this population, as we recently tested; however, the effects may be different for different stages of the disease. For example, there may be a need for L-arginine (and citrulline) before the onset of the disease to avoid the possible reduction of the availability of this amino acid induced by the local islet inflammation (i.e., macrophage activation and arginase secretion). However, the effects of their supplementation after the beta-cell death may direct affect other cells such as endothelial cells, increasing nitric oxide availability, lowering oxidative stress, and improving mitochondrial function.

References

1. Newsholme P, Stenson L, Sulvucci M, Sumayao R, Krause M. Amino acid metabolism. 2nd ed. London: Elsevier; 2011.
2. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37(1):153–68.
3. Matthews GD, Gould RM, Vardimon L. A single glutamine synthetase gene produces tissue-specific subcellular localization by alternative splicing. FEBS Lett. 2005;579(25):5527–34.
4. Krause Mda S, de Bittencourt Jr PI. Type 1 diabetes: can exercise impair the autoimmune event? The L-arginine/glutamine coupling hypothesis. Cell Biochem Funct. 2008;26(4):406–33.
5. Vermeulen MA, van de Poll MC, Ligthart-Melis GC, et al. Specific amino acids in the critically ill patient--exogenous glutamine/L-arginine: a common denominator? Crit Care Med. 2007;35(9 Suppl):S568–76.
6. Preli RB, Klein KP, Herrington DM. Vascular effects of dietary L-arginine supplementation. Atherosclerosis. 2002;162(1):1–15.
7. Clarkson P, Adams MR, Powe AJ, et al. Oral L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults. J Clin Invest. 1996;97(8):1989–94.
8. Fayh AP, Krause M, Rodrigues-Krause J, et al. Effects of L-arginine supplementation on blood flow, oxidative stress status and exercise responses in young adults with uncomplicated type I diabetes. Eur J Nutr. 2014;52(3):975–83.
9. Ziegler TR, Gatzen C, Wilmore DW. Strategies for attenuating protein-catabolic responses in the critically ill. Annu Rev Med. 1994;45:459–80.
10. Barbul A. L-Arginine: biochemistry, physiology, and therapeutic implications. JPEN J Parenter Enteral Nutr. 1986;10(2):227–38.
11. Krause M, Rodrigues-Krause J, O'Hagan C, et al. Differential nitric oxide levels in the blood and skeletal muscle of Type 2 diabetic subjects may be consequence of adiposity: a preliminary study. Metabolism. 2012;61:1528–37.

12. Krause MS, McClenaghan NH, Flatt PR, de Homem de Bittencourt PI, Murphy C, Newsholme P. L-arginine is essential for pancreatic beta-cell functional integrity, metabolism and defense from inflammatory challenge. J Endocrinol. 2011;211(1):87–97.
13. Newsholme P, Homem De Bittencourt PI, O'Hagan C, De Vito G, Murphy C, Krause MS. Exercise and possible molecular mechanisms of protection from vascular disease and diabetes: the central role of ROS and nitric oxide. Clin Sci (Lond). 2009;118(5):341–9.
14. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol. 1990;30:535–60.
15. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43(2):109–42.
16. Melendez-Ramirez LY, Richards RJ, Cefalu WT. Complications of type 1 diabetes. Endocrinol Metab Clin North Am. 2010;39(3):625–40.
17. Krause M, Rodrigues-Krause J, O'Hagan C, et al. The effects of aerobic exercise training at two different intensities in obesity and type 2 diabetes: implications for oxidative stress, low-grade inflammation and nitric oxide production. Eur J Appl Physiol. 2014;114(2):251–60.
18. Menge BA, Schrader H, Ritter PR, et al. Selective amino acid deficiency in patients with impaired glucose tolerance and type 2 diabetes. Regul Pept. 2009;160(1–3):75–80.
19. Pieper GM, Dondlinger LA. Plasma and vascular tissue L-arginine are decreased in diabetes: acute L-arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. J Pharmacol Exp Ther. 1997;283(2):684–91.
20. Maxwell AJ. Mechanisms of dysfunction of the nitric oxide pathway in vascular diseases. Nitric Oxide. 2002;6(2):101–24.
21. Fu WJ, Haynes TE, Kohli R, et al. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr. 2005;135(4):714–21.
22. Vasilijevic A, Buzadzic B, Korac A, Petrovic V, Jankovic A, Korac B. Beneficial effects of L-arginine nitric oxide-producing pathway in rats treated with alloxan. J Physiol. 2007;584(Pt 3):921–33.
23. El-Missiry MA, Othman AI, Amer MA. L-Arginine ameliorates oxidative stress in alloxan-induced experimental diabetes mellitus. J Appl Toxicol. 2004;24(2):93–7.
24. Mendez JD, Balderas F. Regulation of hyperglycemia and dyslipidemia by exogenous L-arginine in diabetic rats. Biochimie. 2001;83(5):453–8.
25. West MB, Ramana KV, Kaiserova K, Srivastava SK, Bhatnagar A. L-Arginine prevents metabolic effects of high glucose in diabetic mice. FEBS Lett. 2008;582(17):2609–14.
26. Mendez JD, Hernandez Rde H. L-arginine and polyamine administration protect beta-cells against alloxan diabetogenic effect in Sprague-Dawley rats. Biomed Pharmacother. 2005;59(6):283–9.
27. Wu G, Collins JK, Perkins-Veazie P, et al. Dietary supplementation with watermelon pomace juice enhances L-arginine availability and ameliorates the metabolic syndrome in Zucker diabetic fatty rats. J Nutr. 2007;137(12): 2680–5.
28. Balderas-Munoz K, Castillo-Martinez L, Orea-Tejeda A, et al. Improvement of ventricular function in systolic heart failure patients with oral L-citrulline supplementation. Cardiol J. 2012;19(6):612–7.
29. Jabecka A, Ast J, Bogdaski P, et al. Oral L-arginine supplementation in patients with mild arterial hypertension and its effect on plasma level of asymmetric dimethylarginine, L-citrulline, L-arginine and antioxidant status. Eur Rev Med Pharmacol Sci. 2012;16(12):1665–74.
30. Lekakis JP, Papathanassiou S, Papaioannou TG, et al. Oral L-arginine improves endothelial dysfunction in patients with essential hypertension. Int J Cardiol. 2002;86(2–3):317–23.
31. Martina V, Masha A, Gigliardi VR, et al. Long-term N-acetylcysteine and L-arginine administration reduces endothelial activation and systolic blood pressure in hypertensive patients with type 2 diabetes. Diabetes Care. 2008;31(5):940–4.
32. Yin WH, Chen JW, Tsai C, Chiang MC, Young MS, Lin SJ. L-arginine improves endothelial function and reduces LDL oxidation in patients with stable coronary artery disease. Clin Nutr. 2005;24(6):988–97.
33. Eizirik DL, Mandrup-Poulsen T. A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia. 2001;44(12):2115–33.
34. Murphy C, Newsholme P. Importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production. Clin Sci (Lond). 1998;95(4):397–407.
35. Zhai Z, Solco A, Wu L, et al. Echinacea increases arginase activity and has anti-inflammatory properties in RAW 264.7 macrophage cells, indicative of alternative macrophage activation. J Ethnopharmacol. 2009;122(1):76–85.
36. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005;5(8):641–54.
37. Stickings P, Mistry SK, Boucher JL, Morris SM, Cunningham JM. Arginase expression and modulation of IL-1beta-induced nitric oxide generation in rat and human islets of Langerhans. Nitric Oxide. 2002;7(4):289–96.

38. McKnight JR, Satterfield MC, Jobgen WS, et al. Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids. 2010;39(2):349–57.
39. Rodrigues-Krause J, Krause M, O'Hagan C, et al. Divergence of intracellular and extracellular HSP72 in type 2 diabetes: does fat matter? Cell Stress Chaperones. 2012;17(3):293–302.
40. Newsholme P, Rebelato E, Abdulkader F, Krause M, Carpinelli A, Curi R. Reactive oxygen and nitrogen species generation, antioxidant defenses, and beta-cell function: a critical role for amino acids. J Endocrinol. 2012;214(1):11–20.
41. Dong JY, Qin LQ, Zhang Z, et al. Effect of oral L-arginine supplementation on blood pressure: a meta-analysis of randomized, double-blind, placebo-controlled trials. Am Heart J. 2011;162(6):959–65.
42. Bai Y, Sun L, Yang T, Sun K, Chen J, Hui R. Increase in fasting vascular endothelial function after short-term oral L-arginine is effective when baseline flow-mediated dilation is low: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2009;89(1):77–84.
43. Lucotti P, Monti L, Setola E, et al. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism. 2009;58(9):1270–6.
44. Monti LD, Setola E, Lucotti PC, et al. Effect of a long-term oral L-arginine supplementation on glucose metabolism: a randomized, double-blind, placebo-controlled trial. Diabetes Obes Metab. 2012;14:893–900.
45. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest. 1997;100(9):2153–7.
46. Romero MJ, Yao L, Sridhar S, et al. l-Citrulline protects from kidney damage in type 1 diabetic mice. Front Immunol. 2013;4:480.
47. Romero MJ, Platt DH, Tawfik HE, et al. Diabetes-induced coronary vascular dysfunction involves increased arginase activity. Circ Res. 2008;102(1):95–102.
48. Beleznai T, Feher A, Spielvogel D, Lansman SL, Bagi Z. Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes. Am J Physiol Heart Circ Physiol. 2011;300(3):H777–83.
49. Romero MJ, Iddings JA, Platt DH, et al. Diabetes-induced vascular dysfunction involves arginase I. Am J Physiol Heart Circ Physiol. 2012;302(1):H159–66.
50. Cvetkovic T, Mitic B, Lazarevic G, Vlahovic P, Antic S, Stefanovic V. Oxidative stress parameters as possible urine markers in patients with diabetic nephropathy. J Diabetes Complications. 2009;23(5):337–42.
51. Huang EA, Gitelman SE. The effect of oral alpha-lipoic acid on oxidative stress in adolescents with type 1 diabetes mellitus. Pediatr Diabetes. 2008;9(3 Pt 2):69–73.
52. Horoz OO, Yuksel B, Bayazit AK, et al. Ambulatory blood pressure monitoring and serum nitric oxide concentration in type 1 diabetic children. Endocr J. 2009;56(3):477–85.
53. Milsom AB, Jones CJ, Goodfellow J, Frenneaux MP, Peters JR, James PE. Abnormal metabolic fate of nitric oxide in Type I diabetes mellitus. Diabetologia. 2002;45(11):1515–22.
54. Wu G, Meininger CJ. L-Arginine nutrition and cardiovascular function. J Nutr. 2000;130(11):2626–9.
55. Wascher TC, Posch K, Wallner S, Hermetter A, Kostner GM, Graier WF. Vascular effects of L-arginine: anything beyond a substrate for the NO-synthase? Biochem Biophys Res Commun. 1997;234(1):35–8.
56. Higashi Y, Oshima T, Ono N, et al. Intravenous administration of L-arginine inhibits angiotensin-converting enzyme in humans. J Clin Endocrinol Metab. 1995;80(7):2198–202.
57. Meeking DR, Browne DL, Allard S, et al. Effects of cyclooxygenase inhibition on vasodilatory response to acetylcholine in patients with type 1 diabetes and nondiabetic subjects. Diabetes Care. 2000;23(12):1840–3.
58. Poston L, Taylor PD. Glaxo/MRS young investigator prize. Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. Clin Sci (Lond). 1995;88(3):245–55.
59. Cowley AJ, Stainer K, Rowley JM, Wilcox RG. Effect of aspirin and indomethacin on exercise-induced changes in blood pressure and limb blood flow in normal volunteers. Cardiovasc Res. 1985;19(3):177–80.

Chapter 32
Oral L-Arginine Supplementation and Glucose Metabolism and Vascular Function

Lucilla D. Monti, Elena Galluccio, Barbara Fontana, Emanuele Bosi, and Piermarco Piatti

Key Points

- L-Arginine has beneficial effects on glucose metabolism and insulin sensitivity.
- L-Arginine supplementation to obese subjects promotes fat reduction and spared lean body mass during weight loss.
- L-Arginine preserves endothelium-dependent vasodilator function.
- L-Arginine supplementation significantly reduces aortic intimal thickening, blocking the production of carotid and coronary intimal plaques.
- L-Arginine-enriched biscuits with low sugar and protein content enhance endothelial function and improve glucose metabolism, insulin sensitivity, and insulin secretion in subjects with impaired glucose tolerance (IGT) and metabolic syndrome.
- The beneficial effect of L-arginine in treating many developmental and health problems is unique among amino acids, and L-arginine may become a useful "nutraceutical" approach.

Keywords L-Arginine supplementation • Glucose metabolism • Vascular function • Nutrition • eNOS

Abbreviations

cGMP	Cyclic GMP
NO	Nitric oxide
ADMA	Asymmetric dimethylarginine
MMP	Matrix metalloproteinase
ARG	L-Arginine
SU	Sucrose
IVGTT	Intravenous glucose tolerance test
BMI	Body mass index

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FFA	Free fatty acid
TIMP-1	Tissue inhibitors of metalloproteinase
IGT	Impaired glucose tolerance
MS	Metabolic syndrome
NGT	Normal glucose tolerance
BP	Blood pressure
eNOS	Endothelial nitric oxide synthase
PTCA	Percutaneous transluminal coronary angioplasty
EDNO	Endothelium-derived nitric oxide
PI-BF	Post-ischemic blood flow

Introduction

It is well known that metabolic syndrome is a cluster of metabolic abnormalities, which includes impaired glucose regulation, hypertension, dyslipidaemia, obesity, and increased cardiovascular disease risk [1]. Metabolic syndrome affects one in five people, and prevalence increases with age; some studies estimate the prevalence in the USA to be nearly 25 % of the population [2].

Previous studies have shown that metabolic syndrome is characterized by endothelial dysfunction with an impairment of endothelial vasodilation and by an altered availability of nitric oxide and/or its second messenger cGMP [3]. It is known that endothelium-derived nitric oxide (EDNO) is a potent endogenous vasodilator that plays a major role in vascular tone [4]. L-Arginine is the endogenous precursor of nitric oxide synthesis, and it has been previously demonstrated that L-arginine potentiates insulin-mediated glucose uptake by increasing blood flow [5]. L-Arginine (2-amino-5-guanidino-pentanoic acid) is a conditionally essential, proteinogenic amino acid that is a natural constituent of dietary proteins. Besides its role in protein metabolism, L-arginine is involved in various metabolic pathways, such as synthesis of creatine, L-ornithine, L-glutamate, and polyamines. Decarboxylation of L-arginine can produce agmatine, a biogenic amine metabolite. L-Arginine is also involved in protein degradation by the ubiquitin-proteasome pathway. A biologically important pathway involves L-arginine as the substrate of a family of enzymes named nitric oxide synthases. The reaction mechanism of NO synthases involves a 2-electron transfer from molecular oxygen via a number of cofactors to L-arginine, resulting in the release of NO and L-citrulline [6]. Experimental studies performed in vitro and in vivo have evaluated the biological effect of L-arginine administration both in humans and animal studies.

Effects of L-Arginine on Glucose Metabolism

Previously, Monti et al. demonstrated that the nitric oxide pathway was an important regulator of hepatic glucose metabolism in rat hepatocytes by modulating glucokinase activity and hepatic glycogen content [7]. Fu et al. studied Zucker diabetic fatty rats submitted to a dietary supplementation of drinking water containing L-arginine (1.51 %) or alanine (2.55 %, isonitrogenous control) for 10 weeks. L-Arginine supplementation significantly reduced weight of retroperitoneal and epididymal adipose tissue by 45 % and 25 %, respectively, as well as circulating levels of glucose by 25 %, triglycerides by 23 %, free fatty acids by 27 %, homocysteine by 26 %, ADMA by 18–21 %, and leptin by 32 % (Table 32.1). Results of the microarray analysis indicated that L-arginine supplementation increased adipose tissue expression of key genes responsible for fatty acid and glucose oxidation, NO synthase-1, AMP-activated protein kinase, and peroxisome proliferator-activated receptor gamma coactivator-1 alpha [8]. Recently it has been published a study to determine whether sucrose-induced insulin resistance could increase the expression of cardiac matrix metalloproteinase (MMPs) and

indices of matrix remodelling and whether the addition of 1.25 g day (−1) of L-arginine (ARG) to a sucrose diet could prevent both the sucrose-induced metabolic abnormalities and elevated cardiac expression of MMPs in an insulin-resistant stage that precedes frank type 2 diabetes. Thirty-eight male Sprague-Dawley rats were involved, 16 rats maintained a standard chow diet (ST), and 12 rats were switched to a sucrose-enriched diet (SU) and 10 rats to a sucrose plus L-arginine [1.25 g day (−1)]-enriched diet (SU+ARG) for a period of 8 weeks. After 8 weeks of different diets, an intravenous glucose tolerance test (IVGTT) was performed. At the end of the study, retroperitoneal fat, heart weight/body weight ratio, fasting plasma glucose, serum insulin, and serum triglyceride levels and integrated insulin area after IVGTT were significantly higher in SU than in SU+ARG and ST. All these parameters were comparable between SU+ARG and ST animals. FFA levels were significantly different among groups, with highest levels in SU and lowest levels in ST. Fasting plasma c-GMP levels and the integrated c-GMP area after IVGTT, an index of nitric oxide activity, were significantly lower in SU than in SU+ARG and ST; the result was similar in SU+ARG; and in ST, MMP-9 protein expression increased 10.5-fold, MMP-2 protein expression increased 2.4-fold, and the expression of tissue inhibitors of metalloproteinase (TIMP-1) increased 1.7-fold in SU rats as compared to ST animals. This was accompanied with a significant increase of cardiac triglyceride concentrations. SU rats developed insulin resistance and hyperlipidaemia, accompanied with increased fat deposition in the heart and enhanced MMP protein expression. Conversely, ARG supplementation prevents these metabolic abnormalities and restored MMP/TIMP-1 balance [9].

In humans, the intravenous infusion 0.5 g/min of L-arginine but not D-arginine increased whole body glucose disposal and blood flow in normal subjects [5]. McConnell et al. performed similar amount of L-arginine infusion in endurance trained males during exercise test [10]. They found that L-arginine determined a significant increase of the whole body glucose disposal and a decrease of free fatty acid levels while insulin levels remained unchanged as compared to saline control study. These data strongly suggest that L-arginine infusion improved muscle glucose utilization modulating free fatty acid concentration during exercise. Insulin sensitivity and insulin-mediated vasodilation were also improved by infusing lower doses of L-arginine (0.52 mg/kg/min) in healthy, obese, and type 2

Table 32.1 Serum concentrations of glucose, triglycerides, cholesterol, NOx, methylarginines, homocysteine, and hormones in ZDF rats randomly assigned to receive drinking water containing either 1.51 % L-arginine-HCl or 2.55 % L-alanine (isonitrogenous control) at week 10 after initiation of L-arginine supplementation

	Week 10	
Variable	Alanine-treated	Arginine-treated
Glucose (mmol/L)	25.7±0.63	19.4±0.58*
Triglycerides (mmol/L)	6.50±0.39	5.01±0.37*
Cholesterol (mmol/L)	5.37±0.46	5.25±0.42
FFA (mmol/L)	1.67±0.11	1.22±0.08*
NOx (μmol/L)	30±2.1	51±3.2*
ADMA (μmol/L)	1.78±0.12	1.40±0.10*
NMMA (μmol/L)	2.06±0.13	1.67±0.09*
SDMA (μmol/L)	1.64±0.10	1.35±0.07*
Homocysteine (μmol/L)	7.38±0.47	5.44±0.32*
Insulin (pmol/L)	251±34	246±29
Growth hormone (pmol/L)	103±6.8	107±9.4
Adiponectin (mg/L)	2.67±0.11	2.76±0.25
Ghrelin (μg/L)	2.46±1.43	2.61±1.58
Leptin (μg/L)	21.6±1.94	14.6±0.67*

Values are means±SEM, $n=6$

*Different from alanine-treated ZDF rats, $p<0.05$

Abbreviations used: *ADMA* asymmetric dimethylarginine, *NMMA* N^G-monomethylarginine, *SDMA* symmetric dimethylarginine)

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diabetic subjects [11]. These effects were confirmed by a double-blind chronic treatment of L-arginine (9 g daily) in non-obese type 2 diabetic patients. After 1 month of L-arginine therapy, peripheral and hepatic insulin sensitivity were significantly improved and cyclic-GMP levels and insulin-mediated vasodilation were normalized [12]. Recently it has published a clinical trial regarding the specific effect of L-arginine on adiposity in humans. This was a 21-day randomized, placebo-controlled trial in 33 hospitalized middle-aged, obese (mean BMI = 39.1 ± 0.5 kg/m^2) subjects with diet-controlled type 2 diabetes mellitus. During the study period, each patient received a low-caloric diet (1000 kcal/day) and a regular exercise training programme (4 min twice a day for 5 days/week). They were randomized to 8.3 g Arg/day (approximately 80 mg/kg body weight per day) or placebo. As expected from the hypocaloric diet, both groups of subjects exhibited reductions in body weight, fat mass, waist circumference, and circulating levels of glucose, fructosamine, and insulin (Table 32.2). Moreover, increase in antioxidant capacity and circulating levels of adiponectin were observed for these patients. Importantly all improvements were significantly greater in L-arginine group than in the placebo group. Additionally fat-free mass was maintained in the L-arginine group but reduced by 1.6 kg in the placebo group. Thus, L-arginine supplementation to obese subjects promoted fat reduction and spared lean body mass during weight loss [13]. The authors evaluated also the effects of long-term oral L-arginine treatment on endothelial dysfunction, inflammation, adipokine levels, glucose tolerance, and insulin sensitivity in no diabetic patients with stable cardiovascular disease (coronary artery disease). Sixty-four patients with cardiovascular disease previously submitted to an aortocoronary bypass and not known for type 2 diabetes mellitus had an oral glucose load to define their glucose tolerance. Thirty-two patients with nondiabetic response were eligible to receive, in a double-blind

Table 32.2 Changes in anthropometric, metabolic, endothelial, and hormonal variables before and after L-arginine and placebo therapy

	L-Arginine		Placebo		*p* value	
	Before	After	Before	After	Time effect	Interaction effect
Weight (kg)	105.8 ± 3.1	102.8 ± 3.0	102.1 ± 3.6	98.4 ± 3.0	<0.0001	<0.35
Fat mass (kg)	49.3 ± 2.2	46.3 ± 2.3	46.8 ± 2.3	44.7 ± 2.4	<0.0001	<0.05
Free-fat mass (kg)	56.5 ± 2.0	56.5 ± 1.8	55.3 ± 2.2	53.6 ± 2.2	<0.02	<0.03
Waist (cm)	121.1 ± 3.0	112.8 ± 2.6	116.7 ± 2.2	113.5 ± 2.4	<0.0001	<0.0001
Systolic blood press (mmHg)	151 ± 3	128 ± 3	149 ± 2	149 ± 2	<0.0001	<0.0001
Diastolic blood press (mmHg)	90 ± 2	78 ± 2	89 ± 2	88 ± 3	<0.0001	<0.0002
Fructosamine (μmol/L)	274.6 ± 15.6	220.4 ± 8.6	280.8 ± 9.4	257.8 ± 8.7	<0.0001	<0.02
Fasting insulin (mU/L)	21.8 ± 3.1	13.6 ± 1.8	18.6 ± 1.7	15.6 ± 1.4	<0.0002	<0.04
Fasting triglycerides (mmol/L)	1.95 ± 0.23	1.34 ± 0.11	2.06 ± 0.16	1.49 ± 0.11	<0.0001	<0.09
Fasting FFA (mmol/L)	0.76 ± 0.6	0.69 ± 0.05	0.77 ± 0.06	0.68 ± 0.03	<0.08	<0.73
Incremental area NOx (μmol/L 6 min)	9.2 ± 5.0	32.8 ± 5.7	9.5 ± 2.6	8.9 ± 2.7	<0.005	<0.002
Fasting cGMP (μmol/mL)	2.30 ± 0.2	3.56 ± 0.3	2.25 ± 0.1	2.70 ± 0.2	<0.005	<0.001
Incremental area cGMP (μmol/mL 6 min)	1.50 ± 0.3	5.45 ± 0.6	1.61 ± 0.2	3.67 ± 0.2	<0.0001	<0.001
Fasting ET-1 (pg/mL)	10.5 ± 0.5	7.4 ± 0.4	9.5 ± 0.4	13.7 ± 1.0	<0.37	<0.0001
Incremental area ET-1 (pg/mL 6 min)	12.4 ± 2.6	0.4 ± 1.1	11.2 ± 2.6	11.1 ± 2.4	<0.01	<0.01
Basal ec-SOD (ng/mL)	84.6 ± 7.0	113.9 ± 12.3	86.8 ± 7.4	77.3 ± 4.9	<0.22	<0.03
Incremental area ec-SOD (mol/mL 6 min)	72.8 ± 31.8	210.5 ± 20.6	37.1 ± 21.9	95.7 ± 10.4	<0.0002	<0.05
Adiponectin (ng/mL)	4.0 ± 0.7	5.6 ± 0.6	4.2 ± 0.6	4.2 ± 0.5	<0.02	<0.03
Leptin (ng/mL)	62.4 ± 7.7	48.0 ± 7.8	53.0 ± 9.5	48.6 ± 9.7	<0.01	<0.07
Leptin-to-adiponectin ratio	21.8 ± 3.9	9.4 ± 1.3	18.1 ± 5.5	13.7 ± 3.2	<0.001	<0.05

*Values are means ± SE. *FFA* free fatty acids, *NOx* nitrate/nitrite, *ET-1* endothelin-1, *ec-SOD* extracellular; superoxide dismutase

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randomized parallel order, L-arginine (6.4 g/day) or placebo for 6 months. An evaluation of insulin sensitivity index during the oral glucose load, markers of systemic nitric oxide bioavailability and inflammation, and blood flow was performed before and at the end of the treatment in both groups. Compared with placebo, L-arginine decreased asymmetric dimethylarginine (ADMA) levels ($p < 0.01$) and increased cyclic guanosine monophosphate ($p < 0.01$), L-arginine to ADMA ratio ($p < 0.0001$), and reactive hyperaemia ($p < 0.05$). Finally, L-arginine increased insulin sensitivity index ($p < 0.05$) and adiponectin ($p < 0.01$) and decreased interleukin-6 and monocyte chemoattractant protein-1 levels. In conclusion L-arginine seems to have anti-inflammatory and metabolic advantages in these patients [14]. An anabolic effect of L-arginine on muscle gain is achieved independent of changes in serum concentrations of insulin or growth hormone. Dietary L-arginine supplementation enhances insulin sensitivity and amplifies its signalling mechanisms on protein synthesis as well as the metabolism of glucose and fatty acids. So L-arginine supplementation regulates the repartitioning of dietary energy to favour muscle over fat gain in the body. Recently Monti LD et al. performed a mono-centre, randomized, double-blind, parallel-group, placebo-controlled, phase III trial (named L-arg trial). In this study, 144 individuals, affected by impaired glucose tolerance (IGT) and metabolic syndrome (MS), received 6.4 g/day of L-arginine or placebo for 18 months plus a 12-month extended follow-up period after study drug termination, in order to prevent or delay type 2 diabetes and to normalize glucose tolerance in individuals at high risk for type 2 diabetes. The results showed that the supplementation of L-arginine for 18 months does not significantly reduce the incidence of diabetes but does significantly increase regression to normal glucose tolerance (NGT) [15].

Effects of L-Arginine on Vascular Function

Experimental studies performed in vitro and in vivo showed that L-arginine improved endothelial vasodilation and delayed atherosclerotic process. In an in vivo study, Girerd et al. measured the responses to acetylcholine of hind limb blood flow before and after acute infusion of intravenous L-arginine in hypercholesterolemic and control rabbits. Compared with acetylcholine alone, the coinfusion of L-arginine improved the blunted endothelial vasodilation of hypercholesterolemic rabbits. Otherwise L-arginine did not affect the vasodilator response to acetylcholine in the control rabbits [16]. Cooke et al. investigated the effects of a prolonged oral L-arginine administration to rabbits on high cholesterol diet. Endothelium-dependent vasodilator response to acetylcholine resulted impaired in cholesterol-fed rabbits, but were markedly improved in those with a dietary L-arginine supplementation; moreover this group presented a reduction of lesion surface area and intimal media thickness [17]. Atherosclerotic plaque extension found in the carotid artery and thoracic aorta of hypercholesterolemic rabbits decreased after a treatment with oral L-arginine compared to controls as demonstrated by Boger et al. during 12 weeks of high cholesterol plus L-arginine diet [18]. Dhawan et al. showed that chronic L-arginine supplementation significantly reduced aortic intimal thickening, blocked the production of carotid and coronary intimal plaques, and preserved endothelium-dependent vasodilator function in atherogenic rhesus monkeys [19]. Hayashi et al. demonstrated that in rabbits fed with a high cholesterol diet, a 12-week oral administration of L-arginine plus L-citrulline, either alone or in combination with antioxidants, caused a marked improvement in endothelium-dependent vasorelaxation and blood flow, dramatic regression in atheromatous lesions, and decrease in superoxide production and oxidation-sensitive gene expression [20]. A systematic review of 25 published controlled studies of L-arginine (3.0–300 mg/kg) and NO donors in experimental stroke has shown that L-arginine induced a beneficial effect on cortical cerebral blood flow although it did not significantly alter lesion volume in both permanent and transient stroke [21]. Bode-Boger et al. investigated healthy volunteers and the effect of an intravenous infusion of L-arginine (a dose of 30 g for 30 min) on blood pressure; they showed that L-arginine reduced blood pressure and that this effect was strikingly evident for diastolic pressure [22].

Dong et al. evaluated the effect of oral L-arginine supplementation on blood pressure by conducting a meta-analysis of 11 randomized, double-blind, placebo-controlled trials. The study involved 387 participants with oral L-arginine intervention ranging from 4 to 24 g/day. Compared with placebo, L-arginine intervention significantly lowered systolic BP by 5.39 mmHg (95 % CI −8.54 to −2.25, $p=0.001$) and diastolic BP by 2.66 mmHg (95 % CI −3.77 to −1.54, $p<0.001$). Sensitivity analyses restricted to trials with a duration of 4 weeks or longer and to trials in which participants did not use antihypertensive medications yielded similar results [23].

Creager et al. demonstrated that in hypercholesterolemic patients, an acute administration of L-arginine (10 mg/kg/min IV) was able to induce an improvement of L-arginine blood vessels' endothelium-dependent vasodilation [24]. Egashira et al. examined the effect of L-arginine on endothelium-dependent coronary vasodilation in patients with microvascular angina throughout an intracoronary infusion of L-arginine (50 mg/mm) on acetylcholine-induced coronary vasomotion. L-Arginine was able to improve endothelium-dependent vasodilation of coronary microcirculation in these patients [25]. An acute infusion of low-dose of L-arginine (0.125 g/min) does not able to stimulate the insulin-induced endothelial-dependent vasodilation, increased forearm blood flow, cyclic-GMP forearm release, and decreased endothelin-1 levels in healthy subjects. As shown by Piatti et al., in patients affected by microvascular angina, similar amount of L-arginine infusion increased forearm blood flow, circulating NOx, and forearm cGMP release and decreased endothelin-1 levels and systolic and diastolic pressure. Moreover, the pre-infusion of low dose of L-arginine restored the endothelin-1, NOx, and forearm cGMP release responses after insulin bolus in these patients [26]. On the other hand, contradictory results are shown in healthy subjects after chronic treatment with L-arginine. Adams et al. did not find a significant ability of oral L-arginine (7 g three times daily for 3 consecutive days) to stimulate endothelial vasodilation although platelet aggregation was significantly inhibited in healthy subjects [27]. However, when endothelial function is impaired as in healthy very old age subjects, oral L-arginine supplementation was able to improved endothelial vasodilation [28]. Clarkson et al. found that L-arginine addition improved endothelium-dependent vasodilation compared to placebo in a double-blind crossover study with 4-week L-arginine supplementation (7 g three times a day) or placebo in hypercholesterolemic young adults [29]. Hambrecht et al. studied the effect of L-arginine (8 g daily) and physical exercise in patients with chronic heart failure. After 4 weeks both L-arginine and physical exercise were able to improve endothelium-dependent vasodilation, but the association of L-arginine plus exercise produced an additive beneficial effect on endothelium-dependent vasodilation [30]. Finally, Palloshi et al. demonstrated that a chronic administration of L-arginine (4 weeks, 6 g daily) in patients with hypertension and microvascular angina determines an improvement of endothelial function and a significant amelioration of symptoms [31] (Table 32.3).

In 1992, Vallance et al. first described the presence of ADMA as an endogenous inhibitor of eNOS in human plasma and urine [32]. ADMA inhibits vascular nitric oxide production within the concentration range found in patients with vascular disease. Elevated ADMA concentrations are present in patients with hypercholesterolaemia, hyperhomocysteinaemia, diabetes mellitus, insulin resistance, atherosclerosis and hypertension, cardiovascular disease, and chronic heart failure [33]. ADMA seem to have also a pivotal role to explain the presence of the "L-arginine paradox." Although it was argued that additional L-arginine could not have any effect on NOS activity, because of enzyme saturation with substrate at physiological levels (the half-saturing L-arginine concentration for eNOS is in the range of 1–10 μM (mean km 2.9 μM while the physiological L-arginine concentrations in the extracellular space are already 20-fold higher (50–200 μM)), L-arginine supplementation was able to positively influence endothelium-dependent vasodilation. The explanation of the "L-arginine paradox" could be that the inhibition of NOS activity induced by ADMA may be overcome by augmented supply of substrate and this could explain how L-arginine may improve endothelial function in patient with vascular disease [23]. It is of notice that in cultured endothelial cells, the L-arginine uptake has been attributed to the cationic amino acid y^+ transporter-1 protein system (CAT-1) for 70–95 %, the

Table 32.3 Data at baseline and after L-arginine treatment

	Baseline	L-Arginine
SBP at rest (mmHg)	166 ± 27	146 ± 12*
DBP at rest (mmHg)	84 ± 17	77 ± 10
Basal FBF (mL/min/100 mL)	2.5 ± 0.8	2.8 ± 0.5
Maximal post-ischemic FBF (mL/min 100 mL)	22 ± 5	31 ± 11*
Nitric oxide (μmol/L)	36 ± 22	35 ± 36
Homocysteine (μmol/L)	17 ± 8	13 ± 4
Cyclic guanosine monophosphate (pmol/mL)	2.30 ± 0.73	2.68 ± 0.86*
Asymmetric dimethylarginine (μmol/L)	0.57 ± 0.12	0.64 ± 0.13
L-Arginine (μmol/L)	54 ± 17.4	71.7 ± 19.7*
L-Arginine/asymmetric dimethylarginine	95 ± 22	113 ± 30*
Endothelin-1 (pg/mL)	6.44 ± 0.23	6.32 ± 0.38

Data are mean ± SD. *DBP* diastolic blood pressure, *SBP* systolic blood pressure
*$p < 0.05$ versus control values
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same transporter utilized by ADMA and symmetrical dimethylarginine (SDMA). Since a competition exists between L-arginine and ADMA and SDMA on this transport system, an excess of L-arginine might overcome this antagonism [34]. Interestingly, an intra-arterial administration of L-arginine can restore L-arginine transport suggesting a possible link between a defective L-arginine transport/NO pathway and the onset of essential hypertension [35]. Plasma ADMA levels are also increased in patients with microvascular angina, and they are correlated with increased endothelin-1 levels and reduced insulin-induced increments in plasma NOx and cGMP, effects that are reversed by intravenous L-arginine infusion (0.125 g/min). These results were confirmed studying a chronic administration of L-arginine (4 weeks, 6 g daily) in patients with microvascular angina that improved both L-arginine/ADMA ratio and cyclic-GMP levels [31].

Several studies were performed to evaluate the role of L-arginine on endothelium dysfunction in patient affected by coronary artery disease. Adams et al. submitted men with premature coronary artery disease to an oral administration of L-arginine (7 g three times per day) or placebo for 3 days each, with a washout period of 10 days. They demonstrated an improvement in endothelium-dependent dilatation and reduction in monocyte/endothelial cell adhesion [36]. Ceremuzynski et al. showed that the same period of L-arginine treatment (6 g daily for 3 days) in patients with stable angina and healed myocardial infarction was also able to increase exercise capacity [37]. Moreover, Fukumoto et al. found that an intracoronary administration of L-arginine (1 μg/kg) at the sites of PTCA induced an increment of the vasodilator response as compared to untreated sites, suggesting that enhancement of local NO production may be clinically useful in preventing restenosis after PTCA [38]. However, probably due to the short period of administration, Skiraki et al. were unable to find any beneficial effects of L-arginine, when administered via a cardiac catheter (500 mg/4 min) before PTCA and via a peripheral vein (30 g/4 h, for 5 days) after PTCA to inhibit restenosis in humans affected by angina pectoris [39]. Lerman et al. found that a chronic administration of L-arginine (9 g daily for 6 months) on patient affected by angina pectoris and mild nonobstructive coronary atherosclerosis markedly improved coronary vasodilator response to acetylcholine and angina symptoms [40]. Oomen et al. investigated by a questionnaire throughout a longitudinal cohort study in a large population (806) of Dutch men how dietary intake of L-arginine could modify coronary heart disease risk in elderly persons [41]. They found no significant effects of dietary L-arginine intake on cardiovascular risk but it is possible that the very low dietary intake (average 4.35 g daily) found in this population could explain these unfavourable results. Blum et al. submitted 30 subjects affected by coronary artery disease to 1 month of L-arginine (9 g/daily) treatment as adjunctive therapy and they did not find any improvement of NO bioavailability and no difference in flow-mediated vasodilation in these patients.

It is important to observe that even before the start of the study, this population presented a normal flow-mediated vasodilation, thanks to an optimized medical treatment before and during the study, as cholesterol lowering and vasoactive drugs, influencing endothelial function [42]. Several authors have shown a benefit of L-arginine in acute study in animal and in human subjects. However, the supplementations of L-arginine in chronic study in humans with normal endothelial function or with atherothrombotic disease have yielded contradictory results [42]. The discrepancy on the efficacy of chronic L-arginine therapy in human also depends on the different amounts of supplemental L-arginine administered that could be inadequate to modulate endothelial nitric oxide production, as suggested by two in vitro studies. In the first study, whereas the simultaneous intramural low dose of L-arginine plus glutamate infusion produced an increase in nitric oxide levels, similar experimental conditions with high L-arginine infusion inhibit NO production [42]. In the second study, incremental concentrations of L-arginine (2.0–8.0 mM) were added to a fixed concentration of methylglyoxal in a buffered lucigenin solution determining a dose-dependent generation of superoxide anion, increasing a redox state [43]. These two studies strongly suggest that only low dose of L-arginine which maintains physiological L-arginine concentrations could positively modulate endothelial function in patients with established atherosclerotic disease while high doses determined a detrimental effect downregulating nitric oxide production by its enzymatic decarboxylation to agmatine, inducing an increase in peroxynitrite and lipid peroxynitrite levels or decreasing the nitric oxide/cyclic-GMP availability. Peroxynitrites both oxidatively modify proteins in the vessel wall and oxidize tetrahydrobiopterin, which uncouples eNOS leading to further decrease in nitric oxide production as well as de novo superoxide production by this enzyme [44]. In summary the mechanisms by which Arg administration may prevent cardiovascular dysfunction include: (1) restoring endothelial NO synthesis and decreasing superoxide production, (2) reducing vascular oxidative damage, and (3) inhibiting platelet adherence and aggregation, leukocyte adherence to the endothelium, and the proliferation of vascular smooth muscle cells.

L-Arginine Supplementation and Nutrition

Maxwell et al. investigated the clinical effects of a food bar enriched with L-arginine and a combination of other nutrients known to enhance the activity of EDNO in individuals with claudication from atherosclerotic peripheral arterial disease. The study was a 2-week, double-blind, placebo-controlled trial of subjects randomized to three groups (two active bars, one active and one placebo bar, and two placebo bars per day) followed by an 8-week open-label period. Subjects ($n=41$) were outpatient volunteers with intermittent claudication. Pain-free and total walking distances were measured by variable-grade, treadmill exercise testing. Quality of life was assessed using the Medical Outcome Survey (SF-36). After 2 weeks of treatment, the pain-free walking distance increased 66 % while the total walking distance increased 23 % in the group taking two active bars/day. The general and emotional/social functioning components of the SF-36 also improved. These effects were not observed in the one active bar/day and placebo groups. The effects were maintained after 10 weeks and, in addition, an improvement in walking distance was observed in the group taking one active bar. These findings reveal that use of a nutrient bar designed to enhance EDNO activity improves pain-free and total walking distance as well as quality of life in individuals with intermittent claudication [45]. Abdelhamed et al. studied the effects of dietary L-arginine supplementation with HeartBars (a medical food rich in L-arginine, Cooke Pharma, Belmont, Calif) on flow-mediated dilation and markers of endothelial function in subjects with hypercholesterolaemia. Forty-seven subjects with hypercholesterolaemia were randomly assigned to receive one HeartBar containing 3.3 g L-arginine each, or a placebo bar, consumed twice daily for 2 weeks. This study showed no favourable effects on endothelial or platelet function [46].

Monti et al. evaluated the effects of a new L-arginine-enriched biscuit on endothelial function, insulin sensitivity/secretion, and body composition. The project was composed of two studies. The first study was an acute pilot postprandial study in seven healthy subjects that evaluated bioavailability and vascular effects of L-arginine-enriched biscuits that contained 6.6 g L-arginine, 21.9 g carbohydrates, 3.6 g protein, 7.5 g fat, and 4.3 g dietary fibre compared with placebo biscuits and 6.6 g powdered L-arginine. Subjects underwent the tests in random order, in at least 14-day intervals. The second study was a double-blind crossover study in 15 obese subjects with IGT and MS. These subjects consumed 6.6 g of L-arginine-enriched biscuits or placebo biscuits in a 1600 kcal diet. Each study period lasted 2 weeks with a 2-week washout in between. Endothelial function, glucose tolerance, insulin sensitivity, and insulin secretion were evaluated at the end of each intervention period. In the first study, the groups that received the L-arginine-enriched biscuits and the powdered L-arginine had similarly increased L-arginine, NOx, and cGMP levels and post-ischemic blood flow (PI-BF). In both cases, these levels were significantly higher than those in the placebo biscuit recipient group. In the second study, the L-arginine-enriched biscuit recipient group displayed increased L-arginine, NOx, cGMP, PI-BF, and Matsuda index levels, whereas their circulating glucose, proinsulin/insulin ratio, and fat mass were decreased compared with the placebo biscuit recipient group. L-Arginine-enriched biscuits with low sugar and protein content enhance endothelial function and improve glucose metabolism, insulin sensitivity, and insulin secretion in subjects with IGT and MS [47].

Conclusions

L-Arginine not only pays a pivotal role on endothelial function but also may provide novel and effective therapy for obesity, diabetes, and metabolic syndrome, decreasing cardiovascular risks in humans. Appropriate use of L-arginine is safe for humans in dietary supplementation and clinical therapy.

In conclusions, the beneficial effect of L-arginine in treating many developmental and health problems is unique among amino acids, and L-arginine may become a useful "nutraceutical" approach.

References

1. Alberti KGMM, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome. A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009;120:1640–5.
2. Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB. The metabolic syndrome: prevalence and associated risk factor findings in the US population from the third national health and nutrition examination survey, 1988–1994. Arch Intern Med. 2003;163:427–36.
3. Piatti P, Fragasso G, Monti LD, Caumo A, Van Phan C, Valsecchi G, Costa S, Fochesato E, Pozza G, Pontiroli AE, Chierchia S. Endothelial and metabolic characteristics of patients with angina and angiographically normal coronary arteries: comparison with subjects with insulin resistance syndrome and normal controls. J Am Coll Cardiol. 1999;34:1452–60.
4. Giugliano D, Ceriello A, Paolisso G. Diabetes mellitus, hypertension and cardiovascular disease: which role for oxidative stress. Metabolism. 1995;44:363–8.
5. Paolisso G, Tagliamonte MR, Marfella R, Verrazzo G, D'Onofrio F, Giugliano D. L-arginine but not D-arginine stimulates insulin-mediated glucose uptake. Metabolism. 1997;46:1068–73.
6. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol. 1990;30:535–60.
7. Monti L, Valsecchi G, Costa S, Sandoli EP, Phan CV, Pontiroli AE, Pozza G, Piatti PM. Effects of endothelin-1 and nitric oxide on glucokinase activity in isolated rat hepatocytes. Metabolism. 2000;49:73–80.

8. Fu WJ, Haynes TE, Kohli R, Hu J, Shi W, Spencer TE, Carroll RJ, Meininger CJ, Wu G. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr. 2005;135:714–21.
9. Monti LD, Galluccio E, Lucotti P, Setola E, Costa S, Fontana B, Oldani M, Merante D, Di Blasi P, Bosi E, Piatti PM. Beneficial role of L-arginine in cardiac matrix remodelling in insulin resistant rats. Eur J Clin Invest. 2008;38:849–56.
10. McConell GK, Huynh NN, Lee-Young RS, Canny BJ, Wadley GD. L-arginine infusion increases glucose clearance during prolonged exercise in humans. Am J Physiol Endocrinol Metab. 2006;290:E60–6.
11. Wascher TC, Graier WF, Dittrich P, Hussain MA, Bahadori B, Waliner S, Topiak H. Effects of low-dose L-arginine on insulin mediated vasodilation and insulin sensitivity. Eur J Clin Invest. 1997;27:690–5.
12. Piatti PM, Monti LD, Valsecchi G, Magni F, Setola E, Marchesi F, Galli-Kienle M, Pozza G, Alberti KG. Long-term oral L-arginine administration improves peripheral and hepatic insulin sensitivity in type 2 diabetic patients. Diabetes Care. 2001;24:875–80.
13. Lucotti P, Setola E, Monti LD, Galluccio E, Costa S, Sandoli EP, Fermo I, Rabaiotti G, Gatti R, Piatti P. Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab. 2006;291:E906–12.
14. Lucotti P, Monti L, Setola E, La Canna G, Castiglioni A, Rossodivita A, Pala MG, Formica F, Paolini G, Catapano AL, Bosi E, Alfieri O, Piatti P. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism. 2009;58:1270–6.
15. Monti LD, Setola E, Lucotti PC, Marrocco-Trischitta MM, Comola M, Galluccio E, Poggi A, Mammì S, Catapano AL, Comi G, Chiesa R, Bosi E, Piatti PM. Effect of a long-term oral L-arginine supplementation on glucose metabolism: a randomized, double-blind, placebo-controlled trial. Diabetes Obes Metab. 2012;14:893–900.
16. Girerd XJ, Hirsch AT, Cooke JP, Dzau VJ, Creager MA. L-arginine augments endothelium-dependent vasodilation in cholesterol-feed rabbits. Circ Res. 1990;67:1301–8.
17. Cooke JP, Singer AH, Tsao P, Zera P, Rowan RA, Billingham ME. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest. 1992;90:1168–72.
18. Boger RH, Bode-Boger SM, Mugge A, Kienke S, Brandes R, Dwenger A, Frolich JC. Supplementation of hypercholesterolemic rabbits with L-arginine reduces vascular release of superoxide anions and restores NO production. Atherosclerosis. 1995;141:31–43.
19. Dhawan V, Handu SS, Nain CK, Ganguly NK. Chronic L-arginine supplementation improves endothelial cell vasoactive functions in hypercholesterolemic and atherosclerotic monkeys. Mol Cell Biochem. 2005;269:1–11.
20. Hayashi T, Juliet PA, Matsui-Hirai H, Miyazaki A, Fukatsu A, Funami J, Iguchi A, Ignarro LJ. L-citrulline and L-arginine supplementation retards the progression of high-cholesterol-diet-induced atherosclerosis in rabbits. Proc Natl Acad Sci USA. 2005;20:13681–6.
21. Willot M, Gray L, Gibson C, Murphy S, Bath PMW. A systematic review of nitric oxide donors and L-arginine in experimental stroke; effects on infarct size and cerebral blood flow. Nitric Oxide. 2005;12:141–9.
22. Boger RH, Bode-Boger SM. The clinical pharmacology of L-arginine. Annu Rev Pharmacol Toxicol. 2001;41:79–99.
23. Dong JY, Qin LQ, Zhang Z, Zhao Y, Wang J, Arigoni F, Zhang W. Effect of oral L-arginine supplementation on blood pressure: a meta-analysis of randomized, double-blind, placebo-controlled trials. Am Heart J. 2011;162(6):959–65.
24. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest. 1992;90:1248–53.
25. Egashira K, Hirooka Y, Kuga T, Mohri M, Takeshita A. Effects of L-arginine supplementation on endothelium-dependent coronary vasodilation in patients with angina pectoris and normal coronary arteriograms. Circulation. 1996;94:130–4.
26. Piatti P, Fragasso G, Monti LD, Setola E, Lucotti P, Fermo I, Paroni R, Galluccio E, Pozza G, Chierchia S, Margonato A. Acute intravenous L-arginine infusion decreases endothelin-1 levels and improves endothelial function in patients with angina pectoris and normal coronary arteriograms: correlation with asymmetric dimethylarginine levels. Circulation. 2003;107:429–36.
27. Adams MR, Forsyth CJ, Jessup W, Robinson J, Celermajer DS. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. J Am Coll Cardiol. 1995;26:1054–61.
28. Bode-Boger SM, Muke J, Surdacki A, Brabant G, Boger RH, Frolich JC. Oral L-arginine improves endothelial function in healthy individuals older than 70 years. Vasc Med. 2003;8:77–81.
29. Clarkson P, Adams MR, Powe AJ, Donald AE, McCredle R, Robinson J, McCarthy SN, Keech A, Celermajer DS, Deanfield JE. Oral L-arginine improves endothelium-dependent vasodilation of hypercholesterolemic young adults. J Clin Invest. 1996;97:1989–94.
30. Hambrecht R, Hilbrich L, Erbs S, Gielen S, Fiehn E, Schoene N, Schuler G. Correction of endothelial dysfunction in chronic heart failure: additional effects of exercise training and oral L-arginine supplementation. J Am Coll Cardiol. 2000;35:706–13.

31. Palloshi A, Fragasso G, Piatti P, Monti LD, Setola E, Valsecchi G, Galluccio E, Chierchia SL, Margonato A. Effect of oral L-arginine on blood pressure and symptoms and endothelial function in patients with systemic hypertension, positive exercise tests, and normal coronary arteries. Am J Cardiol. 2004;93:933–5.
32. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992;339:572–5.
33. Abbasi F, Asagami T, Cooke JP, Lamendola C, McLaughin T, Reaven GM, Stuehlinger M, Tsao PS. Plasma concentrations of asymmetric dimethylarginine are increased in patients with type 2 diabetes mellitus. Am J Cardiol. 2001;88:1201–3.
34. Closs EI, Basha FZ, Habermeier A, Forstermann U. Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. Nitric Oxide. 1997;1:65–73.
35. Schlaich MP, Parnell MM, Ahlers BA, Finch S, Marshall T, Zhang WZ, Kaye DM. Impaired L-arginine transport and endothelial function in hypertensive and genetically predisposed normotensive subjects. Circulation. 2004;110:3680–6.
36. Adams MR, McCredie R, Jessup W, Robinson J, Sullivan D, Celermajer DS. Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. Atherosclerosis. 1997;129:261–9.
37. Cerumuzynski L, Chamiec T, Herbaczynska-Cedro K. Effect of supplemental oral L-arginine on exercise capacity in patients with stable angina pectoris. Am J Cardiol. 1997;80:331–3.
38. Fukumoto Y, Urabe Y, Kubo T, Kaku T, Egashira K, Shimokawa H, Takeshita A. Augmented vasodilator response to L-arginine after coronary angioplasty may attenuate restenosis. Heart Vessels. 2002;16:171–4.
39. Shiraki T, Takamura T, Kajiyama A, Oka T, Saito D. Effect of short-term administration of high dose L-arginine on restenosis after percutaneous transluminal coronary angioplasty. J Cardiol. 2004;44:13–20.
40. Lerman A, Burnett Jr JC, Higano ST, McKinley LJ, Holmes Jr DR. Long term L-arginine supplementation improves small-vessel coronary endothelial function in humans. Circulation. 1998;97:2123–8.
41. Oomen CM, van Erk MJ, Feskens EJ, Kok FJ, Kromhout D. Arginine intake and risk of coronary heart disease mortality in elderly men. Arterioscler Thromb Vasc Biol. 2000;20:2134–9.
42. Blum A, Hathaway L, Mincemoyer R, Schenke WH, Kirby M, Csako G, Waclawiw MA, Panza JA, Cannon 3rd RO. Oral L-arginine in patients with coronary artery disease on medical management. Circulation. 2000;101:2160–4.
43. Tsai CH, Pan TL, Lee YS, Tai YK, Liu TZ. Evidence that high-dose L-arginine may inappropriate for use by diabetic patients as prophylactic blocker of methylglyoxal glycation. J Biomed Sci. 2004;11:692–6.
44. Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH, Polak JM. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes formation and activity of peroxynitrite. Lab Invest. 1996;75:77–85.
45. Maxwell AJ, Anderson BE, Cooke JP. Nutritional therapy for peripheral arterial disease: a double-blind, placebo-controlled, randomized trial of HeartBarReg. Vasc Med. 2000;5(1):11–9.
46. Abdelhamed AI, Reis SE, Sane DC, Brosnihan KB, Preli RB, Herrington DM. No effect of an L-arginine-enriched medical food (HeartBars) on endothelial function and platelet aggregation in subjects with hypercholesterolemia. Am Heart J. 2003;145(3):E15.
47. Monti LD, Casiraghi MC, Setola E, Galluccio E, Pagani MA, Quaglia L, Bosi E, Piatti P. L-arginine enriched biscuits improve endothelial function and glucose metabolism: a pilot study in healthy subjects and a cross-over study in subjects with impaired glucose tolerance and metabolic syndrome. Metabolism. 2013;62(2):255–64.

Chapter 33
L-Arginine-Enriched Apples and Diabetic Control

Andrea Escudero, Jorge Moreno, Jesenia Acurio, and Carlos Escudero

Key Points

- Health consciousness worldwide has been one of the most stimulating factors for rapid global growth of functional food industry.
- Consumption of aliments with high content of L-arginine or L-citrulline is associated with improvement of both metabolic status and vascular dysfunction in diabetes.
- Apple is consumed worldwide, and its ingestion has been associated with reduction in the risk of chronic diseases.
- Apple is very porous, being an ideal matrix for inserting bioactive molecules including vitamins, minerals, or amino acids such as L-arginine.
- Supplementation with apple enriched with L-arginine improves survival rate in diabetic rats.
- Ingestion of apples enriched by L-arginine may offer more benefits than L-arginine by itself.
- Food engineering using apples might offer an opportunity for designing massive nutritional strategies with a therapeutic view.

Keywords Functional foods • Apple • L-Arginine • Diabetes • Endothelial dysfunction

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Abbreviations

Ang II	Angiotensin II
ACE	Angiotensin-converting enzyme
A+LA	Apple+L-Arginine
AP	Apple procyanidins
hCAT1	Cationic amino acid transporter type 1
hCAT2B	Cationic amino acid transporter type 2B
cGMP	Cyclic guanosine-monophosphate
eNOS	Endothelial NOS
EDHF	Endothelium-derived hyperpolarizing factor
FMD	Flow-mediated dilatation
GFBS	Glycated protein
HbA1c	Glycosylated hemoglobin
HDL	High-density lipoprotein
HUVEC	Human umbilical vein endothelial cell
HUVEC	Human umbilical vein endothelial cell
iNOS	Inducible NOS
VLDL	Low-density lipoprotein
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthases
NOx	Nitrosylated species+nitrite
NFkB	Nuclear factor kappa B
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor alpha
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VLDL	Very low-density lipoprotein

Introduction

Functional foods are aliments that are thought to have physiological benefits and/or reduce the risk of chronic disease, beyond their basic nutritional functions, which could be produced by adding new ingredients or more of existing ingredients using food engineering techniques. In this regard, increasing health consciousness worldwide has been one of the most stimulating factors for rapid global growth of the functional food industry [1]. Then, development of functional food products is a growing research niche worldwide.

From the point of view of food engineering, apple is a very noble fruit, since it is one of the most widely cultivated tree fruits; apples are consumed worldwide (see Fig. 33.1) and its consumption has been associated with reduction in the risk of chronic diseases including lung cancer [2, 3], cardiovascular disease [4], asthma [5], and type 2 diabetes mellitus (T2DM) [6] in population-based studies. In addition, apples are relatively easy to process due to their high porosity. Apple is an ideal matrix for inserting bioactive molecules including vitamins, minerals, or amino acids such as L-arginine [7]. In turn, L-arginine is a cationic and semi-essential amino acid [8], which is involved in many metabolic processes including protein synthesis and insulin secretion, and, importantly it is the substrate for nitric oxide (NO) synthesis, a gas with potent vasodilatory, metabolic, and neurotransmission effects [9].

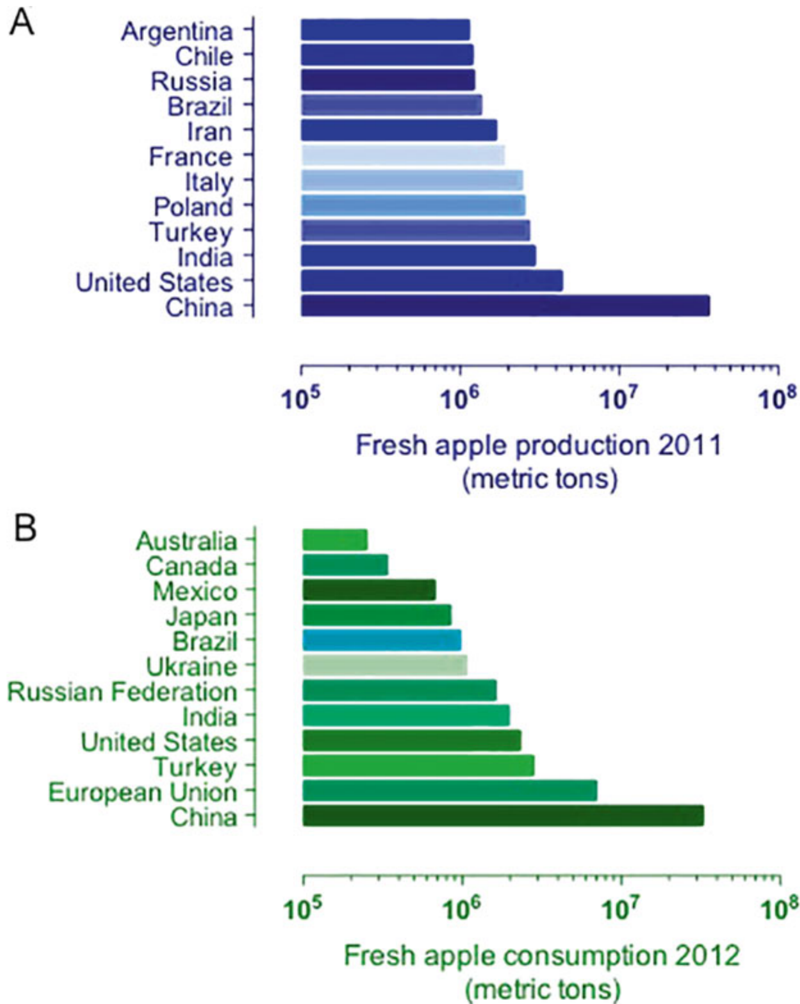

Fig. 33.1 Twelve top countries in the apple market. Top 12 of countries with the highest (**a**) Apple production in 2011 and (**b**) Apple consumption in 2012 according to United States Department of Agriculture

Among many metabolic alteration observed in diabetic patients, they also exhibited a relative deficiency of L-arginine [10, 11], which supports the need for supplementation with this amino acid [10, 12]. L-arginine supplementation either by itself [13–19] or by using fresh food with high content of L-arginine [20, 21] has been related with improvement in the metabolic control of diabetic animals, or in patients with T2DM [12]. However, despite potential beneficial effect for metabolic control in diabetes—and other cardiovascular and metabolic diseases—the supplementation with L-arginine is not always traduced into a continued elevation of plasma levels due to high catabolism. Indeed, it is well known that the more L-arginine is introduced, the more is metabolized [22]. Nevertheless, an issue during L-arginine supplementation in humans is the unpleasant taste of this amino acid, which hinders adherence to supplementation. Then, approaches aimed to enhance availability and ingestion of L-arginine appear as exceptional options for preventing and/or controlling metabolic alteration in diabetes.

We decided to experimentally create a product that adds the beneficial properties of apple and L-arginine. Then, using food engineering techniques we have generated an apple enriched with L-arginine [7] and used it for supplementing diabetic rats in a short-term period (17 days). In those animals, we observed that consumption of apple enriched with L-arginine (A+LA) may offer additional benefits than L-arginine by itself, since favorable effects on plasma levels of L-arginine, insulin, and nitrite are longer than those observed with L-arginine by itself. Interestingly, these metabolic

changes in the A+LA group were associated with a better survival rate. Using this background in this chapter we analyze potential implication of our findings for human health.

Diabetes as a Public Health Problem

Diabetes mellitus is a chronic noncommunicable disease that describes a number of metabolic disorders characterized by chronic increased levels of blood sugar (i.e., hyperglycemia) associated with alterations in the metabolism of carbohydrates, fats, and proteins that result from an initial defect in the production (type 1 diabetes mellitus, T1DM) and/or action (T2DM) of insulin [23]. According to the World Health Organization (WHO), prevalence of diabetes is 10 % in adults aged 25+ years [24]. In numbers, global population with diabetes in 2010 was approximately 285 million, and it may increase to 439 million by 2030 [23], and importantly low- and middle-income countries are the most affected by this disease [24]. Also, another aggravating situation is that obesity, a major risk factor for T2DM in adults, is affecting ~25 of the population, whereas in children between 9 and 12 years the rate of obesity is as high as 40 % in population studies in middle-income countries [25]. This becomes more complex when we consider that 52 % of obese individuals develop T2DM [23]. Then, diabetes or risk of developing diabetes is a growing worldwide public health problem.

Generally speaking, it is known that diabetic complications are associated with damage of blood vessels, either small vessels (such as those identified in the retina or in the kidney) or large vessels (as the aorta, carotid, and cerebral arteries). In this regard, it is known that about 2 % of patients with diabetes develop blindness and ~10 % develop severe visual impairment, whereas ~50 % of diabetic patients die due to cardiovascular disease (primarily heart disease and stroke) and 10–20 % by renal causes [23]. Consequently, a potential strategy to reduce the morbidity and mortality associated with diabetes should include the improvement of vascular function.

Diabetes, Endothelial Function, and L-Arginine

Although ambiguous, endothelial dysfunction has been defined as a pathological state characterized by an imbalance between vasodilators and vasoconstrictors produced by endothelial cells [26]. In particular, it is translated as the inability of these cells to generate vasodilating agents, such as NO [27].

Nitric oxide is synthesized by the action of a family of enzymes named nitric oxide synthases (NOS). At least three isoforms have been described, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). All NOS produce NO and L-citrulline in equimolar concentrations by using L-arginine as a substrate. In turn, L-citrulline is the substrate of L-arginine forming a loop of regulation between L-arginine:NO:L-citrulline synthesis. In addition, it is well described that NO synthesis requires L-arginine uptake via membrane transporters such as human cationic amino acid transporter type 1 (hCAT1) or hCAT2B in the case of endothelial cells. Nevertheless, bioavailability of NO is controlled by several mechanisms, including substrate availability, reaction with reactive oxygen species (ROS), and cellular content of antioxidants. The underlying mechanisms associated with NO-dependent vasorelaxation include elevation of intracellular levels of cyclic guanosine-monophosphate (cGMP) and activation/inhibition of target proteins via direct reaction with tyrosine residues (i.e., nitrotyrosine) [27].

Despite the vasodilatory role of L-arginine, via nitric oxide (NO) synthesis, this amino acid has several other functions in the body [8, 9], including weight control [28], regeneration of pancreatic beta cells [13–19], and thereby production and release of insulin. Interestingly, diabetes is associated with deficiency of L-arginine. Thus, it has been reported that the plasma concentration of L-arginine

in normal and diabetic rats was ~190 μM and ~65 μM, respectively [10, 11]. These reduced plasma levels have been also described in patients with T2DM [29]. In order to recover this deficiency of L-arginine and considering the potential benefits for vascular and metabolic control in patients with diabetes, several clinical trials focused on L-arginine supplementation have been developed.

Supplementation with L-Arginine or L-Citrulline in Diabetes: Focus in Nutritional Strategies

Supplementation with either L-arginine [13–19], or foods with high content of L-arginine [20, 21] or L-citrulline [22, 30] improves many metabolic parameters in diabetic animals compared to respective controls. Thus, it has been described that L-arginine supplementation normalizes plasma glucose level and vascular function in streptozotocin-induced diabetic rats [15], improves metabolic control (i.e., reducing glucose and lipids levels) [13], prevents β cell damage [31], and produces β cell regeneration in alloxan-induced diabetes [14]. Accordingly, L-arginine supplementation improves control of glycemia by increasing insulin sensitivity in T2DM patients [12].

Other studies including diabetic rodents [30, 32, 33], or in patients with diabetes [12, 34–39], showed that supplementation with L-arginine [12, 32] or L-citrulline [30, 34–37] was associated with either increased production of NO or improvement of vascular function, compared with non-supplemented controls. With regard to functional foods, it has been described that ingestion of pulp of watermelon, a source of L-arginine and citrulline (representing 71 % of total free amino acids) [30], was associated with improvement in vascular reactivity and NO production in obese mice [30], as well as with improvement of hemodynamic parameters in healthy individuals and individuals with hypertension or obesity [34–37]. Together with this evidence, it has been shown that L-citrulline supplementation improved erectile dysfunction in diabetic patients [40], a phenomenon associated with endothelial dysfunction. While these studies are promising, conducting large-scale studies is necessary in order to confirm the capacity of functional food for recovering endothelial function in patients with diabetes.

Consumption of Apples to Restore Endothelial Function

Human Studies

Apples are one of the most popular and frequently consumed fruits in the world (see Fig. 33.1). As stated above apple consumption was been related to reduction of chronic diseases such as cancer, cardiovascular disease, asthma, T2DM, or obesity [2–6, 41]. For instance, epidemiological studies suggest that apple consumption is associated with a 20–33 % reduction in the risk of T2DM [6].

Several underlying mechanisms have been proposed as responsible for beneficial effect of apple ingestion, which in turn are associated with different chemical components of this fruit. For instances, apples have high content of micronutrients (i.e., antioxidants), as well as a mixture of macronutrients such as protein, lipids, carbohydrates, and fiber (largely pectin). Indeed, apple pectin prolongs intestinal transit time, which may affect the microbiota (prebiotic effects) and therefore could modify intestinal structure and microenvironment.

Apples are a rich dietary source of antioxidants such as flavanols (mainly epicatechin and epicatechin-rich procyanidins), which may reach 0.1–45 mg per 100 g fresh fruit [42]. In addition, other flavonoids such as quercetins (conjugated, glycosides, or free) and triterpenes are present

mostly in apple peels, which in turn contain three- to sixfold more flavonoids than the flesh [43]. Due to its wide consumption in humans, apples may represent a better source of antioxidants in human diets than cocoa or dark chocolate, and even better, apple peel, which normally is discarded during apple processing, may constitute a potential source of extractable flavanols for inclusion in functional foods [42].

Apple consumption (120 g of apple flesh with 80 g of apple skins or apple flesh only) increased the levels of nitrite and total NOx (nitrosylated species + nitrite) after 140 min post-ingestion, phenomena associated with high flow-mediated vasodilation in the brachial artery (i.e., FMD, a marker for endothelial-dependent vasodilation) [44]. Also, these results were accompanied by a reduction in 3 mmHg in systolic blood pressure [44]. Authors in this last study suggest that this observation may be related to high content of flavonoids (including quercetin and epicatechin), since those antioxidants augment NO status acutely in healthy men [45]. In addition, consumption of apple puree or apple juice (both providing 70 mg epicatechin) was associated with elevation in the epicatechin plasma concentration at 2 h, being faster (0.9 vs. 1.7 h) and higher (3.5 vs. 2.1 μmol/L) in the drink preparation than puree [46]. Authors also investigated whether those changes in plasma epicatechin induced by intake of apple-based drink or puree preparations were associated with elevation in the plasma or urinary level of nitrate and nitrite (i.e., NO metabolites). Despite slightly increased nitrate plasma levels after 30-min post-ingestion using both preparations, changes were not statistically significant in any time point analyzed. However, authors report that when a high dose of apple epicatechin was used as drink preparation (140 mg), an elevation in the urinary nitrate excretion (1.5 vs. 1.1 μmol nitrate/mg creatinine) compared with placebo was found, suggesting that under these conditions NO availability may be increased after apple ingestion.

Contrary to these results observed in healthy volunteers, Auclair and colleagues [47] studied hypercholesterolemic volunteers who were included in a double-blind, randomized crossover trial, where they successively consumed 40 g of lyophilized apples (providing either 1.43 or 0.21 g polyphenols per day during two 4-week periods). These authors did not find significant changes in FMD or any other cardiovascular disease risk factors, including plasma lipids, homocysteine, and antioxidant capacity in the apple-supplemented group compared with controls. Despite many methodological differences in human studies, it seems that at least in healthy volunteers apple ingestion may improve vascular function and particularly NO synthesis.

Apple consumption (300 g of Golden Delicious apple per day for 8 weeks) was associated with reduction in triglycerides and VLDL plasma levels, without changes in total cholesterol, LDL, and HDL in hyperlipidemic and overweight men [48]. On the other hand, other studies have shown that apple cider vinegar consumption increased glucose plasma levels, without changes in the lipids profile in healthy volunteers [49]. Then, apple ingestion may have cholesterol-lowering ability at least in men with cardiovascular risk factors (see details in Ref. [50]).

Animal Studies

Many animal studies suggest the potential beneficial effects of ingestion of apple extraction on the improvement of cardiovascular parameters. For instance, using three different apple varieties containing distinctive phenolic compounds and fiber content, Serra and colleagues [50] showed that ingestion of apple (i.e., Bravo de Esmolfe, 5 g/day per 30 days) significantly reduced serum levels of total cholesterol, LDL (both native and oxidized), and total triglycerides in rats fed with high cholesterol diet. Interestingly, reductive effect on lipid levels was correlated with total phenolic content, in particular catechin, epicatechin, and procyanidin B1, as well as with the antioxidant properties of apples, but not with fiber content of the analyzed apple varieties. These observations confirm previous studies using

rabbits fed with high cholesterol diet, in which ingestion of apple juice (10 mL per 60 days) was associated with reduction in both LDL and atherosclerotic thickness, whereas an increase in HDL cholesterol, without changes in inflammatory markers or nitrite + nitrate levels in apple juice-supplemented animals compared with control non-supplemented [51].

Shishehbor and colleagues [52] showed that administration of apple cider vinegar for 4 weeks was associated with reduction in HbA1c and triglyceride plasma levels, while HDL levels were elevated, but no changes in fasting glucose levels were observed in streptozotocin-induced diabetes. According to these results, authors suggest that consumption of this apple preparation may improve metabolic control in diabetes. Despite this evidence, it is unclear whether apple consumption might be translated into improvement of metabolic control in diabetic patients.

Cellular Mechanisms Underlying Beneficial Effect of Apple Ingestion

Cellular mechanisms behind cardiovascular benefit related to apple consumption have been mainly related to the antioxidant properties of apple extractions. Indeed, it has been estimated that polyphenols extracted from apple exhibited antioxidant activity in a similar extent to superoxide dismutase [53]. In particular, apple procyanidins (AP) induced endothelium-dependent vasorelaxation on aorta ring from rats via activation of both NO/cGMP pathway and endothelium-dependent hyperpolarization [53]. Compatible with those results, using rat aortic endothelial cell line, it was found that incubation with AP induced cell hyperpolarization in concentration-dependent manner, an effect that was prevented by the nonspecific K^+ channel inhibitor, TEA, or by iberiotoxin (BK_{Ca} inhibitor), or 4-aminopyridine (K_v inhibitor), or $BaCl_2$ (Kir inhibitor) or glibenclamide (K_{ATP} inhibitor), suggesting participation of those K^+ channels in the vasodilatory effect of AP. In addition to those findings, AP increased the mRNA levels of eNOS, but not iNOS, as well as increased both NO synthesis and intracellular cGMP levels. Interestingly, both K^+ channel activation and NO/cGMP pathway were associated with antiproliferative effect of AP [54], suggesting that apple-derived procyanidins may improve the synthesis of both endothelium-dependent vasodilators, NO and endothelium-derived hyperpolarizing factor (EDHF).

Protective effect of flavonoids derived from apples has been also related to the capacity for inhibiting the angiotensin-converting enzyme (ACE), which in turn is responsible for the synthesis of angiotensin II (Ang II), a powerful endogenous vasoconstrictor peptide. Thus, apple peel extracts inhibit the activity of rabbit-purified ACE in a concentration-dependent manner. In addition, these extracts also inhibited ACE activity in human umbilical vein endothelial cell (HUVEC), an effect related to the high content of the lipophilic component of apple peel extract, the quercetin-3-*O*-glucoside [55].

Also, underlying mechanisms behind anti-atherogenic and hypolipidemic effects of apple ingestion have been related to anti-inflammatory activity, since apple extract decreased the activation of the pro-inflammatory nuclear factor kappa B (NFkB) in HUVEC either at basal condition or after treatment with tumor necrosis factor alpha (TNF-α) [56]. Then, these results suggest that downregulation of the NFkB signaling may account for the beneficial effect of apple extract on the recovery of endothelial dysfunction.

In addition, also in HUVEC, it has been shown that fresh apple extract prevents lipid peroxidation induced by glycated protein (GFBS), a well-described pro-oxidant observed in diabetes, in a concentration-dependent manner [57]. Then, it is feasible that apple ingestion may prevent oxidative damage of endothelial cells during diabetes. In addition, apple peel extract inhibited oxidation of human LDL, an effect related to high content of quercetin and triterpenes [43]. Altogether these results suggest that apple antioxidants may be participating in the recovery of endothelial function, which in turn may help to prevent cardiovascular disease in diabetic patients.

Apple + L-Arginine: A Good Combination for Improving Diabetes Control?

Apple has been widely used in the food processing industry, due to its healthy and productive properties. For example, apples were used for generating apples enriched with bioactive molecules such as vitamin E, calcium, and selenium [58] which have offered functional foods for clinical applications. In addition, considering all the healthy properties of apple and L-arginine separately, we decided to develop a functional food that combines both nutrients. According to our hypothesis, this product may (1) deliver L-arginine that could recover selective deficiency of this amino acid in diabetes, (2) improve adherence to supplementation with L-arginine avoiding its unpleasant taste, (3) improve metabolic control of diabetes and recovery of endothelial function as a strategy for reducing vascular complications, and (4) offer scientific information to open new forms of production and marketing for apple [7]. Then, we aim to determine whether supplementation with apples enriched with L-arginine offers additional benefits compared with L-arginine by itself in the metabolic control and survival of alloxan-induced diabetes.

Firstly, we confirm previous observation [10, 11, 59] describing low L-arginine plasma level in diabetic rats (67 μM) compared with control (109 μM) [7]. Causes for this reduction have been related to hepatic metabolism in both gluconeogenesis and urea synthesis pathways observed after insulin deficiency [59], but the underlying mechanisms are still unclear. In order to overcome this deficiency and using food engineering techniques, we increased about 150 times the L-arginine content of apples, getting a final concentration of 10 mg L-arginine per gram of apple (see Table 33.1), which means that this functional food would cover at least 2 % of the requirements of L-arginine in humans (0.8–2 g/kg/day).

Then, we used this apple enriched with L-arginine (A+LA) in order to feed normoglycemic and alloxan-induced diabetic rats. Also, controls without supplementation were included. Firstly, since L-arginine supplementation is associated with high catabolism of this amino acid [22], we investigated whether our strategy might avoid this effect. We found that supplementation with either L-arginine or A+LA did not change significantly the plasma levels of L-arginine in controls, but in diabetic animals L-arginine or A+LA showed a partial recovery in the plasmatic L-arginine level, an effect that was maintained until the end of supplementation (17 days) only in animals that received A+LA (see Table 33.2).

After that we discovered that L-arginine supplementation or A+LA has no apparent harmful effects on rats, since all nondiabetic animals survive during supplementation period (10 days). However, A+LA supplementation potentiates weight loss in diabetic animals, without any effect in control animals. Reasons for this reduction are unclear, but L-arginine supplementation can reduce adiposity via retarding growth of white adipose tissue [28].

Interestingly, in diabetic animals, high mortality rate observed in animals without supplementation was reverted by L-arginine or A+LA, survival rate being 50 % and 100 %, respectively, while in non-supplemented diabetic group no animals were able to finish the study ($Chi^2=8.8$; df 2, $P=0.01$). Improvement in survival rate in diabetic animals supplemented with L-arginine and A+LA groups was associated with recovery in relative insulin levels (see Fig. 33.2). Thus, whereas in nondiabetic animals supplementation with L-arginine did not change the relative insulin levels, an increase (1.7-fold) was observed in the normoglycemic A+LA group. Furthermore in the diabetic group, L-arginine group exhibited an increase (24-fold) in the relative level of insulin at 3 days of supplementation, but this effect was not maintained until the end of follow-up (10 days of supplementation). Likewise, in the A+LA group, the elevation of insulin was ~9-fold and ~20-fold at 3 and 10 days of supplementation, respectively. No significant effect of L-arginine or A+LA was observed on glycemia in control or diabetic rats.

Trying to understand potential underlying mechanisms linked with improvement in diabetic animal' survival during A+LA supplementation, we measured L-arginine and nitrite concentration in diabetic animals supplemented during 3 days, which also received a new single oral dose of L-arginine or A+LA. Plasma level of L-arginine and nitrites was significantly elevated (1–4 h after ingestion) in animals that received A+LA compared with those who receiving L-arginine by itself. These elevations reached statistical significance at 1- and 2-h post-ingestion. These changes were also associated

Table 33.1 L-arginine incorporation in apples

	L-arginine concentration (mg/g)
Fresh apple	0.064 ± 0.011
Control	0.055 ± 0.016
VI	4.668 ± 0.825*†
VI-OH	10.14 ± 1.11*†‡

Table modified from Escudero et al. [7]. Fresh apples were exposed to vacuum impregnation (VI, 50 mbar, 5 min) and VI plus ohmic heating (VI-OH, 100 V, 30 °C) in apple-isotonic solution. Control corresponds to apples exposed to VI without L-arginine. Values are in Mean ± SEM, *$P < 0.05$ versus fresh apple, †$P < 0.05$ versus control, ‡$P < 0.05$ versus VI

Table 33.2 Apple enriched with L-arginine partially reverts reduced L-arginine plasma level in diabetic rats

		LA	A+LA	LA	A+LA
	Basal	3 Days		10 Days	
Normal (μM)	108.5 ± 1.9	78.6 ± 6.1	114.3 ± 3.1	92.7 ± 18.9	94.0 ± 8.5
Diabetics (μM)	67.0 ± 8.2*	79.6 ± 17.4	78.6 ± 6.1	62.4 ± 11.8*	75.7 ± 14.0

L-arginine plasma levels were measured after 3 or 10 days of supplementation in Control (Normal) and diabetic (Diabetics) animals without supplementation (basal) or supplemented with either L-arginine (LA) or apple + L-arginine (A+LA). Values are in means ± SEM

*$P < 0.05$ versus basal condition in controls

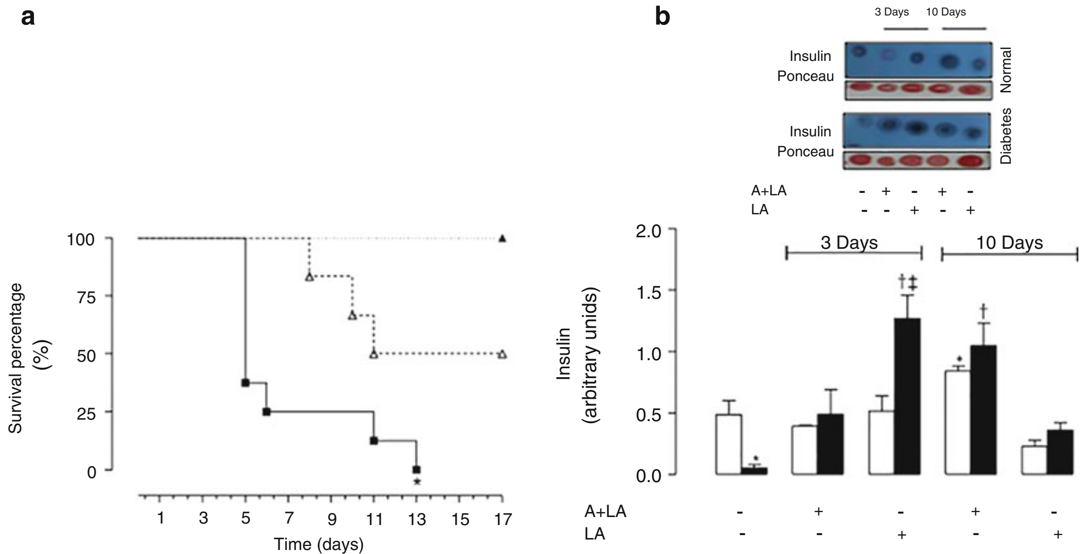

Fig. 33.2 Apple + L-arginine ingestion improves survival rate and insulinemia in alloxan-induced diabetes. Figure modified from Escudero et al. [7] In (**a**) Kaplan–Meier analysis in diabetic animals divided into three groups: without supplementation (*filled square*) or supplemented with either L-arginine (LA, *opened triangle*) or apple + L-arginine (A+LA, *filled triangle*) during 10 days. In (**b**) Plasma proteins (100 μg) from normal (*opened square*) and diabetic (*filled square*) animals with (*plus*) or without (*minus*) supplementation of L-arginine (LA) or apple + L-arginine (A+LA) during 3 or 10 days were subjected to dot-blot assays to estimate insulin relative levels. In (**a**), Wilcoxon Test Chi^2 8.8 and $P = 0.01$. In (**b**); *$P < 0.05$ versus Normal (baseline), †$P < 0.05$ versus diabetics (baseline), and ‡$P < 0.05$ versus corresponding value in normoglycemic group

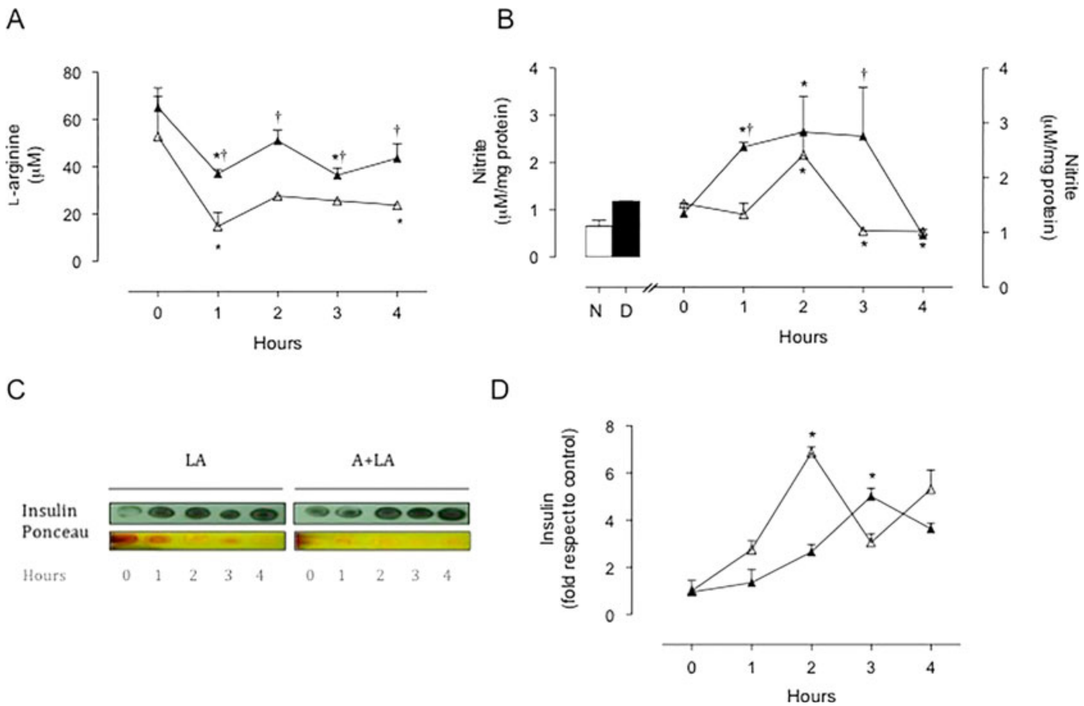

Fig. 33.3 Apple+L-arginine enhances availability of L-arginine in diabetic animals. Figure modified from Escudero et al. [7]. Diabetic animals supplemented with either L-arginine (LA, *opened triangle*) or apple+L-arginine (A+LA, *filled triangle*) during 3 days and that received a new single dose of either L-arginine or apple+L-arginine were used for determining L-arginine bioavailability. In (**a**) L-arginine plasma levels immediately after new single dose (time 0) and after 1–4 h. (**b**) Nitrite plasma level in normoglycemic (*unfilled bar*) and diabetic animals (*filled bar*) without supplementation. (**c**) and (**d**) Insulin plasma level as indicated in (**a**). In (**a**, **b**, and **c**) *$P<0.05$ versus respective value at time 0. †$P<0.05$ versus respective value in animals receiving L-arginine by itself

with a significant increase in the insulin plasma level in diabetic animals that received L-arginine or A+LA, respectively. Nevertheless, a shift in the elevation of insulin level was observed in the group of A+LA compared with the L-arginine group; since in the former elevation was observed at 3 h, while in the latter it was observed at 2 h (see Fig. 33.3).

Therefore, our results agree with previous publications in terms of recovery of insulin production in animals supplemented with L-arginine [14, 31]. However, dynamics of this increase differ between L-arginine and A+LA groups, suggesting that impregnation of L-arginine in apples might offer a "protection" for breakdown and perhaps improve the L-arginine availability in plasma and therefore intracellular action of this amino acid. Also, we concluded that the created functional food, apple enriched with L-arginine, improves the survive of alloxan-induced diabetic rats, a phenomenon associated with partial recovery of L-arginine plasma levels toward normal values, as well as increase in NO and insulin level, suggesting that this product would offer a safe and cost-effective nutrient for improving metabolic profile in diabetes.

Concluding Remarks

We have presented an overview regarding potential beneficial effect of L-arginine and apple consumption for human health (see Fig. 33.4). This is a topic where different edges such as agriculture, food processing, basic sciences, and public health converge. Considering that apple is one of the most

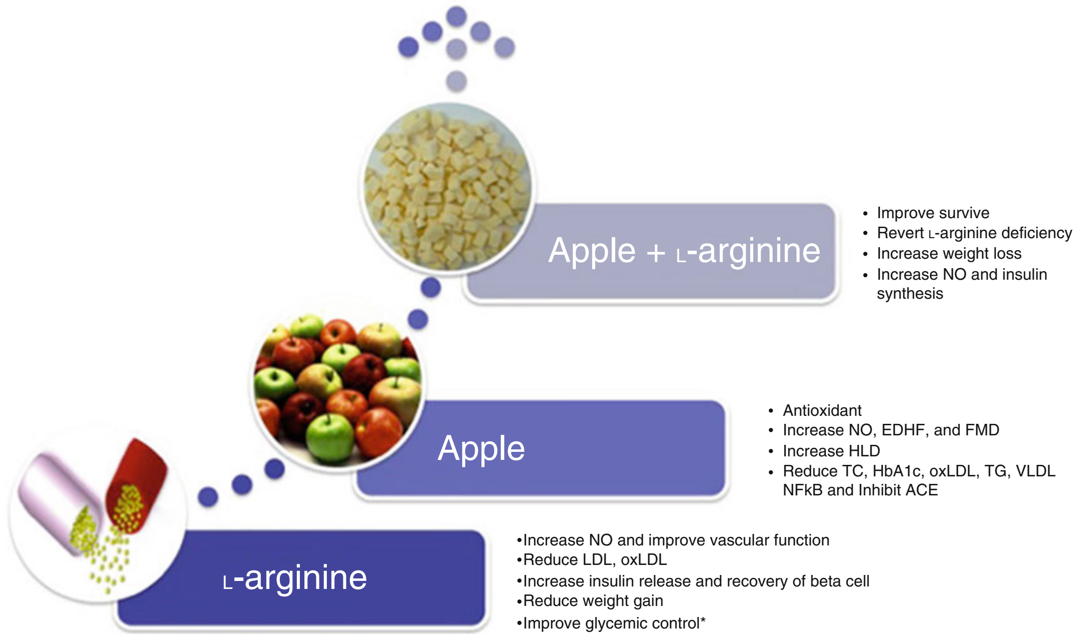

Fig. 33.4 Healthy effects of L-arginine, apple, or apple + L-arginine ingestion. It describes the hypothesis that was tested by our group [7], in which according to literature L-arginine supplementation (mainly aliments with elevated content of L-arginine and/or L-citrulline) is associated with several benefits in metabolic and vascular dysfunction recovery, such as listed in the graph. In addition, apple by itself also has many potential beneficial effects on similar targets. Therefore, our study created an apple enriched with L-arginine in order to generate a functional food able to revert L-arginine deficiency in diabetic rats. For details, see main text. Potential underlying mechanism is summarized in the cartoon. *Observation in diabetes

consumed fruit worldwide, it might offer an opportunity for designing massive nutritional strategies with a therapeutic view. In this scenario, we developed a pilot preclinical study in which we enhanced the healthy properties of L-arginine and apples. According to our results, this strategy might be better than apple or L-arginine alone at least in the recovery of insulin and NO synthesis, as well as L-arginine deficiency in diabetic animals. As far as we know, our study is pioneering in adding apple + L-arginine; therefore, there is limited knowledge regarding potential impact of this strategy for human health. We acknowledge that our study has many limitations; however, in this chapter, we have presented direct evidence in order to support our study. We hope that our manuscript will contribute to the awareness, among the scientific community, of this important issue and stimulate further investigation into this area.

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References

1. Hasler CM. The changing face of functional foods. J Am Coll Nutr. 2000;19 Suppl 5:499S–506S.
2. Feskanich D, Ziegler RG, Michaud DS, Giovannucci EL, Speizer FE, Willett WC, Colditz GA. Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women. J Natl Cancer Inst. 2000;92(22):1812–23.

3. Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E, Aromaa A. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol. 1997;146(3):223–30.
4. Sesso HD, Gaziano JM, Liu S, Buring JE. Flavonoid intake and the risk of cardiovascular disease in women. Am J Clin Nutr. 2003;77(6):1400–8.
5. Woods RK, Walters EH, Raven JM, Wolfe R, Ireland PD, Thien FC, Abramson MJ. Food and nutrient intakes and asthma risk in young adults. Am J Clin Nutr. 2003;78(3):414–21.
6. Wedick NM, Pan A, Cassidy A, Rimm EB, Sampson L, Rosner B, Willett W, Hu FB, Sun Q, van Dam RM. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. Am J Clin Nutr. 2012;95(4):925–33.
7. Escudero A, Petzold G, Moreno J, Gonzalez M, Junod J, Aguayo C, Acurio J, Escudero C. Supplementation with apple enriched with L-arginine may improve metabolic control and survival rate in alloxan-induced diabetic rats. Biofactors. 2013;39:564–74.
8. Morris Jr SM. L-Arginine: beyond protein. Am J Clin Nutr. 2006;83(2):508S–12S.
9. Heffernan KS, Fahs CA, Ranadive SM, Patvardhan EA. L-arginine as a nutritional prophylaxis against vascular endothelial dysfunction with aging. J Cardiovasc Pharmacol Ther. 2010;15(1):17–23.
10. Pieper GM, Dondlinger LA. Plasma and vascular tissue L-arginine are decreased in diabetes: acute L-arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. J Pharmacol Exp Ther. 1997;283(2):684–91.
11. Pieper GM, Siebeneich W, Moore-Hilton G, Roza AM. Reversal by L-arginine of a dysfunctional L-arginine/nitric oxide pathway in the endothelium of the genetic diabetic BB rat. Diabetologia. 1997;40(8):910–5.
12. Lucotti P, Setola E, Monti LD, Galluccio E, Costa S, Sandoli EP, Fermo I, Rabaiotti G, Gatti R, Piatti P. Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab. 2006;291(5):E906–12.
13. Mendez JD, Balderas F. Regulation of hyperglycemia and dyslipidemia by exogenous L-arginine in diabetic rats. Biochimie. 2001;83(5):453–8.
14. Vasilijevic A, Buzadzic B, Korac A, Petrovic V, Jankovic A, Korac B. Beneficial effects of L-arginine nitric oxide-producing pathway in rats treated with alloxan. J Physiol. 2007;584(Pt 3):921–33.
15. Kohli R, Meininger CJ, Haynes TE, Yan W, Self JT, Wu G. Dietary L-arginine supplementation enhances endothelial nitric oxide synthesis in streptozotocin-induced diabetic rats. J Nutr. 2004;134(3):600–8.
16. Mendez JD, Balderas FL. Inhibition by L-arginine and spermidine of hemoglobin glycation and lipid peroxidation in rats with induced diabetes. Biomed Pharmacother. 2006;60(1):26–31.
17. Mendez JD, Hernandez Rde H. L-arginine and polyamine administration protect beta-cells against alloxan diabetogenic effect in Sprague-Dawley rats. Biomed Pharmacother. 2005;59(6):283–9.
18. Petrovic V, Buzadzic B, Korac A, Vasilijevic A, Jankovic A, Korac B. L-Arginine supplementation induces glutathione synthesis in interscapular brown adipose tissue through activation of glutamate-cysteine ligase expression: the role of nitric oxide. Chem Biol Interact. 2009;182(2–3):204–12.
19. Vasilijevic A, Vojcic L, Dinulovic I, Buzadzic B, Korac A, Petrovic V, Jankovic A, Korac B. Expression pattern of thermogenesis-related factors in interscapular brown adipose tissue of alloxan-treated rats: beneficial effect of L-arginine. Nitric Oxide. 2010;23(1):42–50.
20. Salil G, Nevin KG, Rajamohan T. L-Arginine-rich coconut kernel diet influences nitric oxide synthase activity in alloxandiabetic rats. J Sci Food Agric. 2012;92:1903–8.
21. Salil G, Nevin KG, Rajamohan T. L-Arginine rich coconut kernel protein modulates diabetes in alloxan treated rats. Chem Biol Interact. 2011;189(1–2):107–11.
22. Wu G, Bazer FW, Cudd TA, Jobgen WS, Kim SW, Lassala A, Li P, Matis JH, Meininger CJ, Spencer TE. Pharmacokinetics and safety of L-arginine supplementation in animals. J Nutr. 2007;137(6 Suppl 2): 1673S–80S.
23. Diabetes. http://www.who.int/mediacentre/factsheets/fs312/es/index.html
24. WHO. Burden: mortality, morbidity and risk factors. In: WHO, editor. Global status report on noncommunicable diseases 2010. Geneva: WHO; 2011. p. 176.
25. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 2000;894:i–xii, 1–253.
26. Brunner H, Cockcroft JR, Deanfield J, Donald A, Ferrannini E, Halcox J, Kiowski W, Luscher TF, Mancia G, Natali A, et al. Endothelial function and dysfunction. Part II: Association with cardiovascular risk factors and diseases. A statement by the working group on endothelins and endothelial factors of the european society of hypertension. J Hypertens. 2005;23(2):233–46.
27. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol. 2006;147 Suppl 1:S193–201.
28. McKnight JR, Satterfield MC, Jobgen WS, Smith SB, Spencer TE, Meininger CJ, McNeal CJ, Wu G. Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids. 2010;39(2):349–57.

29. Menge BA, Schrader H, Ritter PR, Ellrichmann M, Uhl W, Schmidt WE, Meier JJ. Selective amino acid deficiency in patients with impaired glucose tolerance and type 2 diabetes. Regul Pept. 2010;160(1–3):75–80.
30. Wu G, Collins JK, Perkins-Veazie P, Siddiq M, Dolan KD, Kelly KA, Heaps CL, Meininger CJ. Dietary supplementation with watermelon pomace juice enhances L-arginine availability and ameliorates the metabolic syndrome in Zucker diabetic fatty rats. J Nutr. 2007;137(12):2680–5.
31. Mohan IK, Das UN. Effect of L-arginine-nitric oxide system on chemical-induced diabetes mellitus. Free Radic Biol Med. 1998;25(7):757–65.
32. Cavanal Mde F, Gomes GN, Forti AL, Rocha SO, Franco Mdo C, Fortes ZB, Gil FZ. The influence of L-arginine on blood pressure, vascular nitric oxide and renal morphometry in the offspring from diabetic mothers. Pediatr Res. 2007;62(2):145–50.
33. Hayashi T, Juliet PA, Matsui-Hirai H, Miyazaki A, Fukatsu A, Funami J, Iguchi A, Ignarro LJ. L-Citrulline and L-arginine supplementation retards the progression of high-cholesterol-diet-induced atherosclerosis in rabbits. Proc Natl Acad Sci U S A. 2005;102(38):13681–6.
34. Figueroa A, Sanchez-Gonzalez MA, Perkins-Veazie PM, Arjmandi BH. Effects of watermelon supplementation on aortic blood pressure and wave reflection in individuals with prehypertension: a pilot study. Am J Hypertens. 2011;24(1):40–4.
35. Figueroa A, Sanchez-Gonzalez MA, Wong A, Arjmandi BH. Watermelon extract supplementation reduces ankle blood pressure and carotid augmentation index in obese adults with prehypertension or hypertension. Am J Hypertens. 2012;25(6):640–3.
36. Figueroa A, Trivino JA, Sanchez-Gonzalez MA, Vicil F. Oral L-citrulline supplementation attenuates blood pressure response to cold pressor test in young men. Am J Hypertens. 2010;23(1):12–6.
37. Figueroa A, Wong A, Hooshmand S, Sanchez-Gonzalez MA. Effects of watermelon supplementation on arterial stiffness and wave reflection amplitude in postmenopausal women. Menopause. 2012;20:573–7.
38. Lucotti P, Monti L, Setola E, La Canna G, Castiglioni A, Rossodivita A, Pala MG, Formica F, Paolini G, Catapano AL, et al. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism. 2009;58(9):1270–6.
39. Huynh NT, Tayek JA. Oral L-arginine reduces systemic blood pressure in type 2 diabetes: its potential role in nitric oxide generation. J Am Coll Nutr. 2002;21(5):422–7.
40. Cormio L, De Siati M, Lorusso F, Selvaggio O, Mirabella L, Sanguedolce F, Carrieri G. Oral L-citrulline supplementation improves erection hardness in men with mild erectile dysfunction. Urology. 2011;77(1):119–22.
41. de Oliveira MC, Sichieri R, Venturim Mozzer R. A low-energy-dense diet adding fruit reduces weight and energy intake in women. Appetite. 2008;51(2):291–5.
42. de Pascual-Teresa S, Moreno DA, Garcia-Viguera C. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. Int J Mol Sci. 2010;11(4):1679–703.
43. Thilakarathna SH, Rupasinghe HP, Needs PW. Apple peel bioactive rich extracts effectively inhibit in vitro human LDL cholesterol oxidation. Food Chem. 2013;138(1):463–70.
44. Bondonno CP, Yang X, Croft KD, Considine MJ, Ward NC, Rich L, Puddey IB, Swinny E, Mubarak A, Hodgson JM. Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. Free Radic Biol Med. 2012;52(1):95–102.
45. Loke WM, Hodgson JM, Proudfoot JM, McKinley AJ, Puddey IB, Croft KD. Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. Am J Clin Nutr. 2008;88(4):1018–25.
46. Hollands WJ, Hart DJ, Dainty JR, Hasselwander O, Tiihonen K, Wood R, Kroon PA. Bioavailability of epicatechin and effects on nitric oxide metabolites of an apple flavanol-rich extract supplemented beverage compared to a whole apple puree: a randomized, placebo-controlled, crossover trial. Mol Nutr Food Res. 2013;57(7):1209–17.
47. Abad C, Antczak DF, Carvalho J, Chamley LW, Chen Q, Daher S, Damiano AE, Dantzer V, Diaz P, Dunk CE, et al. IFPA meeting 2010 workshop report I: immunology; ion transport; epigenetics; vascular reactivity; epitheliochorial placentation; proteomics. Placenta. 2011;32 Suppl 2:S81–9.
48. Vafa MR, Haghighatjoo E, Shidfar F, Afshari S, Gohari MR, Ziaee A. Effects of apple consumption on lipid profile of hyperlipidemic and overweight men. Int J Prev Med. 2011;2(2):94–100.
49. Salbe AD, Johnston CS, Buyukbese MA, Tsitouras PD, Harman SM. Vinegar lacks antiglycemic action on enteral carbohydrate absorption in human subjects. Nutr Res. 2009;29(12):846–9.
50. Serra AT, Rocha J, Sepodes B, Matias AA, Feliciano RP, de Carvalho A, Bronze MR, Duarte CM, Figueira ME. Evaluation of cardiovascular protective effect of different apple varieties—correlation of response with composition. Food Chem. 2012;135(4):2378–86.
51. Setorki M, Asgary S, Eidi A, Rohani AH, Esmaeil N. Effects of apple juice on risk factors of lipid profile, inflammation and coagulation, endothelial markers and atherosclerotic lesions in high cholesterolemic rabbits. Lipids Health Dis. 2009;8:39.
52. Shishehbor F, Mansoori A, Sarkaki AR, Jalali MT, Latifi SM. Apple cider vinegar attenuates lipid profile in normal and diabetic rats. Pak J Biol Sci. 2008;11(23):2634–8.

53. Matsui T, Korematsu S, Byun EB, Nishizuka T, Ohshima S, Kanda T. Apple procyanidins induced vascular relaxation in isolated rat aorta through NO/cGMP pathway in combination with hyperpolarization by multiple K+ channel activations. Biosci Biotechnol Biochem. 2009;73(10):2246–51.
54. Byun EB, Korematsu S, Ishikawa T, Nishizuka T, Ohshima S, Kanda T, Matsui T. Apple procyanidins induce hyperpolarization of rat aorta endothelial cells via activation of K+ channels. J Nutr Biochem. 2012;23(3):278–86.
55. Balasuriya N, Rupasinghe HP. Antihypertensive properties of flavonoid-rich apple peel extract. Food Chem. 2012;135(4):2320–5.
56. Davis PA, Polagruto JA, Valacchi G, Phung A, Soucek K, Keen CL, Gershwin ME. Effect of apple extracts on NF-kappaB activation in human umbilical vein endothelial cells. Exp Biol Med (Maywood). 2006;231(5):594–8.
57. Nishigaki I, Rajkapoor B, Rajendran P, Venugopal R, Ekambaram G, Sakthisekaran D, Nishigaki Y. Effect of fresh apple extract on glycated protein/iron chelate-induced toxicity in human umbilical vein endothelial cells in vitro. Nat Prod Res. 2010;24(7):599–609.
58. Allali HM, Marchal L, Vorubiev E. Effects of vacuum impregnation and ohmic heating with citric acid on the behaviour of osmotic dehydration and structural changes of apple fruit. Biosyst Eng. 2010;106(1):6–13.
59. Prior RL, Smith SB. Role of insulin in regulating amino acid metabolism in normal and alloxan-diabetic cattle. J Nutr. 1983;113(5):1016–31.

Chapter 34
Beneficial Impact of Cod Protein, L-Arginine, and Other Amino Acids on Insulin Sensitivity

Véronique Ouellet, S. John Weisnagel, Denis R. Joanisse, Charles Lavigne, Junio Dort, André Marette, and Hélène Jacques

Key Points

- Cod protein has been shown to improve insulin sensitivity, glucose metabolism, lipid profile, and inflammation.
- Cod protein may exert its beneficial effects by a direct action of its constituent amino acids.
- Cod protein consumption/digestion results in higher plasma concentrations of L-arginine and taurine, but a lower plasma concentration of branched-chain amino acids.
- Evidence indicates that L-arginine and taurine play a role in the improvement in insulin sensitivity and glucose metabolism, whereas branched-chain amino acids exert a deleterious effect.

Keywords Cod protein • Insulin sensitivity • Glucose metabolism • Inflammation • Lipid profile • L-Arginine • Taurine • Branched-chain amino acids

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Abbreviations

BCAA	Branched-chain amino acids
BPVEM	Lean beef, pork, veal, eggs, milk, and milk products
CRP	C-reactive protein
HOMA-IR	Homeostasis model assessment of insulin resistance
IL	Interleukin
IRS-1	Insulin receptor substrate-1
JNK	c-jun NH_2-terminal kinase
mTOR/p70S6K	Mammalian target of rapamycin/p70 ribosomal S6 kinase
OGTT	Oral glucose tolerance test
PI 3-kinase	Phosphatidylinositol 3-kinase
PUFA	Polyunsaturated fatty acids
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor-α

Introduction

With the increasing rates of obesity, the prevalence of insulin resistance and its related diseases is likely to increase significantly in the coming years. It is therefore essential to find effective strategies to slow or prevent the progression of insulin resistance. Many intervention studies have shown that early intervention to improve insulin resistance successfully prevents progression to type 2 diabetes (T2D) [1]. The composition of the diet is undoubtedly very important. While our understanding of the effects of fat and carbohydrates on glucose metabolism and insulin sensitivity has greatly increased over the past decades, the role of proteins and the mechanisms behind their effects are less well characterized. However, studies aiming to demystify their potential effects have shown promising results.

One protein of interest is cod protein as it has been shown to exert beneficial effects on insulin sensitivity, glucose metabolism, lipid profile, and inflammation [2–7]. Differences in the amino acid composition of proteins could account for their variable effects on metabolic health. L-Arginine, glycine, lysine, and taurine levels are higher in cod protein, whereas casein contains more branched-chain amino acids (BCAA).

In this chapter, we explore the beneficial effects and the mechanisms by which cod protein and its amino acids, especially L-arginine, modulate glucose metabolism, insulin sensitivity, lipid profile, and inflammation.

Cod Protein

Effects on Insulin Sensitivity and Glucose Metabolism

Epidemiological studies among Inuit populations of Greenland and Alaska have shown that these populations consuming large amounts of fish and marine mammals had a lower incidence of diabetes [8–10]. Moreover, regular fish consumption has been associated with a two times lower risk of developing glucose intolerance over a 4-year follow-up in elderly men and women [11]. In the Finnish and Dutch cohorts of men from the Seven Countries Study, it was also observed that an increase in fish

consumption during the 20-year follow-up was inversely related to 2-h post-OGTT (oral glucose tolerance test) glucose levels [12].

The beneficial effects of fish consumption were first attributed to fish oil, especially to the *n*-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, studies so far have shown no consistent effects of *n*-3 PUFA supplementation on glycemic control, insulin sensitivity, or T2D incidence in human subjects [13, 14]. Others have rather suggested that another component of fish may protect against the development of diabetes. As regular consumption of small amounts of lean fish (24 g/day) providing only 140 mg *n*-3 PUFA was inversely associated with the incidence of glucose intolerance and diabetes, and because protein is the most abundant component of lean fish (excluding water), it was suggested that the beneficial effects observed with the consumption of lean fish might be attributable to fish protein [11].

This hypothesis was first tested in normoglycemic rats fed purified diets containing as the sole source of protein casein, soy, or cod protein [15]. The results showed that consumption of cod protein reduced fasting plasma glucose and tended to decrease insulin levels compared with casein. Another study in rats showed that a sucrose-rich diet containing cod protein induced lower fasting plasma glucose and insulin concentrations than casein [16]. Furthermore, after an intravenous glucose tolerance test and a test meal, the cod protein-fed rats showed lower incremental areas under the glucose and insulin curves compared with casein-fed animals. Finally, consumption of cod protein resulted in an improvement of peripheral insulin sensitivity. Together, these results suggest that cod protein improves glucose tolerance and insulin sensitivity in rats when compared with casein. A subsequent study showed that cod protein completely prevented the development of insulin resistance in skeletal muscle of rats fed a high-fat diet [17]. These beneficial effects occurred even in the presence of body weight gain and increased visceral adipose tissue mass, suggesting that cod protein can uncouple obesity from insulin resistance. Investigation of the mechanisms underlying the improvement of skeletal muscle insulin sensitivity in cod protein-fed rats revealed that cod protein completely prevented the development of insulin resistance in muscle by normalizing insulin activation of PI 3-kinase and of its proximal effector Akt (PKB) [18]. This normalization of PI 3-kinase/Akt activation was associated with improved translocation of GLUT4 glucose transporters to the T-tubules, a unique component of the muscle cell surface.

Studies in humans also support a modulatory role for cod protein on glucose metabolism and insulin sensitivity. In six healthy men, consumption of a meal containing cod fillets resulted in lower postprandial plasma insulin levels than a meal containing beef [19]. In agreement with these results, we recently reported beneficial effects on glycemic and insulinemic responses during an OGTT after 4 weeks of cod protein consumption in nine insulin-resistant women with polycystic ovary syndrome (PCOS) [20]. Indeed, one cod protein meal per day for 3 months induced lower glycemic and insulinemic responses during the early phase of the OGTT as compared with the BPVEM (beef, pork, veal, eggs, and milk) meal. In another long-term study, Vikoren et al. [5] examined the effects of consuming either cod protein supplements (providing 3 g/day of fish protein for the first 4 weeks and 6 g/day for the last 4 weeks) or placebo tablets for 8 weeks in 34 overweight adults. The consumption of cod protein supplements led to lower fasting glucose, 2 h postprandial glucose, and glucose area under the curve compared with placebo. Improved glucose tolerance in the fish protein group was suggested to be a consequence of an increased early postprandial insulin secretion rather than an increased insulin sensitivity based on the fact that 30 min postprandial insulin and C-peptide serum concentrations were increased, while HOMA-IR was unchanged after 8 weeks. A more accurate method to measure insulin sensitivity is the hyperinsulinemic-euglycemic clamp. Using a 120-min hyperinsulinemic-euglycemic clamp, we addressed the effects of consuming either a cod protein diet or a BPVEM diet for 4 weeks in 19 insulin-resistant men and women [3]. Subjects consumed either two cod protein meals per day or lean beef, pork, veal, eggs, milk, and milk products (BPVEM) in a crossover design. Our results showed that cod protein improved insulin sensitivity by about 30 % compared with BPVEM (Fig. 34.1a). Moreover, a strong tendency ($P = 0.055$) for a greater increase in the disposition

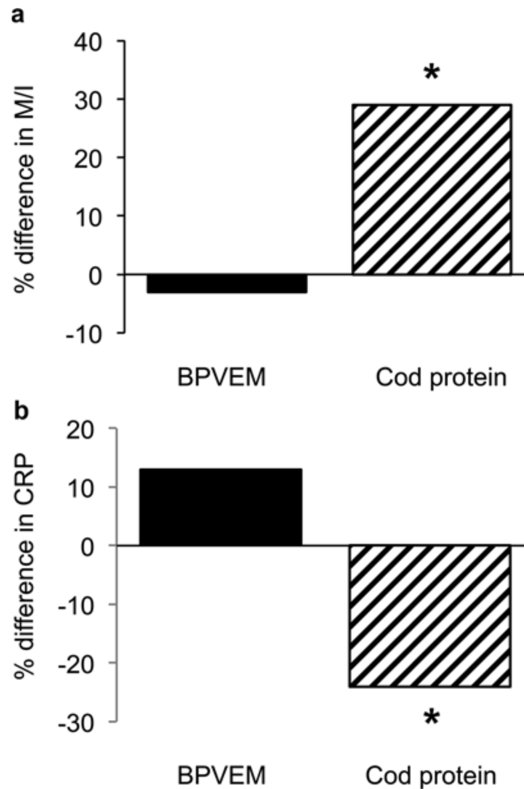

Fig. 34.1 Percent change from baseline in (**a**) insulin sensitivity (M/I) and (**b**) fasting plasma C-reactive protein (CRP) concentrations in insulin-resistant men and women after consumption of cod protein compared with other animal proteins (BPVEM) for 4 weeks in a crossover design. $n = 19$ for M/I and $n = 18$ for CRP. *Different from BPVEM, $P < 0.05$

index with the cod protein diet was observed. As the disposition index reflects the capacity of β-cells to increase insulin secretion in order to adequately compensate for insulin resistance, this suggests that the cod protein diet has a greater potential to decrease the risk of progression from normal glucose tolerance to T2D. In the above study, we further investigated the mechanisms underlying the improvement of insulin sensitivity following the consumption of the cod protein diet. In this respect, skeletal muscle insulin signaling pathways were examined by taking muscle biopsies from the *vastus lateralis* after each experimental diet in the basal state and after insulin stimulation (before and after the hyperinsulinemic-euglycemic clamp). As in rats, we assessed the effect of cod protein on insulin-stimulated PI 3-kinase activity. In the basal state, PI 3-kinase activity was not different between both diets (Fig. 34.2a). IRS-1-associated PI 3-kinase was significantly increased following insulin stimulation in muscle of subjects having consumed the cod protein diet (+57 %, $P = 0.001$) whereas it tended to be less so (+28 %, $P = 0.06$) after consumption of the BPVEM diet. Thus, the increase in PI 3-kinase activity from basal tended to be greater following the cod protein diet compared with the BPVEM diet ($P = 0.13$). We next investigated the effects of the cod protein diet on phosphorylation of Akt on Ser473, since the kinase activity of this downstream effector of PI 3-kinase was improved by cod protein in the high fat-fed rat model [18]. While insulin significantly induced Akt phosphorylation on Ser473 with both diets (+233 %, $P = 0.006$ for the cod protein diet, and +144 %, $P = 0.002$ for the BPVEM diet; Fig. 34.2b), the increase following insulin stimulation was greater after the CP diet compared with the BPVEM diet ($P = 0.04$). Third, we also measured the phosphorylation of IRS-1 on Ser636/9 as a marker of mTORC1/S6K1 activation. We previously hypothesized that the ability of

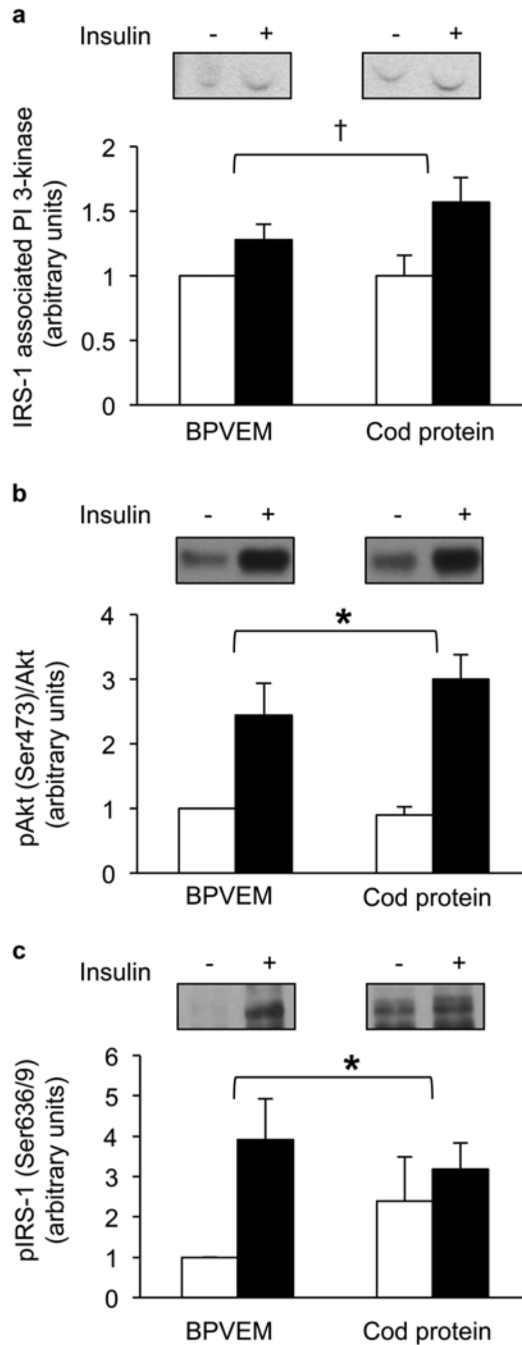

Fig. 34.2 Effects of cod protein compared with other animal proteins (BPVEM) on skeletal muscle insulin signaling pathway. (**a**) IRS-1–associated PI 3-kinase activity. Quantification of ^{32}P incorporated into PIP_3 was expressed relative to BPVEM basal values. The means ± SEM of 13 subjects are shown. (**b**) Akt phosphorylation on Ser473, the downstream effector of PI 3-kinase. Quantification of phosphorylation was expressed relative to BPVEM basal values. The means ± SEM of nine subjects are shown. (**c**) IRS-1 phosphorylation on Ser636/9, a marker of mTORC1/S6K1 activation which is implicated in a negative feedback loop that inhibits insulin signaling pathway. Quantification of phosphorylation of IRS-1 on Ser636/9 was expressed relative to BPVEM basal values. The means ± SEM of 11 subjects are shown. *Open square*, basal; *filled square*, insulin. $^{*}P < 0.05$ and $\dagger P = 0.13$, for BPVEM versus cod protein (increase between basal and insulin). *IRS-1* insulin receptor substrate-1, *PI 3-kinase phosphatidylinositol 3-kinase*, *PIP3* phosphatidylinositol 3,4,5-trisphosphate, *mTOR/p70S6K* mammalian target of rapamycin/p70 ribosomal S6 kinase, *Ser* serine

cod protein to improve insulin sensitivity could be linked to reduced mTORC1/S6K1 activation [18], a component of insulin signaling and nutrient sensing pathway especially activated when dietary proteins are rich in BCAA [17]. In this respect, whereas basal IRS-1 S636/9 phosphorylation was not significantly different between the cod protein group and the BPVEM group ($P = 0.31$), insulin stimulation induced a significant increase in IRS-1 Ser636/9 phosphorylation ($P = 0.01$) with the BPVEM diet whereas there was no change with the CP diet ($P = 0.97$), leading to a significant difference between the BPVEM and CP diet ($P = 0.05$; Fig. 34.2c). Finally, since some studies have reported that insulin resistance correlates with muscle fiber type distribution, and more specifically with reduced type I fibers and increased type IIx fibers [21], we examined muscle typology. The proportion of the type IIx MHC isoform was lower in the skeletal muscle of subjects consuming the CP diet compared with the BPVEM diet (Table 34.1), thus potentially contributing to improved insulin sensitivity.

Effects on Lipid Profile

In addition to its effect on insulin sensitivity and glucose metabolism, fish protein was also shown to affect plasma lipids in humans. A series of studies were conducted in our laboratory in premenopausal [22] and postmenopausal [23] women as well as in normolipidemic [24] and hypercholesterolemic [25] men. In these studies, lean white fish (pollack, cod, sole, haddock) was incorporated into a low-fat diet (30 %) with less than 0.45 % of total energy intake coming from *n*-3 PUFA. The lean white fish diet was compared to a BPVEM (beef, pork, veal, eggs, and milk) or a lean beef diet. We observed that the consumption of lean fish increased plasma HDL_2 cholesterol in men [24, 25] and decreased plasma VLDL triglycerides in women [22]. In another study, the effects of consuming salmon, cod, fish oil capsules, or sunflower oil capsules (control) for 8 weeks on blood lipid concentration in 262 overweight and obese individuals were examined [6]. Consumption of fish (salmon or cod) or fish oil resulted in a reduction in plasma TG concentration. The greatest decrease in TG was observed with consumption of salmon, which was the group receiving the largest amount of *n*-3 PUFA (2 g/day), in agreement with the well-documented TG-lowering effects of *n*-3 PUFA. However, interestingly, the cod diet lowered TG concentrations to a similar degree as fish oil, although *n*-3 PUFA content was lower (0.26 vs. 1 g/day). This suggests that another component in fish, likely protein, also has a TG-lowering effect, as reported in rat studies. Indeed, studies in rats reported lower plasma TG concentration [15, 16] as well as lower hepatic TG concentration and a lower rate of TG secretion into the blood [26] in cod protein-fed rats compared to their casein-fed counterparts. In addition to the TG-lowering effect, a reduction in total cholesterol was observed in both groups receiving fish (cod or salmon) compared with the control group, while no change was observed in the fish oil group. The hypocholesterolemic effect of fish protein is supported by studies showing little effect of *n*-3 PUFA on total cholesterol, LDL cholesterol, and HDL cholesterol levels (reviewed in Ref. [27]). In the study by Vikoren et al. [5], fish protein supplements

Table 34.1 Human skeletal muscle (*vastus lateralis*) typology after consuming cod protein and other animal proteins (BPVEM) diets for 4 weeks in a crossover design

	Myosin heavy chain relative content (%)		
	Cod protein	BPVEM	*P* value
Type I	50.4 ± 2.0	49.5 ± 3.2	0.66
Type IIa	34.1 ± 1.9	30.4 ± 2.6	0.14
Type IIx	15.5 ± 1.7	19.7 ± 2.4	0.05

Data are means ± SEM; $n = 14$

consumed for 8 weeks reduced LDL cholesterol compared to placebo in overweight adults. Moreover, an increase in HDL:LDL ratio was also seen after fish protein supplements. In contrast, we found that consumption of the BPVEM diet induced a greater decrease in plasma total cholesterol and tended to induce a greater decrease in LDL cholesterol as well as in total apolipoprotein B compared with cod protein in insulin-resistant individuals [4]. We suggested that the cholesterol-lowering effect of *n*-6 PUFA was counteracted when combined with dietary fish protein, as observed in rabbits [28]. We also suggested that the higher initial hsCRP concentrations before the cod protein diet (median = 2.15 mg/L) compared with the BPVEM diet (median = 1.85 mg/L) affected the response to the cholesterol-lowering diet.

Effects on Inflammation

In some epidemiological studies, fish consumption has been associated with lower circulating levels of CRP, IL-6, and TNF-α [29, 30], and it has been suggested that intake of EPA and DHA is responsible for the beneficial effects observed. For example, in the Nurses' Health Study I, it was observed that CRP levels were 29 % lower and those of IL-6 were 23 % lower among women in the highest quintile of *n*-3 PUFA intake (from fish) compared with those in the lowest quintile [31]. While there is epidemiological evidence to suggest an anti-inflammatory effect of EPA and DHA, intervention studies in humans have produced conflicting results (reviewed in [32]).

Studies on the effects of fish/cod protein on inflammation in humans are scarce, but they suggest a protective effect against inflammation. We examined in an intervention study the effects of a cod protein or a BPVEM diet on some inflammatory markers in insulin-resistant men and women [4]. Plasma CRP decreased by 24 % following the cod protein diet, while the BPVEM diet tended to cause an increase of 13 %. However, no difference between the two diets was observed for plasma levels of IL-6, TNF-α, and adiponectin (Fig. 34.1b). In agreement with these results, consumption of 2 weekly portions (150 g) of either farmed salmon (fatty fish) or icelandic cod (lean fish) for 6 months in addition to dietary advice resulted in a 25–30 % decrease in serum CRP concentration compared with the control group that received dietary advice alone [2]. In yet another study, the effects of consuming salmon, cod, fish oil capsules, or sunflower oil capsules for 8 weeks on CRP, IL-6, glutathione reductase, and prostaglandin F2 alpha (PGEF2alpha) in overweight and obese individuals were examined [7]. Both salmon and cod consumption led to a decrease in CRP and IL-6, while PGEF2alpha was decreased only after salmon consumption. Both fish and sunflower oil capsules had no effect on any inflammatory markers. In contrast, in the study on cod protein supplements, no significant changes were observed in CRP levels [5]; however, in this study all subjects had low baseline CRP levels.

Although studies in humans are scarce, animal and in vitro studies do support the notion that fish/cod protein has anti-inflammatory properties. A study in rats examined the effects of bonito, herring, mackerel, or salmon proteins on the expression of TNF-α and IL-6 in visceral adipose tissue [33]. The results showed that after 28 days of feeding, expression of both inflammatory markers was reduced in visceral adipose tissue of all fish protein groups fed a diabetogenic/obesogenic diet compared with casein-fed groups. In agreement, feeding dietary sardine protein for 2 months led to a reduction in TNF-α in rats with fructose-induced metabolic syndrome [34]. In an in vitro study, human macrophages were incubated with casein hydrolysates or fish protein hydrolysates with or without *n*-3 PUFA and compared with *n*-3 PUFA alone [35]. They found that the combination of fish protein hydrolysate and *n*-3 PUFA synergistically decreased expression levels of TNF-α compared with *n*-3 PUFA or fish protein alone [35].

L-Arginine and Other Amino Acids

Cod Protein and Amino Acid Composition

Cod protein may exert its beneficial effects on lipid profile, insulin sensitivity, glucose metabolism, and inflammation through the direct action of its constituent amino acids. This hypothesis is supported by an in vitro study with cultured L6 myocytes. The myocytes were treated with an amino acid mixture corresponding to the concentration of plasma amino acids in rats fed chow, casein, cod protein, or soy protein [17]. The myocytes treated with the cod-derived amino acid mixture were more insulin sensitive than those treated with the mixtures corresponding to rats fed casein or soy protein (Fig. 34.3). Interestingly, these effects were observed in the total absence of *n*-3 PUFA and were similar to what was observed when the rats were fed the intact proteins [17].

Individual amino acids or groups of amino acids may be responsible for the observed effects. For instance, lysine, methionine, and L-arginine content was higher, whereas glutamine and BCAA content was lower following the in vitro digestion of fish protein compared to beef protein. Moreover, we examined the relationships between fasting and postprandial amino acid profiles with glucoregulatory hormones which are indices of insulin sensitivity and glucose tolerance. Male Wistar rats were fed high-sucrose diets containing either casein or cod protein as the sole protein source, and after 4 weeks, rats were submitted to a 5 g test meal of similar content to the chronic diet given over the 4-week period. Amino acid concentrations and glucoregulatory hormones were measured before (fasting state) as well as 30 and 120 min after the test meal. In the fasting state, histidine concentration was

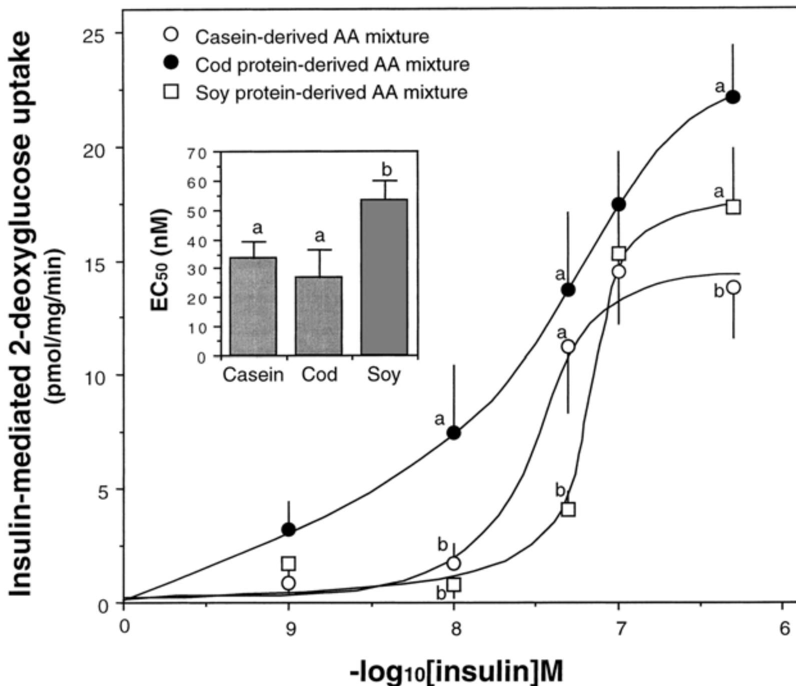

Fig. 34.3 Effects of casein-, cod protein-, or soy protein-derived amino acid (AA) mixtures on insulin-stimulated glucose uptake in L6 myocytes. Muscle cells were incubated for 1 h with AA before measurements of glucose uptake. Results are expressed as insulin minus basal glucose uptake values. Inset, insulin sensitivity index (EC_{50}) calculated from individual dose–response curves. Data are means ± SE of 4–5 separate experiments performed in triplicate. Groups bearing different letters are significantly different at $P < 0.05$. Reproduced with permission from [17]

lower whereas taurine concentration was higher in cod protein-fed rats compared to those fed casein (Table 34.2). In the postprandial state, alanine, BCAA, and the lysine:L-arginine ratio were lower whereas L-arginine and taurine were higher at both 30 and 120 min in cod protein-fed rats compared to those fed casein (Table 34.2). Glutamine concentration was also lower in cod protein-fed rats but only after 30 min. Another study also showed higher plasma taurine levels in sardine protein-fed rats compared with their casein-fed counterparts [34], although no other plasma amino acids levels were measured.

In humans, a study in six healthy men reported that consumption of a meal containing cod fillets resulted in plasma concentrations of L-arginine and lysine which were higher and of histidine which was lower than when consuming beef [19]. For our part, we observed that only taurine plasma concentrations differed in cod protein-fed subjects compared with BPVEM-fed subjects after 4 weeks [4]. Taurine plasma concentrations remained unchanged following cod protein consumption whereas it decreased with the BPVEM diet (Table 34.3). However, plasma amino acid concentration was measured in the fasting state in this study, potentially masking the effects; indeed, evidence from our study in rats demonstrated that only postprandial plasma L-arginine concentration was higher after cod protein than casein feeding, whereas fasting concentration was similar (Table 34.2).

Regulation of Insulin Sensitivity and Glucose Metabolism by L-Arginine and Other Amino Acids

Individual amino acids or certain classes of amino acids exert distinct effects on insulin sensitivity. Indeed, long-term oral L-arginine supplementation has been shown to improve insulin sensitivity in individuals with impaired glucose tolerance [36], T2D [37, 38] or coronary artery disease [39]. The improvement in insulin sensitivity was associated with normalization of insulin-mediated vasodilatation and an increase in blood flow [37]. As a substrate for NO production, L-arginine can increase NO bioavailability which then increases vasodilatation and blood flow. This increases skeletal muscle

Table 34.2 Plasma amino acid concentrations in fasting state, 30 min, and 120 min after a test meal (μmol/L) in male Wistar rats

	Fasting		30-min		120-min	
	Cod protein	Casein	Cod protein	Casein	Cod protein	Casein
Alanine	301±12	326±19	563±35*	663±52	526±36*	614±51
L-Arginine	147±15	183±24	194±17*	150±13	172±14*	109±19
Glutamine	1060±152	1398±230	926±119*	1263±205	1019±162	1129±201
Glycine	269±18	231±15	255±21	215±17	246±12	197±11
Histidine	54±6*	70±6	67±8	76±8	73±12	84±6
Isoleucine	69±6	75±6	90±8	115±9	96±7	109±13
Leucine	106±7	119±9	122±9*	170±18	128±6*	165±23
Lysine	391±28	398±23	440±32	470±32	440±25	468±35
Methionine	62±10	65±8	102±14	98±10	95±10	87±8
Taurine	128±11*	95±7	156±10*	104±11	129±8*	79±6
Valine	131±8	163±14	163±16*	230±23	163±11*	219±25
BCAA	302±20	356±25	375±33*	515±49	386±25*	493±61
Lysine:L-arginine ratio	2.8±0.2	2.3±0.2	2.3±0.1*	3.2±0.2	2.7±0.1*	5.1±1.0

Values are means±SEM. The test meals consisted of 5 g of food in which either casein or cod protein was the sole source of protein. *$P<0.05$ versus casein for same time point. *BCAA* branched-chain amino acids (sum of isoleucine, leucine, and valine)

Table 34.3 Changes in fasting plasma amino acid concentrations after consuming cod protein and other animal proteins (BPVEM) diets for 4 weeks in a crossover design

	Cod protein (μmol/L)	BPVEM (μmol/L)	*P* value
Alanine	−7.40 ± 4.61	−20.00 ± 4.22	0.064
L-Arginine	−8.77 ± 3.39	−10.05 ± 2.96	0.741
Asparagine	−2.37 ± 1.34	−4.51 ± 2.29	0.418
Aspartic acid	−7.19 ± 2.59	−8.45 ± 3.24	0.763
Glutamic acid	−11.67 ± 6.88	−21.01 ± 5.92	0.323
Glutamine	−37.68 ± 19.01	−41.84 ± 27.96	0.902
Glycine	−3.38 ± 3.81	−2.25 ± 5.68	0.879
Histidine	−12.92 ± 3.23	−8.32 ± 4.61	0.419
Isoleucine	−6.62 ± 1.90	−8.39 ± 2.71	0.575
Leucine	−14.51 ± 3.32	−13.34 ± 4.76	0.867
Lysine	−5.66 ± 4.36	−14.65 ± 7.04	0.274
Methionine	−1.55 ± 0.70	−3.60 ± 0.80	0.078
Phenylalanine	−4.72 ± 2.30	−7.60 ± 3.38	0.484
Serine	−0.27 ± 3.30	−5.22 ± 3.66	0.328
Taurine	3.12 ± 3.51	−14.71 ± 5.55	0.010
Threonine	−8.36 ± 5.28	−14.65 ± 7.36	0.490
Tryptophan	−3.58 ± 1.36	−4.43 ± 1.85	0.684
Tyrosine	−12.89 ± 2.56	−9.57 ± 3.08	0.471
Valine	−13.54 ± 5.62	−11.89 ± 8.27	0.900
EAA	−56.50 ± 17.81	−74.75 ± 28.65	0.557
BCAA	−34.67 ± 10.28	−33.62 ± 15.32	0.985

Values are means ± SEM; $n = 19$. EAA, essential amino acids (sum of histidine, isoleucine, leucine, methionine, lysine, phenylalanine, threonine, tryptophan, valine); *BCAA* branched-chain amino acids (sum of isoleucine, leucine, and valine). Adapted with permission from [4]

perfusion, providing better insulin and glucose supply which, in turn, contributes to better glucose disposal.

In addition to L-arginine, taurine has also been suggested to have beneficial effects on insulin sensitivity. Indeed, as reviewed by Ito et al. [40], studies in genetic and diet-induced animal models of insulin resistance and T2D have reported an improvement in insulin sensitivity and a reduction of hyperglycemia and hyperinsulinemia with taurine supplementation. In humans, there are only a few clinical studies on taurine supplementation in patients with diabetes. Some of these studies have failed to show beneficial effects of taurine on insulin sensitivity and glucose metabolism, which is inconsistent with animal studies [40]. In contrast, a 2-week pretreatment with 3 g/day of taurine improved the deleterious effects on insulin sensitivity of a 48-h intravenous lipid infusion in overweight or obese men [40]. Taurine pretreatment also prevented the rise in lipid peroxidation products, suggesting that the improvement in insulin sensitivity might be partly due to reduced oxidative stress. In agreement with the latter study, 1 g/day for 30 days taurine supplementation in diabetic patients led to reduced plasma glucose [40]. Several factors (small number of subjects, short-term duration, metabolic status of subjects, presence of metabolic complications, use of other medication, etc.) may have led to these divergent results across studies.

In contrast to the beneficial effects of L-arginine and taurine on insulin sensitivity and glucose metabolism, BCAA have been implicated in the development of insulin resistance. For instance, metabolomic profiling of obese (median BMI = 37 kg/m^2) insulin-resistant versus lean (median BMI = 23 kg/m^2) insulin-sensitive subjects revealed that BCAA were strongly associated with insulin resistance (HOMA) [41]. Newgard further reported that supplementation of a high-fat diet with BCAA in rats promoted insulin resistance by activation of the mTOR pathway and inflammation as

shown by the upregulation of JNK [41]. In line with these observations, five metabolites (leucine, isoleucine, valine, phenylalanine, and tyrosine) showed the strongest association with incident diabetes in the Framingham longitudinal cohort where 189 subjects who developed T2D over 12 years of follow-up were compared to 189 control subjects who did not develop T2D [41]. As reviewed by Lu et al. [42], evidence from interventional studies also supports a role for BCAA in development of insulin resistance. Indeed, in the Weight Loss Maintenance (WLM) trial, metabolomic profiling of 500 subjects who had lost ≥4 kg during phase I showed that baseline BCAA and related catabolites predicted the improvement in insulin resistance (HOMA), independent of the amount of weight loss. In another study, comparison of circulating amino acids in subjects who lost 10 kg with either gastric bypass surgery or dietary intervention revealed that BCAA significantly decreased after gastric bypass surgery but not after dietary intervention. Moreover, BCAA were uniquely correlated with insulin resistance (HOMA).

Regulation of Inflammation by L-Arginine and Other Amino Acids

In the Third National Health Nutrition and Examination Survey, a high intake of L-arginine (>7.5 g/day) was associated with lower levels of CRP, even after controlling for confounding factors [43]. Moreover, in patients having unstable angina, oral administration of L-arginine for 3 months led to a decrease in IL-1β and IL-6 but not in TNF-α [44]. In line with the above results, supplementation with 6.4 g of L-arginine daily for 6 months led to a 47 % decrease in IL-6, a 19 % decrease in monocyte chemoattractant protein-1, and a 37 % increase in adiponectin levels in patients with cardiovascular disease [39]. In contrast, some studies found that markers of inflammation were not affected by oral supplementation of L-arginine [45–47]. Therefore, it is still unclear whether L-arginine supplementation has beneficial effects on inflammation. It is thus possible that other amino acids, such as taurine and/or glycine, are also involved in the beneficial effects of cod protein consumption on inflammation.

Indeed, taurine is another amino acid which has been suggested to have anti-inflammatory properties, although studies in animals and humans are still very limited. Reduced plasma taurine levels and increased CRP levels have been observed in obese women compared with normal weight women [48]. Moreover, 8 weeks of taurine supplementation (3 g/day) in the obese women led to a significant increase in taurine plasma levels (97 %) which was accompanied by a reduction in CRP (29 %) and an increase in adiponectin (12 %) levels [48]. However, changes in IL-6 and TNF-α levels were not different between the supplementation and placebo groups. Several in vitro studies have shown that taurine chloramine, which is the result of the reaction between taurine and hypochlorous acid (HOCl) generated by monocytes and activated neutrophils, can suppress the production of pro-inflammatory mediators (TNF-α, IL-1β, IL-6) by macrophage cell lines, by activated macrophages from mice and rats, as well as by human monocytes [49]. It has also been suggested that taurine may reduce the mRNA expression of TNF-α and iNOS [49]. It has further been suggested that taurine interferes with the signaling pathways responsible for the production of pro-inflammatory cytokines, particularly with the activation of NFκB [49].

In a context of muscle regeneration, we have shown that cod protein can also decrease the inflammatory response in rat skeletal muscles following injury [50]. We hypothesized that the beneficial effects of cod protein on the resolution of inflammation and muscle regeneration after injury were attributable to its high content of L-arginine, glycine, taurine, and lysine. To test this hypothesis, we formulated a diet (casein$^+$) consisting of casein supplemented with L-arginine, glycine, taurine, and lysine, corresponding to their respective levels in cod protein. In agreement with our hypothesis, the addition of cod protein levels of L-arginine, glycine, lysine, and taurine to casein reproduced the anti-inflammatory effect observed with the cod protein, thus highlighting the contribution of these amino acids to the anti-inflammatory properties of cod protein [50].

Regulation of Lipid Profile by L-Arginine and Other Amino Acids

Some studies have shown that L-arginine supplementation in animal models of insulin resistance and T2D leads to a decrease in total cholesterol, LDL cholesterol, and TG [51–53]. It has further been suggested that the lysine:L-arginine ratio was important in regulating cholesterol levels: a low lysine:L-arginine ratio resulting in lower levels of total cholesterol and LDL cholesterol than a high lysine:L-arginine ratio [54, 55]. In contrast to animal studies, intervention studies in humans have observed that L-arginine supplementation has no effect on lipid profile in young hypercholesterolemic adult men [46], healthy postmenopausal women [56], and individuals with T2D [38]. However, when L-arginine supplementation was combined with a statin (simvastatin), there was a sharp decrease in TG concentrations compared with placebo plus statin [57]. Once again, it may be that other amino acids in cod protein, such as taurine, exert the beneficial effects on lipids.

An epidemiological study in middle-aged Japanese living in Japan or Brazil showed an inverse association between fish intake and the prevalence of hypercholesterolemia and this association was partly influenced by taurine [58]. Only a few controlled studies in humans have evaluated the effect of taurine on lipid profile. For instance, a decrease in TG and atherogenic index [(total cholesterol − HDL cholesterol)/HDL cholesterol] was observed in 30 overweight or obese young adults following supplementation with 3 g/day of taurine for 7 weeks [59]. In another study, 22 healthy young male consumed a high-fat, high-cholesterol diet with either 6 g/day of taurine powder or placebo capsules for 3 weeks [60]. Taurine supplementation attenuated the increase in total cholesterol and LDL cholesterol induced by the high-fat, high-cholesterol diet but resulted in an increase in VLDL cholesterol and TG. Animal studies have demonstrated that the cholesterol-lowering effect of taurine is mainly due to an upregulation of LDL receptors in the liver and accelerated bile acids' production via increased activation of 7α-hydroxylase, a key enzyme in the conversion of cholesterol to bile acids [61].

Conclusion

A significant number of studies have shown that the consumption of cod protein is beneficial in improving insulin sensitivity, inflammation, and lipid profile. The underlying mechanisms are still largely unknown. However, it seems likely that individual amino acids or groups of amino acids, such as L-arginine, taurine, and BCAA, are specifically implicated. Indeed, studies on amino acids show that L-arginine and taurine, found in greater quantities in fish than in beef or pork, have beneficial effects on insulin sensitivity and inflammation, whereas BCAA, which are lower in fish, have deleterious effects. Therefore, individuals could benefit from including dietary fish protein in their diet in order to improve insulin sensitivity and decrease its metabolic complications, helping to prevent T2D.

References

1. Crandall JP, Knowler WC, Kahn SE, et al. The prevention of type 2 diabetes. Nat Clin Pract Endocrinol Metab. 2008;4:382–93.
2. Pot GK, Geelen A, Majsak-Newman G, et al. Increased consumption of fatty and lean fish reduces serum C-reactive protein concentrations but not inflammation markers in feces and in colonic biopsies. J Nutr. 2010;140:371–6.
3. Ouellet V, Marois J, Weisnagel SJ, Jacques H. Dietary cod protein improves insulin sensitivity in insulin-resistant men and women: a randomized controlled trial. Diabetes Care. 2007;30:2816–21.

4. Ouellet V, Weisnagel SJ, Marois J, et al. Dietary cod protein reduces plasma C-reactive protein in insulin-resistant men and women. J Nutr. 2008;138:2386–91.
5. Vikoren LA, Nygard OK, Lied E, Rostrup E, Gudbrandsen OA. A randomised study on the effects of fish protein supplement on glucose tolerance, lipids and body composition in overweight adults. Br J Nutr. 2012;31:1–10.
6. Gunnarsdottir I, Tomasson H, Kiely M, et al. Inclusion of fish or fish oil in weight-loss diets for young adults: effects on blood lipids. Int J Obes (Lond). 2008;32:1105–12.
7. Ramel A, Martinez JA, Kiely M, Bandarra NM, Thorsdottir I. Effects of weight loss and seafood consumption on inflammation parameters in young, overweight and obese European men and women during 8 weeks of energy restriction. Eur J Clin Nutr. 2010;64:987–93.
8. Mouratoff GJ, Carroll NV, Scott EM. Diabetes mellitus in Athabaskan Indians in Alaska. Diabetes. 1969;18:29–32.
9. Kromann N, Green A. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950–1974. Acta Med Scand. 1980;208:401–6.
10. Bang HO, Dyerberg J, Sinclair HM. The composition of the Eskimo food in north western Greenland. Am J Clin Nutr. 1980;33:2657–61.
11. Feskens EJ, Bowles CH, Kromhout D. Inverse association between fish intake and risk of glucose intolerance in normoglycemic elderly men and women. Diabetes Care. 1991;14:935–41.
12. Feskens EJ, Virtanen SM, Rasanen L, et al. Dietary factors determining diabetes and impaired glucose tolerance. A 20-year follow-up of the Finnish and Dutch cohorts of the Seven Countries Study. Diabetes Care. 1995;18:1104–12.
13. Hartweg J, Perera R, Montori V, Dinneen S, Neil HA, Farmer A. Omega-3 polyunsaturated fatty acids (PUFA) for type 2 diabetes mellitus. Cochrane Database Syst Rev. 2008;23:CD003205.
14. Zhang M, Picard-Deland E, Marette A. Fish and marine omega-3 polyunsaturated fatty acid consumption and incidence of type 2 diabetes: a systematic review and meta-analysis. Int J Endocrinol. 2013;2013:501015.
15. Hurley C, Galibois I, Jacques H. Fasting and postprandial lipid and glucose metabolisms are modulated by dietary proteins and carbohydrates: role of plasma insulin concentrations. J Nutr Biochem. 1995;6:540–6.
16. Lavigne C, Marette A, Jacques H. Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. Am J Physiol Endocrinol Metab. 2000;278:E491–500.
17. Lavigne C, Tremblay F, Asselin G, Jacques H, Marette A. Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. Am J Physiol Endocrinol Metab. 2001;281:E62–71.
18. Tremblay F, Lavigne C, Jacques H, Marette A. Dietary cod protein restores insulin-induced activation of phosphatidylinositol 3-kinase/Akt and GLUT4 translocation to the T-tubules in skeletal muscle of high-fat-fed obese rats. Diabetes. 2003;52:29–37.
19. Soucy J, Leblanc J. The effects of a beef and fish meal on plasma amino acids, insulin and glucagon levels. Nutr Res. 1999;19:17–24.
20. Talbot E, Weisnagel SJ, Marois J, Jacques H. Impact of cod protein on insulin sensitivity in women with polycystic ovary syndrome. Can J Diabetes. 2013;37:62.
21. Lillioja S, Young AA, Culter CL, et al. Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. J Clin Invest. 1987;80:415–24.
22. Gascon A, Jacques H, Moorjani S, Deshaies Y, Brun LD, Julien P. Plasma lipoprotein profile and lipolytic activities in response to the substitution of lean white fish for other animal protein sources in premenopausal women. Am J Clin Nutr. 1996;63:315–21.
23. Jacques H, Noreau L, Moorjani S. Effects on plasma lipoproteins and endogenous sex hormones of substituting lean white fish for other animal-protein sources in diets of postmenopausal women. Am J Clin Nutr. 1992;55:896–901.
24. Lacaille B, Julien P, Deshaies Y, Lavigne C, Brun LD, Jacques H. Responses of plasma lipoproteins and sex hormones to the consumption of lean fish incorporated in a prudent-type diet in normolipidemic men. J Am Coll Nutr. 2000;19:745–53.
25. Beauchesne-Rondeau E, Gascon A, Bergeron J, Jacques H. Plasma lipids and lipoproteins in hypercholesterolemic men fed a lipid-lowering diet containing lean beef, lean fish, or poultry. Am J Clin Nutr. 2003;77:587–93.
26. Demonty I, Deshaies Y, Lamarche B, Jacques H. Cod protein lowers the hepatic triglyceride secretion rate in rats. J Nutr. 2003;133:1398–402.
27. Balk EM, Lichtenstein AH, Chung M, Kupelnick B, Chew P, Lau J. Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: a systematic review. Atherosclerosis. 2006;189:19–30.
28. Bergeron N, Deshaies Y, Lavigne C, Jacques H. Interaction between dietary proteins and lipids in the regulation of serum and liver lipids in the rabbit. Effect of fish protein. Lipids. 1991;26:759–64.
29. Zampelas A, Panagiotakos DB, Pitsavos C, et al. Fish consumption among healthy adults is associated with decreased levels of inflammatory markers related to cardiovascular disease: the ATTICA study. J Am Coll Cardiol. 2005;46:120–4.

30. Nakamura Y, Ueno Y, Tamaki S, et al. Fish consumption and early atherosclerosis in middle-aged men. Metabolism. 2007;56:1060–4.
31. Lopez-Garcia E, Schulze MB, Manson JE, et al. Consumption of (n-3) fatty acids is related to plasma biomarkers of inflammation and endothelial activation in women. J Nutr. 2004;134:1806–11.
32. Robinson LE, Mazurak VC. N-3 polyunsaturated fatty acids: relationship to inflammation in healthy adults and adults exhibiting features of metabolic syndrome. Lipids. 2013;48:319–32.
33. Pilon G, Ruzzin J, Rioux LE, et al. Differential effects of various fish proteins in altering body weight, adiposity, inflammatory status, and insulin sensitivity in high-fat-fed rats. Metabolism. 2011;60:1122–30.
34. Madani Z, Louchami K, Sener A, Malaisse WJ, Ait Yahia D. Dietary sardine protein lowers insulin resistance, leptin and TNF-alpha and beneficially affects adipose tissue oxidative stress in rats with fructose-induced metabolic syndrome. Int J Mol Med. 2012;29:311–8.
35. Rudkowska I, Marcotte B, Pilon G, Lavigne C, Marette A, Vohl MC. Fish nutrients decrease expression levels of tumor necrosis factor-alpha in cultured human macrophages. Physiol Genomics. 2010;40:189–94.
36. Monti LD, Setola E, Lucotti PC, et al. Effect of a long-term oral L-arginine supplementation on glucose metabolism: a randomized, double-blind, placebo-controlled trial. Diabetes Obes Metab. 2012;14:893–900.
37. Piatti PM, Monti LD, Valsecchi G, et al. Long-term oral L-arginine administration improves peripheral and hepatic insulin sensitivity in type 2 diabetic patients. Diabetes Care. 2001;24:875–80.
38. Lucotti P, Setola E, Monti LD, et al. Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab. 2006;291:E906–12.
39. Lucotti P, Monti L, Setola E, et al. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism. 2009;58:1270–6.
40. Ito T, Schaffer SW, Azuma J. The potential usefulness of taurine on diabetes mellitus and its complications. Amino Acids. 2012;42:1529–39.
41. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab. 2012;15:606–14.
42. Lu J, Xie G, Jia W. Insulin resistance and the metabolism of branched-chain amino acids. Front Med. 2013;7:53–9.
43. Wells BJ, Mainous 3rd AG, Everett CJ. Association between dietary L-arginine and C-reactive protein. Nutrition. 2005;21:125–30.
44. Blum A, Porat R, Rosenschein U, et al. Clinical and inflammatory effects of dietary L-arginine in patients with intractable angina pectoris. Am J Cardiol. 1999;83:1488–90.
45. Blum A, Hathaway L, Mincemoyer R, et al. Effects of oral L-arginine on endothelium-dependent vasodilation and markers of inflammation in healthy postmenopausal women. J Am Coll Cardiol. 2000;35:271–6.
46. West SG, Likos-Krick A, Brown P, Mariotti F. Oral L-arginine improves hemodynamic responses to stress and reduces plasma homocysteine in hypercholesterolemic men. J Nutr. 2005;135:212–7.
47. Bogdanski P, Suliburska J, Grabanska K, et al. Effect of 3-month L-arginine supplementation on insulin resistance and tumor necrosis factor activity in patients with visceral obesity. Eur Rev Med Pharmacol Sci. 2012;16:816–23.
48. Rosa FT, Freitas EC, Deminice R, Jordao AA, Marchini JS. Oxidative stress and inflammation in obesity after taurine supplementation: a double-blind, placebo-controlled study. Eur J Nutr. 2014;53:823–30.
49. Schuller-Levis GB, Park E. Taurine: new implications for an old amino acid. FEMS Microbiol Lett. 2003;226:195–202.
50. Dort J, Leblanc N, Maltais-Giguere J, Liaset B, Cote CH, Jacques H. Beneficial effects of cod protein on inflammatory cell accumulation in rat skeletal muscle after injury are driven by its high levels of L-arginine, glycine, taurine and lysine. PLoS One. 2013;8:e77274.
51. Popov D, Costache G, Georgescu A, Enache M. Beneficial effects of L-arginine supplementation in experimental hyperlipemia-hyperglycemia in the hamster. Cell Tissue Res. 2002;308:109–20.
52. Kawano T, Nomura M, Nisikado A, Nakaya Y, Ito S. Supplementation of L-arginine improves hypertension and lipid metabolism but not insulin resistance in diabetic rats. Life Sci. 2003;73:3017–26.
53. Miguez I, Marino G, Rodriguez B, Taboada C. Effects of dietary L-arginine supplementation on serum lipids and intestinal enzyme activities in diabetic rats. J Physiol Biochem. 2004;60:31–7.
54. Sugano M, Ishiwaki N, Nakashima K. Dietary protein-dependent modification of serum cholesterol level in rats. Significance of the L-arginine/lysine ratio. Ann Nutr Metab. 1984;28:192–9.
55. Gudbrandsen OA, Wergedahl H, Liaset B, Espe M, Berge RK. Dietary proteins with high isoflavone content or low methionine-glycine and lysine-L-arginine ratios are hypocholesterolaemic and lower the plasma homocysteine level in male Zucker fa/fa rats. Br J Nutr. 2005;94:321–30.
56. Blum A, Cannon 3rd RO, Costello R, Schenke WH, Csako G. Endocrine and lipid effects of oral L-arginine treatment in healthy postmenopausal women. J Lab Clin Med. 2000;135:231–7.

57. Schulze F, Glos S, Petruschka D, et al. L-Arginine enhances the triglyceride-lowering effect of simvastatin in patients with elevated plasma triglycerides. Nutr Res. 2009;29:291–7.
58. Mizushima S, Moriguchi EH, Ishikawa P, et al. Fish intake and cardiovascular risk among middle-aged Japanese in Japan and Brazil. J Cardiovasc Risk. 1997;4:191–9.
59. Zhang M, Bi LF, Fang JH, et al. Beneficial effects of taurine on serum lipids in overweight or obese non-diabetic subjects. Amino Acids. 2004;26:267–71.
60. Mizushima S, Nara Y, Sawamura M, Yamori Y. Effects of oral taurine supplementation on lipids and sympathetic nerve tone. Adv Exp Med Biol. 1996;403:615–22.
61. Yamori Y, Taguchi T, Hamada A, Kunimasa K, Mori H, Mori M. Taurine in health and diseases: consistent evidence from experimental and epidemiological studies. J Biomed Sci. 2010;17 Suppl 1:S6.

Chapter 35
Obese Subjects and Supplemental L-Arginine

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Key Points

- L-arginine influences metabolism in obesity.
- The action of L-arginine in adiposity is related to fat reduction, glucose and fatty acid oxidation, increases in lipolysis, inhibition of lipogenic processes, and changes in fat tissue endocrine secretion.
- L-arginine supplementation improves insulin sensitivity in obese patients.
- Nitric oxide signaling, mitochondrial biogenesis, the growth of brown adipose tissue, and the regulation of fat metabolic gene expression are the main mechanisms underlying the beneficial effect of L-arginine in obesity.
- L-arginine can play a crucial role in preventing and treating obesity and metabolic syndrome.

Keywords L-Arginine • Obesity • Fat metabolism • Brown adipose tissue • White adipose tissue • Insulin sensitivity

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Abbreviations

ACCα	Acetyl-CoA carboxylase α
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Arg	L-arginine
ATP	Adenosine triphosphate
cGMP	Cyclic guanosine monophosphate
DIO	Diet-induced obese
GLUT4	Glucose transporter type 4
GSS	Glutathione synthetase
HO-3	Heme oxygenase-3
HSL	Hormone-sensitive lipase
NO	Nitric oxide
NOS	NO synthase
NOS-1	NO synthase-1
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PPAR-α	Peroxisome proliferator-activated receptor-α
TAS	Total antioxidant status
ZDF	Zucker diabetic fatty

Introduction

Obesity and overweight are constantly growing health problems throughout the world. According to the World Health Organization, nearly one billion adults are overweight, and 300 million people are obese [1]. Although it is widely accepted that obesity is associated with dyslipidemia, hypertension, atherosclerosis, stroke, insulin resistance, and some types of cancers, pharmacological treatment for this chronic disease is limited. The alarming increase in the occurrence of obesity and overweight has compelled researchers to seek new forms of drug therapy. Emerging evidence from both experimental and clinical studies shows that L-arginine (Arg) holds great promise for the prevention and treatment of adiposity and associated metabolic disorders in humans and animals. An anti-obesity effect of Arg is yet to be demonstrated, and the mechanisms responsible for the beneficial effects of Arg are very complex and involve nitric oxide (NO) signaling, enhancing of mitochondrial biogenesis, growth of brown adipose tissue and stimulation of thermogenesis, regulation of fat metabolic gene expression, and changes in fat tissue endocrine secretion.

Crosstalk Between L-Arginine and Metabolism in Obesity

There are several mechanisms that may be involved in the biochemical changes responsible for the effect of Arg treatment on overweight and obese subjects. In experimental studies, it has been found that dietary Arg supplementation decreases adipose cell size without leading to a reduction in adipose cell numbers. It can be suggested that Arg reduces abdominal fat by decreasing triglycerides deposition in adipose cell and not through any influence on the differentiation or proliferation of adipose cells [2]. It has been demonstrated in clinical studies that the use of Arg in central obesity results in a significant decrease in waist circumference.

Table 35.1 Possible mechanisms of L-arginine influence on fat mass loss

Mechanism	Effect
Increases brown adipose tissue Increases blood flow to organs	Increases energy expenditure through enhanced oxidation of glucose and fatty acids
Modulates gene expression to enhance energy substrate oxidation	Reduces white fat accretion in insulin-sensitive tissues
Regulates fat metabolic genes in skeletal muscle and white adipose tissue; increases lipogenesis in muscle and lipolysis in adipose tissue	Regulates lipid distribution between muscle tissue and adipose tissue
Increases activity of lipoprotein lipase in skeletal muscle	
Increases content of oleic acid in skeletal muscle	Stimulates glucose uptake by skeletal muscle for oxidation and reduces glucose availability for fatty acid synthesis in other tissues

Arg regulates the metabolism of energy substrates—such as fatty acids, amino acids, and glucose—partly through the production of NO. This mechanism may be involved in the decreases in fat deposition and the increase in muscle growth and protein gain in the body [3]. In experimental studies, it has been shown that enhancing Arg availability stimulates muscle protein synthesis. In animal studies, increases in the proportional weight of skeletal muscle have been observed following Arg supplementation [4]. The growth of muscle tissue in conjunction with the decrease in body fat constitutes the main beneficial effect of Arg, especially in obese people whose muscle mass is relatively reduced. It seems possible that Arg may regulate lipid distribution between muscle and adipose tissue. We suggest that enhanced concentration of Arg upregulates the expression of lipogenic genes in skeletal muscle, while in white adipose tissue, Arg supplementation downregulates the expression of lipogenic genes and increases the expression of lipolytic genes. This idea is supported by the increased activity of lipoprotein lipase observed in skeletal muscle after Arg supplementation. Muscle lipoprotein lipase phosphorylation is regulated by Arg or NO. This enzyme activity provides a substrate for fat biosynthesis in skeletal muscle [5]. Another possible mechanism of Arg's effect on fat content is the increase in the concentration of oleic acid in skeletal muscle. It is known that oleic acid stimulates glucose uptake by skeletal muscles for oxidation and potentially reduces the availability of glucose for fatty acid synthesis in other tissues [5].

It has been found that the anabolic effect of Arg is independent of the insulin level in the blood. In experiments on pigs, Arg supplementation increased muscle mass and reduced white fat content without affecting the body mass. This suggests that Arg may regulate intracellular protein turnover, contributing to the accumulation of protein in muscle tissue. Through this, Arg—as a biological precursor of NO—may increase insulin sensitivity in muscle cells and amplify the signaling mechanisms to enhance protein deposition in skeletal muscle [2].

Arg may also reduce body mass by increasing the content of brown adipose tissue or by increasing blood flows. The main mechanisms through which Arg might reduce fat mass in the body are summarized in Table 35.1.

Experimental and Clinical Studies Concerning L-Arginine Supplementation in Obesity

Animal Studies

The first report on the role of dietary Arg supplementation in reducing fat mass in obese animals with non-insulin-dependent diabetes mellitus emerged in 2005, when Fu et al. observed a loss of weight and adipose tissue mass in supplemented Zucker diabetic fatty (ZDF) rats. They noted

that 10 weeks of oral administration of Arg (1.25 % in drinking water) was highly effective in enhancing NO production, lipolysis, and oxidation of glucose in the abdominal and epididymal adipose tissues. Compared to the placebo group, at the end of the supplementation period, the weight of the epididymal and retroperitoneal adipose tissue in the Arg-treated ZDF rats was 25 % and 45 % lower, respectively. The results of this study indicate that Arg supplementation reduced the serum levels of glucose, free fatty acids, triglycerides, homocysteine, and leptin. The expression of the key genes responsible for fatty acid and glucose oxidation in adipose tissue—among them NO synthase-1 (NOS-1), heme oxygenase-3 (HO-3), adenosine monophosphate (AMP)-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)—was upregulated in the study [4]. In another study, the effect of Arg was observed after 4 weeks supplementation of watermelon pomace juice using 0.2 % L-citrulline, which is converted into Arg in the animals. ZDF rats supplemented with watermelon pomace juice exhibited increased serum concentration of Arg and increased brown adipose tissue mass, along with reduced excess white fat mass and enhanced NO-dependent vessel reactivity [6]. Studies conducted on diet-induced obese (DIO) rats have shown that 12 weeks supplementation with 1.51 % L-arginine–HCl in drinking water reduced the white fat gain, increased the skeletal muscle mass, and decreased the serum concentration of glucose and triglycerides. Improvements in insulin sensitivity were also observed [2].

In studies conducted on growing–finishing pigs, it was found that supplementation with 1.0 % Arg reduced fat accretion and promoted muscle gains. Following 60 days of supplementation, Arg was seen to increase the average daily weight gain and carcass skeletal muscle content by 6.5 % and 5.5 %, respectively, and to decrease carcass fat content by 11 %, compared with the control group. In the Arg-supplemented pigs, serum triglyceride concentration was 20 % lower, while the glucagon level was 36 % greater than in the control pigs [7]. The improvement in metabolic profile in the growing pigs was also detected by metabolomic analysis of serum samples [8]. Interestingly, in a more recent study, Go et al. were unable to demonstrate the depressing lipid synthesis effect of Arg in either subcutaneous or retroperitoneal adipose tissue in growing–finishing pigs. This effect was supported by the dramatic increase in adipocyte volumes. The net effect of supplementation with 1.0 % Arg was an increase in backfat thickness, leading to fatter carcasses [9].

Human Studies

Although the effect of Arg supplementation in human subjects has been dealt with in multiple studies, it seems to be limited mainly to its effect on endothelial function. Bai et al. summarized 13 previously randomized placebo-controlled trials and concluded that short-term oral Arg supplementation is effective at improving the fasting endothelial function [10].

The data from the literature show that the reduction in body weight in clinical studies is not as spectacular as in the experimental studies (Table 35.1). It seems that the greatest advantage of using Arg in obese people is associated with the fact that this compound promotes fat reduction and spares lean body mass during weight loss. In few studies, it was noted that supplementation with Arg in an amino acid mixture led to a reduction in white adipose tissue in both children and adults [11, 12]. However, only one clinical trial regarding the specific effect of Arg alone on adiposity in humans has been conducted. In that trial, a long-term oral Arg treatment was added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetes patients. Thirty-three patients with type 2 diabetes were treated with Arg (8.3 g/day) or placebo for 21 days. During the study, each patient received a low-calorie diet (1000 kcal/day) and took part in a regular exercise training program (45 min twice a day for 5 days per week). In both groups, the authors observed a decrease in body weight, waist circumference, and daily

Table 35.2 Body mass and fat content due to L-arginine supplementation in obesity

References	Study group	Dose of L-arginine	Duration of the supplementation	Body mass loss (BMI)	Fat mass loss
Clinical studies					
Lucotti et al. [13]	Obese, insulin-resistant type 2 diabetes adults	L-arginine 8.3 g (oral treatment with low-calorie diet and a regular exercise training program)	21 days	Body mass ↓ 2.8 % (NS)	Fat mass ↓ 6.1 % ($p<0.05$)
Monti et al. [14]	Subjects with impaired glucose tolerance and metabolic syndrome	L-arginine 6.6 g (oral treatment)	14 days	Body mass ↓ 2.4 % ($p<0.05$)	Fat mass ↓ 6.7 % ($p<0.05$)
Bogdanski et al. [15]	Obese adults	L-arginine 9 g (oral treatment)	3 months	BMI ↓ 0.8 % (NS)	Fat content ↓ 0.7 % (NS)
Suliburska et al. [16]	Obese adults	L-arginine 9 g (oral treatment)	6 months	BMI ↓ 2 % (NS)	Fat content ↓ 1.4 % (NS)
Hurt et al. [17]	Obese adults	L-arginine 9 g (oral treatment)	12 weeks	Body mass ↓ 2.9 % ($p<0.05$)	
Experimental studies					
Fu et al. [4]	Zucker diabetic fatty rats[a]	L-Arginine–HCl (1.51 %) in drinking water	10 weeks	Body weight ↓ 16.0 % ($p<0.05$)	Abdominal fat ↓ 44.5 % ($p<0.05$)
Jobgen et al. [2]	Sprague–Dawley rats	L-Arginine–HCl (1.51 %) in drinking water	12 weeks	Low-fat diet: Body weight ↓ 68.1 % ($p<0.05$) High-fat diet: Body weight ↓ 40.7 % ($p<0.05$)	Low-fat diet: White fat ↓ 64.8 % ($p<0.05$) High-fat diet: White fat ↓ 63.4 % ($p<0.05$)
Suliburska et al. [18]	Wistar male rats	L-arginine 20 g/kg diet (with high-fat diet)	6 weeks	Body weight ↓ 2.9 % (NS)	

NS not significant
[a]Animal model for human type 2 diabetes mellitus with obesity

glucose profiles, while an improvement was seen in insulin sensitivity; however, in the Arg-supplemented group, the improvement was significantly greater (p values <0.0001 for most variables) [13].

The results of selected experimental and clinical studies are presented in Table 35.2.

The Effect of L-Arginine Supplementation on Insulin Sensitivity

Arg may affect the endocrine system. It was observed that elevated plasma levels of Arg correlate with alteration in the secretion of numerous cytokines and hormones. Those alterations, in turn, may affect insulin sensitivity and glucose and lipid metabolism [19].

The possible effect of Arg on insulin resistance is presently under discussion, and the results of previous clinical and experimental studies are not clear. The study performed by Wascher et al. indicated an improvement in insulin sensitivity during Arg supplementation. The study group consisted of seven healthy subjects, nine patients with obesity, and nine non-insulin-dependent diabetes mellitus

individuals. In order to assess insulin sensitivity, the authors measured insulin-mediated vasodilatation by venous occlusion plethysmography during the insulin suppression test. Experiments were performed twice on each subject in the presence or absence of a concomitant infusion of Arg (0.52 mg/kg/min). The authors concluded that insulin sensitivity was improved significantly in all three groups by the infusion of Arg [20]. During the long-term administration of Arg (9 g/day), improvements in peripheral and hepatic insulin sensitivity, acting through a normalization of the NO/cGMP pathway, have also been observed in diabetic patients [21]. Amelioration of insulin sensitivity was also emphasized by Luccotti et al. [13]. Other studies have shown that, in patients with cardiovascular disease, supplementation with Arg (6.4 g/day) serves to increase the insulin sensitivity index and adiponectin level. Following 6 months of treatment, a decrease in interleukin-6 and monocyte chemoattractant protein-1 levels was observed [22]. Suliburska et al. also provided evidence of the impact of Arg on insulin sensitivity in obese patients. Arg treatment (9 g/day) resulted in a significant increase in insulin sensitivity [16]. The beneficial effect of Arg on insulin sensitivity in patients with obesity was confirmed by Bogdanski et al. After 6 months of Arg supplementation (9 g/day), significant increases in NO, total antioxidant status (TAS), and insulin sensitivity level were noticed [23]. Interestingly, there are also studies in which improvement in insulin sensitivity during Arg supplementation was not observed [24]. Hormones released from white adipose tissue play a pivotal role in energy partitioning and influence insulin sensitivity [25]. Some clinical observations support the idea that rational manipulation of adipocytokines is a promising avenue for the therapy of obesity and associated metabolic abnormalities [26].

Mechanisms of the Favorable Effects of L-Arginine in Obesity

The mechanism responsible for the beneficial effects of Arg involves NO signaling, mitochondrial biogenesis, the growth of brown adipose tissue, and the regulation of fat metabolic gene expression (Fig. 35.1).

The NO Pathway in Lipid Metabolism

There is growing interest in NO, which is synthesized from Arg in almost all mammalian cells by NO synthase (NOS), in order to regulate energy and lipid metabolism [27]. Studies with knockout endothelial NOS mice have shown the importance of synthase in body fat accumulation [28]. Khedara et al., after feeding rats with the NOS inhibitor L-*N*(omega)nitroarginine for 8 weeks, observed a reduced combustion of body fat, leading to an increase in total body fat. The inhibition of NOS in this study was reversed by the addition of 4 % Arg to the rodent diet [29]. These findings providing evidence that NO may affect adiposity were also confirmed by Fu et al., who indicated that the increase in NO availability by Arg can improve lipolysis, as well as fatty acid and glucose oxidation [4]. It is believed that physiological levels of NO have a beneficial influence on the homeostasis of energy substrates involving fatty acid and glucose oxidation and affect energy metabolism in the whole body [30]. The underlying mechanism behind the stimulation of oxidation of the energy substrates may involve multiple cyclic guanosine monophosphate (cGMP)-dependent pathways in insulin-sensitive tissues [31]. Dai et al. highlighted the following functions of NO [30]. NO stimulates the phosphorylation of AMPK, which acts as a sensor of cellular energy. AMPK is activated by a rise in the intracellular AMP/adenosine triphosphate (ATP) ratio within the cell [32] and influences glucose transport by increasing GLUT4 translocation. It also decreases concentrations of malonyl-CoA (thus increasing the transport of long-chain fatty acids into mitochondria) and decreases the expression of genes related

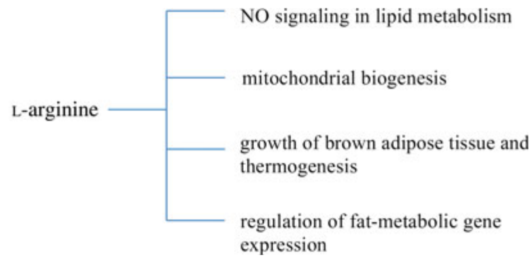

Fig. 35.1 Mechanism of the effects of L-arginine in obesity

to lipogenesis and gluconeogenesis. NO plays an important role in increasing the phosphorylation of hormone-sensitive lipase (HSL). HSL, which is considered to be a regulatory enzyme in lipolysis [33], is activated by cGMP-dependent protein kinase (PKG). This activation results in the translocation of the lipase to neutral lipid droplets and the stimulation of lipolysis in white adipose tissue [31]. NO is also a modulator of PGC-1α. The activation of PGC-1α by NO increases mitochondrial markers, demonstrating the induction of biosynthesis of functional mitochondria able to generate ATP via oxidative phosphorylation [34, 35]. The systemic role of NO (including interorgan cooperation) is to stimulate blood flow (enhancing the transport of fatty acids, glucose, and oxygen to insulin-sensitive tissues), promoting substrate uptake and product removal via circulation and improving mitochondrial oxidation of energy substrates [31, 36]. Arg increases blood flow to organs and thus enhances the uptake of energy substrates for oxidation and ATP production in various tissues in the body. This hemodynamic improvement is at least partly via NO-mediated mechanism. NO causes vasodilatation in blood vessels, and consequently blood pressure decreases and blood flow is improved. Moreover, hemodynamics may lead to improved exercise capacity, and it enables increased physical activity, which is an important aspect of the nonpharmacological treatment of obesity [12].

Mitochondrial Biogenesis

Mitochondria are sources of energy in their role producing ATP for cell metabolism. They are the major organelles for the complete oxidation of energy substrates and play a pivotal role in modifying adipocyte lipid metabolism and adipogenesis [31, 37]. It is known that PGC-1α, whose expression is influenced by NO [4], can induce mitochondrial biogenesis [38, 39]. During in vitro studies and experimental studies conducted on eNOS knockout mice, researchers have emphasized the crucial role of the NO-cGMP-dependent pathway for mitochondrial biogenesis and body energy balance [28, 35]. It is worth noting that Arg may upregulate nuclear transcription factors 1 and 2 expression and thereby enhance mitochondrial biogenesis [40]. An analysis of previous studies provides compelling evidence that Arg can stimulate mitochondrial biogenesis and brown adipose tissue development, while improving whole-body energy metabolism [19, 41] (Fig. 35.2).

Brown Adipose Tissue and Thermogenesis

Brown adipose tissue plays a crucial role in the oxidation of glucose, fatty acids, and some amino acids. It is also responsible for nonshivering thermogenesis in mammals. In contrast to white adipose tissue, brown adipose tissue consists of small brown adipocytes, containing a much greater number of

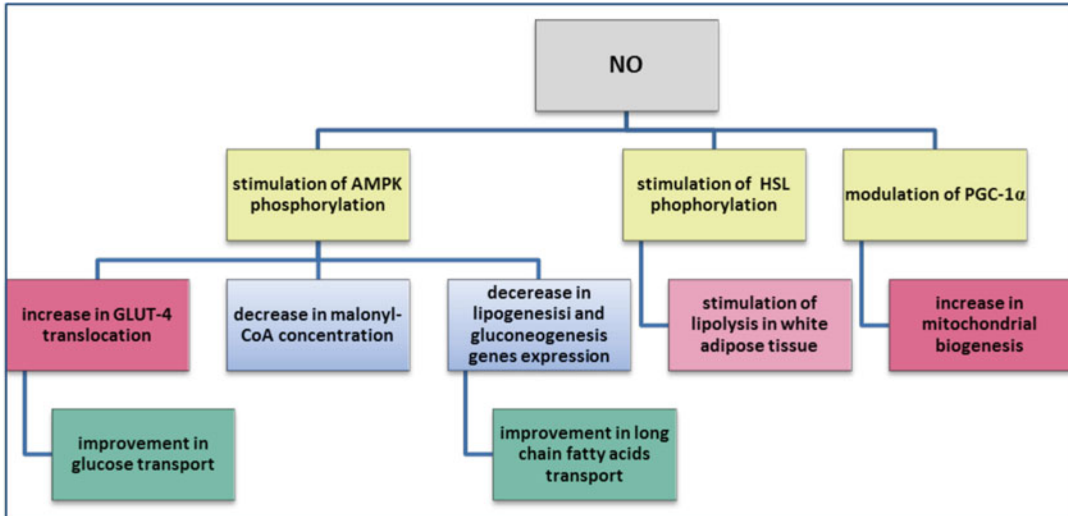

Fig. 35.2 Mechanism of NO to regulate energy, lipid, and glucose metabolism

mitochondria than in white adipose tissue. Mitochondria are also greater in size in brown adipose tissue [42, 43]. Brown adipose tissue is highly vascularized with blood vessels and can produce more heat than adipose tissue and other organs [44]. Interestingly, current evidence shows that brown adipose tissue exists in adult humans and can also play an important role in overall energy expenditure and heat production in adults, not just in neonates [45]. It has also been observed that brown adipose tissue activity is reduced in overweight and obese humans and is positively correlated with resting metabolic rate [46].

It has been observed in experimental and clinical studies that dietary Arg supplementation increases brown adipose tissue mass and mitochondrial biogenesis and thus ameliorates obesity. Observation of ZDF and DIO rats indicates that Arg supplementation significantly reduces white adipose tissue and increases brown adipose tissue mass [2, 4, 6]. These results are consistent with those obtained using growing–finishing pigs. During this study, it was indicated that lipid metabolism varies with the anatomical location of white adipose tissue [7]. Studies on animal models and studies with type 2 diabetes patients indicated the role of Arg in brown adipose tissue alteration [13]. Emerging reports propose that dietary supplementation with Arg stimulates brown adipose tissue growth and development through enhanced syntheses of NO, polyamines, and cyclic AMP [41].

Regulation of Fat Metabolic Gene Expression

A growing body of evidence emphasizes the role of Arg in the regulation of expression of various genes. Jobgen et al. analyzed global changes in gene expression by microarray in DIO rats supplemented with Arg. After 12 weeks of supplementation, they found that high-fat feeding decreased mRNA levels for lipogenic enzymes, AMPK, glucose transporters, HO-3, glutathione synthetase (GSS), superoxide dismutase 3, peroxiredoxin 5, glutathione peroxidase 3, and stress-induced protein. Conversely, the transcripts for carboxypeptidase A, peroxisome proliferator-activated receptor (PPAR)-α, caspase 2, caveolin 3, and diacylglycerol kinase were found to be upregulated in this study. Administration of Arg reduced mRNA levels for fatty acid-binding protein 1, glycogenin, protein

phosphatase 1B, caspases 1 and 2, and hepatic lipase but increased expression of PPARγ, HO-3, GSS, insulin-like growth factor 2, sphingosine-1-phosphate receptor, and stress-induced protein [40]. Changes in lipid metabolic gene expression have also been observed in studies conducted on pigs. Dietary Arg supplementation increased mRNA levels for HSL and decreased mRNA levels for lipoprotein lipase, GLUT4, and ACCα in subcutaneous adipose tissue. There were higher mRNA levels for fatty acid synthase in the skeletal muscle of Arg-supplemented pigs, compared to the control. These findings reveal an upregulation of lipogenic gene expression in skeletal muscle and a downregulation of lipogenic genes and an increase in lipolytic gene expression in white adipose tissue [5]. Together, these results indicate that Arg beneficially modulates gene expression to enhance energy substrate oxidation and to reduce white fat accretion in tissues [3].

Conclusions and Future Perspectives

Arg supplementation may represent a safe and efficient nutritional treatment for obesity. It has been demonstrated that Arg is stable under sterilization conditions and is not toxic to mammalian cells. A level of 85 mg/kg body mass of Arg is physiologically attainable when the human diet is supplemented with this amino acid. The Arg supplementation should be taken in divided doses each day to prevent gastrointestinal tract discomfort through the production of large amounts of NO, to increase the availability of circulating Arg over a longer period of time, and to avoid a potential imbalance among dietary amino acids [3].

Observations from recent studies indicate that Arg supplementation markedly reduces obesity in humans and animals. It beneficially alters hemodynamics in white adipose tissue and brown adipose tissue by increasing the oxidation of energy substrates. Studies have shown that Arg improves insulin sensitivity. Although a number of biochemical and molecular mechanisms have been proposed to explain the role of Arg in metabolism, the likely mechanisms are stimulation of NO signaling, mitochondrial biogenesis, growth of brown adipose tissue, regulation of fat metabolic gene expression, and fat tissue endocrine secretion. There are very promising reports, especially considering the good tolerance of Arg, its availability, and the low cost of treatment. Arg is inexpensive and readily available from natural foods. It seems that careful modulation of the Arg metabolic pathway through dietary supplementation may be beneficial in preventing and treating obesity, a problem that is currently growing worldwide. Although the present findings are very promising, further studies are necessary on the potential therapeutic role of Arg in obesity (Fig. 35.3).

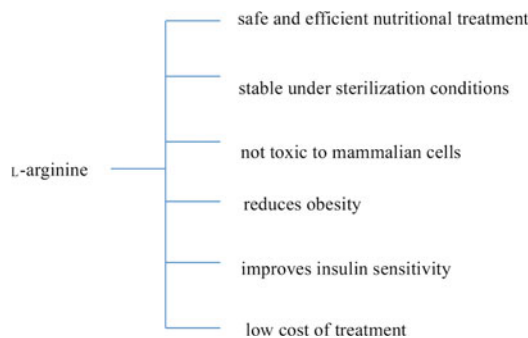

Fig. 35.3 Benefits to use L-arginine

References

1. World Health Statistics. http://www.who.int/gho/publications/world_health_statistics/en/. Accessed 12 Apr 2014.
2. Jobgen WS, Meininger CJ, Jobgen SC, et al. Dietary L-arginine supplementation reduces white fat gain and enhances skeletal muscle and brown fat masses in diet-induced obese rats. J Nutr. 2009;139:230–7.
3. McKnight JR, Satterfield MC, Jobgen WS, et al. Beneficial effects of L-arginine on reducing obesity: potential mechanisms and important implications for human health. Amino Acids. 2010;39:349–57.
4. Fu WJ, Haynes TE, Kohli R, et al. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr. 2005;135:714–21.
5. Tan B, Yin Y, Liu Z, et al. Dietary L-arginine supplementation differentially regulates expression of lipid-metabolic genes in porcine adipose tissue and skeletal muscle. J Nutr Biochem. 2011;22:441–5.
6. Wu G, Collins JK, Perkins-Veazie P, et al. Dietary supplementation with watermelon pomace juice enhances L-arginine availability and ameliorates the metabolic syndrome in Zucker diabetic fatty rats. J Nutr. 2007;137:2680–5.
7. Tan B, Yin Y, Liu Z, et al. Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. Amino Acids. 2009;37:169–75.
8. He QH, Kong XF, Wu G, et al. Metabolomic analysis of the response of growing pigs to dietary L-arginine supplementation. Amino Acids. 2009;37:199–208.
9. Go G, Wu G, Silvey D, et al. Lipid metabolism in pigs fed supplemental conjugated linoleic acid and/or dietary L-arginine. Amino Acids. 2012;43:1713–26.
10. Bai Y, Sun L, Yang T, et al. Increase in fasting vascular endothelial function after short-term oral L-arginine is effective when baseline flow-mediated dilation is low: a meta-analysis of randomised controlled trials. Am J Clin Nutr. 2009;89:77–84.
11. van Vught AJ, Heitmann BL, Nieuwenhuizen AG, et al. Association between dietary protein and change in body composition among children (EYHS). Clin Nutr. 2009;28:684–8.
12. Michishita T, Kobayashi S, Katsuya T, et al. Evaluation of the antiobesity effects of an amino acid mixture and conjugated linoleic acid on exercising healthy overweight humans: a randomized, double-blind, placebo-controlled trial. J Int Med Res. 2010;38:844–59.
13. Lucotti P, Setola E, Monti LD, et al. Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab. 2006;291:906–12.
14. Monti LD, Casiraghi MC, Setola E, Galluccio E, et al. L-arginine enriched biscuits improve endothelial function and glucose metabolism: a pilot study in healthy subjets and a cross-over study in subjects with impaired glucose tolerance and metabolic syndrome. Metabolism. 2013;62:255–64.
15. Bogdanski P, Suliburska J, Grabanska K, et al. Effect of 3-month L-arginine supplementation on insulin resistance and tumor necrosis factor activity in patients with visceral obesity. Eur Rev Med Pharmacol Sci. 2012;16:816–23.
16. Suliburska J, Bogdanski P, Szulinska M, et al. Changes in mineral status are associated with improvements in insulin sensitivity in obese patients following L-arginine supplementation. Eur J Nutr. 2014;53:387–93.
17. Hurt RT, Ebbert JO, Schroeder DR, et al. L-arginine for the treatment of centrally obese subjects: a pilot study. J Diet Suppl. 2014;11:40–52.
18. Suliburska J, Bogdanski P, Krejpcio Z, et al. The effects of L-arginine, alone and combined with vitamin C, on mineral status in relation to its antidiabetic, anti-inflammatory, and antioxidant properties in male rats on a high-fat diet. Biol Trace Elem Res. 2014;157:67–74.
19. Tan B, Li X, Wu Z, et al. Regulatory roles for L-arginine in reducing white adipose tissue. Front Biosci. 2012;17:2237–46.
20. Wascher TC, Graier WF, Dittrich P, et al. Effects of low-dose L-arginine on insulin-mediated vasodilatation and insulin sensitivity. Eur J Clin Invest. 1997;27:690–5.
21. Piatti PM, Monti LD, Valsecchi G, et al. Long-term oral L-arginine administration improves peripheral and hepatic insulin sensitivity in type 2 diabetic patients. Diabetes Care. 2001;24:875–80.
22. Lucotti P, Monti L, Setola E, et al. Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. Metabolism. 2009;58:1270–6.
23. Bogdanski P, Szulinska M, Suliburska J, et al. Supplementation with L-arginine favorably influences plasminogen activator inhibitor type 1 concentration in obese patients. A randomized, double blind trial. J Endocrinol Invest. 2013;36:221–6.
24. Kawano T, Nomura M, Nisikado A, et al. Supplementation of L-arginine improves hypertension and lipid metabolism but not insulin resistance in diabetic rats. Life Sci. 2003;73:3017–26.
25. Dyck DJ, Heigenhauser GJ, Bruce CR. The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity. Acta Physiol. 2006;186:5–16.
26. Cao H. Adipocytokines in obesity and metabolic disease. J Endocrinol. 2014;220:47–59.

27. Joffin N, Niang F, Forest C, et al. Is there NO help for leptin? Biochimie. 2012;94:2104–10.
28. Nisoli E, Clementi E, Paolucci C, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science. 2003;299:896–9.
29. Khedara A, Goto T, Morishima M, et al. Elevated body fat in rats by the dietary nitric oxide synthase inhibitor, L-N omega nitroarginine. Biosci Biotechnol Biochem. 1999;63:698–702.
30. Dai Z, Wu Z, Yang T, et al. Nitric oxide and energy metabolism in mammals. Biofactors. 2013;39:383–91.
31. Jobgen WS, Fried SK, Fu WJ, et al. Regulatory role for the L-arginine-nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem. 2006;17:571–88.
32. Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest. 2006;116:1776–83.
33. Kraemer FB, Shen WJ. Hormone-sensitive lipase: control of intracellular tri-(di)acylglycerol and cholesteryl ester hydrolysis. J Lipid Res. 2002;43:1585–94.
34. Lira VA, Brown DL, Lira AK, et al. Nitric oxide and ampk cooperatively regulate pgc-1 in skeletal muscle cells. J Physiol. 2010;588:3551–66.
35. Nisoli E, Falcone S, Tonello C, et al. Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci USA. 2004;101:16507–12.
36. Dillon EL, Casperson SL, Durham WJ, et al. Muscle protein metabolism responds similarly to exogenous amino acids in healthy younger and older adults during NO-induced hyperemia. Am J Physiol Regul Integr Comp Physiol. 2011;301:1408–17.
37. Vankoningsloo S, Piens M, Lecocq C, et al. Mitochondrial dysfunction induces triglyceride accumulation in 3T3-L1 cells: role of fatty acid beta-oxidation and glucose. J Lipid Res. 2005;46:1133–49.
38. Lehman JJ, Barger PM, Kovacs A, et al. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J Clin Invest. 2000;106:847–56.
39. Karamitri A, Shore AM, Docherty K, et al. Combinatorial transcription factor regulation of the cyclic AMP-response element on the Pgc-1α promoter in white 3T3-L1 and brown HIB-1B preadipocytes. J Biol Chem. 2009;284:20738–52.
40. Jobgen WJ, Fu WJ, Gao H, et al. High fat feeding and dietary L-arginine supplementation differentially regulate gene expression in rat white adipose tissue. Amino Acids. 2009;3:187–98.
41. Wu Z, Satterfield MC, Bazer FW, et al. Regulation of brown adipose tissue development and white fat reduction by L-arginine. Curr Opin Clin Nutr Metab Care. 2012;15:529–38.
42. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84:277–359.
43. Tupone D, Madden CD, Morrison SF. Autonomic regulation of brown adipose tissue thermogenesis in health and disease: potential clinical applications for altering BAT thermogenesis. Front Neurosci. 2014;8:14.
44. Satterfield MC, Wu G. Brown adipose tissue growth and development: significance and nutritional regulation. Front Biosci (Landmark Ed). 2011;16:1589–608.
45. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. Am J Physiol Endocrinol Metab. 2007;293:444–52.
46. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med. 2009;360:1500–8.

Chapter 36
Mitochondrial Cardiomyopathy and Usage of L-Arginine

Kenichiro Arakawa, Masamichi Ikawa, Hiroshi Tada, Hidehiko Okazawa, and Makoto Yoneda

Key Points

- Cardiomyopathy is present in 17–40 % of patients with mitochondrial disease and is one of the major causes of death in such patients.
- MELAS is a syndrome caused by an A-to-G transition at nucleotide position 3243 in tRNA-Leu of mtDNA and is the most common type of mitochondrial disease.
- In vivo functional imaging makes it possible to evaluate aspects of energy metabolism such as membrane potential and TCA cycle kinetics in MELAS patients noninvasively.
- L-Arg therapy is a promising approach for controlling the stroke-like episode of MELAS because of its vasodilative effect.
- L-Arg also has the potential to accelerate TCA cycle activity, irrespective of its vasodilative effect, and this can be used for treatment of mitochondrial cardiomyopathy.

Keywords Cardiomyopathy • MELAS • SPECT • PET • L-Arginine • TCA cycle

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Abbreviations

MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
mtDNA	Mitochondrial DNA
ATP	Adenosine triphosphate
LVH	Left ventricular hypertrophy
Arg	L-Arginine
NOx	Nitric oxide
SPECT	Single-photon emission computed tomography
^{99m}Tc-MIBI	Technetium 99 m methoxyisobutylisonitrile
^{123}I-BMIPP	Iodine-123-labeled 15-4-iodophenyl-3-(*R*,*S*)-methyl-pentadecanoic acid
PET	Positron emission tomography
TCA	Tricarboxylic acid
MBF	Myocardial blood flow

Introduction

It is well known that the most common morphology of cardiomyopathy is hypertrophy of the left ventricle. Practically, it is diagnosed as idiopathic hypertrophic cardiomyopathy, although occasionally it occurs secondary to systemic disease. The etiology of hypertrophic cardiomyopathy varies and can include ischemia, valve disease, inflammation, muscle dystrophy, toxemia, collagen disease, and metabolic diseases such as amyloidosis, Fabry's disease, and mitochondrial disease [1]. Accordingly, the treatment and prognosis of each individual disease differ, making a correct diagnosis important.

A recent epidemiological study has revealed that the prevalence or risk of developing mitochondrial DNA (mtDNA) disease is 12.48 per 100,000 individuals in the general population [2]. Moreover, pathogenic mtDNA mutations that can potentially cause disease are detected in at least one in 200 live births, indicating that mtDNA is not as rare a disease as once thought previously [3].

The human mitochondrial genome disorders discovered up to the present are cited in MITOMAP (URL: http://www.mitomap.org/), and more than 40 mutations of mtDNA or nuclear DNA associated with structural mitochondrial cardiomyopathy have been reported (Tables 36.1, 36.2, and 36.3). Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is the most common type of mitochondrial disease and is also related to familial cardiomyopathy, which is caused by an A-to-G transition at position 3243 (A3243G) in tRNA-Leu of the mtDNA [4, 5]. This mutation reduces the activity of NADH–ubiquinone oxidoreductase (complex I), leading to impairment of respiratory chain function with consequent reduction of adenosine triphosphate (ATP) production [6]. Furthermore, this mutant and wild-type mtDNA coexist in each individual cell (heteroplasmy), and the proportion of mutant mtDNA must exceed a certain fixed level in order to result

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Table 36.1 mtDNA mutations in rRNA/tRNA regions causing cardiomyopathy

Position	Locus	Disease	Allele	RNA	Homoplasmy	Heteroplasmy
1391	MT-RNR1	HCM	T1391C	12S rRNA	+	–
1556	MT-RNR1	HCM	C1556T	12S rRNA	+	–
1644	MT-TV	HCM + MELAS	G1644A	tRNA Val	–	+
3242	MT-TL1	MM/HCM + renal tubular dysfunction	G3242A	tRNA-Leu (UUR)	+	+
3243	MT-TL1	DMDF/MIDD/SNHL/FSGS/ cardiac + multiorgan dysfunction	A3243G	tRNA-Leu (UUR)	–	+
3260	MT-TL1	MMC/MELAS	A3260G	tRNA-Leu (UUR)	–	+
3303	MT-TL1	MMC	C3303T	tRNA-Leu (UUR)	+	+
4269	MT-TI	FICP	A4269G	tRNA Ile	–	+
4295	MT-TI	MHCM/maternally inherited hypertension	A4295G	tRNA Ile	+	+
4316	MT-TI	HCM with hearing loss/poss. hypertension factor	A4316G	tRNA Ile	+	+
4317	MT-TI	FICP/poss. hypertension factor	A4317G	tRNA Ile	+	–
5545	MT-TW	HCM severe multisystem disorder	C5545T	tRNATrp	–	+
8296	MT-TK	DMDF/MERRF/HCM/epilepsy	A8296G	tRNA Lys	+	+
8348	MT-TK	Cardiomyopathy/SNHL/poss. hypertension factor	A8348G	tRNA Lys	+	+
8363	MT-TK	MICM+DEAF/MERRF/autism/ LS/ataxia + lipomas	G8363A	tRNA Lys	–	+
9997	MT-TG	MHCM	T9997C	tRNAGly	nd	+
12297	MT-TL2	Dilated cardiomyopathy/LS/ failure to thrive and LA	T12297C	tRNA-Leu (CUN)	+	+
12308	MT-TL2	CPEO/stroke/CM/breast and renal and prostate cancer risk/altered brain pH	A12308G	tRNA-Leu (CUN)	+	+
15923	MT-TT	Infantile CM	A15923G	tRNAThr	–	+
16032	MT-TP	Dilated cardiomyopathy	[a]	tRNA Pro	–	+

HCM hypertrophic cardiomyopathy, *MM* mitochondrial myopathy, *DMDF* diabetes mellitus + deafness, *MIDD* maternally inherited diabetes and deafness, *SNHL* sensorineural hearing loss, *FSGS* focal segmental glomerulosclerosis, *MMC* maternal myopathy and cardiomyopathy, *FICP* fatal infantile cardiomyopathy + a MELAS-associated cardiomyopathy, *MHCM* maternally inherited hypertrophic cardiomyopathy, *MERRF* myoclonic epilepsy and ragged-red muscle fibers, *MICM* maternally inherited cardiomyopathy, *DEAF* maternally inherited deafness or aminoglycoside-induced deafness, *LS* Leigh syndrome, *LA* lactic acidemia, *CPEO* chronic progressive external ophthalmoplegia, *CM* cardiomyopathy

[a]T16032TTCTCTGTTCTTTCAT (15 bp dup) (cited from MITOMAP and adapted to the text contents)

in clinically apparent respiratory chain failure [7, 8]. Thus, energy production differs from tissue to tissue and also among organs, markedly energy-dependent organs tending to be affected most significantly. The distinct clinical features of MELAS patients are systemic and include myopathy, lactic acidosis, stroke-like episodes, hearing loss, diabetes mellitus, gastrointestinal manifestations, renal failure, and cardiomyopathies [4, 7, 8].

Mitochondrial cardiomyopathy often results in concentric left ventricular hypertrophy (LVH), and the severity of the LVH correlates with the burden of mitochondrial disease (Fig. 36.1). The reasons for development of LVH have been investigated using knockout mice with a deficiency in the mitochondrial adenine nucleotide translocator [9]. Like MELAS patients, these experimental mice show

Table 36.2 mtDNA mutations in the coding/control genes causing cardiomyopathy

Position	Locus	Disease	Allele	Nucleotide change	Amino acid change	Homoplasmy	Heteroplasmy
3337	MT-ND1	Cardiomyopathy	G3337A	G-A	V-M	+	–
3395	MT-ND1	HCM with hearing loss	A3395G	A-G	Y-C	–	+
3397	MT-ND1	ADPD/possibly LVNC cardiomyopathy associated	A3397G	A-G	M-V	+	–
3407	MT-ND1	HCM/muscle involvement	G3407A	G-A	R-H	+	–
5001	MT-ND2	Developmental delay, seizure, cardiomyopathy, lactic acidosis	A5001AA	A-AA	Frameshift	–	+
8528	MT-ATP8/6	Infantile cardiomyopathy	T8528C	T-C	W-R (ATP); M(start)-T(ATP6)	+	+
8558	MT-ATP8/6	Possibly LVNC cardiomyopathy associated	C8558T	C-T	P-S(ATP8); A-V(ATP6)	+	–
9058	MT-ATP6	Possibly LVNC cardiomyopathy associated	A9058G	A-G	T-A	+	–
15498	MT-CYB	HCM/WPW, DEAF	G15498A	G-A	G-D	–	+
15693	MT-CYB	Possibly LVNC cardiomyopathy associated	T15693C	T-C	M-T	+	–

ADPD Alzheimer's disease and Parkinson's disease, *LVNC* left ventricular noncompaction, *WPW* Wolff–Parkinson–White syndrome (cited from MITOMAP and adapted to the text contents)

Table 36.3 Nuclear DNA mutations causing mitochondrial cardiomyopathy

Gene	Chromosome function	Chromosome	Inheritance	Clinical phenotype
Structural gene				
NDUFV2	FP fraction	18p11	AR	Cardiomyopathy, hypotonia, encephalopathy
Complex assembly				
NDUFAF1 (CIA30)	Assembly	15q13.3	AR	Cardioencephalopathy
SCO2	Copper transport	22q13	AR	Neonatal cardioencephalomyopathy
COX10	Heme A farnesyltransferase	17p12–p11.2	AR	Neonatal tubulopathy and encephalopathy, LS, cardiomyopathy
COX15	Heme A synthesis	10q24	AR	Early-onset hypertrophic cardiomyopathy
TMEM70	Assembly	8q21.11	AR	Neonatal encephalopathy, cardiomyopathy
Mitochondrial import				
DNAJC19	Protein import	3q26.3	AR	Cardiomyopathy, ataxia
Mt protein synthesis				
MRPS22	Mitochondrial translation	3q23	AR	Cardiomyopathy, tubulopathy
Iron homeostasis				
BOLA3	Iron–sulfur cluster biosynthesis	2p13.1	AR	Encephalomyopathy, cardiomyopathy
CoQ10 biosynthesis				
COQ9	CoQ10 deficiency	16q13	AR	Neonatal lactic acidosis, seizures, cardiomyopathy
Chaperon function				
G4.5 (tafazzin)	Cardiolipin defect	Xq28	X linked	Barth syndrome, X-linked dilated cardiomyopathy

FP flavin protein, *AR* autosomal recessive, *CoQ* coenzyme Q (cited from MITOMAP and adapted to the text contents)

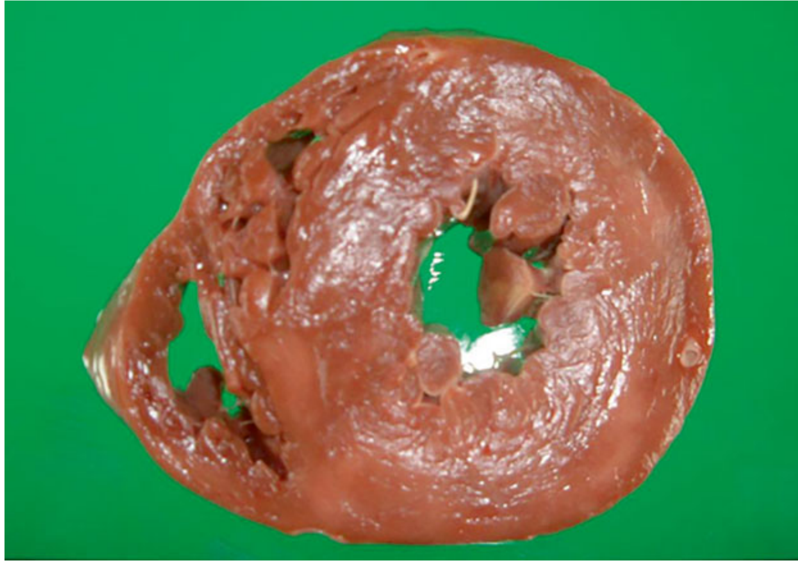

Fig. 36.1 Representative photograph of hypertrophic cardiomyopathy of a patient with mitochondrial disease

ragged-red muscle fibers, lactic acidosis, and cardiac hypertrophy, suggesting that deficiency of ATP production plays an important role in these conditions. On the other hand, a rare form of dilated-type mitochondrial cardiomyopathy has also been reported [10, 11]. A subset of patients with LVH progress to the dilated phase, which resembles idiopathic hypertrophic cardiomyopathy [12], but in some cases dilated cardiomyopathy is already present in childhood [13]. This discrepancy has been explained using a transgenic mouse model of mtDNA mutations, in which increased production of mitochondrial reactive oxygen species during the aging process leads to initiation of apoptosis and plays a crucial role in the development of dilated cardiomyopathy [14].

The frequency of cardiomyopathy in patients with mitochondrial disease is reported to be 17–40 % and is one of the major causes of death in affected patients [15–17]. Unfortunately no effective therapies for cardiomyopathy have been found to date. Koga et al. reported that L-arginine (Arg) infusion during the acute phase of the stroke-like episodes in MELAS patients dramatically improved all of the stroke-like symptoms within 30 min [18]. Moreover, oral administration of L-Arg during the interictal phase significantly decreased the frequency and severity of stroke-like episodes in MELAS patients [19]. L-Arg therapy is therefore now a promising approach for controlling the stroke-like episode of MELAS. Here we further investigated the therapeutic effect of L-Arg infusion in patients with cardiomyopathy and the possible mechanisms responsible.

In Vivo Functional Imaging of Mitochondrial Cardiomyopathy

Although the histopathologic abnormalities of mitochondrial cardiomyopathy have been clearly revealed using autopsied and/or biopsied tissue samples, the pathogenesis of cardiomyopathy has been discussed largely on the basis of the experimental studies [9, 14, 20]. Here we evaluated energy states in the myocardium of patients with MELAS using in vivo functional imaging.

Evaluation of Mitochondrial Membrane Potential and the Anaerobic Pathway Using Single-Photon Emission Computed Tomography (SPECT)

Technetium 99 m methoxyisobutylisonitrile (^{99m}Tc-MIBI) is incorporated and retained in the mitochondria of myocardial cells, a process that depends on mitochondrial membrane potential [21]. This tracer is not retained in necrotic or irreversibly ischemic myocardium and therefore can be used for assessing myocardial perfusion and myocardial cell viability [22].

Iodine-123-labeled 15-4-iodophenyl-3-(*R*,*S*)-methyl-pentadecanoic acid (^{123}I-BMIPP) is converted to acyl-CoA, a common pathway of myocardial fatty acid metabolism, but is not metabolized via beta-oxidation, which reflects the enhanced triglyceride pool [23]. An increasing number of studies have reported that patients with idiopathic hypertrophic cardiomyopathy show reduced uptake of ^{123}I-BMIPP and that this is related to impairment of the plasma membrane of cardiac myocytes [24].

Using these two tracers, we recently reported that in MELAS patients, the ^{99m}Tc-MIBI washout rate (WOR) was increased, resulting in decreased uptake of ^{99m}Tc-MIBI (Fig. 36.2) [25]. In contrast, ^{123}I-BMIPP uptake increased according to the severity of left ventricular function (Fig. 36.2) [25]. These findings confirmed that respiratory chain failure leads to a continuous energy shift from the aerobic to the anaerobic (glycolytic) pathway, resulting in the lactic acidemia that is observed in MELAS patients. To ameliorate the over-reduction stress resulting from respiratory chain failure, reduction of dihydroxyacetone phosphate to glycerol-3-phosphate occurs in order to oxidize superfluous nicotinamide adenine dinucleotide [NADH] to [NAD$^+$], the excess glycerol-3-phosphate being utilized for synthesis of triglyceride. Accumulation of ^{123}I-BMIPP in MELAS patients was provoked by this enhanced triglyceride pool (Fig. 36.2) [25].

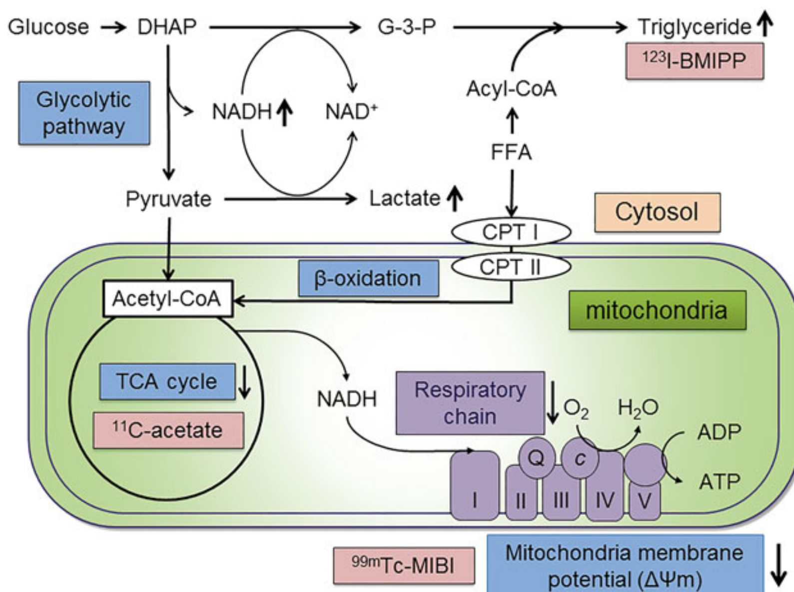

Fig. 36.2 Schematic illustration of energy production pathways in which functional imaging can be adapted. ^{99m}Tc-MIBI is incorporated and retained in the mitochondria depending on mitochondrial membrane potential created by the respiratory chain. ^{123}I-BMIPP is incorporated into the TG pool, associated with an excess of glycerol-3-phosphate (G-3-P), and is enhanced by increased glucose utilization. ^{11}C-acetate PET is responsible for the flux of TCA cycle. *CPT* carnitine palmitoyltransferase, *FFA* free fatty acid (cited from Ref. [25] with modifications)

Evaluation of TCA Cycle Kinetics Using Positron Emission Tomography (PET)

Radiolabeled ^{11}C-acetate kinetics demonstrated by PET are closely correlated with myocardial oxygen consumption [26, 27]. The acetate is known to be a substrate that can be utilized readily by the heart and is incorporated directly into the tricarboxylic acid (TCA) cycle after conversion to acetyl CoA. Therefore, ^{11}C-acetate can be used to measure the flux of the TCA cycle without being affected by conditions of energy production in the heart such as normoxemia, ischemia, and reperfusion, which advantages over other conventional tracers such as ^{18}F-deoxyglucose and ^{11}C-palmitate [28].

^{11}C-acetate PET also has the potential for detecting myocardial blood flow (MBF) using the early-phase (0–3 min after tracer injection) kinetics of ^{11}C-acetate [29]. Since the flux of the TCA cycle was measured using the delayed-phase (7–20 min after injection) kinetics of ^{11}C-acetate, these two parameters can be measured in exactly the same location in the heart.

Our SPECT study in MELAS patients with cardiomyopathy demonstrated a shift in energy production from the aerobic to the anaerobic pathway [25], although TCA cycle activity, which is of central importance in oxidative metabolism, was not fully evaluated. We therefore applied ^{11}C-acetate PET to MELAS patients and compared the findings with those in healthy controls [30]. The results revealed that TCA cycle activity tended to be lower in the patients than in the controls, thus confirming a shift of energy production to the anaerobic pathway according to impairment of electron transport and oxidative phosphorylation resulting from respiratory chain failure (Fig. 36.2) [25].

Effect of L-Arginine Administration on Mitochondrial Cardiomyopathy Evaluated by ^{11}C-Acetate PET

As described at the beginning of this chapter, L-Arg administration is now a promising therapy for the acute and interictal phase of the stroke-like episodes in MELAS patients [19]. One suggested mechanism is that L-Arg, which is a precursor of nitric oxide (NOx), may increase blood flow in the cerebral microcirculation and reduce ischemic damage to the brain. From the fact that the concentrations of L-Arg, citrulline, and NOx were low in the acute phase of the stroke-like episodes in MELAS patients, it seems plausible to supplement the amounts of these substances [19]. An improvement of endothelial function in MELAS patients was also observed after oral L-Arg supplementation, which would explain the long-term outcome [31]. As the impact of L-Arg administration on mitochondrial cardiomyopathy has not yet been reported, we recently evaluated the acute effect of L-Arg administration on cardiomyopathy using ^{11}C-acetate PET [30].

We performed ^{11}C-acetate PET before and after L-Arg infusion (0.5 g/kg, within 30 min) in six patients with clinically and genetically diagnosed MELAS. After L-Arg injection, TCA cycle activity (expressed as K_{mono}) of the entire heart did not increase significantly, although four of the six patients showed improvement after L-Arg administration. Due to heteroplasmy, mitochondrial dysfunction occurs in various tissues to varying degrees, a phenomenon known as “mosaicism of mitochondrial disease.” Therefore, we further divided the heart into nine segments. TCA cycle activity was improved after L-Arg injection among six to eight segments in four responders, whereas it was five segments in two nonresponders. On the other hand, MBF increased in two patients, decreased in two patients, and remained the same in two patients after L-Arg infusion. To analyze the relationship between TCA cycle activity and MBF, we prepared a bull’s-eye map of these two parameters before and after L-Arg injection. Figure 36.3 shows representative data for a MELAS patient who showed an increase of TCA cycle activity after L-Arg infusion. Surprisingly, the regions of improved TCA cycle activity did not correspond to the regions of increased MBF.

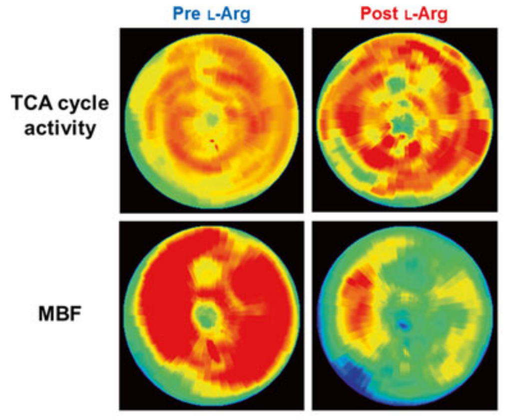

Fig. 36.3 Representative bull's-eye map of TCA cycle activity (*upper deck*) and myocardial blood flow (MBF; *lower deck*) before and after L-arginine administration in MELAS patients (cited from Ref. [30])

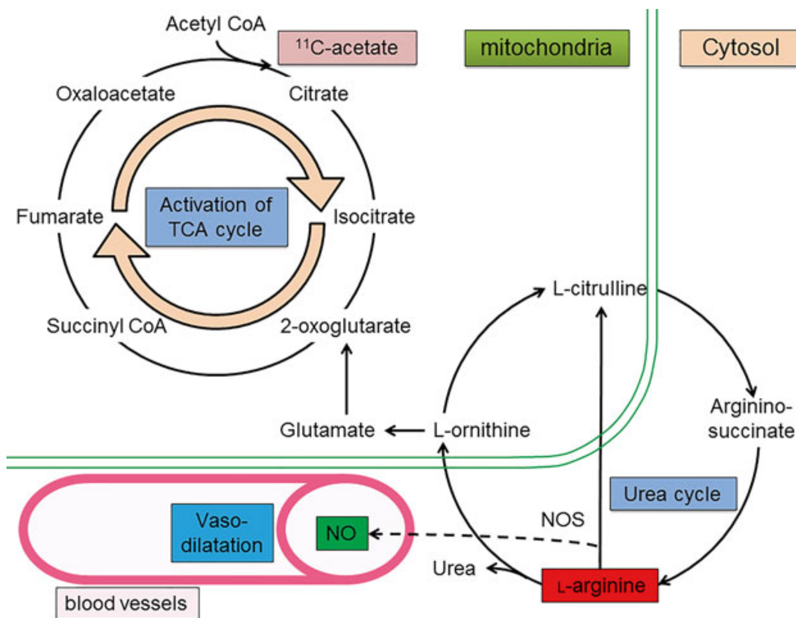

Fig. 36.4 Schematic illustration of L-Arg catabolism. Nitric oxide (NO) is synthesized from L-Arg catalyst of nitric oxide synthase (NOS). L-Arg has another potential to enter the TCA cycle by conversion to 2-oxoglutarate

L-Arg is a well-known precursor of NOx affected by endothelial nitric oxide synthase, a strong endogenous vasodilator [32, 33]. Accordingly, we expected that the regions of improved TCA cycle activity would match the regions of increased MBF, but no such relationship was observed. Although the reason for this remains obscure, Arg has a wide range of biological roles, such as a precursor for synthesis of urea, NOx, citrulline, ornithine, creatine, and agmatine. Furthermore, ornithine generates polyamine, proline, and particularly glutamate, which undergoes conversion to 2-oxoglutarate and enters the TCA cycle (Fig. 36.4). Therefore, an excess of 2-oxoglutarate in the TCA cycle induced by L-Arg injection could be responsible for acceleration of TCA cycle activity with little relevance to the coronary microcirculation.

The primary cause of the stroke-like episodes in MELAS patients remains uncertain but is thought to involve angiopathy, cytopathy, or both. Potential therapeutic effects of L-Arg for strokes are mainly thought to contribute to amelioration of angiopathy through its vasodilative effect and improvement of endothelial function. The logic of this approach is result from the loss of NOx in vascular endothelial and smooth muscle cells. However, the concentration of NOx was quite elevated in the interictal phase of stroke-like episodes [19]. Moreover, an in vitro experimental study has revealed that the synthesis of NOx was increased in cybrid cells carrying the A3243G mutation, which supports this condition [34]. Our study suggests that L-Arg enhances TCA cycle activity irrespective of vasodilation, which rescues the cytopathy (over-reduction stress) of MELAS patients. A recent study has also revealed that L-Arg improved the activity of complex I activity, a nonvascular system, in cybrid cells harboring A3243G mutation, thus strongly supporting our hypothesis regarding the metabolic effect of L-Arg [35].

Accordingly, our study has clearly demonstrated that L-Arg has dual pharmaceutical effects—vasodilatation (angiopathy) and acceleration of the TCA cycle (cytopathy)—which can be used as a treatment for patients with mitochondrial cardiomyopathy.

Conclusions

Mitochondrial cardiomyopathy is caused by respiratory chain failure due to mtDNA mutation, one of the key conditions that determine the prognosis of patients with mitochondrial disease. Functional imaging modalities such as SPECT and PET enable evaluation of in vivo energy production and the efficacy of treatment for patients with MELAS. It was clearly revealed that TCA cycle activity was markedly suppressed, resulting in a change in oxidative metabolism from an aerobic to an anaerobic state. L-Arg has the potential to enhance TCA cycle activity without being affected by any vasodilative effect, suggesting dual pharmaceutical effects that could be applied for treatment of mitochondrial cardiomyopathy.

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Conflict of Interest None.

References

1. Richardson P, Mckenna W, Bristow M, et al. Report of the 1995 World Health Organization/International society and federation of cardiology task force on the definition and classification of cardiomyopathies. Circulation. 1996;93:841–2.
2. Chinnery PF, Johnson MA, Wardell TM, et al. The epidemiology of pathogenic mitochondrial DNA mutations. Ann Neurol. 2000;48:188–93.
3. Elliott HR, Samuels DC, Eden JA, et al. Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet. 2008;83:254–60.
4. Pavlakis SG, Phillips PC, DiMauro S, et al. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes: a distinctive clinical syndrome. Ann Neurol. 1984;16:481–8.
5. Förster C, Hübner G, Müller-Höcker J, et al. Mitochondrial angiopathy in a family with MELAS. Neuropediatrics. 1992;23:165–8.
6. Ichiki T, Tanaka M, Nishikimi M, et al. Deficiency of complex I and mitochondrial encephalomyopathy. Ann Neurol. 1988;23:287–94.

7. Holt IJ, Harding AE, Morgan-Hughes JA. Deletion of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature. 1988;331:717–9.
8. Schon EA, Bonilla E, DiMauro S. Mitochondrial DNA mutations and pathogenesis. J Bioenerg Biomembr. 1997;29:131–49.
9. Graham BH, Waymire KG, Cottrell B, et al. A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. Nat Genet. 1997;16:226–34.
10. Majamaa-Voltti K, Peuhkurinen K, Kortelainen ML, et al. Cardiac abnormalities in patients with mitochondrial DNA mutation 3243A>G. BMC Cardiovasc Disord. 2002;2:12.
11. Chinnery PF. Mitochondrial disorders overview. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle, WA: University of Washington; 1993–2014
12. Ten Cate FJ, Roelandt J. Progression to left ventricular dilatation in patients with hypertrophic obstructive cardiomyopathy. Am Heart J. 1979;97:762–5.
13. Vilarinho L, Santorelli FM, Osas MJ, et al. The mitochondrial A3243G mutation presenting as severe cardiomyopathy. J Med Genet. 1997;34:607–9.
14. Wallace DC. Mitochondrial defects in cardiomyopathy and neuromuscular disease. Am Heart J. 2000;139:70–85.
15. Holmgren D, Wahlander H, Eriksson BO, et al. Cardiomyopathy in children with mitochondrial disease. Eur Heart J. 2003;24:280–8.
16. Scaglia F, Towbin JA, Craigen WJ, et al. Clinical spectrum, morbidity, and mortality in 113 pediatric patients with mitochondrial disease. Pediatrics. 2004;114:925–31.
17. Anan R, Nakagawa M, Miyata M, et al. Cardiac involvement in mitochondrial disease: a study on 17 patients with documented mitochondrial DNA defects. Circulation. 1995;91:955–61.
18. Koga Y, Akita Y, Nishioka J, et al. L-arginine improves the symptom of strokelike episodes in MELAS. Neurology. 2005;64:710–2.
19. Koga Y, Akita Y, Nishioka J, et al. MELAS and L-arginine therapy. Mitochondrion. 2007;7:133–9.
20. Ban S, Mori N, Saito K, et al. An autopsy case of mitochondrial encephalomyopathy (MELAS) with special reference to extra-neuromuscular abnormalities. Acta Pathol Jpn. 1992;42:818–25.
21. Carvalho PA, Chiu ML, Kronauge JF, et al. Subcellular distribution and analysis of technetium-99m-MIBI in isolated perfused rat hearts. J Nucl Med. 1992;33:1516–22.
22. Crane P, Laliberte R, Heminway S, et al. Effect of mitochondrial viability and metabolism on technetium-99m-sestamibi myocardial retention. Eur J Nucl Med. 1993;20:20–5.
23. Knapp Jr FF, Ambrose KR, Goodman MM. New radioiodinated methyl-branched fatty acids for cardiac studies. Eur Nucl Med. 1986;12:39–44.
24. Nakamura T, Suguhara H, Kinoshita N, et al. Serum carnitine concentrations in patients with idiopathic hypertrophic cardiomyopathy: relationship with impaired myocardial fatty acid metabolism. Clin Sci. 1999;97:493–501.
25. Ikawa M, Kawai Y, Arakawa K, et al. Evaluation of respiratory chain failure in mitochondrial cardiomyopathy by assessments of ^{99m}Tc-MIBI washout and ^{123}I-BMIPP/^{99m}Tc-MIBI mismatch. Mitochondrion. 2007;7:164–70.
26. Klein LJ, Visser FC, Knaapen P, et al. Carbon-11 acetate as a tracer of myocardial oxygen consumption. Eur J Nucl Med. 2001;28:651–68.
27. Buxton DB, Nienaber CA, Luxen A, et al. Noninvasive quantitation of regional myocardial oxygen consumption in vivo with [1-^{11}C]acetate and dynamic positron emission tomography. Circulation. 1989;79:134–42.
28. Brown M, Marshall DR, Sobel BE, et al. Delineation of myocardial oxygen utilization with carbon-11-labeled acetate. Circulation. 1987;76:687–96.
29. Kudo T, Hata T, Kagawa S, et al. Simple quantification of myocardial perfusion by pixel-by-pixel graphical analysis using carbon-11 acetate and nitrogen-13 ammonia. Nucl Med Commun. 2008;29:679–85.
30. Arakawa K, Kudo T, Ikawa M, et al. Abnormal myocardial energy-production state in mitochondrial cardiomyopathy and acute response to L-arginine infusion. Circ J. 2010;74:2702–11.
31. Koga Y, Akita Y, Junko N, et al. Endothelial dysfunction in MELAS improved by L-arginine supplementation. Neurology. 2006;6:1766–9.
32. Cooke JP, Andon NA, Girerd XJ, et al. L-Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta. Circulation. 1991;83:1118–20.
33. Tsao PS, McEvoy LM, Drexler H, et al. Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. Circulation. 1994;89:2176–82.
34. Gamba J, Gamba LT, Rodrigues GS, et al. Nitric oxide synthesis is increased in cybrid cell with m.3243A>G mutation. Int J Mol Sci. 2013;14:394–410.
35. Desquiret-Dumas V, Gueguen N, Barth M, et al. Metabolically induced heteroplasmy shifting and L-arginine treatment reduce the energetic defect in a neuronal-like model of MELAS. Biochim Biophys Acta. 2012; 1822:1019–29.

Chapter 37
L-Arginine Measurement and Concentrations in Hypertension and Other Cardiovascular Disease

Geoffrey P. Candy and Cameron Naidoo

Key Points

- Extracellular L-arginine regulates nitric oxide production in many cell types.
- Impaired uptake of L-arginine into cells has been demonstrated in various cardiovascular diseases.
- L-Arginine concentrations are increased in patients with hypertension and in the spontaneously hypertensive rat once they develop hypertension through a mechanism which is not known.
- Increased L-arginine concentrations in hypertension explain why the global L-arginine bioavailability ratio is not diminished.
- Other measures such as increased urinary orotic acid suggest that L-arginine is limited in hypertension.

Keywords L-Arginine • Cationic amino acids • Hypertension • Bioavailability • Orotic acid • Spontaneously hypertensive rat

Abbreviations

ACQ 6-Aminoquinolyl-*N*-hydroxy-*N*-hydroxysuccinimidyl carbamate
arg L-Arginine
BP Blood pressure
CAD Coronary artery disease
DBP Diastolic blood pressure
ECG Electrocardiogram
FMD Flow mediated vasodilation
FMOC 9-Fluorenyl-methyloxycarbonyl

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GABR	Global L-arginine bioavailability ratio
HPLC	High performance liquid chromatography
K_m	Michaelis constant (half the substrate concentration of maximal uptake rate)
LBBB	Left branch bundle block
Lys	Lysine
mm Hg	Millimetres mercury
mmol/L	Millimole per litre
MS	Mass spectrometry
NO	Nitric oxide
NOS	Nitric oxide synthase
OPA	*o*-Phthaldialdehyde
OR	Odds ratio
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
WKY	Wistar Kyoto rats
μmol/L	Micromoles per litre

Introduction

High blood pressure (BP) or hypertension is the major risk factor for cardiovascular events including stroke as well as kidney and heart failure [1]. An estimated 600 million persons worldwide are affected by hypertension [2] with the prevalence increasing rapidly in sub-Saharan Africa [1]. Between 2011 and 2025, the projected cost of non-communicable disease to the economy of the United States was estimated at $7.28 billion with cardiovascular disease accounting for more than half of this cost [3]. The prevalence of hypertension in South Africa is between 14.6 and 24.4 %, and in 1991 the cost to the economy was estimated at $400 million or 7.5 % of healthcare expenditure [4, 5]. Of concern is that of patients on treatment, only 30 % have blood pressures adequately controlled, with patients inadequately controlled still at excess risk for adverse cardiovascular events [1]. Furthermore, traditional risk factors do not fully account for the burden and risk for cardiovascular disease seen in patients.

Blood pressure is determined by the balance between vasoconstrictors (e.g. adrenaline in a fight-or-flee response) and vasodilators (e.g. nitric oxide (NO)). NO is the most important vasodilator and is produced by endothelial cells from the 'semi-essential' amino acid L-arginine and oxygen in a reaction catalysed by the enzyme NO synthase (NOS). NO dilates arteries and regulates blood pressure, improves endothelial function and mediates a host of other effects [6] that are beyond the scope of this chapter. Although L-arginine concentrations are in excess of the K_m of NOS, NO production is dependent on extracellular L-arginine [7].

Factors affecting the concentration of the L-arginine, its synthesis, metabolism and transport, in the context of hypertension and cardiovascular disease, have not been fully elucidated. Given the underlying endothelial dysfunction in hypertension and commonly associated obesity, diabetes and hyperlipidaemia, the measurement of L-arginine, metabolites and associated amino acids may provide further insight into this dysfunction. This chapter presents these data for L-arginine, its metabolites and related cationic amino acids, as other aspects have been expertly reviewed in accompanying chapters. Such studies emphasise an important role for L-arginine in hypertension and other cardiovascular diseases.

L-Arginine Synthesis and Metabolism

L-Arginine synthesis and metabolism have been reviewed [6, 8]. De novo synthesis of L-arginine occurs in the intestines, kidneys and the liver. At birth, the enterocytes of the small intestine are the major sites of L-arginine synthesis and as the gut matures there is an increase in arginase activity, a catabolic enzyme that converts L-arginine to ornithine and urea. Ornithine is converted to citrulline locally in the enterocytes. This citrulline is then filtered by the kidneys and converted to L-arginine in the proximal tubules. The kidneys are responsible for approximately 60 % of L-arginine synthesis and the majority of L-arginine synthesis, in adults, occurs through this intestinal–renal axis [6, 9, 10]. Although the highest rates of L-arginine synthesis occur within the hepatic urea cycle, the net production of L-arginine from the liver is small compared with the intestinal–renal axis.

Studies have been conducted to determine whether L-arginine supplementation can reverse the underlying endothelial dysfunction common to cardiovascular diseases. In cardiovascular disease, oral L-arginine supplementation (expertly reviewed in the chapter by Sourij and Tripolt) was beneficial in heart failure [11], increased coronary small vessel endothelial function [12], inhibited atherogenesis [12, 13], improved haemodynamic responses in hypercholesterolaemic men [14], and improved endothelium-dependent vasodilation [15]. However, other studies have shown no improvement in endothelial function and even possible harm and increased mortality in patients with pre-existing cardiovascular disease with L-arginine supplementation [16–19]. The approach of L-arginine supplementation to reverse endothelial dysfunction may therefore not be benign. However, most studies were not randomised with small sample size and were of short duration.

A meta-analysis of randomised oral L-arginine supplementation versus placebo-controlled trials ($n = 12$ trials of short duration) in patients with coronary artery disease, hypercholesterolaemia, heart failure, peripheral artery disease, children in chronic renal failure and elderly patients asymptomatic for cardiovascular diseases, as well as healthy individuals has been undertaken. The analysis found only individuals with initially low baseline flow mediated vasodilation (FMD) improved FMD (OR: 1.98 [0.47–3.48] ($p = 0.01$) [20].

A recent meta-analysis analysed randomised, double-blinded, placebo-controlled studies ($n = 11$), supplementing between 2 and 24 g L-arginine/day (median 9 g/day) in patients diagnosed with hypertension. The median duration of the studies was 9 weeks and included 387 participants (range 12–79 patients per study). L-Arginine supplementation reduced systolic blood pressure (SBP) by 5.4 mmHg (95 % CI −8.5 to −2.3; $p = 0.001$) and diastolic blood pressure (DBP) by 2.7 mmHg (95 % CI −3.77 to −1.54; $p < 0.001$) [21].

Measurement of L-Arginine, Amino Acids, and Metabolites

Sample preparation. Samples should be immediately cooled, centrifuged and plasma frozen within 30 min. This may be the reason for the narrower range of reported values of earlier studies (80–100 μmol/L) [22], compared to more recent studies [23]. Furthermore, immediate removal of protein by precipitation or by using low molecular weight sample filters is recommended.

Amino acid analysis. Amino acid analysis started in the late 1950s using ion-exchange chromatography with post-column reaction with ninhydrin. The analysis of 0.5 mL of urine took 750 min to resolve 175 ninhydrin reactive compounds [24]. Subsequently, the development of high performance liquid chromatography (HPLC) allowed significantly faster separations. Although still used with HPLC ion-exchange column chromatography, ninhydrin has become less popular with the development of rapid

and milder derivatisation with *o*-phthaldialdehyde (OPA), dansyl chloride, 6-aminoquinolyl-*N*-hydroxy-*N*-hydroxysuccinimidyl carbamate (ACQ) and 9-fluorenyl-methyloxycarbonyl (FMOC). However, the stability of these derivatives may be limited and requires rapid separation of the amino acid derivatives. Although often used, OPA does not react with secondary amino acids, requiring the important metabolite of L-arginine, proline, to be oxidised prior to quantification. The derivatives of the 22 major amino acids can be separated within a reasonable time by reversed phase HPLC. The separation of the 40 physiological amino acids is challenging particularly as the concentrations span three orders of magnitude (<1 to >1000 μmol/L). Therefore, rapid methods to analyse amino acids directly related to L-arginine, the precursors, and metabolites have been developed [25].

Developments using HPLC with detection by mass spectrometry (MS) rely on good calibration and accurate calculation of mass of the amino acids allowing several amino acids to be detected simultaneously. Derivatisation using alkyl chloroformates forms the basis for a commercially available kit for amino acid analysis (EZ:faast. Phenomenex, Torrance, CA). The gas chromatographic–MS version of this kit does not detect L-arginine.

Interpretation of data. Differences between techniques used to measure amino acid have yielded different values for control and disease states, and direct comparison between reported values should be regarded with caution. Furthermore, concentrations expressed as μg/mL or μmol/L are subject to the effects of dilution, and correlations with other amino acids and phenotypic parameters (blood pressure) may be spurious. Amino acid concentrations should, therefore, also be expressed relative to total amino acid nitrogen, or urinary concentrations relative to that of creatinine concentrations (Fig. 37.1).

Although providing interesting information, reporting changes or differences in the concentrations of a list of individual amino acids may be limited. Associations between amino acid concentrations have been shown for L-arginine, L-arginine precursors, metabolites and those using common transport systems. Arranging the amino acids according to their respective membrane transport, i.e. characteristics or functional groups (cationic transport), may provide insight into mechanism of such changes.

An example of reporting data relative to total amino acid nitrogen and the measurement of similar cationic amino acids in hypertension is illustrated in Fig. 37.1. As a semi-essential amino acid, plasma concentrations of L-arginine are determined by a number of pathways as the body is capable of endogenous synthesis, the metabolic pathways, and renal handling. In contrast, the concentration of the essential amino acid lysine, which uses the same cationic transport, depends only on dietary intake, protein turnover and recovery from the renal tubules. In African patients, L-arginine expressed as either μmol/L or as μmol/mg total amino acid nitrogen shows little significant relationship with mean daytime ambulatory systolic blood pressure (Fig. 37.1). In contrast, direct lysine concentrations (as μmol/L) correlated with 24 h, daytime and nocturnal blood pressures. Moreover, these correlations were much stronger when corrected for total amino acid nitrogen (Fig. 37.1 only data for mean daytime systolic blood pressure shown; data unpublished).

L-Arginine and Metabolite Concentrations

Fasting L-arginine concentrations. Plasma L-arginine concentrations vary among different population groups, diseases, gender and age. Cultural dietary profiles and geographical availability of food products may further contribute to this variation. Values of plasma L-arginine concentrations range from normal 59.6 μmol/L [26] to 210 μmol/L [6]. In elderly men and women, the averages are approximately 113.7 ± 19.8 μmol/L and 88.0 ± 7.8 μmol/L, respectively [27, 28]. Young men had lower (not statistically

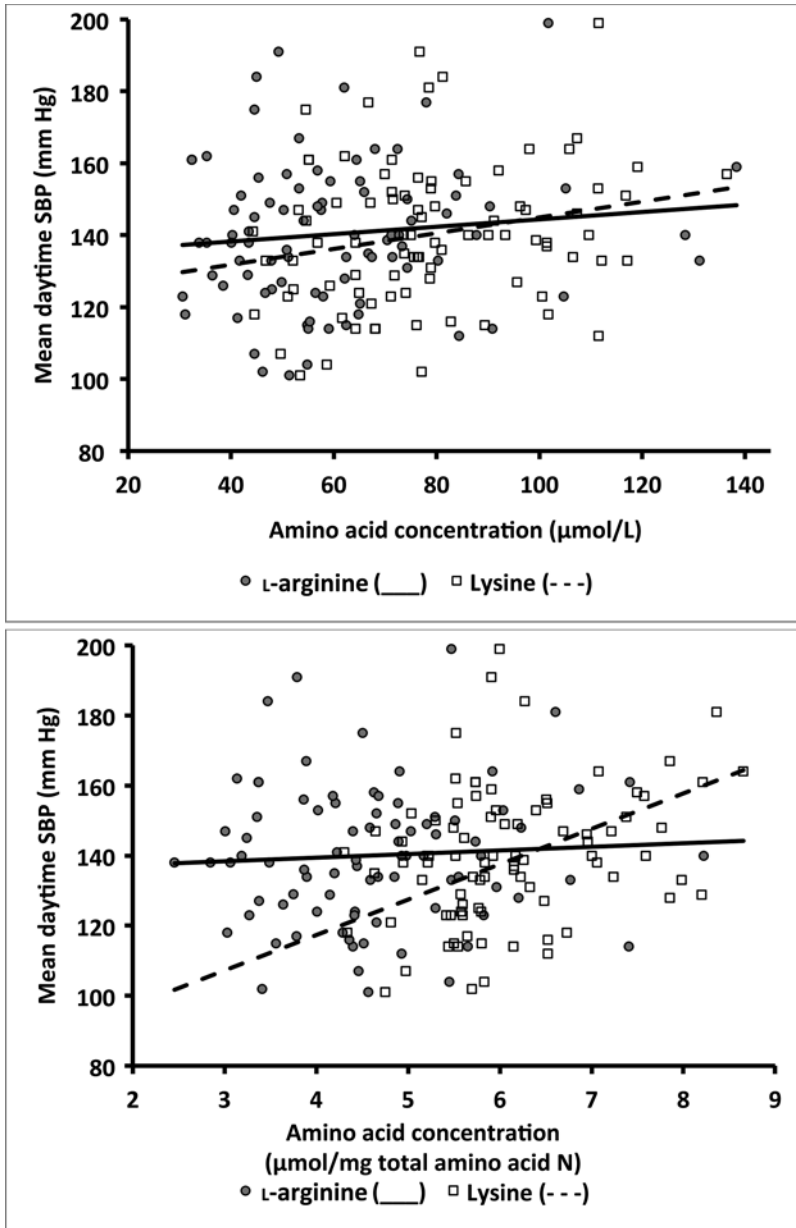

Fig. 37.1 Relationships between mean daytime ambulatory systolic blood pressure and plasma L-arginine and lysine concentrations in African patients ($n=85$) expressed as (**a**) µmol/L and (**b**) µmol/mg total amino acid nitrogen. Concentrations as µmol/L: L-arginine $r=0.113$ ($p=0.30$); lysine $r=0.22$ ($p<0.04$). Concentrations as µmol/mg amino acid nitrogen: L-arginine $r=0.06$ ($p=0.59$); lysine $r=0.30$ ($p=0.0054$) (GP Candy unpublished data)

different) L-arginine concentrations, 81.6±7.3 mmol/L, compared to older men whereas younger and older women have similar L-arginine concentrations, 72.4±6.7 µmol/L [27, 28] (Table 37.1).

Although endogenous synthesis of L-arginine maintains relatively constant circulating concentrations, low L-arginine concentrations may occur [29], particularly in the setting of infectious diseases, such as cerebral malaria [30] and sepsis [31]. Studies have shown increased concentrations of L-arginine and related metabolites in humans with essential hypertension [26, 32, 33]. L-Arginine

Table 37.1 Reported concentrations of amino acids related to L-arginine and L-arginine metabolism in control subjects and patients with hypertension and commonly associated metabolic diseases

	Population/group	L-Arginine	Lysine	Citrulline	Ornithine	Proline	ADMA	Reference
Men (young)	Sweden	82 ± 7	166 ± 14	40 ± 8	97 ± 8	192 ± 15	nd	[27, 28]
Men (elderly)		114 ± 20	220 ± 19	49 ± 6	116 ± 7	235 ± 45	nd	
Women (young)		72 ± 7						
Women (elderly)		88 ± 8						
Hypertension								
Controls	African (South Africa)	54 ± 15	74 ± 19	13 ± 4	17 ± 13	69 ± 18		[26]
Hypertensive		61 ± 17*	81 ± 19	17 ± 10	17 ± 16	103 ± 13		
Controls	Italy	26.0 ± 8.1					0.40 ± 0.09	[33]
Hypertensive		47.4 ± 18.5**					0.59 ± 0.14***	
Controls	Brazil	137 ± 8	123 ± 4	49 ± 3	35 ± 2			[32]
Hypertensive		175 ± 19*	176 ± 8*	57 ± 3	80 ± 17*			

*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$

Naidoo et al. [26]: African ancestry; mean day ambulatory systolic pressure > 140mmHg

Perticone et al. [33]: hypertension diagnosed from clinic pressure measurements

concentrations also showed a positive correlation with diastolic blood pressure [26, 34]. As L-arginine supplementation decreases blood pressure in hypertension [21], the reports of raised L-arginine concentrations in this cardiovascular disease are unexpected. As supplementation would further increase L-arginine concentrations, these data may suggest that in patients with hypertension, either cellular uptake by the cationic transporters [9, 32] was reduced or that subsequent NO synthesis was impaired [32]. Ornithine and lysine have been shown to be elevated in hypertension [32] (Table 37.1) and although these amino acids were not statistically increased in African patients with hypertension, lysine was shown to correlate with 24 h and nocturnal ambulatory blood pressures [26], again implicating reduced activity of cationic transport or NO synthesis.

One of the only longitudinal studies in the spontaneously hypertensive rat (SHR) demonstrated that L-arginine concentrations were reduced until the rat developed hypertension at around 8 weeks of age [34]. Thereafter, L-arginine concentrations increased and approached those of the control Wistar Kyoto rats (WKY). Changes in lysine concentrations paralleled those of L-arginine, remaining lower than the WKY rats once hypertension manifest, but were not different at the end of the study period (Fig. 37.2) [34]. The common changes in L-arginine and lysine concentrations implicate altered handling of cationic amino acids in this model of hypertension and can be partly explained by the common transport. Furthermore, these data can be explained if during the development of hypertension, the SHRs are capable of upregulating L-arginine synthesis, whereas lysine, as an essential amino acid, in vivo biosynthesis does not occur. Chein et al. [35] delayed the onset and attenuated hypertension in SHRs with citrulline and nitrate in young pre-hypertensive SHRs. However, this study was of short duration and comparison to the results of Jones [34] may not be possible.

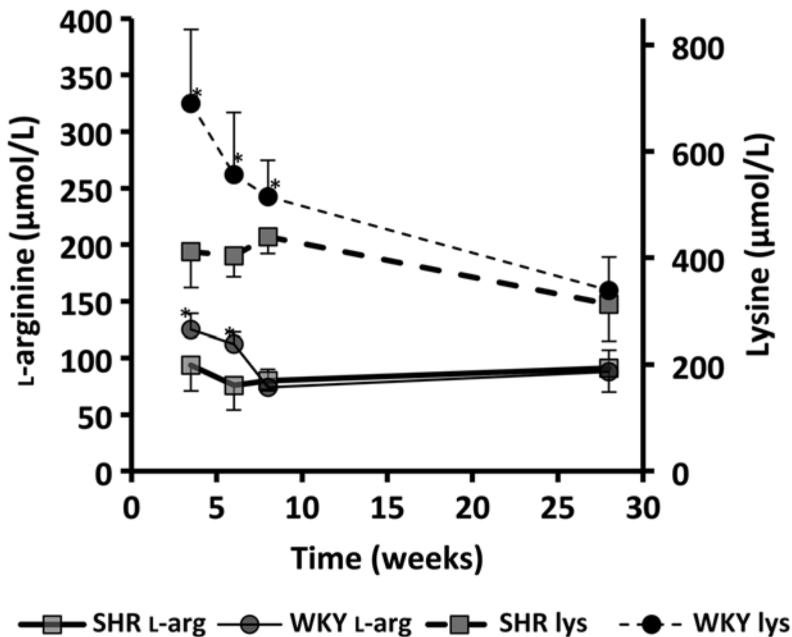

Fig. 37.2 Changes in cationic amino acids, L-arginine (arg) and lysine (lys), over time in the spontaneously hypertensive rat (SHR) compared to the Wistar Kyoto (WKY) rats. The SHRs develop hypertension around 8 weeks of age. Results as mean ± standard deviation; *$p < 0.05$ between SHR and WKY values. Adapted from Jones [34] (with permission from the American Nutrition Society). Abbreviations: *arg* L-arginine, *lys* lysine

Table 37.2 Reported concentrations of amino acids related to L-arginine and L-arginine metabolism in syndrome X, heart failure, and supplementation in vitamin B_6 deficiency

	L-Arginine	Lysine	Citrulline	Ornithine	GABR	Reference
Controls	154±34					[36]
Syndrome X	143±27					
Controls	117±23	207±48		125±42		[38]
Heart failure	59±3*	113±13*		83±9		
Obstructive atherosclerotic CAD						
Controls	74±24		18 (14–26)	36 (26–49)	1.27 (0.96–1.73)	[37]
CAD	68±20***		23*** (18–31)	41*** (30–57)	1.06*** (0.75–1.31)	
B_6 deficiency						[50]
Deficiency	71±20					
Supplementation						
B_6 7 days	110±23*					
B_6 14 days	103±24					

*$p<0.05$, **$p<0.005$; ***$p<0.0005$ compared to control subjects or pre-vitamin B_6 supplementation
CAD coronary artery disease
Bellany [36]: Syndrome X: patients with effort-related angina; positive exercise stress test; normal coronary angiogram; no other cardiovascular diseases (BP>150/90 mmHg; raised cholesterol >6.5 mmol/L; homocysteine >15 μmol/L) Hypertension (57±3 μmol/L compared with 49±3 μmol/L in control)
Tang [37]: patients: all women were postmenopausal, one on hormone replacement therapy. All resting ECGs were normal except for one patient with LBBB (angina, normal coronary arteriogram, and positive exercise test history of stable effort-related angina and positive exercise stress test)

In metabolic syndrome (Syndrome X) L-arginine concentrations were no different to controls (Table 37.2) [36]. However, in obstructive CAD L-arginine concentrations were reduced [37] and once heart failure developed, plasma L-arginine concentrations were significantly reduced (Table 37.2) [38].

L-Arginine Transport and Availability of L-Arginine

L-Arginine transport. NO production is dependent on extracellular L-arginine despite L-arginine concentrations in excess of the K_m of NOS [7]. L-Arginine is transported across cell membranes by several transporters [39] and several research groups have reviewed the data demonstrating reduced L-arginine transport in hypertension and cardiovascular diseases [40, 41]. The demonstration of impaired L-arginine transport in cardiovascular disease has led to the suggestion that the membrane transporters are potentially important therapeutic targets in cardiovascular disease [9]. In contrast in heart failure L-arginine concentrations were lower than controls, but L-arginine transport was increased [38].

Transport by low capacity, high affinity y+L transport appears to be important for NO production [41, 42]. In the absence of selective inhibitors/activators for such transport, the importance of this transport in NO production requires further investigation.

L-Arginine to asymmetrical dimethyl L-arginine Asymmetrical dimethyl L-arginine (ADMA) is an endogenous inhibitor of NOS and a risk factor for cardiovascular disease [43]. As L-arginine

concentrations were elevated in hypertension, the value of ADMA measurement as a risk factor in hypertension may be limited.

Global L-arginine bioavailability ratio (GABR) The ratio of concentration of L-arginine to that of (citrulline + ornithine) has been proposed as a measure of the bioavailability of L-arginine in cardiovascular disease (Table 37.2) [37]. Low L-arginine concentrations reduced the GABR in obstructive coronary artery disease (CAD) compared to patients without CAD [37] and a low GABR was associated with mortality in patients undergoing coronary angiography [44]. Low GABR has also been reported in patients with pulmonary hypertension associated with systolic heart failure [45]. However, GABR appears not to have been determined in essential hypertension. Given that L-arginine concentrations were reported as increased in hypertension [26, 32, 33], the ratio may not be reduced and therefore would not reflect reduced bioavailability of L-arginine.

Orotic acid Increased urinary orotic acid is a sensitive indicator of ornithine transcarbamylase activity and has been used as a marker of L-arginine deficiency and of inborn errors of the ornithine cycle [46] (Fig. 37.3). Increased orotic acid concentrations have been reported in patients with hypertension, stroke and heart failure suggesting these conditions were L-arginine deficient [47]. Furthermore, a

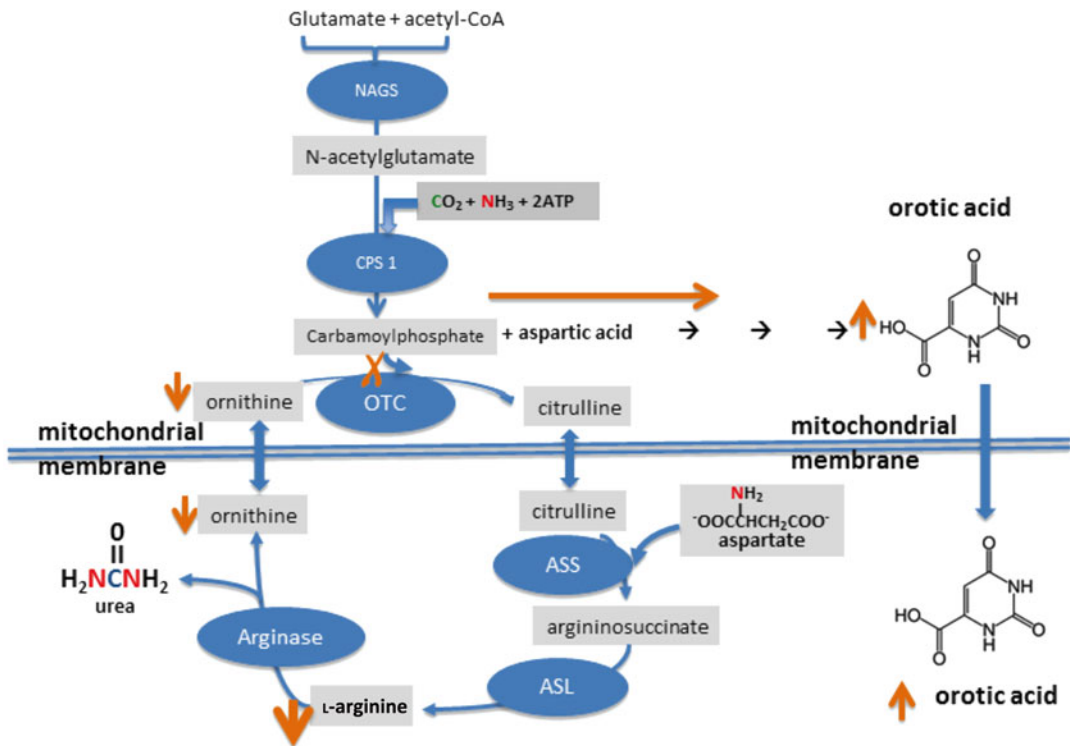

Fig. 37.3 Synthetic route of orotic acid. *Arrows* indicate when L-arginine concentrations are limiting, ornithine production is reduced and is insufficient to react with carbamoyl phosphate. Carbamoyl phosphate is diverted to orotic acid, which accumulates and is increased in the urine. Although the presence of orotic acid is a sensitive measure of ornithine transcarbamylase activity, it is useful in diagnosing genetic abnormalities of the urea cycle and derangements in L-arginine metabolism. *NAGS* N-acetylglutamate synthase; *CPS1* carbamoylphosphate synthase; *OTC* ornithine transcarbamylase; *ASS* argininosuccinate synthase; *ASL* L-arginine-succinate lyase

single report of patients with pregnancy-induced hypertension has found lower third trimester urinary orotic acid levels compared to normal pregnancy. In normal pregnancy, orotic acid levels were elevated in the second and third trimesters compared to the first trimester ($p < 0.01$) [48]. Although orotic acid is stable and provides an indication of L-arginine deficiency, it has been infrequently measured in hypertension and cardiovascular disease.

Vitamin B_6 An animal model of hypertension was developed by feeding rats a vitamin B_6-deficient diet [49]. Kang-Yoon and Kirksey [50] supplemented women on a low vitamin B_6 diet with pyridoxine. Plasma L-arginine and urea concentrations were shown to increase significantly within 1 week, but blood pressures were not measured in this study (Table 37.2). These observations and the underlying mechanisms appear not to have been investigated further.

Conclusion

Although traditional risk factors for cardiovascular disease, such as fasting glucose concentrations and lipid profiles, are routinely measured to access cardiovascular risk, amino acid profiles are not. L-Arginine is the precursor of the vasodilator nitric oxide (NO) and extracellular concentrations of L-arginine determine the availability of NO. Therefore, the measurement of L-arginine, L-arginine precursor and metabolite concentrations may provide useful data and insight into the mechanisms underlying cardiovascular disease.

In patients with hypertension, there is little information available on amino acid profiles or the bioavailability of L-arginine. A single study in the spontaneously hypertensive rat (SHR) shows prior to the onset of hypertension, cationic amino acids, including L-arginine and lysine, are reduced in concentration. Once hypertension develops in the SHR, L-arginine concentrations are equal to those of control animals. In human patients L-arginine and lysine concentrations are increased. These data suggest the capacity to increase L-arginine, altered cationic amino acid transport in humans with hypertension or reduced production or availability of NO.

Reduced global L-arginine bioavailability ratio (GABR) has been demonstrated in coronary artery disease and heart failure even though in the latter, L-arginine transport was increased. The GABR may not apply in hypertension as L-arginine concentrations were increased, and therefore alternative measures such as increased orotic acid have suggested hypertension to be an L-arginine-deficient state. Very limited data suggest a role for vitamin B_6 in hypertension with rats developing elevated blood pressures on a vitamin B_6-deficient diet. A human study has shown altered L-arginine concentrations following vitamin B_6 supplementation, but such observations require further investigation.

The development of newer techniques and rapid HPLC/MS techniques will allow easier measurement of amino acid profiling in cardiovascular disease and may provide further insight into the role of L-arginine in cardiovascular disease.

References

1. Addo J, Smeeth L, Leon DA. Hypertension in sub-Saharan Africa: a systematic review. Hypertension. 2007;50: 1012–8.
2. Vasdev S, Gill V. The antihypertensive effect of L-arginine. Int J Angiol. 2008;17:7–22.
3. World Health Organisation. A global brief on hypertension. Document WHO/DCO/WHD/2013.2. www.apps.who/int/iris/bitstream/10665/1/WHO_DCO_WHD_2013.2_eng.pdf. Downloaded 12 Aug 2014.

4. Pestana JAX, Steyn K, Leiman A, Hartzenberg GM. The direct and indirect costs of cardiovascular disease in South Africa in 1991. S Afr Med J. 1996;86:679–84.
5. Dougherty J, McIntyre D, Bloom G. Value for money in South African health care: finding of a review of health care and finance. Cent Afr J Med. 1996;42:21–4.
6. Wu G, Morris SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336:1–17.
7. Hardy TA, May JM. Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. Free Radic Biol Med. 2002;32:122–31.
8. Popolo A, Adesso S, Pinto A, et al. L-Arginine and its metabolites in kidney and cardiovascular disease. Amino Acids. 2014;46:2271–86.
9. Chin-Dusting JPF, Willems L, Kaye DM. L-Arginine transporters in cardiovascular disease: a novel therapeutic target. Clin Pharmacol Ther. 2007;117:428–36.
10. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
11. Rector TS, Bank AJ, Mullen KA, et al. Randomised, double-blind, placebo controlled study on supplemental oral L-arginine in patients with heart failure. Circulation. 1996;93:2135–41.
12. Cooke JP. L-Arginine: a new therapy for atherosclerosis? Circulation. 1997;95:311–2.
13. Napoli C. Nitric oxide and atherosclerotic lesion progression: an overview. J Card Surg. 2002;17:355–62.
14. West SG, Likos-Krick A, Brown P, et al. Oral L-arginine improves haemodynamic responses to stress and reduced plasma homocysteine in hypercholesterolemic men. J Nutr. 2005;135:212–7.
15. Creager MA, Gallagher SJ, Girerd XJ, et al. L-Arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest. 1992;90:1248–53.
16. Thorne S, Mullen MJ, Clarkson P, et al. Early endothelial dysfunction in adults at risk from atherosclerosis: different responses to L-arginine. J Am Coll Cardiol. 1998;32:110–6.
17. Blum J, Hathaway L, Mincemoyer R, et al. Oral L-arginine in patients with coronary artery disease on medical management. Circulation. 2000;101:2126–9.
18. Schulman SP, Becker LC, Kass DA, et al. L-Arginine therapy in acute myocardial infarction: Vascular INTeraction with AGE in myocardial infarction (VINTAGE MI) randomized clinical) trial. JAMA. 2006;295:58–64.
19. Wilson AM, Harada R, Nair N, et al. L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. Circulation. 2007;116:188–95.
20. Bai Y, Sun L, Tang T, et al. Increase in fasting vascular endothelial function after short-term oral L-arginine is effective when baseline flow-mediated dilation is low: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2009;89:77–84.
21. Dong J-Y, Qin L-Q, Zhang Z, et al. Effect of oral L-arginine supplementation on blood pressure. A meta-analysis of randomized, double-blind, placebo-controlled trials. Am Heart J. 2011;162:959–65.
22. Van Eijk HM, Dejong CH, Deutz NE, Soeters PB. Influence of storage conditions on normal plasma amino acid concentrations. Clin Nutr. 1994;13:374–80.
23. Luneburg N, Xanthakis V, Schwedhelm E, et al. Reference intervals for plasma L-arginine and the L-arginine:asymmetric dimethylarginine ratio in the Framingham Offspring Cohort. J Nutr. 2011;141:186–2190.
24. Hamilton PB. Ion exchange chromatography of amino acids—a single column, high resolving, fully automatic procedure. Anal Chem. 1963;35:2055–64.
25. Zhang W-Z, Kaye DM. Simultaneous determination of L-arginine and seven metabolites in plasma by reversed-phase liquid chromatography with time-controlled *ortho*-phthaldialdehyde precolumn derivatization. Anal Biochem. 2004;326:87–92.
26. Naidoo C, Cromarty AD, Snyman T, Sliwa E, Libhaber E, Essop MR, Candy GP. Relationships between fasting plasma amino acid concentrations and ambulatory blood pressure in a group of African descent in South Africa. SA Heart J. 2009;6:142–7.
27. Möller P, Bergström J, Eriksson S, Furst P, Hellström K. Effects of aging on free amino acids and electrolytes in leg skeletal muscle. Clin Sci. 1979;56:427–32.
28. Möller P, Alvestrand A, Bergström J, Furst P, Hellström K. Electrolytes and free amino acids in leg skeletal muscle of young and elderly women. Gerontology. 1983;29:1–8.
29. Hallemeesch MM, Lamers WH, Deutz NE. Reduced L-arginine availability and nitric oxide production. Clin Nutr. 2002;21:273–9.
30. Lopansri BK, Anstey NM, Weinberg JB, et al. Low plasma L-arginine concentrations in children with cerebral malaria and decreased nitric oxide production. Lancet. 2003;361:676–8.
31. Luiking YC, Poeze M, Deong CH, Ramsey G, Deutz NE. Sepsis: an L-arginine deficient state? Crit Care Med. 2004;32:2135–45.
32. Moss MB, Brunini TM, Soares De Moura R, Novaes Malagris LE, Roberts NB, Ellory JC, Mann GE, Mendes Ribeiro AC. Diminished L-arginine bioavailability in hypertension. Clin Sci (Lond). 2004;107:391–7.
33. Perticone T, Sciacqua A, MAio R, et al. Asymmetrical dimethylarginine, L-arginine and endothelial dysfunction in essential hypertension. J Am Coll Cardiol. 2005;46:518–23.

34. Jones MR. Free amino acid pools in spontaneously hypertensive rat: a longitudinal study. J Nutr. 1988;118: 579–87.
35. Chein SJ, Lin K-M, Kuo H-C, Huang C-F, Lin Y-J, Huang L-T, Tain Y-L. Two different approaches to restore renal nitric oxide and prevent hypertension in young spontaneously hypertensive rats: L-citrulline and nitrate. Transl Res. 2014;163:43–52.
36. Bellamy MF, Goodfellow J, Tweddel AC, et al. Syndrome X and endothelial dysfunction. Cardiovasc Res. 1998; 40:410–7.
37. Tang WHW, Wang Z, Cho L, et al. Diminished global L-arginine bioavailability and increased L-arginine catabolism as metabolic profile of increased cardiovascular risk. J Am Coll Cardiol. 2009;53:2061–7.
38. Hanssen H, Brunini TM, Conway M, et al. Increased L-arginine transport in human erythrocytes in chronic heart failure. Clin Sci (Lond). 1998;94(1):43–8.
39. Hatzoglou M, Fernandez J, Yaman I, Closs E. Regulation of cationic amino acid transport: the story of the CAT-1 transporter. Annu Rev Nutr. 2004;24:377–99.
40. Gokce N. L-Arginine and hypertension. J Nutr. 2004;134:S2807S–11.
41. Mendes Ribeiro AC, Brunini TMC. L-Arginine transport in disease. Curr Med Chem. 2004;2:123–31.
42. Arancibia-Garavilla Y, Toledo F, Casanello P, Sobrevia L. Nitric oxide synthesis requires activity of the cationic and neutral amino acid transport system y+L in human umbilical vein endothelium. Exp Physiol. 2003;88(6):699–710.
43. Bode-Böger S, Scalera F, Ignarro LJ. The L-arginine paradox: importance of the L-arginine/asymmetrical dimethylarginine ratio. Pharmacol Ther. 2007;114:295–306.
44. Sourij H, Meinitzer A, Pilz S, et al. L-Arginine bioavailability ratios are associated with cardiovascular mortality in patients referred to coronary angiograph. Atherosclerosis. 2011;218(1):220–5.
45. Shao Z, Wang Z, Shrestha K, et al. Dysregulated L-arginine metabolism and importance of compensatory dimethylarginine dimethylaminohydrolase-1 in pulmonary hypertension associated with advanced systolic heart failure. J Am Coll Cardiol. 2012;59:1150–8.
46. Brosnan ME, Brosnan JT. Orotic acid excretion and L-arginine metabolism. J Nutr. 2007;137:1656S–61S.
47. Sumi S, Kidouchi K, Imaeda M, et al. Urinary orotic acid in healthy adults and patients with various diseases. Clin Chim Acta. 1997;266:195–7.
48. Furutani K, Ochi H, Matsubara K, et al. Urinary orotic acid levels in normal pregnancy and pregnancy induced hypertension. Gynecol Obstet Invest. 2000;50:33–5.
49. Dakshinamurti K, Paulose CS, Viswanathan M. Vitamin B6 and hypertension. Ann N Y Acad Sci. 1990;585: 241–9.
50. Kang-Yon SA, Kirksey A. Relation of short-term pyridoxine-HCI supplementation to plasma vitamin B-6 vitamers and amino acid concentrations in young women. Am J Clin Nutr. 1992;55:865–72.

Chapter 38
L-Arginine and Cardiovascular Disease

Norbert J. Tripolt and Harald Sourij

Key Points

- L-arginine is a weak predictor for incident cardiovascular events.
- The global L-arginine bioavailability ratio accounts for the metabolic products of L-arginine and is calculated as follows: L-arginine/(L-citrulline + L-ornithine).
- A low global L-arginine bioavailabilty ratio is predictive for future major adverse cardiovascular events and mortality in subjects undergoing coronary angiography and with sickle cell disease.
- Animal and human mechanistic studies suggest cardiovascular benefits from L-arginine supplementation.
- Large-scale trials with L-arginine supplementation investigating cardiovascular endpoints are needed.

Keywords L-arginine • Oral supplementation • Infusion • Animal studies • Human trials • Coronary artery disease • Endothelial function

Abbreviations

ADMA Asymmetric dimethylarginine
AMI Acute myocardial infarction
CAD Coronary artery disease
CC Case-controlled
cGMP Cyclic guanosine monophosphate
CO Controlled
CVD Cardiovascular disease
DB Double-blind

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eNOS	Endothelial nitric oxide synthase
FMD	Flow-mediated dilatation
GABR	Global L-arginine bioavailability ratio
HR	Hazard ratio
IDDM	Insulin-dependent diabetes mellitus
IGT	Impaired glucose tolerance
L-NAME	*N*-nitro L-arginine methyl ester
MACE	Major adverse cardiovascular events
MetS	Metabolic syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NMD	Nitroglycerin-mediated dilatation
NO	Nitric oxide
NO3	Urinary nitrate
PC	Placebo-controlled
Pts	Patients
R	Randomized
T2DM	Type 2 diabetes mellitus
TIA	Transient ischemic attack

Introduction

Cardiovascular disease (CVD) encompasses a group of disorders of the heart and blood vessels, including coronary heart, cerebrovascular, peripheral artery disease, or deep vein thrombosis and pulmonary embolism [1]. Although the mortality rates of CVD decreased substantially over the last decade, still almost a half of all deaths in Europe are attributable to CVD [2].

A healthy endothelium is capable of thoroughly regulating pivotal functions such as vascular tone, platelet aggregation, coagulation, or fibrinolysis. One critical signaling molecule involved in this blood vessel homeostasis is nitric oxide (NO). Reduced bioavailability of NO, also known as endothelial dysfunction, has been demonstrated to play a crucial role in the pathogenesis of atherosclerosis and was subsequently associated with CVD events [3]. Since the semi-essential amino acid L-arginine represents the unique source for NO production, it is not surprising that the role of L-arginine in cardiovascular disease and outcome is of great interest to many research groups.

NO is produced by the endothelial nitric oxide synthase (eNOS) in a complex reaction requiring cofactors such as tetrahydrobiopterin and nicotinamide adenine dinucleotide phosphate (NADPH) [4] (reviewed in more detail in the Chapter 1). Endothelial cell-derived NO subsequently leads to relaxation of smooth muscle cells and it inhibits their proliferation and migration; NO increases proliferation of endothelial cells and angiogenesis, reduces expression of adhesion molecules on endothelial cells, and decreases aggregation and adhesion of platelets [4]. Therefore, it has been postulated that increasing blood L-arginine levels might directly increase NO production, leading to improved cardiovascular outcome. However, due to the complex L-arginine metabolism, total plasma L-arginine levels turned out not to be different in subjects with cardiac syndrome X as compared to a control group [5] and blood L-arginine levels are only weak predictors for future myocardial infarction or stroke and not predictive for mortality or major adverse cardiovascular events (MACE) [6]. Therefore, it has been suggested to take the complexity of L-arginine metabolism into account when looking into cardiovascular effects of this amino acid and its use in cardiovascular risk prediction.

It has been shown that the quantitatively more important pathway of L-arginine catabolism occurs via the enzyme arginase, which metabolizes L-arginine to urea and ornithine (for more details, see Chap. 14). Ornithine, however, competes with L-arginine for the same transporter [7], leading to reduced intracellular L-arginine bioavailability. Higher levels of ornithine occur in conditions associated with arginase upregulation such as inflammation, coronary artery disease, cystic fibrosis, asthma, or diabetes mellitus [6, 8].

In 2005, Morris et al. suggested the concept of the "L-arginine bioavailability" which takes alternative pathways such as the arginase pathways and the levels of the metabolites on the bioavailability of L-arginine for vascular function into account [9]. In the proposed "global L-arginine bioavailability ratio" (GABR), total L-arginine levels are adjusted for the major catabolic products: L-ornithine and L-citrulline (L-arginine/(L-ornithine + L-citrulline)). They demonstrated in a cohort of 228 subjects with sickle cell anemia an increased mortality (HR (95 % CI): 3.6 (1.5–8.3), $p < 0.001$) in those in the lowest GABR quartile compared to the highest. Tang et al. performed an analysis of the Cleveland Clinic GENEBANK, a prospective cohort study, and they demonstrated that subjects with coronary artery disease (CAD) had significantly lower GABR levels than those without CAD (Median (IQR) 1.06 (0.75, 1.31) versus 1.27 (0.96, 1.73); $p < 0.001$) [6]. The lowest GABR quartile was associated with an increased risk for incident MACE during the 3 years of follow-up (adjusted hazard ratio (HR) 1.98, $p = 0.025$) as compared to the highest, while L-arginine levels themselves were not predictive for future cardiovascular events in the adjusted logistic regression models.

This finding was confirmed in an analysis of 2236 subjects referred to coronary angiography who participated in the prospective Ludwigshafen Risk and Cardiovascular Health (LURIC) study [8]. Subjects in the lowest GABR quartile had a significantly increased risk for cardiovascular mortality (adjusted HR (95 % CI): 1.75 (1.24–2.45)) compared to those in the highest GABR quartile and the frequency of confirmed coronary artery disease was inversely associated with quartiles of GABR (p for trend < 0.002). In addition, subjects with type 2 diabetes mellitus had a lower GABR than those without diabetes (0.88 ± 0.23 vs. 0.94 ± 0.24, $p < 0.001$). The available data suggest that GABR seems to be a better predictor for cardiovascular events than L-arginine alone (see Table 38.1). Tripolt et al. [10] added the important finding that intensified risk factor intervention improves GABR in patients with type 2 diabetes.

However, besides the question of which of the parameters is more suitable for risk prediction, the even more important question is whether L-arginine supplementation has an impact on cardiovascular disease or outcome and can be considered as a therapeutic option.

Animal Studies of L-Arginine Supplementation (Summarized in Table 38.2)

In 1990, Girerd et al. [11] investigated the impact of L-arginine supplementation on endothelium-dependent vasodilatation in cholesterol-fed rabbits. Vasodilatator responses of the hind limb to intra-arterial infusion of acetylcholine and nitroprusside were measured before and during intravenous infusion of L-arginine (10 mg/kg/min) and in controls, respectively. This trial demonstrated an improved endothelial-dependent relaxation by L-arginine infusion.

Table 38.1 Global L-arginine bioavailability ratio (GABR) for cardiovascular risk prediction

Publication	Patient cohort	*n*	Outcome	Results
Morris et al. [9]	Sickle cell disease	228	All-cause mortality	1st GABR quartile vs. 4th quartile: HR (95 % CI) 3.6 (1.5–8.3)
Tang et al. [6]	Pts. undergoing coronary angiography	1010	MACE	1st GABR quartile vs. 4th quartile: HR (95 % CI) 1.98 (1.09–3.59)
Sourij et al. [8]	Pts. undergoing coronary angiography	2236	Cardiovascular mortality	1st GABR quartile vs. 4th quartile: HR (95 % CI) 1.75 (1.24–2.45)

Table 38.2 Results of experimental L-arginine supplementation studies in animals (in order of publication year)

First author	Year	Subjects	Number of animals	Measured outcome(s)	Study design	Results	Conclusions
Girerd [11]	1990	Rabbits	28	Endothelium-dependent vasodilatation	2 groups: L-arginine (10 mg/kg/min) intravenous to 16 animals; 12 controls	+	L-arginine normalizes the endothelium-dependent vasodilatation
Cooke [12]	1992	Rabbits	54	Aortic atherosclerosis Aortic endothelial function	3 groups: fed chow, high-chol, or high-chol + L-arginine × 10 weeks	+	L-arginine improved endothelium-dependent vasorelaxation and reduced atherogenesis
Wang [13]	1994	Rabbits	28	Intimal area of left main and left anterior descending artery	4 groups: fed chow, high-chol, high-chol + L-arginine, or high-chol + methionine	+	L-arginine prevented intimal thickening in coronary arteries
Hamon [14]	1994	Rabbits	26	Endothelial function	Fed chow or chow + L-arginine after arterial injury × 4 weeks	+	L-arginine improved neoendothelial-dependent relaxation
Tarry [15]	1994	Rabbits	26	Endothelial function	2 groups fed chow or chow + L-arginine × 7 weeks, iliac artery angioplasty at 3 weeks	+	L-arginine enhanced NO production at sites of vascular healing and may reduce intimal hyperplasia
Singer [16]	1995	Rabbits	18	Aortic atherosclerosis, vascular reactivity	3 groups: fed chow, high-chol, or high-chol + L-arginine in water × 10 weeks	+/–	L-arginine reduced intimal thickening 8-fold. Endothelium-dependent relaxation to acetylcholine impaired in hypercholesterolemic animals
Boger [17]	1995	Rabbits	32	Vascular reactivity, carotid artery plaque area	4 groups: fed chow, high-chol, high-chol + L-arginine, or high-chol + L-NAME × 12 weeks	+	L-arginine restored endothelial function in hypercholesterolemia
Davies [20]	1995	Rabbits	30	Right carotid vein bypass graft	3 groups: fed chow, high-chol, high-chol + L-arginine × 6 weeks	+	L-arginine preserved acetylcholine-mediated relaxation

Davies [19]	1996	Rabbits	24	Jugular vein isometric tension and contractile response	3 groups: fed chow, high-chol, high-chol + L-arginine × 8 weeks	+	L-arginine may ameliorate hypercholesterolemia-induced functional abnormalities in endothelial cells
Aji [22]	1997	LDL receptor knockout mice	40	Aortic atherosclerosis and NOS, xanthomas	4 groups: fed chow, high-chol + L-arginine, or *N*-nitro-L-arginine × 6 months	+	L-arginine prevented xanthoma formation and reduced atherosclerosis. L-arginine may be beneficial in familial hypercholesterolemia
Boger [18]	1998	Rabbits	32	Aortic atherosclerosis	4 groups: fed chow, high-chol, high-chol + vit. E in water, high-chol + L-arginine in water	+	L-arginine improved endothelium-dependent relaxation
Davies [21]	1999	Rabbits	30	Right carotid vein bypass graft	3 groups: fed chow, high-chol, high-chol + L-arginine × 6 weeks	+	L-arginine improved endothelial cell function

Cooke and colleagues [12] investigated the effect of L-arginine supplementation on endothelium-dependent relaxation in hypercholesterolemic rabbits. Fifty-four male rabbits were divided into three groups receiving normal rabbit chow, 1 % cholesterol diet, or 1 % cholesterol diet supplemented by 2.25 % L-arginine in drinking water. In this trial, L-arginine supplementation in hypercholesterolemic rabbits decreased the surface area and reduced intimal thickness of atheromatous lesion in the thoracic aorta.

The hypothesis that long-term oral supplementation of L-arginine would inhibit atherogenesis in hypercholesterolemic rabbits was further tested by Wang and colleagues [13]. They fed male New Zealand white rabbits with normal chow, 1 % cholesterol chow, or 1 % cholesterol chow with dietary L-arginine or methionine supplementation. This trial demonstrated a prevention of coronary artery intima thickening by the supplementation of L-arginine.

Hamon et al. [14] demonstrated that L-arginine reduced neointimal thickening after balloon denudation and improved neoendothelial-dependent acetylcholine-induced relaxation. Therefore, 26 rabbits were fed either with a standard diet or a diet supplemented with L-arginine (2.25 %) in their drinking water 3 days before and 4 weeks after balloon denudation. These findings were confirmed by Tarry and Makhoul [15] and Singer et al. [16] in two other trials with rabbits.

Boger et al. [17] investigated endothelium-dependent relaxation induced by acetylcholine in isolated aortic rings of male rabbits. Rabbits were divided into four groups of eight receiving a normal chow diet, a high-cholesterol diet, a high-cholesterol diet plus L-arginine, or a high-cholesterol diet plus L-NAME for 12 weeks. This study showed that the supplementation of L-arginine in cholesterol-fed rabbits restores the diminished systemic NO production, as assessed by endothelium-dependent relaxations of aortic rings. In contrast, chronic administration of L-NAME resulted in a further decrease in urinary nitrate excretion and completely removed endothelium-dependent relaxations by acetylcholine. In another trial, Boger et al. [18] reported that both L-arginine and vitamin E supplementation significantly reduced atherosclerosis formation in the thoracic aorta and common carotid arteries of hypercholesterolemic rabbits.

Davies et al. [19–21] confirmed that L-arginine-rich diet ameliorated hypercholesterolemia-induced functional abnormalities in endothelial cells.

In 2007, Aji et al. [22] showed that L-arginine had a beneficial effect on vascular endothelium, but the effect was reversed by an L-arginine analogue that blocked NO production. The study group performed experiments in an LDL-receptor-deficient mouse model of familial hypercholesterolemia. Forty mice were divided into four groups of ten that received regular chow, a high-cholesterol diet, a high-cholesterol diet plus L-arginine, or a high-cholesterol diet plus *N*-nitro-L-arginine and L-arginine. After 6 months, the mean atherosclerotic lesion area in the L-arginine group was significantly less than in the high-cholesterol group, and the lesion area in the L-arginine plus *N*-nitro-L-arginine group was larger than in the high-cholesterol group.

This plethora of studies (see Table 38.2) suggests beneficial effects of L-arginine supplementation on endothelial function and atherosclerosis in animals.

Human Studies of Oral L-Arginine Supplementation (for Summary See Tables 38.3 and 38.4)

Endothelial Function

In the mid-1990s, Adams and colleagues [23] investigated for the first time the effect of oral L-arginine on endothelial function in 12 healthy young men. In this prospective, double-blind, randomized crossover trial young men took 7 g L-arginine or placebo for 3 consecutive days, separated by a washout period of 7–14 days. No change in flow-mediated brachial artery dilatation could be measured. However, the same investigators [24] showed a few years later in a prospective, double-blind, randomized crossover study of ten young men with CAD, which oral L-arginine (7 g L-arginine three

Table 38.3 Clinical studies using intra-arterial or intravenous infusions of L-arginine in humans (in order of publication year)

First author	Year	Treatment nature (L-arginine)	Study population	Number of patients	Outcome
Creager [43]	1992	Intravenous infusion, 10 mg/kg/min	Healthy humans	11	L-arginine did not improve endothelium-dependent vasodilation
Creager [43]	1992	Intravenous infusion, 10 mg/kg/min	Patients with hypercholesterolemia	14	L-arginine improved endothelium-dependent vasodilation
Chowienczyk [44]	1994	Intra-arterial infusion, 10 mg/min	Patients with hypercholesterolemia	26	Improvement of acetylcholine-induced vasodilatation of brachial artery in males but not in females
Thorne [45]	1998	Intravenous infusion, 0.1 g/kg	Patients with hypercholesterolemia	9	Improvement of FMD
Cross [47]	2001	Intra-arterial infusion, 50 μmol/min	Predialysis patients	8	L-arginine did not improve endothelial function
Cross [47]	2001	Intravenous infusion, 10 g	Hemodialysis patients	18	L-arginine did not improve endothelial function
Kawano [46]	2002	Intra-arterial infusion, 30 g for 1 h	Patients with hypercholesterolemia	17	Improvement of endothelial function in the brachial artery
Koifman [48]	2006	Intravenous infusion, 20 g	Patients with heart failure	9	Positive effects on hemodynamic variables and endothelium-dependent vasodilatation

times daily for 3 days) was able to increase endothelial-dependent vasodilatation and reduce monocyte adhesion to endothelial cells.

Coronary Artery Disease

Lerman et al. [25] reported the beneficial effects of oral supplementation of L-arginine in 26 patients with small vessel CAD. Coronary blood flow reserve in response to acetylcholine was assessed at baseline and 6-month intervals. The L-arginine group demonstrated in this blinded, controlled trial a significant increase in coronary blood flow and decreased endothelin levels compared with placebo.

In the years 1999 and 2000, Blum and colleagues published the results of three clinical trials dealing with the supplementation of oral L-arginine: The first trial [26] reported the benefits of oral L-arginine therapy (9 g/day for 30 days) in a crossover, double-blind study of ten patients with class IV angina. Oral L-arginine supplementation resulted in clinical improvement in seven patients (from angina pectoris class IV to class II). In their second trial, Blum et al. [27] reported an increase in plasma L-arginine levels after 1 month of oral L-arginine therapy in 30 patients with coronary artery

Table 38.4 Clinical studies of oral L-arginine supplementation (in order of publication year)

First author	Year	L-arginine dose (g/day)	Study population	Study duration (day)	Number of patients	Outcome	Study design
Adams [23]	1995	21	Healthy young men	3	12	Endothelial function (FMD)	R, PC, DB
Clarkson [49]	1996	21	Hypercholesterolemic subjects	28	27	Endothelial function (FMD, NMD)	R, PC, DB
Adams [24]	1997	21	Patients with CAD	3	10	Endothelial function (FMD)	R, PC, DB
Blum [26]	1999	9	Patients with angina pectoris	90	10	Clinical events	CC
Blum [28]	2000	9	Postmenopausal women	30	10	No augmentation in NO synthesis and release	R, DB, CO
Blum [27]	2000	9	Stable CAD	30	30	Endothelial function (FMD, NMD, brachial artery diameter)	R, PC, DB
Maxwell [30]	2002	6	Stable CAD	14	36	Endothelial function (FMD)	R, PC, DB
Lekakis [32]	2002	6	Patients with essential hypertension	1	18	Endothelial function (FMD, NMD, Intima media thickness)	R, PC, DB
Regensteiner [37]	2003	9	Women with type 2 diabetes	7	20	Endothelial function (FMD, basal forearm blood flow)	R, CO
Bode-Böger [42]	2003	16	Healthy individuals	14	12	Endothelial function (FMD)	R, PC, DB
Abdelhamed [31]	2003	3.3	Hypercholesterolemic patients	14	47	Endothelial function (FMD)	R, PC, DB
Yin [29]	2005	15	Stable CAD	28	31	Endothelial function (FMD)	R, PC, DB
Bednarz [34]	2005	9	Patients with acute myocardial infarction	30	774	Clinical events (all-cause mortality, myocardial reinfarction, successful resuscitation, shock/pulmonary edema, recurrent myocardial ischemia, and hospitalization for heart failure)	R, PC, DB
Lucotti [38]	2006	8.3	Obese type 2 diabetic patients	21	33	Endothelial function (Endothelin-1, Adiponectin)	R, PC, DB
Schulman [33]	2006	9	Patients with acute myocardial infarction	180	153	Endothelial function (arterial stiffness, arterial elastance, pulse wave velocity, radial artery compliance)	R, PC, DB

(continued)

Table 38.4 (continued)

First author	Year	L-arginine dose (g/day)	Study population	Study duration (day)	Number of patients	Outcome	Study design
Böger [41]	2007	3	Clinically asymptomatic elderly subjects	21	98	Endothelial function (FMD)	R, PC
Wilson [36]	2007	3	Peripheral arterial disease	180	133	Endothelial function	R, PC, DB
Siasos [40]	2008	6	Healthy individuals who smoked	3	10	Endothelial function (FMD, pulse wave velocity, augmentation index)	R, PC, DB
Monti [39]	2012	6.6	Obese subjects	14	15	Endothelial function (basal forearm blood flow, post-ischemic forearm blood flow)	R, PC, DB

disease, but no difference in flow-mediated dilatation was seen between the two groups. Finally, in a randomized, double-blind, crossover study, the same investigators [28] examined ten postmenopausal women receiving L-arginine (9 g) or placebo for 1 month. No effect of L-arginine supplementation was seen compared to placebo.

Yin et al. [29] also noted an improvement in endothelial function in patients with CAD after oral L-arginine supplementation. In this study, 31 subjects with stable CAD were randomly assigned to oral L-arginine (10 g) or vitamin C (500 mg) daily for 4 weeks, with crossover to the alternate therapy after 2 weeks off therapy. The study group demonstrated that consumption of L-arginine or vitamin C significantly increased brachial artery FMD.

Maxwell and colleagues [30] determined vascular and clinical effects of a medical food bar 162 enriched with L-arginine and a combination of other nutrients ON endothelium-derived NO PRODUCTION in 36 patients 163 with stable angina pectoris. In this randomized, double-blind, placebo-controlled, crossover trial, the medical food improved flow-mediated vasodilatation and quality of life scores. Another investigation with an enriched medical food (HeartBar) was performed in the year 2002 by Abdelhamed and colleagues which demonstrated no effect on FMD [31].

In a prospective, randomized, double-blind trial by Lekakis et al. [32], 35 subjects with essential hypertension were examined before and 1.5 h after administration of L-arginine or placebo. L-arginine supplementation (6 g) significantly improved flow-mediated dilatation while placebo did not change this parameter.

Schulman and colleagues [33] randomly assigned 153 patients, following a first ST-segment elevation myocardial infarction to receive L-arginine (9 g daily) or placebo for 6 months. In this trial, L-arginine supplementation had no effect on vascular stiffness measurements. A Polish workgroup [34] randomized 774 subjects with AMI to oral L-arginine (9 g daily for 30 days) or placebo on top of routine therapy. In this trial, the composite endpoint of 30-day cardiovascular death, reinfarction, successful resuscitation, shock/pulmonary edema, or recurrent myocardial ischemia occurred in 24 % patients with L-arginine and 27 % with placebo (RR 0.89, 95 % CI 0.70–1.13). Six months clinical events, including death, myocardial infarction, and hospitalization for heart failure, occurred in 12 (17 %) patients in the L-arginine group compared with 7 (10 %) in the placebo group. The event rate varied between these two trials and none of the trials showed a significant difference between the L-arginine and placebo group. Sun and colleagues [35] combined the results of these trials in a

meta-analysis. There was a 7 % reduction in mortality in the L-arginine group (105/459, 22.9 %) compared with the placebo group (111/455, 24.4 %), which did not reach statistical significance. Important limitations of this meta-analysis were that only two studies have been combined and that one of which was over five times the size of the other. So the results would be overwhelmed by the larger one.

Peripheral Artery Disease

The Nitric Oxide in Peripheral Arterial Insufficiency (NO-PAIN) trial [36] was a randomized, placebo-controlled study of L-arginine (3 g daily) over 6 months in 133 subjects with intermittent claudication. L-arginine supplementation significantly augmented plasma L-arginine levels. However, flow-mediated vasodilatation and vascular compliance were reduced or not improved compared to placebo.

Diabetes Mellitus and Impaired Glucose Tolerance

Regensteiner et al. [37] investigated ten premenopausal women with type 2 diabetes (T2DM) and ten premenopausal women without T2DM and randomized them to either L-arginine (9 g daily) or vitamin E (1800 mg) and vitamin C (1000 mg). At baseline, subjects with T2DM had reduced brachial artery dilatator responses to post-ischemic hyperemia when compared to nondiabetic controls. Administration of L-arginine resulted in a 50 ± 12 % increase in FMD in T2DM ($p < 0.05$). No significant changes were seen in controls.

Impaired Glucose Tolerance and Diabetes Mellitus

Lucotti and colleagues investigated oral L-arginine treatment in insulin-resistant type 2 diabetic patients [38]. Thirty-three patients with T2DM participated in a hypocaloric diet plus an exercise training program for 21 days and were randomized to either L-arginine (8.3 g daily) or placebo. L-arginine treatment reduced endothelin-1 levels by 30 % and an increase of cGMP levels by 35 %, whereas in the placebo group these parameters remained unchanged.

Recently, Monti et al. [39] examined the effects of L-arginine-enriched biscuits containing 6.6 g L-arginine compared to placebo biscuits and 6.6 g powdered L-arginine in 15 subjects with impaired glucose tolerance (IGT) and metabolic syndrome (MetS). L-arginine-enriched biscuits enhanced endothelial function in both subjects with IGT and those with MetS.

Smoking

Siasos et al. [40] studied in a randomized, placebo-controlled, double-blind, crossover trial the short-term effect of a 3-day oral supplementation of L-arginine (7 g daily) in ten healthy smokers. L-arginine led to an increase in FMD ($p < 0.05$ at day 2), compared to placebo; however, statistical significance was lost at day 3.

Elderly Subjects

Böger and colleagues [41] performed a study in 98 clinically asymptomatic elderly patients. Those in the highest and lowest quartiles of ADMA distribution were eligible to receive, in a random order, simvastatin (40 mg daily), L-arginine (3 g daily), or both, each for 3 weeks. L-arginine alone improved endothelial function either alone or in combination with simvastatin. In subjects with high ADMA, simvastatin had no effect on endothelial function, whereas simvastatin plus L-arginine significantly improved FMD. In the group with low ADMA levels, simvastatin improved FMD when given alone or in combination with L-arginine. The main finding of this trial is that the addition of L-arginine to simvastatin improves FMD in patients with elevated ADMA levels, whereas simvastatin alone had no significant effect.

Bode-Böger et al. [42] investigated in a prospective, randomized, double-blind, crossover trial the effect of L-arginine supplementation on endothelial function in patients older than 70 years. Twelve putatively healthy subjects took L-arginine (16 g daily) or placebo for 14 days each, separated by a washout period of 14 days. L-arginine significantly improved FMD and increased plasma levels of L-arginine, whereas placebo had no effect on these parameters.

Hypercholesterolemia

Creager et al. [43] examined whether infusion of L-arginine improves endothelium-dependent vasodilatation in hypercholesterolemic patients and healthy subjects. Intravenous infusion of L-arginine augmented forearm blood flow response to methacholine in patients with hypercholesterolemia but not in healthy subjects.

Chowienczyk et al. [44] demonstrated an improvement of acetylcholine-induced vasodilatation of brachial artery in males but not in females with hypercholesterolemia.

A research group from United Kingdom [45] examined endothelial response to L-arginine in three groups with isolated risk factors, smoking, insulin-dependent diabetes mellitus (IDDM), and hypercholesterolemia. Although flow-mediated dilatation is impaired in hypercholesterolemic subjects, smokers, and patients with diabetes, these risk factors respond differently to L-arginine infusion (0.1 g/kg). Indeed, FMD was improved in subjects with hypercholesterolemia and smokers, but remained unchanged in diabetic subjects.

In a study by Kawano and colleagues [46], 17 men with hypercholesterolemia were found to have increased FMD in 1 h post-infusion of L-arginine (30 g infused over 1 h).

Other Patient Groups

Cross and colleagues [47] investigated the effect of L-arginine by intravenous infusion (10 g) in 18 hemodialysis patients or by intra-arterial infusion (50 μmol/min) in eight predialysis patients on endothelial function. This trial demonstrated that neither acute local nor systemic administration of L-arginine improved endothelial function in conduit or resistance arteries in patients with chronic heart failure.

Koifman et al. [48] investigated the effects of losartan and the combination of losartan and L-arginine on endothelial function in patients with heart failure. Nine patients were given losartan 50 mg orally on 2 consecutive days. On the second day, 1 h after losartan administration, L-arginine (20 g) was given by intravenous infusion. Combination therapy significantly improved FMD. A trend toward improved

FMD was also observed with losartan alone. However, combination of L-arginine and losartan seems to have superior effects on endothelium-dependent vasodilatation compared with losartan alone.

Conclusions

L-arginine plays a pivotal role in vascular health and cardiovascular disease since it is the unique source for NO production. While total L-arginine blood levels turn out to be of limited prognostic value for future cardiovascular outcome, indices of L-arginine bioavailability, accounting for the metabolites ornithine and citrulline, proved to be more useful for risk prediction.

Although there is a plethora of animal and human studies suggesting cardiovascular benefit from L-arginine supplementation, large-scale outcome trials are needed to potentially prove beneficial effects on patient-relevant outcome measurements. This could be facilitated by currently ongoing trials as an adjunct using a factorial trial design.

References

1. Definition of cardiovascular diseases. http://www.euro.who.int/en/health-topics/noncommunicable-diseases/cardiovascular-diseases/cardiovascular-diseases2/definition-of-cardiovascular-diseases. Accessed 30 Oct 2014.
2. Nichols M, Townsend N, Scarborough P, Rayner M. Trends in age-specific coronary heart disease mortality in the European Union over three decades: 1980-2009. Eur Heart J. 2013;34:3017–27.
3. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation. 2002;106:653–8.
4. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation. 2004;109:III27–32.
5. Okyay K, Cengel A, Sahinarslan A, Tavil Y, Turkoglu S, Biberoglu G, Hasanoglu A. Plasma asymmetric dimethylarginine and L-arginine levels in patients with cardiac syndrome x. Coron Artery Dis. 2007;18:539–44.
6. Tang WH, Wang Z, Cho L, Brennan DM, Hazen SL. Diminished global L-arginine bioavailability and increased L-arginine catabolism as metabolic profile of increased cardiovascular risk. J Am Coll Cardiol. 2009;53:2061–7.
7. Closs EI. Expression, regulation and function of carrier proteins for cationic amino acids. Curr Opin Nephrol Hypertens. 2002;11:99–107.
8. Sourij H, Meinitzer A, Pilz S, Grammer TB, Winkelmann BR, Boehm BO, Marz W. L-Arginine bioavailability ratios are associated with cardiovascular mortality in patients referred to coronary angiography. Atherosclerosis. 2011;218:220–5.
9. Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V, Hazen SL, Vichinsky EP, Morris Jr SM, Gladwin MT. Dysregulated L-arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. JAMA. 2005;294:81–90.
10. Tripolt NJ, Meinitzer A, Eder M, Wascher TC, Pieber TR, Sourij H. Multifactorial risk factor intervention in patients with type 2 diabetes improves L-arginine bioavailability ratios. Diabet Med. 2012;29:e365–8.
11. Girerd XJ, Hirsch AT, Cooke JP, Dzau VJ, Creager MA. L-arginine augments endothelium-dependent vasodilation in cholesterol-fed rabbits. Circ Res. 1990;67:1301–8.
12. Cooke JP, Singer AH, Tsao P, Zera P, Rowan RA, Billingham ME. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest. 1992;90:1168–72.
13. Wang YX, Pang CC. Ng-nitro-L-arginine contracts vascular smooth muscle by an endothelium-independent mechanism. J Cardiovasc Pharmacol. 1994;24:59–63.
14. Hamon M, Vallet B, Bauters C, Wernert N, McFadden EP, Lablanche JM, Dupuis B, Bertrand ME. Long-term oral administration of L-arginine reduces intimal thickening and enhances neoendothelium-dependent acetylcholine-induced relaxation after arterial injury. Circulation. 1994;90:1357–62.
15. Tarry WC, Makhoul RG. L-arginine improves endothelium-dependent vasorelaxation and reduces intimal hyperplasia after balloon angioplasty. Arterioscler Thromb. 1994;14:938–43.
16. Singer AH, Tsao PS, Wang BY, Bloch DA, Cooke JP. Discordant effects of dietary L-arginine on vascular structure and reactivity in hypercholesterolemic rabbits. J Cardiovasc Pharmacol. 1995;25:710–6.
17. Boger RH, Bode-Boger SM, Mugge A, Kienke S, Brandes R, Dwenger A, Frolich JC. Supplementation of hypercholesterolaemic rabbits with L-arginine reduces the vascular release of superoxide anions and restores no production. Atherosclerosis. 1995;117:273–84.

18. Boger RH, Bode-Boger SM, Phivthong-ngam L, Brandes RP, Schwedhelm E, Mugge A, Bohme M, Tsikas D, Frolich JC. Dietary L-arginine and alpha-tocopherol reduce vascular oxidative stress and preserve endothelial function in hypercholesterolemic rabbits via different mechanisms. Atherosclerosis. 1998;141:31–43.
19. Davies MG, Barber E, Dalen H, Hagen PO. L-arginine supplementation improves venous endothelial cell but not smooth muscle cell dysfunction induced by prolonged diet-induced hypercholesterolemia. J Invest Surg. 1996;9:415–22.
20. Davies MG, Dalen H, Kim JH, Barber L, Svendsen E, Hagen PO. Control of accelerated vein graft atheroma with the nitric oxide precursor: L-arginine. J Surg Res. 1995;59:35–42.
21. Davies MG, Fulton GJ, Huynh TT, Barber L, Svendsen E, Hagen PO. Combination therapy of cholesterol reduction and L-arginine supplementation controls accelerated vein graft atheroma. Ann Vasc Surg. 1999;13:484–93.
22. Aji W, Ravalli S, Szabolcs M, Jiang XC, Sciacca RR, Michler RE, Cannon PJ. L-arginine prevents xanthoma development and inhibits atherosclerosis in ldl receptor knockout mice. Circulation. 1997;95:430–7.
23. Adams MR, Forsyth CJ, Jessup W, Robinson J, Celermajer DS. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. J Am Coll Cardiol. 1995;26:1054–61.
24. Adams MR, McCredie R, Jessup W, Robinson J, Sullivan D, Celermajer DS. Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. Atherosclerosis. 1997;129:261–9.
25. Lerman A, Burnett Jr JC, Higano ST, McKinley LJ, Holmes Jr DR. Long-term L-arginine supplementation improves small-vessel coronary endothelial function in humans. Circulation. 1998;97:2123–8.
26. Blum A, Porat R, Rosenschein U, Keren G, Roth A, Laniado S, Miller H. Clinical and inflammatory effects of dietary L-arginine in patients with intractable angina pectoris. Am J Cardiol. 1999;83:1488–90, A1488.
27. Blum A, Hathaway L, Mincemoyer R, Schenke WH, Kirby M, Csako G, Waclawiw MA, Panza JA, Cannon 3rd RO. Oral L-arginine in patients with coronary artery disease on medical management. Circulation. 2000;101:2160–4.
28. Blum A, Hathaway L, Mincemoyer R, Schenke WH, Kirby M, Csako G, Waclawiw MA, Panza JA, Cannon 3rd RO. Effects of oral L-arginine on endothelium-dependent vasodilation and markers of inflammation in healthy postmenopausal women. J Am Coll Cardiol. 2000;35:271–6.
29. Yin WH, Chen JW, Tsai C, Chiang MC, Young MS, Lin SJ. L-arginine improves endothelial function and reduces ldl oxidation in patients with stable coronary artery disease. Clin Nutr. 2005;24:988–97.
30. Maxwell AJ, Zapien MP, Pearce GL, MacCallum G, Stone PH. Randomized trial of a medical food for the dietary management of chronic, stable angina. J Am Coll Cardiol. 2002;39:37–45.
31. Abdelhamed AI, Reis SE, Sane DC, Brosnihan KB, Preli RB, Herrington DM. No effect of an L-arginine-enriched medical food (HeartBars) on endothelial function and platelet aggregation in subjects with hypercholesterolemia. Am Heart J. 2003;145:E15.
32. Lekakis JP, Papathanassiou S, Papaioannou TG, Papamichael CM, Zakopoulos N, Kotsis V, Dagre AG, Stamatelopoulos K, Protogerou A, Stamatelopoulos SF. Oral L-arginine improves endothelial dysfunction in patients with essential hypertension. Int J Cardiol. 2002;86:317–23.
33. Schulman SP, Becker LC, Kass DA, Champion HC, Terrin ML, Forman S, Ernst KV, Kelemen MD, Townsend SN, Capriotti A, Hare JM, Gerstenblith G. L-arginine therapy in acute myocardial infarction: the vascular interaction with age in myocardial infarction (vintage mi) randomized clinical trial. JAMA. 2006;295:58–64.
34. Bednarz B, Jaxa-Chamiec T, Maciejewski P, Szpajer M, Janik K, Gniot J, Kawka-Urbanek T, Drozdowska D, Gessek J, Laskowski H. Efficacy and safety of oral L-arginine in acute myocardial infarction. Results of the multicenter, randomized, double-blind, placebo-controlled ARAMI pilot trial. Kardiol Pol. 2005;62:421–7.
35. Sun T, Zhou WB, Luo XP, Tang YL, Shi HM. Oral L-arginine supplementation in acute myocardial infarction therapy: a meta-analysis of randomized controlled trials. Clin Cardiol. 2009;32:649–52.
36. Wilson AM, Harada R, Nair N, Balasubramanian N, Cooke JP. L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. Circulation. 2007;116:188–95.
37. Regensteiner JG, Popylisen S, Bauer TA, Lindenfeld J, Gill E, Smith S, Oliver-Pickett CK, Reusch JE, Weil JV. Oral L-arginine and vitamins e and c improve endothelial function in women with type 2 diabetes. Vasc Med. 2003;8:169–75.
38. Lucotti P, Setola E, Monti LD, Galluccio E, Costa S, Sandoli EP, Fermo I, Rabaiotti G, Gatti R, Piatti P. Beneficial effects of a long-term oral L-arginine treatment added to a hypocaloric diet and exercise training program in obese, insulin-resistant type 2 diabetic patients. Am J Physiol Endocrinol Metab. 2006;291:E906–12.
39. Monti LD, Casiraghi MC, Setola E, Galluccio E, Pagani MA, Quaglia L, Bosi E, Piatti P. L-arginine enriched biscuits improve endothelial function and glucose metabolism: a pilot study in healthy subjects and a cross-over study in subjects with impaired glucose tolerance and metabolic syndrome. Metabolism. 2013;62:255–64.
40. Siasos G, Tousoulis D, Vlachopoulos C, Antoniades C, Stefanadi E, Ioakeimidis N, Andreou I, Zisimos K, Papavassiliou AG, Stefanadis C. Short-term treatment with L-arginine prevents the smoking-induced impairment of endothelial function and vascular elastic properties in young individuals. Int J Cardiol. 2008;126:394–9.

41. Boger GI, Rudolph TK, Maas R, Schwedhelm E, Dumbadze E, Bierend A, Benndorf RA, Boger RH. Asymmetric dimethylarginine determines the improvement of endothelium-dependent vasodilation by simvastatin: effect of combination with oral L-arginine. J Am Coll Cardiol. 2007;49:2274–82.
42. Bode-Boger SM, Muke J, Surdacki A, Brabant G, Boger RH, Frolich JC. Oral L-arginine improves endothelial function in healthy individuals older than 70 years. Vasc Med. 2003;8:77–81.
43. Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest. 1992;90:1248–53.
44. Chowienczyk PJ, Watts GF, Cockcroft JR, Brett SE, Ritter JM. Sex differences in endothelial function in normal and hypercholesterolaemic subjects. Lancet. 1994;344:305–6.
45. Thorne S, Mullen MJ, Clarkson P, Donald AE, Deanfield JE. Early endothelial dysfunction in adults at risk from atherosclerosis: different responses to L-arginine. J Am Coll Cardiol. 1998;32:110–6.
46. Kawano H, Motoyama T, Hirai N, Kugiyama K, Yasue H, Ogawa H. Endothelial dysfunction in hypercholesterolemia is improved by L-arginine administration: possible role of oxidative stress. Atherosclerosis. 2002;161:375–80.
47. Cross JM, Donald AE, Kharbanda R, Deanfield JE, Woolfson RG, MacAllister RJ. Acute administration of L-arginine does not improve arterial endothelial function in chronic renal failure. Kidney Int. 2001;60:2318–23.
48. Koifman B, Topilski I, Megidish R, Zelmanovich L, Chernihovsky T, Bykhovsy E, Keren G. Effects of losartan + L-arginine on nitric oxide production, endothelial cell function, and hemodynamic variables in patients with heart failure secondary to coronary heart disease. Am J Cardiol. 2006;98:172–7.
49. Clarkson P, Adams MR, Powe AJ, Donald AE, McCredie R, Robinson J, McCarthy SN, Keech A, Celermajer DS, Deanfield JE. Oral L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults. J Clin Invest. 1996;97:1989–94.

Chapter 39
L-Arginine Therapy in Sickle Cell Disease

Claudia R. Morris

Key Points

- Sickle cell disease is an L-arginine deficiency syndrome.
- Hemolysis and the release of erythrocyte-arginase into circulation contribute to an altered L-arginine metabolome in sickle cell disease.
- Low nitric oxide bioavailability contributes to vasculopathy in sickle cell disease.
- Low global L-arginine bioavailability is associated with severe pain, pulmonary hypertension risk, and early mortality in sickle cell disease.
- L-Arginine therapy shows promise in preliminary studies for the treatment of leg ulcers, pulmonary hypertension, and vaso-occlusive pain episodes in patients with sickle cell disease.
- Intravenous L-arginine therapy decreased total opioid use by greater than 50 % and was associated with lower pain scores at discharge in children with sickle cell disease hospitalized for pain compared to placebo in a recently published randomized, placebo-controlled trial.

Keywords Arginase • L-Arginine • Cell-free hemoglobin • Global L-arginine bioavailability ratio • Hemolysis • Nitric oxide • Oxidative stress • Sickle cell disease • Vaso-occlusive pain episodes

Abbreviations

ACS	Acute chest syndrome
ADMA	Asymmetric dimethylarginine
CAT	Cationic amino acid transporter
CSCC	Comprehensive sickle cell centers
ED	Emergency department

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GAB	Global L-arginine bioavailability
HU	Hydroxyurea
K_m	Michaelis affinity constant
LDH	Lactate dehydrogenase
MACE	Major adverse cardiovascular events
NADP	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOHA	*N*-hydroxy-L-arginine
NOS	Nitric oxide synthase
NO*x*	NO metabolites
PICU	Pediatric intensive care unit
VOE	Vaso-occlusive painful episodes

Introduction

Sickle cell disease (SCD) affects nearly 100,000 people in the USA and millions worldwide. Genetically, SCD is caused by an amino acid substitution of valine for glutamic acid in the sixth position of the β subunits of hemoglobin. This structural change results in intracellular polymerization of the deoxygenated hemoglobin molecules under hypoxic conditions. Intracellular polymer increases erythrocyte rigidity and ultimately damages and distorts the erythrocyte membrane. This produces a rigid "sickled" red cell with altered rheological and adhesive properties that becomes entrapped in the microcirculation and gives rise to the vaso-occlusive events characteristic of the disease. The clinical phenotype of SCD varies widely and is characterized by anemia, severe pain, and potentially life-threatening complications such as bacterial sepsis, splenic sequestration, acute chest syndrome (ACS), stroke, and chronic organ damage. These and other manifestations result from acute and chronic hemolysis and intermittent episodes of vascular occlusion that cause tissue injury and organ dysfunction [1, 2].

Although polymerization is an important contributor toward disease pathology, SCD is as much a disease of endothelial dysfunction [3] as it is a hemoglobinopathy. Increased expression of adhesion molecules on erythrocytes and endothelial cells, interactions with leukocytes, increased levels of circulating inflammatory cytokines, enhanced microvascular thrombosis, and endothelial damage are all thought to contribute to obstruction of the arterioles by sickled erythrocytes [4, 5].

SCD is an L-arginine deficiency syndrome [6, 7]. Normal L-arginine metabolism is impaired through various mechanisms (Fig. 39.1) that contribute to endothelial dysfunction, vaso-occlusion, pulmonary complications, risk of leg ulcers, and early mortality [8, 9]. Since low global L-arginine bioavailability (GAB) is associated with a growing number of SCD-related complications [6, 10], L-arginine therapy represents a promising option for SCD [8].

L-Arginine is a semi-essential cationic amino acid involved in multiple pathways in health and disease. It becomes essential, however, under conditions of stress and catabolic states when the capacity of endogenous L-arginine synthesis is exceeded, including trauma, sepsis, burns, and in particular, SCD which is the focus of this chapter. L-Arginine serves as a substrate for protein synthesis and is the precursor to nitric oxide (NO), polyamines, proline, glutamate, creatine, and agmatine. Since it is involved in multiple metabolic processes, an L-arginine deficiency has the potential to disrupt many cellular and organ functions [7].

L-Arginine is derived from dietary protein intake, body protein breakdown, or endogenous de novo L-arginine production in the kidneys. Approximately 2–7 g of L-arginine is ingested daily in a normal Western diet. Common dietary sources are meat, poultry, nuts, fish, and watermelon. It is also a safe nutritional supplement that has been studied extensively in human and animal trials, including a growing number of trials in SCD [8].

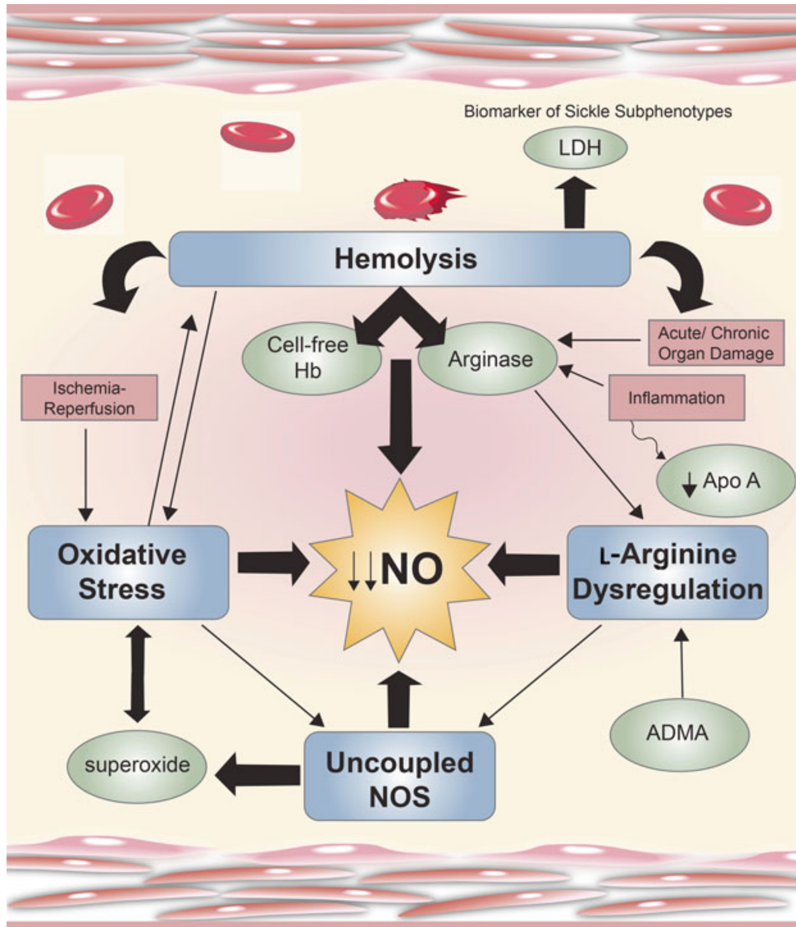

Fig. 39.1 Mechanisms of vasculopathy in SCD. Hemolysis, L-arginine dysregulation, oxidative stress, uncoupled NOS, and damage from redox-active heme are key mechanisms that contribute to the complex vascular pathophysiology of SCD. These events limit NO bioavailability through several paths that ultimately provoke increased consumption and decreased production of the potent vasodilator, NO. During hemolysis, cell-free hemoglobin and arginase are simultaneously released from the erythrocyte and profoundly contribute to low NO bioavailability. LDH is also released from the erythrocyte and represents a convenient biomarker of hemolysis that delineates the subphenotypes of SCD. Reproduced with permission [9]

Alterations in the L-Arginine–Nitric Oxide Pathway in Sickle Cell Disease

Low Nitric Oxide Bioavailability

NO is one of the most potent vasodilators known and is essential to vascular homeostasis. As the obligate substrate for NO production, L-arginine plays a crucial role in endothelial function. NO is produced by a family of enzymes, the nitric oxide synthases (NOS) that metabolize L-arginine through the intermediate *N*-hydroxy-L-arginine (NOHA) to form NO and L-citrulline. A number of cofactors are necessary for normal NOS function, including oxygen, nicotinamide adenine dinucleotide phosphate (NADP), tetrahydrobiopterin, and sufficient glutathione availability [11, 12]. NO has properties that can impact every aspect of SCD, from decreasing platelet activation [13] and adhesion receptor expression on the vascular endothelium to decreasing vascular smooth muscle proliferation [14], limiting ischemia–reperfusion injury [15], modulating endothelial proliferation [3], and regulating inflammation [16].

Although NOS expression and activity is increased [17], SCD is characterized by a state of NO resistance and increased NO inactivation [18, 19]. Impaired NO bioavailability in this disorder is demonstrated by blunted response to endothelium-dependent vasodilators in sickle cell mouse models [20, 21], as well as by a reduced flow-mediated vasodilation in human patients with SCD [22–24]. NO metabolites (NO*x*) are elevated in patients with SCD at steady state compared to normal controls [25], while peripheral vascular resistance and resting blood pressures are low. Adding to this paradox, a "relative hypertension" occurs in SCD since blood pressures, while lower than in normal subjects, are higher than in other types of anemia [26]. Under conditions of increased hemolysis, inflammation, and/or oxidative stress, the compensatory upregulation of NO likely becomes overwhelmed and ineffective. NO*x* levels drop during times of stress including vaso-occlusive pain episodes (VOE) [27, 28] and ACS [27], varying inversely with degree of pain [29].

Low Global L-Arginine Bioavailability

Adults with SCD are L-arginine deficient at steady state [27, 30, 31], while children have plasma levels that are similar to normal controls [27]. An L-arginine deficiency develops over time and is influenced by acute events [27]. Although the altered L-arginine metabolome differs in children compared to adults, plasma L-arginine concentration decreases significantly in both adults and children during VOE and ACS and is associated with low NO*x* levels [27, 32, 33]. Both L-arginine and NO*x* levels returned to baseline during convalescence in the hospital. Ongoing intermittent vaso-occlusion may lead to a chronic depletion of L-arginine stores that are worsened by acute events. Of interest, low plasma L-arginine levels predicted clinical need for admission in children with SCD and pain presenting for emergency care, while NO*x* levels did not [27], suggesting a role for L-arginine bioavailability during pain events that goes beyond NO production.

Intracellular L-arginine transport is another component of L-arginine dysregulation in SCD. Plasma L-arginine concentration in adults with SCD is approximately 40–50 μM at baseline, low compared to normal controls (80–100 μM) and well below the affinity constant (K_m) for the cationic amino acid transporter (CAT) protein (100–150 μM) responsible for intracellular L-arginine uptake. Therefore, even modest fluctuations in extracellular L-arginine concentration may significantly impact cellular L-arginine uptake and bioavailability. In addition, ornithine and lysine use the same CAT protein for intracellular transport. High concentrations of these amino acids will competitively inhibit intracellular L-arginine transport [8].

More recently, focus has turned to the pathway of L-arginine catabolism by arginase as important in regulating endogenous NO production. The NOS and arginase enzymes can be expressed simultaneously under a wide variety of inflammatory conditions, resulting in competition for their common substrate [34]. Arginase is a urea cycle enzyme that catalyzes the hydrolysis of L-arginine to urea and L-ornithine. Both Arginase I and II isoforms are found in many cell types and constitutively expressed in the human airways; Arginase I is cytosolic and highly expressed in the liver, while arginase II is mitochondrial and extrahepatic. Arginase-1 is also present in human erythrocytes, which has significant implications for hemolytic disorders like SCD, where it is aberrantly released into plasma in active form as the red blood cells rupture. While the affinity (K_m) of L-arginine for arginase is in the low micromolar range compared to the low millimolar range for NOS, substrate competition does occur between arginase and NOS because the V_{max} of arginase is 1000-fold higher [35]. By competing for a common substrate, arginase reduces the bioavailability of L-arginine for NOS, therefore limiting NO production.

Plasma arginase activity is elevated in SCD as a consequence of inflammation, liver dysfunction, and, most significantly, by the release of erythrocyte arginase during intravascular hemolysis [6], which has been demonstrated by the strong correlation between plasma arginase levels and cell-free hemoglobin levels [6] together with other markers of increased hemolytic rate including lactate

dehydrogenase (LDH) [6, 36]. Whether inflammatory or hemolytic in origin, arginase will redirect the metabolism of L-arginine to ornithine and the formation of polyamines and proline, which are essential to form smooth muscle cell growth and collagen synthesis. By creating a shift toward ornithine metabolism, arginase can trigger a process that contributes to the vascular smooth muscle proliferation and airway remodeling. These are features of asthma and pulmonary hypertension, common comorbidities found in SCD that are also associated with increased mortality risk and low GAB [6, 37, 38]. This is particularly important given the implications of pulmonary disease in SCD, where hypoxemia will trigger a cycle of erythrocyte sickling.

An L-arginine deficiency in SCD is associated with elevated arginase activity and a low L-arginine–ornithine ratio [6, 39] (Fig. 39.2) that correlates to markers of hemolysis [6, 8, 36]. The L-arginine–ornithine ratio also correlates to mortality in SCD and may represent an easily attainable blood biomarker of arginase activity and disease severity. Given de novo synthesis of L-arginine occurs from

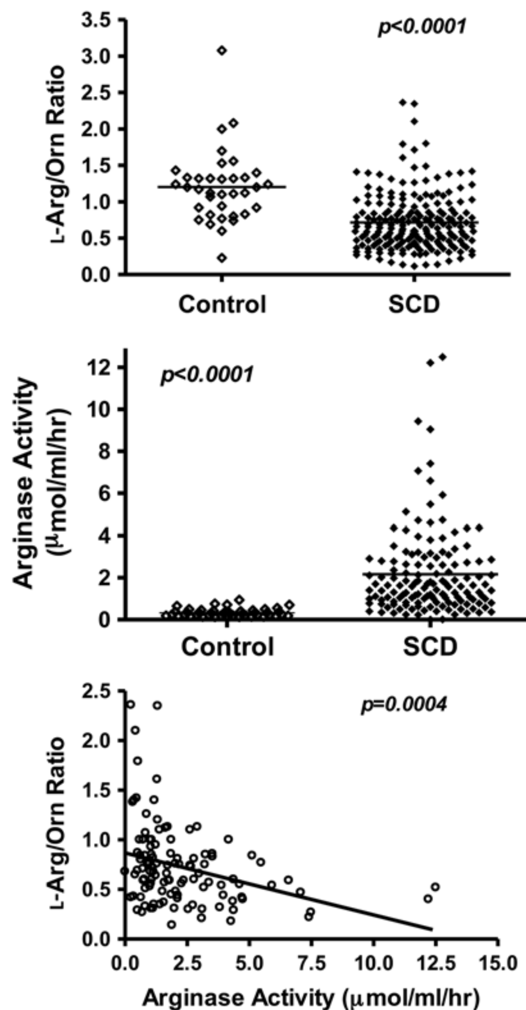

Fig. 39.2 Association of L-arginine–ornithine ratio with plasma arginase activity in patients with SCD. (**a**) L-Arginine–ornithine ratio in controls vs. patients with SCD. (**b**) Plasma arginase activity in controls vs. patients with SCD. (**c**) Correlation of plasma arginase activity to L-arginine–ornithine ratio. Reproduced with permission from the American Medical Association [6]

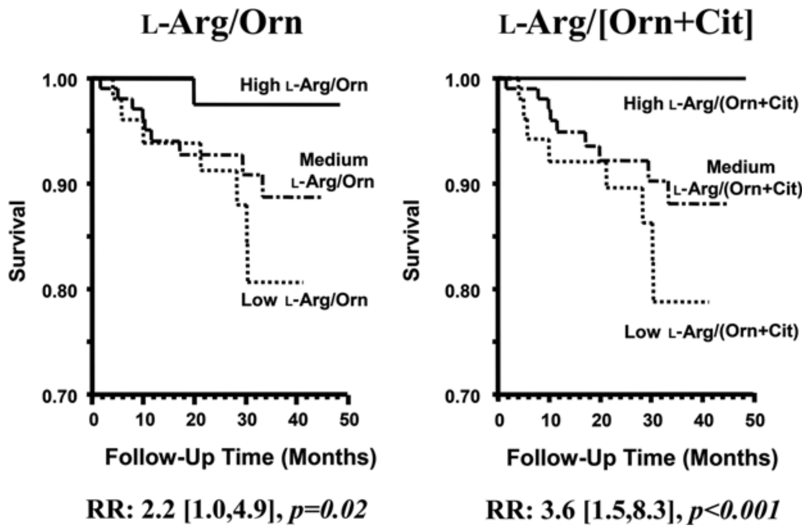

Fig. 39.3 Association of global L-arginine bioavailability ratios (GABR) with mortality in sickle cell disease: Kaplan–Meier survival plots. (**a**) Survival for three categories of L-arginine–ornithine ratio: "high" = upper quartile, >0.8690; "medium" = 25th to 75th percentiles, >0.4385 and ≤0.8690; "low" = lower quartile, ≤0.4385. (**b**) Survival for three categories of L-arginine-(ornithine + citrulline) ratio: "high" = upper quartile, >0.6254; "medium" = 25th to 75th percentiles, >0.3245 and ≤0.6254; "low" = lower quartile, ≤0.3245. Reproduced with permission from the American Medical Association [6]

citrulline in the kidneys, including citrulline in the ratio (L-arginine/[ornithine+citrulline]) escalates the value of this analysis to identify increased risk of death by taking into account the impact of renal dysfunction on L-arginine bioavailability [6, 40] (Fig. 39.3). Low GAB may be exacerbated further by the presence of elevated asymmetric dimethylarginine (ADMA), which is a competitive inhibitor of L-arginine transport and all NOS isozymes. Circulating ADMA levels are elevated in several conditions of endothelial dysfunction, including SCD, and are also linked to increased mortality [41].

Low L-arginine bioavailability itself may contribute to increased consumption and decreased production of NO. Under conditions of low L-arginine or tetrahydrobiopterin availability [42], NOS is uncoupled, producing reactive oxygen species in lieu of NO [43], potentially further reducing NO bioavailability. An imbalance between endothelial NOS-derived NO and superoxide generation has been established in SCD by Wood et al. [44]. These authors were also the first to suggest that abnormal tetrahydrobiopterin function or availability may be yet another mechanism contributing to dysregulation of the L-arginine-NO pathway in SCD [44]. Upregulation of NOS would therefore enhance oxidative stress when L-arginine, tetrahydrobiopterin, or other NOS cofactors like glutathione are deficient [11, 12, 45] and NOS becomes uncoupled. This process is supported by studies in transgenic sickle cell mice that demonstrate that NOS activity is paradoxically increased [46] and uncoupled [47] in a disease state involving a marked decrease in NO bioavailability.

Hemolysis: A Path Toward L-Arginine Dysregulation

Hemolysis will drive L-arginine consumption. Accumulating evidence supports a paradigm of hemolysis-associated endothelial dysfunction that is particularly relevant for SCD [9, 48–52]. Rapid consumption and decreased production of NO is a fundamental aspect of this model. Hemoglobin is decompartmentalized from the erythrocyte during the process of hemolysis and released into plasma

where it rapidly reacts with and destroys NO [18]. This results in abnormally high NO consumption, the formation of reactive oxygen species, and a state of NO resistance [19]. The simultaneous release of erythrocyte-arginase will metabolize L-arginine during hemolysis [6] and further diminished NO bioavailability (Fig. 39.4). Formation of superoxide from enzymatic oxidases such as NADPH oxidase, xanthine oxidase [53], and uncoupled endothelial NOS [47] will also react with and scavenge NO, further amplifying a state of NO resistance. Consequently, smooth muscle guanylyl cyclase is not activated and vasodilation is inhibited. NO destruction by hemoglobin can also cause further impairment in vascular endothelial function via transcriptional activation of adhesion molecules and potent vasoconstrictors such as endothelin-1 [19]. Intravascular hemolysis also has the potential to drive a pro-coagulant state, as NO has properties that inhibit platelet activation, tissue factor expression, and thrombin generation [19]. This phenomenon has now been implicated as a mechanism of NO depletion in the red cell storage lesion [54] and other hemolytic conditions such as thalassemia, malaria, and paroxysmal nocturnal hemoglobinuria [55–58]. Clinically, decreased NO bioavailability ultimately contributes to the development of the hemolytic subphenotypes of SCD [6, 36], which include pulmonary hypertension, priapism, cutaneous leg ulceration, stroke, renal dysfunction, and possibly asthma [37, 49, 59] (Fig. 39.5).

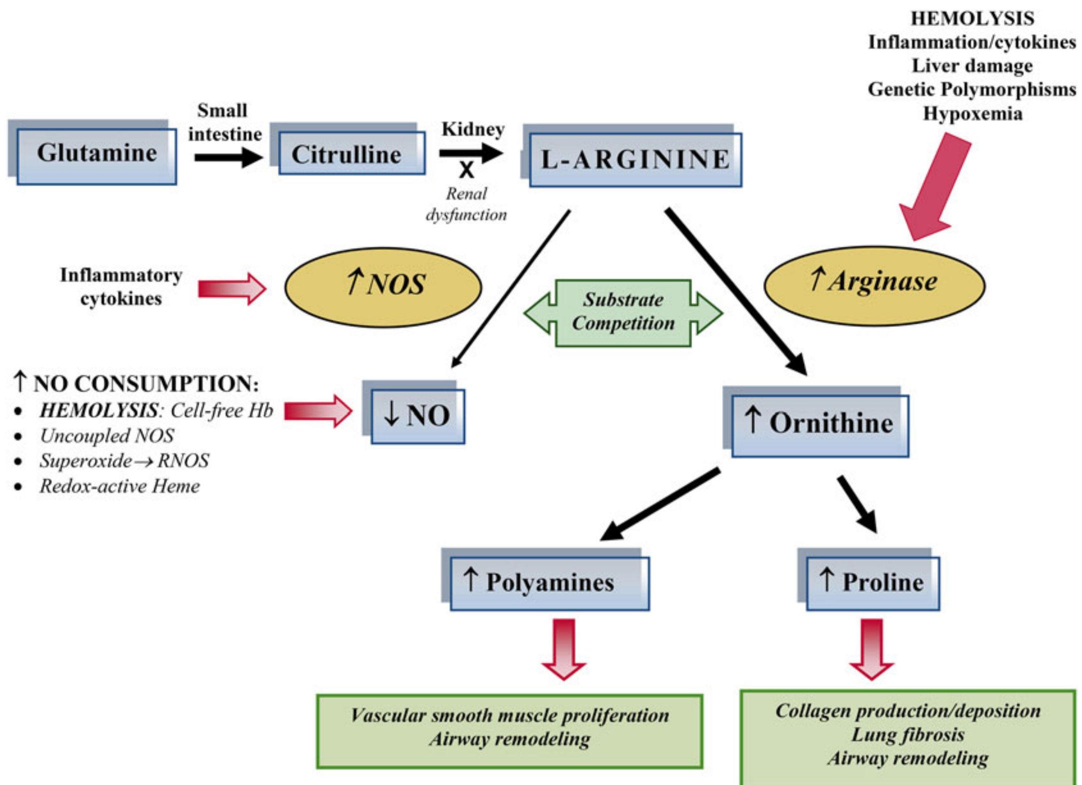

Fig. 39.4 Altered L-arginine metabolism in hemolysis. Dietary glutamine serves as a precursor for the de novo production of L-arginine through the citrulline–L-arginine pathway. L-Arginine is synthesized endogenously from citrulline primarily via the intestinal–renal axis. Arginase and NOS compete for L-arginine, their common substrate. In SCD, bioavailability of L-arginine and NO are decreased by several mechanisms linked to hemolysis and oxidative stress. Endothelial dysfunction resulting from NO depletion and increased levels of the downstream products of ornithine metabolism (polyamines and proline) likely contribute to the pathogenesis of lung injury, fibrosis, and pulmonary hypertension. This disease paradigm has implications for all hemolytic processes. Reproduced with permission [9]

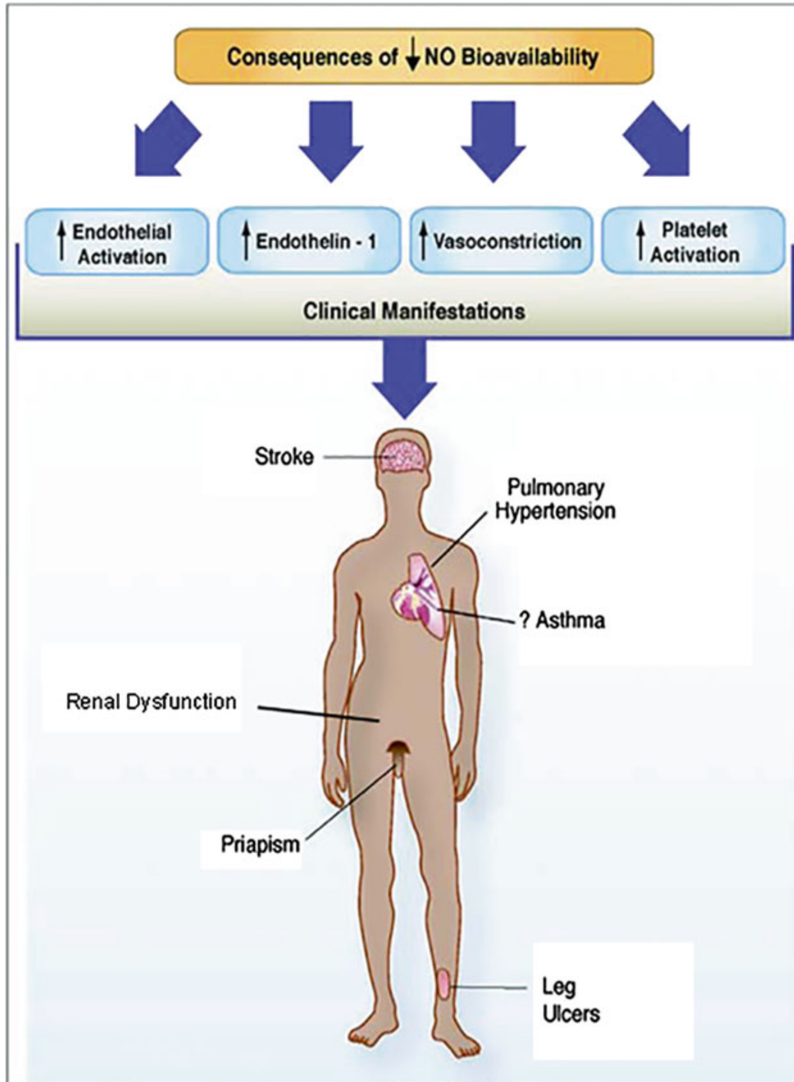

Fig. 39.5 Consequences of low NO bioavailability. NO bioavailability is particularly vulnerable to the effects of hemolysis. The consequences of decreased NO bioavailability include endothelial cell activation, upregulation of the potent vasoconstrictor endothelin-1, vasoconstriction, platelet activation, increased tissue factor, and activation of coagulation pathways, all of which ultimately translates into the clinical manifestations of SCD. Reproduced with permission [9]

Therapeutic Potential of L-Arginine Therapy in Sickle Cell Disease

Although mechanisms of L-arginine dysregulation are complex and multifactorial [9], they can be overcome through L-arginine supplementation, a phenomenon known as the "L-arginine paradox" [60]. The exact mechanisms responsible for the benefits of L-arginine therapy in SCD remain unknown but likely are not limited to NO production alone. In transgenic mouse models of SCD, L-arginine supplementation inhibits the red cell Gardos channels [61], reduces red cell density [61], improves perfusion, and reduces inflammation [62], lung injury, microvascular vaso-occlusion, and mortality [47, 63, 64]. L-Arginine also increases erythrocyte glutathione levels in both mouse [63] and human

trials [65]. In addition, L-arginine is a key substrate in creatine synthesis, an important metabolic pathway not yet sufficiently studied in SCD that may be impacted by an L-arginine-deficient state. Although the role of NO in SCD has become controversial [66, 67], these studies further demonstrate that the mechanistic impact of L-arginine may go beyond NO production.

Independent of SCD, low GAB is associated with major adverse cardiovascular events (MACE) including mortality in patients screened for cardiovascular disease [40] (Fig. 39.6), mortality risk in malaria [56], and is associated with pulmonary hypertension risk [68–70]. Rapid healing of leg ulcers was reported with oral ***L-arginine***-hydrochloride [39] and intravenous ***L-arginine***-butyrate in both SCD and thalassemia [71]. A randomized controlled phase-2 trial of intravenous *L-arginine*-butyrate for patients with SCD and chronic recalcitrant leg ulcers confirmed the initial anecdotal observations [72]. Short-term L-arginine therapy improved pulmonary hypertension in SCD [39] (Fig. 39.7) and acutely increased both plasma and exhaled NO when administered to ethnically matched normal controls and patients hospitalized for pain [32, 73]. When L-arginine is given to SCD patients at steady state, a paradoxical decrease in NO*x* occurs that is not overcome by higher doses [32], clearly indicating that L-arginine is metabolized differently in SCD compared to control subjects. However, when L-arginine is given during VOE, a robust dose-dependent increase in NO*x* is observed. [32] This indicates that L-arginine is also metabolized differently in SCD at steady-state compared to times of acute illness including pain and ACS [27, 32, 73]. These early observations may account for the negative outcome of the unpublished Comprehensive Sickle Cell Centers' (CSCC) prophylactic L-arginine trial, particularly since the primary outcome measure of that study was an increase in plasma NO*x* levels, when published data in fact demonstrated a decrease in NO*x* with L-arginine supplementation in SCD patients at baseline. Ultimately nutritional therapies like L-arginine may possess the greatest benefit potential during a deficient state.

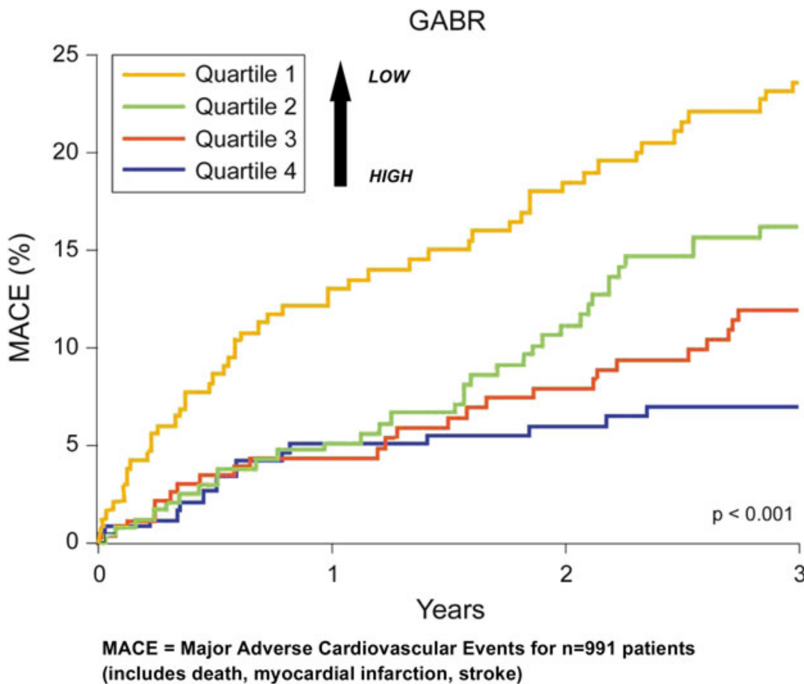

Fig. 39.6 Kaplan–Meier survival analysis for patients with 3-year incidence of major adverse cardiac events (MACE) according to global L-arginine bioavailability ratio (GABR) by L-arginine quartiles. Low GABR is associated with increased risk of nonfatal myocardial infarction, nonfatal stroke, or death within 3 years of follow-up. Reproduced with permission [40]

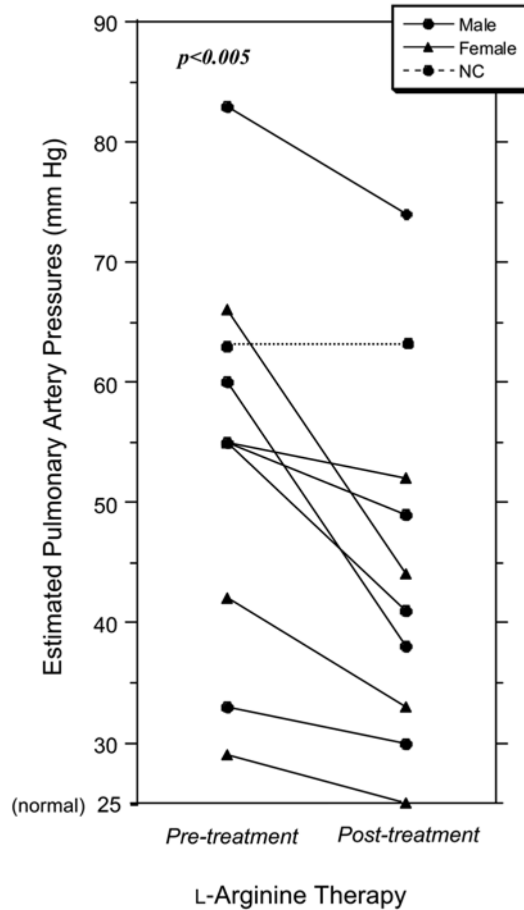

Fig. 39.7 Changes in estimated mean pulmonary artery systolic pressures (mmHg) measured by Doppler echocardiography in patients with SCD and pulmonary hypertension risk. Measurements are taken before L-arginine therapy is started (*pretreatment*) and after completion of 15 doses of L-arginine (*posttreatment*). Male patients are represented by *circle*s, and females are represented by *triangles*. The *dotted line* represents the only patient found to be noncompliant (NC) based on posttreatment plasma L-arginine levels. L-Arginine supplementation significantly decreases estimated pulmonary artery systolic pressures ($n = 10$; $p < 0.005$). Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society [39]

The capacity of L-arginine supplementation to increase NO*x* production in SCD during VOE is dose-dependent [32]. Low-dose L-arginine therapy is therefore likely to be subtherapeutic in SCD and may represent an additional flaw in the CSCC prophylactic L-arginine trial design, as doses used were close to placebo based on the cardiovascular literature [74, 75]. Previous studies have shown that low-dose L-arginine is unlikely to impact NO synthesis [75], an observation confirmed in the CSCC study. Higher levels of plasma L-arginine are likely needed to overcome multifactorial effects including impact of arginase and ADMA on GAB and accelerated L-arginine consumption during pain events compared to baseline. However, the long-term safety of doses greater than 100 mg/kg/dose given three times a day is unknown in SCD, although a one-time dose of 30 g IV is safe and commonly used for growth hormone stimulation testing [76]. Since the L-arginine formula is L-arginine hydrochloride, the wisdom of higher doses over time is questionable given the potential to induce acidosis and must be taken into consideration. However, this author believes that using a one-time loading dose upon initial presentation for pain should be safe based on endocrine experience with growth hormone

stimulation testing using 500 mg/kg/dose for children, would avoid risks of repeated higher doses and may theoretically be more efficacious by quickly overcoming effects of excess arginase and ADMA. This is the rationale behind investigating clinical effects of a loading dose of 200 mg/kg upon initial presentation for pain in pediatric SCD studies that are underway.

Based on preliminary pharmacokinetic studies [32, 73], peak plasma L-arginine concentration after oral L-arginine (100 mg/kg) is significantly higher during SCD steady state compared to patients experiencing VOE, although levels are similar by 4 h. Normal controls reach a peak L-arginine level between 1 and 2 h that is maintained at 4 h, and does not trend down as in SCD [32]. Accelerated L-arginine metabolism or consumption occurs during pain events compared to steady-state despite the same oral L-arginine dose given. Similar observations were made with respect to L-arginine pharmacokinetics in moderate compared to severe malaria [77, 78], suggesting that a greater consumption of L-arginine may occur when the disease state or hemolytic rate is more severe. An L-arginine infusion significantly improved endothelial function and maintained plasma L-arginine concentration above the K_m for CAT-1 for the duration of the infusion compared to bolus dosing in patients with malaria [77, 79]. Bolus dosing provided concentrations above the K_m for 50 % of the patients at 2 h and only 25 % at 3 h. It remains to be determined if L-arginine infusions are superior to bolus dosing of L-arginine in SCD; however, this question will be addressed in future clinical trials in SCD.

Combination Therapy: L-Arginine Plus Hydroxyurea

Coadministration of oral L-arginine with hydroxyurea (HU) ameliorated the paradoxical decrease in plasma NO*x* observed in patients with SCD at steady state compared with L-arginine monotherapy [73]. A recently published study performed in Brazil adds to the growing body of literature in support of L-arginine coadministration with HU. Twenty-one adult patients with SCD were randomized to receive HU alone (500–1500 mg/day; $n=9$) or HU + L-arginine (250 mg/day; $n=12$) for 12 weeks. An increase in levels of nitrite and fetal hemoglobin were observed in the L-arginine/HU arm compared with patients receiving HU alone [80], despite the low dose of L-arginine used. L-Arginine therapy together with HU may be superior to either single intervention. This is important information to consider when designing clinical trials, particularly since up to 50 % of patients with Hb-SS may be on HU therapy. Stratification by HU use is important; however, patients on HU should not be excluded from L-arginine trials.

L-Arginine Therapy for Pain

Pain is a clinical hallmark of SCD and a significant problem in emergency medicine. VOEs are the leading cause of hospitalizations, ED visits, and missed school and are associated with an increased mortality rate [81]. Nationally, 78 % of the nearly 200,000 annual emergency department (ED) visits for SCD are for a complaint of pain. There is no effective therapy that targets the underlying mechanisms of sickle-related pain. Symptomatic relief with analgesics and hydration is the only currently available treatment, and this has not changed in decades. Episodic periods of severe pain lead to high use of health care resources, with high readmission rates even in patients initially hospitalized for pain management. Hospital admission rates are particularly high for children with SCD presenting to ED with pain and are commonly greater than 60 % [81]. Although the reason for high pediatric admission rates are unknown, many children with SCD live with daily pain to some extent that their families try to control at home through various methods. It is when the pain becomes acutely worse, and unbearable, that they present to the ED, often in acute distress. Novel approaches to treatment of acute pain for SCD that can be utilized in the ED as well on the hospital ward are critically needed. Interventions that target underlying mechanisms of SCD pain in addition to providing symptomatic relief would be ideal and are worth pursuing.

Since an acute L-arginine deficiency and low NO bioavailability develops during VOEs, it is intuitive to explore the potential of L-arginine supplementation as a potential new treatment for sickle-related pain. It is interesting to note that low GAB has recently been reported in patients with complex regional pain syndrome, suggesting a role for L-arginine bioavailability in pain beyond SCD [82]. We have now completed a single-center randomize, double-blinded, placebo-controlled trial of L-arginine therapy in children with SCD and pain requiring hospitalization [83]. Thirty-eight children with SCD admitted for 56 episodes of VOE were randomized to receive oral or parenteral L-arginine (100 mg/kg three times per day) or placebo for 5 days or until discharge. A significant reduction in total parenteral opioid use by 54 % (1.9 ± 2.0 mg/kg vs. 4.1 ± 4.1 mg/kg, $p = 0.02$) and lower pain scores at discharge (1.9 ± 2.4 vs. 3.9 ± 2.9, $p = 0.01$) were observed in the treatment arm receiving L-arginine compared with placebo (Fig. 39.8). There was no significant difference in hospital length of stay (4.1 ± 01.8 vs. 4.8 ± 2.5 days, $p = 0.34$), although a trend favored the L-arginine arm, and total opioid use correlated strongly to length of admission ($r = 0.86$, $p < 0.0001$; Fig. 39.9). In future studies, delivering L-arginine therapy as early as possible in the ED or clinic may have a greater impact on time to

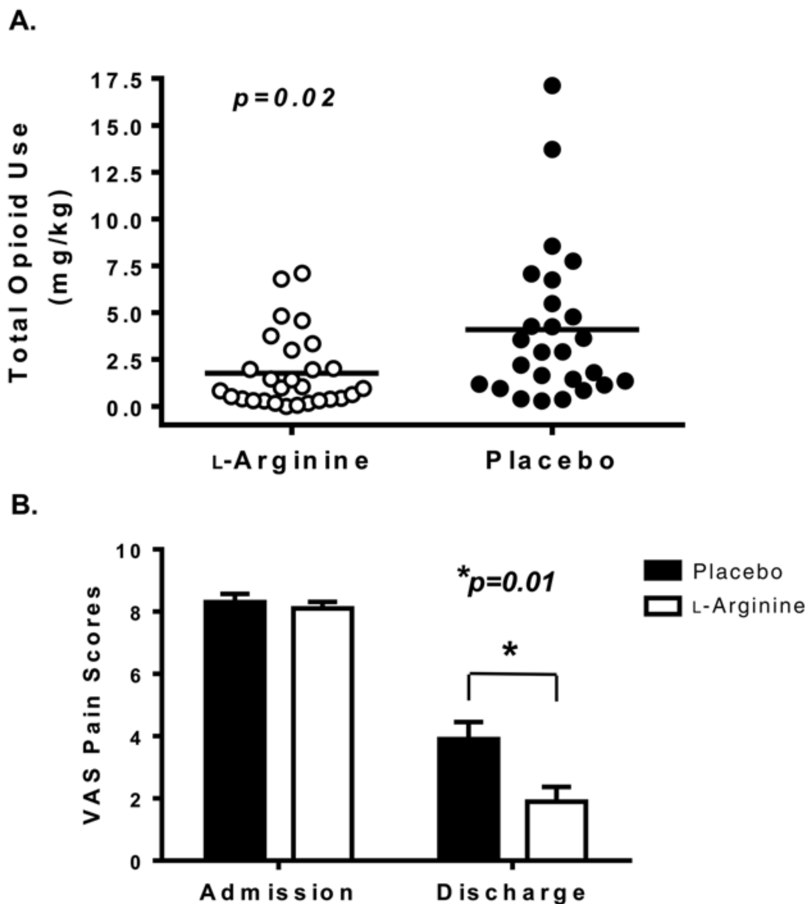

Fig. 39.8 Impact of L-arginine therapy on total opioid use (mg/kg) and pain scores in children sickle cell disease hospitalized for vaso-occlusive pain. (**a**) L-Arginine supplementation (*unfilled circles*) led to a significant and clinically relevant reduction in total opioid use by 54 % over the course of the hospital stay compared to total opioid use in the placebo group (*filled circles*). The difference remains significant even when the two outliers with the largest total opioid use in the placebo arm are excluded from the analysis ($p = 0.04$). (**b**) 10-cm visual analog scale (VAS) pain scores were similar at the time of admission in both groups, but were significantly lower at discharge in the L-arginine group compared to placebo by 2 cm ($p = 0.01$). Reproduced with permission [83]

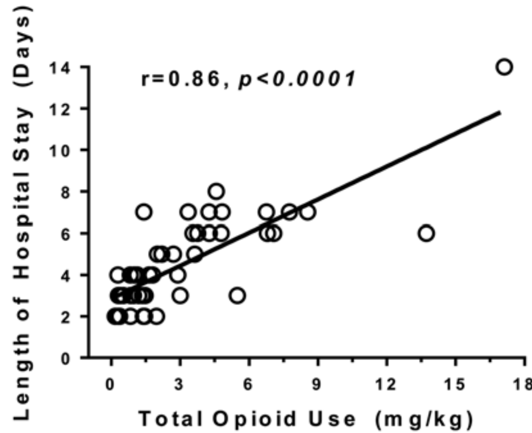

Fig. 39.9 Pearson correlation between total opioid use (mg/kg) and total length of hospital stay (days). Total opioid use (mg/kg) is directly correlated to length of hospital stay ($r=0.86$, $p<0.0001$). Total opioid use may be a surrogate for length of hospital stay as an outcome measure for patients with SCD and pain. Reproduced with permission [83]

pain crisis resolution because many patients in the above L-arginine study received their first dose of study medication more than 24 h after presenting to the ED in pain [83].

No drug-related adverse events were observed. One patient experienced clinical deterioration associated with ACS requiring emergent transfusion and a transfer to the pediatric intensive care unit (PICU) in the placebo arm. No clinical deterioration or PICU transfers occurred in the L-arginine arm [83]. Although a large-scale multicenter trial is needed to confirm these promising observations, L-arginine may be a beneficial adjunct to standard pain therapy for VOE that could reduce suffering and improve emergency care.

Conclusions

An altered L-arginine metabolome occurs in SCD and is associated with acute pain, pulmonary hypertension, leg ulcers, and early mortality. However, the utility of L-arginine supplementation to treat this L-arginine deficiency syndrome remains controversial. The failure of other NO-based therapies in SCD, including the use of inhaled NO for treatment of sickle-related pain [84], and sildenafil for the treatment of pulmonary hypertension [85] has significantly dampened enthusiasm in the field for this therapeutic approach. Nevertheless, promising data from phase-2 randomized controlled trials for treatment of chronic refractory leg ulcers and vaso-occlusive pain in patients with SCD support the need for further investigation. A greater than 50 % decrease in total opioid use in children hospitalized for pain is remarkable. This is the first successful intervention for SCD-related pain that targets the underlying mechanism of VOE through a promising NO-based therapy. L-arginine is a safe and efficacious intervention with narcotic-sparing effects in pediatric SCD patients with pain. Experience with L-arginine therapy in SCD has been growing over the last decade [32, 39, 63, 69, 73, 86]. No serious adverse events have been reported and extensive safety data has been maintained with the United States Food and Drug Administration. Interventions that target underlying mechanisms of sickle-related pain beyond simply providing symptomatic relief are ideal. A large, multicenter phase-3 study is now warranted; plans for such a trial are under way by this author and colleagues.

References

1. Stuart MJ, Nagel RL. Sickle-cell disease. Lancet. 2004;364(9442):1343–60.
2. Gladwin MT, Vichinsky E. Pulmonary complications of sickle cell disease. N Engl J Med. 2008;359(21): 2254–65.
3. Hebbel RP, Osarogiagbon KD. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. Microcirculation. 2004;11:129–51.
4. Frenette PS. Sickle cell vaso-occlusion: multistep and multicellular paradigm. Curr Opin Hematol. 2002;9(2):101–6.
5. Parise LV, Telen MJ. Erythrocyte adhesion in sickle cell disease. Curr Hematol Rep. 2003;2(2):102–8.
6. Morris CR, Kato GJ, Poljakovic M, et al. Dysregulated L-arginine metabolism, hemolysis-associated pulmonary hypertension and mortality in sickle cell disease. JAMA. 2005;294(1):81–90.
7. Morris Jr SM. Arginases and L-arginine deficiency syndromes. Curr Opin Clin Nutr Metab Care. 2012;15(1):64–70.
8. Morris CR. Alterations of the L-arginine metabolome in sickle cell disease: a growing rationale for L-arginine therapy. Hematol Oncol Clin North Am. 2014;28(2):301–21.
9. Morris CR. Mechanisms of vasculopathy in sickle cell disease and thalassemia. Hematology Am Soc Hematol Educ Program. 2008;2008:177–85.
10. Cox SE, Makani J, Komba AN, et al. Global L-arginine bioavailability in Tanzanian sickle cell anaemia patients at steady-state: a nested case control study of deaths versus survivors. Br J Haematol. 2011;155(4):522–4.
11. Stuehr DJ, Kwon N, Nathan CF, Griffith OW, Felman PL, Wiseman J. N-Hydroxyl-L-arginine is an intermediate in the biosynthesis of nitric oxide for L-arginine. J Biol Chem. 1991;266:6259–63.
12. Stuehr DJ, Kwon NS, Nathan CF. FAD and GSH participate in macrophage synthesis of nitric oxide. Biochem Biophys Res Commun. 1990;168(2):558–65.
13. Adams MR, Forsyth CJ, Jessup W, Robinson J, Celermajer DS. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young men. J Am Coll Cardiol. 1995;26:1054–61.
14. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329:2002–12.
15. Kaul DK, Hebbel RP. Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. J Clin Invest. 2000;106:411–20.
16. Peng H-B, Spiecker M, Liao J. Inducible nitric oxide: an autoregulatory feedback inhibitor of vascular inflammation. J Immunol. 1998;161:1970–6.
17. Reiter CD, Gladwin MT. An emerging role for nitric oxide in sickle cell disease vascular homeostatis and therapy. Curr Opin Hematol. 2003;10:99–107.
18. Reiter C, Wang X, Tanus-Santos J, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle cell disease. Nat Med. 2002;8:1383–9.
19. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA. 2005;293:1653–62.
20. Aslan M, Ryan TM, Adler B, et al. Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. Proc Natl Acad Sci U S A. 2001;98(26):15215–20.
21. Kaul DK, Liu XD, Fabry ME, Nagel RL. Impaired nitric oxide-mediated vasodilation in transgenic sickle mouse. Am J Physiol Heart Circ Physiol. 2000;278:H1799–806.
22. Eberhardt RT, McMahon L, Duffy SJ, et al. Sickle cell anemia is associated with reduced nitric oxide bioactivity in peripheral conduit and resistance vessels. Am J Hematol. 2003;74:104–11.
23. Gladwin M, Schechter A, Ognibene F, et al. Divergent nitric oxide bioavailability in men and women with sickle cell disease. Circulation. 2003;107:271–8.
24. Belhassen L, Pelle G, Sediame S, et al. Endothelial dysfunction in patients with sickle cell disease is related to selective impairment of shear stress-mediated vasodilation. Blood. 2001;97:1584–9.
25. Rees DC, Cervi P, Grimwade D, et al. The metabolites of nitric oxide in sickle-cell disease. Br J Haematol. 1995;91:834–7.
26. Pegelow CH, Colangelo L, Steinberg M, et al. Natural history of blood pressure in sickle cell disease: risks for stroke and death associated with relative hypertension in sickle cell anemia. Am J Med. 1997;102(2):171–7.
27. Morris CR, Kuypers FA, Larkin S, Vichinsky E, Styles L. Patterns of L-arginine and nitric oxide in sickle cell disease patients with vaso-occlusive crisis and acute chest syndrome. J Pediatr Hematol Oncol. 2000;22:515–20.
28. Lopez BL, Barnett J, Ballas SK, Christopher TA, Davis-Moon L, Ma X. Nitric oxide metabolite levels in acute vaso-occlusive sickle-cell crisis. Acad Emerg Med. 1996;3:1098–103.
29. Lopez B, Davis-Moon L, Ballas S. Sequential nitric oxide measurements during the emergency department treatment of acute vasoocclusive sickle cell crisis. Am J Hematol. 2000;64:15–9.
30. Enwonwu CO. Increased metabolic demand for L-arginine in sickle cell anaemia. Med Sci Res. 1989;17:997–8.
31. Waugh W, Daeschner C, Files B, Gordon D. Evidence that L-arginine is a key amino acid in sickle cell anemia—a preliminary report. Nutr Res. 1999;19:501–18.

32. Morris CR, Kuypers FA, Larkin S, et al. L-Arginine therapy: a novel strategy to increase nitric oxide production in sickle cell disease. Br J Haematol. 2000;111:498–500.
33. Lopez B, Kreshak A, Morris CR, Davis-Moon L, Ballas S, Ma X. L-arginine levels are diminished in adult acute vaso-occlusive sickle cell crisis in the emergency department. Br J Haematol. 2003;120:532–4.
34. Takemoto K, Ogino K, Shibamori M, et al. Transiently, paralleled upregulation of arginase and nitric oxide synthase and the effect of both enzymes on the pathology of asthma. Am J Physiol Lung Cell Mol Physiol. 2007;293(6):L1419–26.
35. Wu G, Morris SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336:1–17.
36. Kato GJ, McGowan V, Machado RF, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. Blood. 2006;107(6):2279–85.
37. Morris CR. Asthma management: reinventing the wheel in sickle cell disease. Am J Hematol. 2009;84(4):234–41.
38. Morris CR, Poljakovic M, Lavisha L, Machado L, Kuypers F, Morris Jr SM. Decreased L-arginine bioavailability and increased arginase activity in asthma. Am J Respir Crit Care Med. 2004;170:148–53.
39. Morris CR, Morris Jr SM, Hagar W, et al. L-Arginine therapy: a new treatment for pulmonary hypertension in sickle cell disease? Am J Respir Crit Care Med. 2003;168:63–9.
40. Tang WH, Wang Z, Cho L, Brennan DM, Hazen SL. Diminished global L-arginine bioavailability and increased L-arginine catabolism as metabolic profile of increased cardiovascular risk. J Am Coll Cardiol. 2009;53(22):2061–7.
41. Kato GJ, Wang Z, Machado RF, Blackwelder WC, Taylor 6th JG, Hazen SL. Endogenous nitric oxide synthase inhibitors in sickle cell disease: abnormal levels and correlations with pulmonary hypertension, desaturation, haemolysis, organ dysfunction and death. Br J Haematol. 2009;145(4):506–13.
42. Berka V, Wu G, Yeh HC, Palmer G, Tsai AL. Three different oxygen-induced radical species in endothelial nitric-oxide synthase oxygenase domain under regulation by L-arginine and tetrahydrobiopterin. J Biol Chem. 2004;279(31):32243–51.
43. Xia Y, Dawson V, Dawson T, Snyder S, Zweier J. Nitric oxide synthase generates superoxide and nitric oxide in L-arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci USA. 1996;93:6770–4.
44. Wood KC, Hebbel RP, Lefer DJ, Granger DN. Critical role of endothelial cell-derived nitric oxide synthase in sickle cell disease-induced microvascular dysfunction. Free Radic Biol Med. 2006;40(8):1443–53.
45. Morris CR, Suh JH, Hagar W, et al. Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease. Blood. 2008;140:104–12.
46. Bank N, Aynedjian H, Qiu J, et al. Renal nitric oxide synthases in transgenic sickle cell mice. Kidney Int. 1996;50:184–9.
47. Hsu LL, Champion HC, Campbell-Lee SA, et al. Hemolysis in sickle cell mice causes pulmonary hypertension due to global impairment in nitric oxide bioavailability. Blood. 2007;109:3088–98.
48. Barnett CF, Hsue PY, Machado RF. Pulmonary hypertension: an increasingly recognized complication of hereditary hemolytic anemias and HIV infection. JAMA. 2008;299(3):324–31.
49. Kato GJ, Gladwin MT, Steinberg MH. Deconstructing sickle cell disease: reappraisal of the role of hemolysis in the development of clinical subphenotypes. Blood Rev. 2007;21(1):37–47.
50. Morris C, Kuypers F, Kato G, et al. Hemolysis-associated pulmonary hypertension in thalassemia. Ann N Y Acad Sci. 2005;1054:481–5.
51. Potoka KP, Gladwin MT. Vasculopathy and pulmonary hypertension in sickle cell disease. Am J Physiol Lung Cell Mol Physiol. 2015;308(4):L314–24. doi:10.1152/ajplung.00252.2014.
52. Gladwin MT, Ofori-Acquah SF. Erythroid DAMPs drive inflammation in SCD. Blood. 2014;123(24):3689–90.
53. Aslan M, Freeman BA. Oxidant-mediated impairment of nitric oxide signaling in sickle cell disease—mechanisms and consequences. Cell Mol Biol (Noisy-le-Grand). 2004;50(1):95–105.
54. Donadee C, Raat NJ, Kanias T, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation. 2011;124(4):465–76.
55. Morris CR, Vichinsky EP. Pulmonary hypertension in thalassemia. Ann N Y Acad Sci. 2010;1202:205–13.
56. Omodeo-Sale F, Cortelezzi L, Vommaro Z, Scaccabarozzi D, Dondorp AM. Dysregulation of L-arginine metabolism and bioavailability associated to free plasma heme. Am J Physiol Cell Physiol. 2010;299(1):C148–54.
57. Morris CR, Kim H, Klings ES, et al. Dysregulated L-arginine metabolism and cardiopulmonary dysfunction in patients with thalassaemia. Br J Haematol. 2015;169(6):887–98 [Epub ahead of print].
58. Morris CR, Kim HY, Wood J, et al. Sildenafil therapy in thalassemia patients with Doppler-defined risk of pulmonary hypertension. Haematologica. 2013;98(9):1359–67.
59. Saraf SL, Zhang X, Kanias T, et al. Haemoglobinuria is associated with chronic kidney disease and its progression in patients with sickle cell anaemia. Br J Haematol. 2014;164(5):729–39.

60. Gornik HL, Creager MA. L-Arginine and endothelial and vascular health. J Nutr. 2004;134(10 Suppl):2880S–7. Discussion 2895S.
61. Romero J, Suzuka S, Nagel R, Fabry M. L-Arginine supplementation of sickle transgenic mice reduces red cell density and Gardos channel activity. Blood. 2002;99:1103–8.
62. Manci EA, Hyacinth HI, Capers PL, et al. High protein diet attenuates histopathologic organ damage and vascular leakage in transgenic murine model of sickle cell anemia. Exp Biol Med (Maywood). 2014;239(8):966–74.
63. Dasgupta T, Hebbel RP, Kaul DK. Protective effect of L-arginine on oxidative stress in transgenic sickle mouse models. Free Radic Biol Med. 2006;41(12):1771–80.
64. Kaul DK, Zhang X, Dasgupta T, Fabry ME. L-Arginine therapy of transgenic-knockout sickle mice improves microvascular function by reducing non-nitric oxide vasodilators, hemolysis, and oxidative stress. Am J Physiol Heart Circ Physiol. 2008;295(1):H39–47.
65. Little JA, Hauser KP, Martyr SE, et al. Hematologic, biochemical, and cardiopulmonary effects of L-arginine supplementation or phosphodiesterase 5 inhibition in patients with sickle cell disease who are on hydroxyurea therapy. Eur J Haematol. 2009;82(4):315–21.
66. Gladwin MT, Barst RJ, Castro OL, et al. Pulmonary hypertension and NO in sickle cell. Blood. 2010; 116(5):852–4.
67. Bunn HF, Nathan DG, Dover GJ, et al. Pulmonary hypertension and nitric oxide depletion in sickle cell disease. Blood. 2010;116(5):687–92.
68. Morris CR, Teehankee C, Kato G, et al. Decreased L-arginine bioavailability contributes to the pathogenesis of pulmonary artery hypertension. In: American College of Cardiology Annual Meeting, Orlando, FL, 6–9 Mar 2005.
69. Morris CR. New strategies for the treatment of pulmonary hypertension in sickle cell disease: the rationale for L-arginine therapy. Treat Respir Med. 2006;5(1):31–45.
70. Morris CR, Gladwin MT, Kato G. Nitric oxide and L-arginine dysregulation: a novel pathway to pulmonary hypertension in hemolytic disorders. Curr Mol Med. 2008;8:81–90.
71. Sher GD, Olivieri NG. Rapid healing of leg ulcers during L-arginine butyrate therapy in patients with sickle cell disease and thalassemia. Blood. 1994;84:2378–80.
72. McMahon L, Tamary H, Askin M, et al. A randomized phase II trial of L-Arginine Butyrate with standard local therapy in refractory sickle cell leg ulcers. Br J Haematol. 2010;151(5):516–24.
73. Morris CR, Vichinsky EP, van Warmerdam J, et al. Hydroxyurea and L-arginine therapy: impact on nitric oxide production in sickle cell disease. J Pediatr Hematol Oncol. 2003;25:629–34.
74. Morris CR. Reduced global L-arginine bioavailability: a common mechanism of vasculopathy in sickle cell disease and pulmonary hypertension. Blood. 2010. http://bloodjournal.hematologylibrary.org/cgi/eletters/blood-2010-02-268193v1 [e-letter] 22 Apr 2010.
75. Maxwell AJ, Cooke JP. Cardiovascular effects of L-arginine. Curr Opin Nephrol Hypertens. 1998;7:63–70.
76. Merimee TJ, Rabinowitz D, Riggs L, Burgess JA, Rimoin DL, McKusick VA. Plasma growth hormone after L-arginine infusion. N Engl J Med. 1967;276:434–9.
77. Yeo TW, Rooslamiati I, Gitawati R, et al. Pharmacokinetics of L-arginine in adults with moderately severe malaria. Antimicrob Agents Chemother. 2008;52(12):4381–7.
78. Yeo TW, Lampah DA, Rooslamiati I, et al. A randomized pilot study of L-arginine infusion in severe falciparum malaria: preliminary safety, efficacy and pharmacokinetics. PLoS One. 2013;8(7):e69587.
79. Yeo TW, Lampah DA, Gitawati R, et al. Safety profile of L-arginine infusion in moderately severe falciparum malaria. PLoS One. 2008;3(6):e2347.
80. Elias DB, Barbosa MC, Rocha LB, et al. L-arginine as an adjuvant drug in the treatment of sickle cell anaemia. Br J Haematol. 2013;160(3):410–2.
81. Brousseau DC, Owens PL, Mosso AL, Panepinto JA, Steiner CA. Acute care utilization and rehospitalizations for sickle cell disease. JAMA. 2010;303(13):1288–94.
82. Alexander GM, Reichenberger E, Peterlin BL, Perreault MJ, Grothusen JR, Schwartzman RJ. Plasma amino acids changes in complex regional pain syndrome. Pain Res Treat. 2013;2013:742407.
83. Morris CR, Kuypers FA, Lavrisha L, et al. A randomized, placebo-controlled trial of L-arginine therapy for the treatment of children with sickle cell disease hospitalized with vaso-occlusive pain episodes. Haematologica. 2013;98(9):1375–82.
84. Gladwin MT, Kato GJ, Weiner D, et al. Nitric oxide for inhalation in the acute treatment of sickle cell pain crisis: a randomized controlled trial. JAMA. 2011;305(9):893–902.
85. Machado RF, Barst RJ, Yovetich NA, et al. Hospitalization for pain in patients with sickle cell disease treated with sildenafil for elevated TRV and low exercise capacity. Blood. 2011;118(4):855–64.
86. Little JA, McGowan VR, Kato GJ, et al. Combination erythropoietin-hydroxyurea therapy in sickle cell disease: experience from the National Institutes of Health and a literature review. Haematologica. 2006;91(8):1076–83.

Part VII
Therapeutic Uses of L-*Arginine*: Cancer, Wound Healing and Infectious Disease

Chapter 40
Role of L-Arginine in Surgical Patients with Head and Neck Cancer

Daniel de Luis, Rocio Aller, and Pablo Casas-Rodera

Key Points

- Significant malnutrition exists in up to 40–50 % of patients with cancer of the head and neck.
- There is evidence that perioperative nutritional supplements with immunonutritional additives can favorably modulate the immune and inflammatory responses both in vitro and in patients with trauma or burns or those undergoing oncological surgery.
- L-Arginine is a semi-essential amino acid and the store can become depleted in times of stress. It plays an important role in T- and B-cell immunity as well as in the production of nitric oxide.
- In tumor patients with head and neck surgery, wound infection and fistula formation are two major complications that could influence the evolution of these postsurgical patients.
- Postsurgical patients with head and neck cancer show an improvement in immunological parameters with L-arginine-enhanced enteral formulas, without reductions in clinical complications.
- Perioperative immunonutrition with L-arginine is associated with reduced length of hospital stay.

Keywords L-Arginine • Head and neck • Immunonutrition • Cancer • Surgery

Abbreviations

BMI	Body mass index
CI	Confidence interval
EORTC-QL	European Organization for Research and Treatment of Cancer Quality of Life Questionnaire
IG	Immunoglobulin
RNA	Ribonucleotide acid
TNF	Tumor necrosis factor

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Introduction

Significant malnutrition exists in up to 40–50 % of patients with cancer of the head and neck [1]. Many factors contribute to malnutrition in these patients, including poor dietary practices, alcoholism, catabolic factors secreted by the tumor, such as the cytokines tumor necrosis factor-α (TNF-α) and interleukins (IL), local tumor effects, anorexia, cancer-induced cachexia, and treatment effects [2]. Nutritional evaluation is a main key in the treatment of these surgical patients (Table 40.1). Patients undergoing surgery because of a head and neck malignancy have a 20–50 % incidence of postoperative complications [3]. These complications include major wound infections, fistula, anastomotic leakage, and septicemia and may lead to not only a prolonged hospital stay but also a poorer prognosis. Several factors may contribute to this morbidity, one of which is malnutrition [4].

Malnutrition is associated with defects in immune function that may impair the host response to malignancy. The alterations in the host defense mechanism make patients highly susceptible to postoperative infections. Multiple components of the diet may affect immune function. There is evidence that perioperative nutritional supplements with immunonutritional additives can favorably modulate the immune and inflammatory responses both in vitro and in patients with trauma or burns or those undergoing oncological surgery [5]. In particular, the important role of amino acids (glutamine and L-arginine), dietary nucleotides (RNA), and lipids (w3 fatty acids) in modulating immune function has been recognized [6].

Standard commercial nutritional supplements are described as polymeric, which means they contain whole protein, partially digested starch and triglycerides with electrolytes, minerals, trace elements and vitamins. They are usually given in liquid form and designed to provide a patient's "complete" nutritional requirements. These formulas could be enriched with immunonutrients, and L-arginine is the most common immunonutrient given to patients with head and neck cancer. L-Arginine is a semi-essential amino acid and the store can become depleted in times of stress. It plays an important role in T- and B-cell immunity as well as in the production of nitric oxide. L-Arginine is able to reduce the production of inflammatory mediators such as IL-1 beta, IL-6, and TNF-α at the site of injury in rat septic models and can accelerate tissue growth after trauma [7]. Dietary supplementation with L-arginine has positive effects on immune function and reparative collagen synthesis [8].

Table 40.1 Useful nutritional parameters in patients with head and neck surgery

Anthropometric parameters
Weight
Loss of weight (percentage)
Body mass index
Tricipital skinfold
Midarm circumference
Biochemical parameters
Albumin
Pre-albumin
Transferrin
Screening test
Malnutrition Screening tool
Nutritional assessment test
Subjective global assessment test

This table shows the most important anthropometric, biochemical parameters, screening test, and nutritional assessment test used in clinical practice

Studies with L-Arginine-Enhanced Enteral Formulas (Randomized Clinical Trials)

Sixteen randomized controlled trials were identified with a total of 873 patients all undergoing surgery for head and neck cancer (Tables 40.2 and 40.3) [10–24]. Three trials compared two types of immunonutrition started at hospital discharge [12, 23, 24] and 13 trials compared polymeric feeds with immunonutrition [10, 11, 13–22].

These patients had different age, sex ratio, and basal body mass index (BMI); these variables must be taken into account to explain differences among studies. All studies used isocaloric and isonitrogenous feed regimens.

The first variables analyzed in these studies are immunological parameters (Table 40.4). These parameters are indirect variables of clinical evolution and its variation could imply clinical differences in the main outcomes. The trials examined reported a lot of biochemical and immunological parameters including C-reactive protein, interleukin-6, tumor necrosis factor-α, T-cell subsets, and total lymphocyte counts. Riso et al. [16] demonstrated an increase in total lymphocytes, CD4, and CD4/CD8 ratio on postoperative day 4. In this study [16], malnourished patients showed reduced preoperative immune status in some variables (IgA, IgG, CD4, CD4/CD8), with some parameters (CD4, CD4/CD8) increasing postoperatively compared with baseline but not between the two groups.

Our group [20] showed no significant intergroup differences in the trend of the two plasma proteins, lymphocytes, and weight. In the three groups that were compared, there was a significant decrease of the transferrin at the seventh postoperative day, in relation to preoperative levels, with a significant increase only in the enriched diet groups, at the 14th postoperative day. The control group showed the highest levels of TNF-α at the 14th postoperative day. The control group showed the lower levels of lymphocytes at the 7th and 14th postoperative day.

In tumor patients with head and neck surgery, wound infection and fistula formation are two major complications that could influence the evolution of these postsurgical patients. The effects of immunonutrition on wound infections and fistula formation are shown in Table 40.5. First of all, occurrence of fistula formation was reported in nine trials and ranged from 0 % (0/23) to 5 % (4/82) in immunonutrition fed groups and from 0 % (0/38) to 18.9 % (7/37) in control groups. Secondly, rate of wound infection was reported in five trials. The risk of wound infection ranged from 0 % (0/45) to 4.8 % (4/82) in immunonutrition fed groups and from 0 % (0/45) to 12.5 % (3/24) in control groups. The effects of immunonutrition in malnourished patients could only be ascertained from the study by Riso et al. [16], where 13 patients were considered malnourished. These patients had reduced wound infections when given immunonutrition.

Despite the importance of immunological markers as well as complications related to surgical wound (infection and fistula), undoubtedly the most interesting clinical variables are hospital stay and survival. Mean postoperative hospital stays were long (Table 40.6) with a huge standard deviations. de Luis et al. [13] reported a significant reduction in postoperative stay, 25.8 days versus 35 days in intervention and control groups, respectively. Riso et al. [16] reported a reduced hospital stay in the intervention group. In a recently systematic review, pooled estimates showed a reduction in length of hospital stay by 3.5 days (95 % CI 0.7 to 6.3 days, $P<0.01$) [25].

The last to relevant clinical variables in this type of patients are locoregional recurrence and long-term survival. Buijs et al. [21] showed that the median overall long-term survival was 34.8 months in the L-arginine-supplemented group and 20.7 months in the control group ($P=0.019$). Disease-specific survival was 94.4 months in the L-arginine-supplemented group and 20.8 months in the control group ($P=0.022$). Locoregional recurrence occurred in 4 of the 17 patients in the L-arginine group and in 9 of the 15 patients in the control group.

Table 40.2 Trial design of the use of L-arginine in surgical patients with head and neck cancer

Study	Inclusion criteria	Outcome	Duration of follow-up
Snyderman 1999 [17]	Curative surgery squamous cell carcinoma (stage II–IV) of oral cavity, pharynx, or larynx	Weight, laboratory parameters, clinical complications (defined), tolerance to feeding, length of hospital stay	1 month post-operation
Riso 2000 [16]	Surgery for carcinoma (stage I–IV) of oral cavity, pharynx, or larynx	Clinical complications (not defined), tolerance to feeding, laboratory parameters	To hospital discharge
van Bokhorst-de van der Schueren 2000/2001 [18, 19]	Recent weight loss undergoing surgery for carcinoma of oral cavity, pharynx, or larynx	Weight, laboratory parameters, clinical complications (not defined), tolerance to feeding, length of hospital stay, quality of life, hand grip	16 months
de Luis 2002 [9]	Surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters, clinical complications (not defined), tolerance to feeding, length of hospital stay	3 months post-hospital discharge
de Luis 2003 [13]	Recent weight loss undergoing surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters	5 days post-operation
de Luis 2004 [12]	Surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters, clinical complications (not defined), tolerance to feeding, length of hospital stay	To hospital discharge
de Luis 2005 [10]	Recent weight loss undergoing surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters	6 days post-operation
Felekis 2005 [15]	Surgery for head and neck cancer	Major and minor complications (not defined), septic complications, mortality	Not stated
de Luis 2005 [11]	Surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters, clinical complications (not defined), tolerance to feeding, length of hospital stay	12 weeks

de Luis 2007 [14]	Surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters, clinical complications (defined), tolerance to feeding, length of hospital stay	Not stated
de Luis 2010 [23]	Surgery for carcinoma of oral cavity or larynx	Weight, laboratory parameters, clinical complications (defined), tolerance to feeding, length of hospital stay	15 days
Casas-Rodera 2008 [20]	Surgery for carcinoma of oral cavity and larynx	Weight, laboratory parameters, clinical complications, tolerance to feeding, length of hospital stay	To hospital discharge
Buijs 2010 [21]	Surgery for head and neck cancer	Long-term survival, locoregional recurrence, distant metastases, and second primary tumors	10 years
Felekis 2010 [22]	Surgery for head and neck cancer	Major and minor complications, tolerance, immunological markers	Not stated
de Luis 2013 [24]	Surgery for head and neck cancer	Weight, laboratory parameters	To hospital discharge

This table shows the type of designs in published trials with L-arginine in surgical patients with head and neck cancer

Table 40.3 Nutritional supplements and regimes examining L-arginine in surgical patients with head and neck cancer

Study	Nº groups	Groups	Control	Intervention with L-arginine	Isocaloric/ isonitrogenous	Duration of supplementation (days)		Post-op day commenced
						Preop	Postop	
Snyderman 1999 [17]	4	1. Active pre & post operative 2. Active postoperative only 3. Control pre- and postoperative 4. Control postoperative only	Polymeric (Replete1, Resource1, Isosource1, Jevity1, Vivonex1, Osmolite1)	Polymeric + L-arginine (Impact1)	Not stated	>5 days	> 7 days	Not stated
Riso 2000 [16]	2	1. Active 2. Control postoperative enteral, both with parenteral nutrition for 3 days	Polymeric (Nutrison protein plus1)	Polymeric + L-arginine (Nutrison intensive1)	Yes	None	> 10 partial > 21 total laryngectomy	Within 24 h
van Bokhorst-de van der Schueren 2000/2001 [18, 19]	3	1. No pre-op + postoperative 2. Pre-op + postoperative 3. L-Arginine supplemented pre-op + postoperative	Polymeric	Polymeric + L-arginine	Yes	7–10 days	> 10 days	Within 24 h
de Luis 2002 [9]	2	Postoperative	Polymeric	Polymeric + L-arginine + fiber	Yes	None	> 10 days	Within 24 h
de Luis 2003 [13]	2	Postoperative	Polymeric	Polymeric + L-arginine	Yes	None	> 10 days	Within 24 h
de Luis 2004 [12]	2	Postoperative	Polymeric +fiber	Polymeric + L-arginine + fiber	Yes	None	> 10 days	Within 24 h
de Luis 2005 [10]	2	Postoperative	Polymeric	Polymeric + L-arginine	Yes	None	> 10 days	Within 24 h
Felekis 2005 [15]	2	Active pre & post operative vs no	Polymeric	Undefined enteral immunonutrition	Yes	6 days	8 days	Not stated
de Luis 2005 [11]	2	Postoperative	Polymeric + L-arginine	Polymeric + v3 fatty acids	Not stated	None	12 weeks	At hospital discharged
de Luis 2007 [14]	2	Postoperative	Polymeric	Polymeric + L-arginine	Yes	None	> 10 days	Within 24 h
de Luis 2010 [23]	2	Active postoperative	Polymeric + medium-dose L-arginine	Polymeric + high-dose L-arginine	Yes	None	15 days	Within 24 h
Casas-Rodera 2008 [20]	3	Postoperative	Polymeric	1. Polymeric + L-arginine 2. Polymeric + L-arginine, RNA, and omega-3 fatty acids	Yes	None	> 10 days	Within 24 h
Buijs 2010 [21]	2	Pre- and postoperative	Polymeric	1. Polymeric 2. Polymeric + L-arginine	Yes	7–10 days	10 days	Within 24 h
Felekis 2010 [22]	2	1. Postoperative 2. Pre and postoperative	Polymeric (Nutrison, Nutricia)	Pre: Polymeric + L-arginine, RNA, and omega-3 fatty acids (impact) Post: Polymeric + L-arginine, RNA, and omega-3 fatty acids (impact)	Yes	Group active for 5 days	8 days	Not stated
de Luis 2013 [24]	2	Postoperative	Polymeric + w3	Polymeric + L-arginine	Yes	None	12 weeks	Not stated

This table shows the type of enteral formula and time course in surgical patients with head and neck cancer

Table 40.4 Immunological and biochemical endpoints in regimes examining L-arginine in surgical patients with head and neck cancer

Study	Biochemical endpoints	Immunological endpoints	Total lymphocytes		IL-6 (pg/ml) Post-op		TNF alpha (pg/ml)		Postoperative day
			Control	Intervention	Control	Intervention	Control	Intervention	
Snyderman 1999 [17]	Albumin, transferrin, hemoglobin	Total lymphocytes	1.29	1.51	–	–	–	–	7
Riso 2000 [16]	Albumin, pre-albumin, transferrin	Total Lymphocytes, T-cell subsets, immunoglobulins	1.0, 1.3, 1.5	1.2, 1.9, 2.2	–	–	–	–	1, 4, 8
van Bokhorst-de van der Schueren 2000/2001 [18, 19]	Albumin	Total white blood cells, total lymphocytes, T-cell subsets, IL-6, and TNF-α after stimulation with lipopolysaccharide, HLA-DR expression	NS	NS	NS	NS	NS	NS	1, 4, 7
de Luis 2002 [9]	Albumin, pre-albumin, transferrin	Total lymphocytes	1.55, 2.27	1.66, 2.10	–	–	–	–	7, 14
de Luis 2003 [13]	Albumin, pre-albumin, transferrin	CRP, lymphocytes, TNF-α, IL-6	1.56	1.63	9.9	35.6	5.8	5.1	5
de Luis 2004 [12]	Albumin, pre-albumin, transferrin	Total lymphocytes	1.55	1.88	–	–	–	–	14
de Luis 2005 [10]	Albumin, pre-albumin, transferrin	Total lymphocytes CRP, TNF-α, IL-6	1.45	1.36	9.9	6.7	6.11	5.6	6
Felekis 2005 [15]	Not measured	None	–	–	–	–	–	–	–
de Luis 2005 [11]	Albumin, pre-albumin, transferrin	Total lymphocytes	2.09	2.27	–	–	–	–	30
de Luis 2007 [14]	Albumin, pre-albumin, transferrin	Total lymphocytes	1.55	1.68	–	–	–	–	12

(continued)

Table 40.4 (continued)

Study	Biochemical endpoints	Immunological endpoints	Total lymphocytes		IL-6 (pg/ml) Post-op		TNF alpha (pg/ml)		Postoperative day
			Control	Intervention	Control	Intervention	Control	Intervention	
de Luis 2010 [23]	Albumin, pre-albumin, transferrin	Total lymphocytes	1.56	1.82	–	–	–	–	10
Casas-Rodera 2008 [20]	Albumin, transferrin	Total lymphocytes CRP, TNF-α, IL-6	1.5	1.93, 1.91	17.57	30.66, 20.26	38.39	39.26, 46.29	7
Buijs 2010 [21]	Albumin, transferrin	Total lymphocytes, CRP, TNF-α, IL-6	1.59	1.86, 1.97	23.6	14.01, 25.17	87.11	35.57, 36.79	14
Felekis 2010 [22]	Not measured	None	–	–	–	–	–	–	–
de Luis 2013 [24]	Albumin, pre-albumin, transferrin	None	–	–	–	–	–	–	–

Albumin (g/dl), pre-albumin (mg/dl), transferrin (mg/dl), lymphocytes (×106 ml/mm^3), interleukin (pg/ml), TNF (pg/ml)
TNF tumor necrosis factor, *CRP* C-reactive protein, *IL* interleukin, *NS* not stated
This table shows immunological and biochemical endpoints
For details on the study design using L-arginine, see Tables 40.2 and 40.3

Table 40.5 Wound infection and fistula endpoints in regimes examining L-arginine in surgical patients with head and neck cancer

Study	Wound infections		Fistula formation	
	Control	Intervention	Control	Intervention
Snyderman 1999 [17]	2	4	1	4
Riso 2000 [16]	3	2	1	1
van Bokhorst-de van der Schueren 2000/2001 [18, 19]	Not stated	Not stated	Not stated	Not stated
de Luis 2002 [9]	3	1	5	0
de Luis 2003 [13]	Not recorded	Not recorded	Not recorded	Not recorded
de Luis 2004 [12]	0	0	5	2
de Luis 2005 [10]	Not recorded	Not recorded	Not recorded	Not recorded
Felekis 2005 [15]	Not stated	Not stated	Not stated	Not stated
de Luis 2005 [11]	0	0	0	0
de Luis 2007 [14]	0	0	7	1
de Luis 2010 [23]	2	2	6	2
Casas-Rodera 2008 [20]	2	2	2	4
Buijs 2010 [21]	Not stated	Not stated	Not stated	Not stated
Felekis 2010 [22]	Not stated	Not stated	2	1
de Luis 2013 [24]	Not stated	Not stated	Not stated	Not stated

This table shows wound infection and fistula endpoints

Only two studies reported the effect of perioperative immunonutrition on quality of life [18, 19]. The disease-specific (EORTC QLQ-C30) and generic questionnaire (COOP-WONCA) with follow up as long as 16 months showed no evidence of benefit with pre- or postoperative immunonutrition.

Finally, our group [26] has studied the effect (in a non randomized clinical trial) of an enteral inmunoenhanced formula with L-arginine and glutamine, too. At hospital discharge, a population of 39 ambulatory postsurgical patients with oral and laryngeal cancer was enrolled. Duration of supplementation was 90.8 + 20 days. A significant increase of albumin (3.1 ± 0.6 g/dl vs. 4.12 + 0.7 g/dl; $P < 0.05$), prealbumin (21.4 ± 6.3 mg/dl vs. 22.4 + 5.9 mg/dl; $P < 0.05$), and transferrin (198.8 ± 45.2 mg/dl vs. 253.8 + 60.7 mg/dl; $P < 0.05$) levels was observed. No differences were detected in weight and other anthropometric parameters. Ten patients (41.3 %) received radiotherapy along the enhanced supplementation period and only 5 (20 % of patients with radiotherapy) developed a clinical oral mucositis.

Table 40.6 Hospital stance and survival in regimens examining L-arginine in surgical patients with head and neck cancer

Study	Length of postoperative hospital stay		Post-op death		Long-term survival		Disease-specific survival		Locoregional recurrence	
	Control	Intervention	Control	Intervention	Control	Intervention	Control	Intervention	Control	Intervention
Snyderman 1999 [17]	17.4	15.3	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
Riso 2000 [16]	28.0	25.0	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
van Bokhorst-de van der Schueren 2000/2001 [18, 19]	41 + 46	31	0 + 1	2	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2002 [9]	31.2	22.8	2	3	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2003 [13]	Not recorded	Not recorded	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2004 [12]	35.0	25.8	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2005 [10]	Not recorded	Not recorded	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
Felekis 2005 [15]	Not stated	Not stated	1	1	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2005 [11]	Not stated	Not stated	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2007 [14]	28.2	27.9	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2010 [23]	25.7	27.2	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
Casas-Rodera 2008 [20]	18.2	20.4	0	0	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
Buijs 2010 [21]	Not stated	Not stated	Not stated	Not stated	20.7 months	34.8 months	20.8 month	94.4 month	9 (15)	4 (17)
Felekis 2010 [22]	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
de Luis 2013 [24]	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated

This table shows hospital stance and survival
For details on the study design using L-arginine, see Tables 40.2 and 40.3

Conclusions

Based on all previous studies, postsurgical patients with head and neck cancer show an improvement in immunological parameters with L-arginine-enhanced enteral formulas, without reductions in clinical complications. Perioperative immunonutrition with L-arginine is associated with reduced length of hospital stay; the mechanism is unclear. Trials analyzed in this chapter were small and adequately powered trials are required to substantiate benefit.

References

1. Goncalves Dias MC, De Fatima Nunes Marucci M, Nadalin W, Waitzberg DL. Nutritional intervention improves the caloric and proteic ingestion of head and neck cancer patients under radiotherapy. Nutr Hosp. 2005;20:320–5.
2. Oloriz Rivas MR, Domínguez Vázquez A. Nutritional support in laryngectomized patients. Nutr Hosp. 1992; 7:282–90.
3. Harries M, Lund VJ. Head and neck surgery in the elderly: a maturing problem. J Laryngol Otol. 1989; 103:306–9.
4. Reilly JJ. Does nutrition management benefit the head and neck cancer patient? Oncology. 1990;4:105–15.
5. Di Carlo V, Gianotti I, Balzano G, Zerbi A, Braga M. Complications of pancreatic surgery and the role of perioperative nutrition. Dig Dis Sci. 1999;16:320–6.
6. Pérez de la Cruz AJ, Abilés J, Pérez Abud R. Perspectives in the design and development of new products for enteral nutrition. Nutr Hosp. 2006;21 Suppl 2:98–108.
7. Yeh CL, Yen SL, Lin MT. Effects of L-arginine-enriched total parenteral nutrition on inflammatory-related mediators and T-cell population in septic rats. Nutrition. 2002;18:631–5.
8. Kirk SJ, Barbul A. Role of L-arginine in trauma, sepsis, and immunity. JPEN J Parenter Enteral Nutr. 1990; 14:226S–9.
9. de Luis DA, Aller R, Izaola O, Cuellar L L, Terroba MC. Postsurgery enteral nutrition in head and neck cancer patients. Eur J Clin Nutr. 2002;56:1126–9.
10. de Luis DA, Arranz M, Aller R, Izaola O, Cuellar L, Terroba MC. Immunoenhanced enteral nutrition, effect on inflammatory markers in head and neck cancer patients. Eur J Clin Nutr. 2005;59:145–7.
11. de Luis DA, Izaola O, Aller R, Cuellar L, Terroba MC. A randomized clinical trial with oral immunonutrition (v3-enhanced formula vs L-arginine-enhanced formula) in ambulatory head and neck cancer patients. Ann Nutr Metab. 2005;49:95–9.
12. de Luis DA, Izaola O, Cuellar L, Terroba MC, Aller R. Randomized clinical trial with an L-arginine-enhanced formula in early postsurgical head and neck cancer patients. Eur J Clin Nutr. 2004;58:1505–8.
13. de Luis DA, Izaola O, Cuellar L, Terroba MC, Arranz M, Fernandez N, Aller R. Effect of C-reactive protein and interleukins blood levels in postsurgery L-arginine-enhanced enteral nutrition in head and neck cancer patients. Eur J Clin Nutr. 2003;57:96–9.
14. de Luis DA, Izaola O, Cuellar L, Terroba MC, Martin T, Aller R. Clinical and biochemical outcomes after a randomized trial with high dose of enteral L-arginine formula in postsurgical head and neck cancer patients. Eur J Clin Nutr. 2007;61:200–4.
15. Felekis. The effect of perioperative enteral immunonutrition on outcome of head and neck cancer patients: a prospective, randomised, controlled clinical trial. Clin Nutr. 2005;24:296.
16. Riso S, Aluffi P, Brugnani M, Farinetti F, Pia F, D'Andrea F. Postoperative enteral immunonutrition in head and neck cancer patients. Clin Nutr. 2000;19:407–12.
17. Snyderman CH, Kachman K, Molseed L, Wagner R, D'Amico F, Bumpous J, Rueger R. Reduced postoperative infections with an immune-enhancing nutritional supplements. Laryngoscope. 1999;109:915–21.
18. van Bokhorst-de van der Schueren MAE, Langendoen SI, Vondeling H, Kuik DJ, Quak JJ, van Leeuwen PAM. Perioperative enteral nutrition and quality of life of severely malnourished head and neck cancer patients: a randomised clinical trial. Clin Nutr. 2000;19:437–44.
19. van Bokhorst-de van der Schueren MAE, Quak JJ, von Blomberg-van der Flier BME, Kuik DJ, Langendoen SI, Snow GB, Green CJ, van Leeuwen PAM. Effect of perioperative nutrition, with and without L-arginine supplementation, on nutritional status, immune function, postoperative morbidity, and survival in severely malnourished head and neck cancer patients. Am J Clin Nutr. 2001;73:323–32.
20. Casas-Rodera P, Gomez-Candela C, Benitez S, Mateo R, Armero M, et al. Immunoenhanced enteral nutrition formulas in head and neck cancer surgery: a prospective, randomized clinical trial. Nutr Hosp. 2008;23:105–10.

21. Buijs N, van Bokhorst-de van der Schueren MA, Langius JA, Leemans CR, Kuik DJ, Vermeulen MA, van Leeuwen PA. Perioperative L-arginine-supplemented nutrition in malnourished patients with head and neck cancer improves long-term survival. Am J Clin Nutr. 2010;92(5):1151–6.
22. Felekis D, Eleftheriadou A, Papadakos G, Bosinakou I, Ferekidou E, Kandiloros D, Katsaragakis S, Charalabopoulos K, Manolopoulos L. Effect of perioperative immuno-enhanced enteral nutrition on inflammatory response, nutritional status, and outcomes in head and neck cancer patients undergoing major surgery. Nutr Cancer. 2010; 62(8):1105–12.
23. de Luis DA, Izaola O, Cuellar L, Terroba MC, Martin T, Ventosa M. A randomized double-blind clinical trial with two different doses of L-arginine enhanced enteral nutrition in postsurgical cancer patients. Eur Rev Med Pharmacol Sci. 2010;14(11):941–5.
24. de Luis DA, Izaola O, Cuellar L, Terroba MC, de la Fuente B, Cabezas G. A randomized clinical trial with two doses of a omega 3 fatty acids oral and L-arginine enhanced formula in clinical and biochemical parameters of head and neck cancer ambulatory patients. Eur Rev Med Pharmacol Sci. 2013;17(8):1090–4.
25. Stableford WB. A systematic review of the role of immunonutrition in patients undergoing surgery for head and neck cancer. Int J Oral Maxillofac Surg. 2009;38:103–10.
26. Izaola O, de Luis DA, Cuellar L, Terroba MC, Ventosa M, Martin T, Aller R. Influence of an immuno-enhanced formula in postsurgical ambulatory patients with head and neck cancer. Nutr Hosp. 2010;25(5):793–6.

Chapter 41
L-Arginine-Incorporated Albumin Mesospheres: A Drug Delivery System for Cancer Therapy

Hung-Yen Lee, Kamal A. Mohammed, and Najmunnisa Nasreen

Key Points

- L-Arginine deprivation has been widely studied as a potential targeted therapy for cancer due to its inhibiting effects on tumor growth via the alternation of metabolic pathways and the dysfunction of immune responses.
- On molecular level, L-arginine has been shown to be effective in inhibiting cancer cell proliferation when an appropriate dose is applied; otherwise, L-arginine facilitates cell proliferation.
- In addition to the apoptosis induced by metabolism of L-arginine, in an ideal solution environment, L-arginine molecules assemble into L-arginine clusters to destroy cancer cells through interrupting cell membranes.
- To make the L-arginine an effective anticancer agent, we incorporated L-arginine with albumin microspheres to provide a localized high concentration of L-arginine and an L-arginine-rich surface on microspheres, which is similar to the L-arginine cluster, to effectively inhibit tumor growth.
- In earlier studies in vitro we showed that L-arginine/albumin microspheres significantly inhibited cell proliferation, cell migration, and tumor growth of non-small cell lung cancer cells and malignant mesothelioma cells, while similar concentration of free L-arginine promoted the tumor growth and migration.
- The L-arginine/albumin microspheres showed more efficient inhibiting effects on lung cancer cells when compared to the free L-arginine, suggesting that the L-arginine/albumin microspheres can be an ideal delivery vehicle for therapeutic interventions against lung cancers.

Keywords L-Arginine • Cancer • Lung cancer • Albumin microspheres • Drug delivery • Controlled release

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Abbreviations

AAMS	L-Arginine/albumin microspheres
ADI	L-Arginine deiminase
AMS	Albumin microspheres
ARG	Arginase
Arg	L-Arginine
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthetase
BSA	Bovine serum albumin
CAT	Cationic amino acid transporter
CPPs	Cell-penetrating peptides
EITC	Endobronchial intratumoral chemotherapy
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSCLC	Non-small cell lung carcinoma (or cancer)
OCT	Ornithine transcarbamyl transferase
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RNA	Ribonucleic acid
SEM	Scanning electron microscopy

Role of L-Arginine in Cancer Biology

Biochemistry of L-Arginine

L-Arginine (2-amino-5-guanidinopentanoic acid), the cationic amino acid that carries most nitrogen atoms per molecule in humans, is the most common amino acids among the 20 amino acids that can be found naturally in vegetables and mammals. L-Arginine is considered as a semi-essential or conditionally essential amino acid because although it can be synthesized in humans, endogenous L-arginine biosynthesis is insufficient to compensate for depletion when cells are under stress or in different development stages [1]. It has been identified as a precursor for initiation of various metabolic pathways in human body. The L-arginine through diet has to be uptaken by intestinal epithelial cells and transported through the cell membrane via the cationic amino acid transporters (CAT) [2]. Half of L-arginine ingested in human body can be efficiently absorbed and converted to ornithine by arginase in urea cycle, as shown in Fig. 41.1 [3, 4]. Then the L-arginine-derived ornithine can be metabolized to glutamate and proline, enzymatically degraded into polyamines, or converted to citrulline by ornithine transcarbamyl transferase (OCT). Through endogenous biosynthesis, besides the recycling of citrulline that is derived from ornithine, most L-arginine is converted from the citrulline produced in glutamine metabolism and released into circulation. Argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) are the cytosolic enzymes catalyzing the two-step sequential conversion of citrulline to L-arginine. The conversion takes place in kidneys.

To elucidate the possible mechanism of action of L-arginine on cancers, the by-products and catalytic enzymes involved in the L-arginine metabolic and catabolic pathways should be fully illustrated. (1) L-Arginine is well known as the biological precursor of nitric oxide (NO), an endogenous signal transduction molecule and endothelium-derived relaxing factor involved in various physiological and

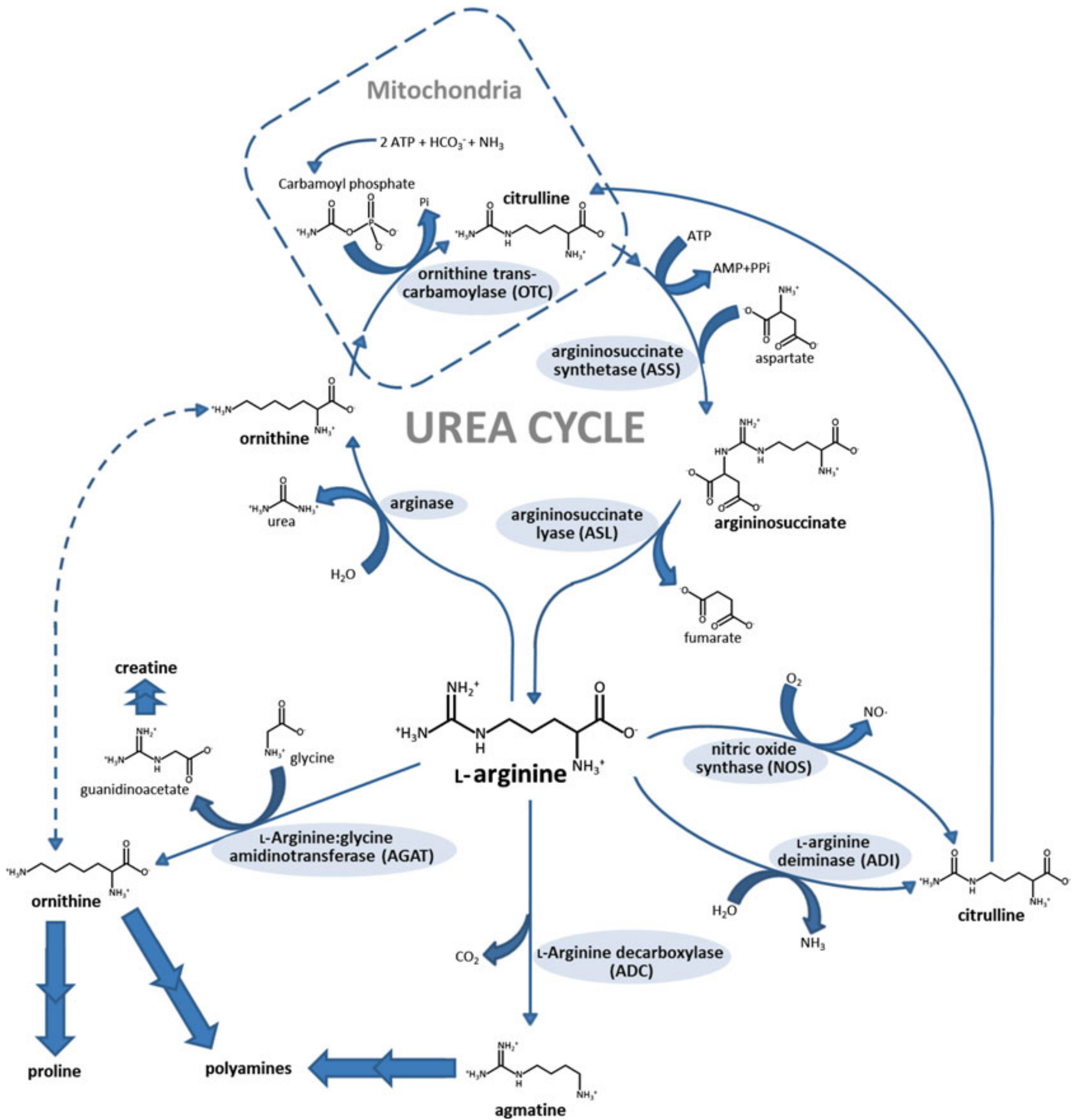


Fig. 41.1 Metabolic pathways of L-arginine. L-Arginine can be metabolized or catabolized into ornithine, citrulline, and agmatine by five different catalytic enzymes. The conversion of L-arginine to ornithine is the last step in urea cycle which converts amine to urea via the bioreactions catalyzed by ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase. The citrulline produced by L-arginine via the catalyses of nitric oxide synthase (NOS) and L-arginine deiminase (ADI) can rejoin into the urea cycle. L-Arginine:glycine amidinotransferase (AGAT), which is the enzyme that catalyzes glycine to guanidinoacetate, the precursor of creatine, converts L-arginine to ornithine and leads to the biosynthesis of polyamines. Agmatine, an intermediate of polyamine biosynthesis, can be produced from L-arginine by the catalysis of L-arginine decarboxylase (ADC)

pathological processes. NO is the by-product generated in the metabolic conversion of L-arginine to citrulline catalyzed by nitric oxide synthase (NOS). (2) Three major enzymes are involved in L-arginine catabolic degradation: arginase, L-arginine decarboxylase, and L-arginine deiminase (ADI). Arginase converts L-arginine to ornithine, L-arginine decarboxylase converts L-arginine to agmatine, an intermediate in polyamine biosynthesis, and L-arginine deiminase converts L-arginine to citrulline with ammonia as by-product. (3) The two enzymes involved in the biosynthesis of L-arginine from citrulline are ASS and ASL, and OCT is the enzyme that converts ornithine to citrulline.

Potential Mechanisms of Action

L-Arginine, especially L-arginine, has been reported to be involved in a number of biosynthetic pathways that influence carcinogenesis and tumor generation [5, 6]. It is generally believed that tumor cells depend on L-arginine for cell growth due to the increasing demand on polyamines derived from L-arginine, and thus L-arginine deprivation has been widely studied as a novel therapeutic method to inhibit tumor growth. In some other reports, L-arginine has also been shown to inhibit cell proliferation in various cancer cells including lung, breast, and gastric cancers [7–9]. However, the mechanisms of cell proliferation inhibition by L-arginine are still not completely clear and the reactions induced by L-arginine in tumor biology are too complex to elucidate.

The nitric oxide (NO) generated through L-arginine metabolism has been widely thought as one of the most important molecules to influence the carcinogenesis, cancer cell proliferation, and tumor growth [10]. L-Arginine-derived NO has been implicated to be involved in carcinogenesis in various cancers including brain, lung, breast, and esophagus cancers. The carcinogenic effects of NO may promote abnormal cell growth through involving mechanisms, including direct DNA and protein damage, or the inhibition of programmed apoptosis [11]. Different types of NOS (inducible iNOS, endothelial eNOS, and neuronal nNOS) have been detected in tumor cells and these NOS isomers may be involved in different stages of tumor progression [12]. NO may also stimulate tumor angiogenesis, including endothelial cell proliferation, vascular permeability, and stimulation of growth factor, to promote tumor growth [5, 13]. However, on the effects of cancer cell proliferation and tumor growth, NO may have significant antitumor potential due to its enhancement on apoptosis, but its clinical application has been limited by the adverse effects such as hypertension and toxicity produced while administered systematically. In addition, the overlapping, conflicting, and complicated roles of L-arginine-derived NO in tumor initiation, promotion, and progression limit the antitumor effectiveness of NO on cancer treatments. Previous studies have shown both stimulation and inhibition effects of NO on cancer cell proliferation, depending upon NO level and cell types [8, 9, 14–16].

Enzymatic Regulation of L-Arginine

The roles of other catalytic enzymes involved in L-arginine metabolism and catabolism on tumor progression have also been investigated in order to elucidate the mechanism of L-arginine on cancer therapy. These enzymes such as arginase and ADI have been attracting attention and tested since 1950. High concentration of arginase, which converts L-arginine to ornithine and urea, has been found in the microenvironment of various malignant tumor tissues, and this enables the tumor cells to escape the immune surveillance [17]. It has been indicated that the high demand for L-arginine is neither due to the high requirements of polyamine synthesis nor the NO production for growth promotion. The main reason for high L-arginine requirement in breast cancer cells is due to the irreversible endogenous conversion of L-arginine to ornithine by arginase, which resulted in L-arginine deprivation leading to inhibition on cell proliferation [6]. However, the development of arginase as an antitumor agent has failed due to its low affinity for L-arginine, and as well for the reason that ornithine accumulation may cause toxicity in normal tissues lacking OCT, the enzyme that catabolizes ornithine to citrulline. On the other hand, the catalytic enzyme that converts L-arginine to citrulline, L-arginine iminohydrolase (L-arginine deiminase, ADI), has attracted interest for its potential application to targeted therapy for cancer tumors lacking ASS expression, including melanoma, hepatocellular carcinoma, mesotheliomas, and renal cancers [18–20]. The ADI isolated from *Mycoplasma* has been reported to show high affinity to L-arginine and have significant in vitro and in vivo antitumor effects in various cancer cells including melanoma, hepatocellular carcinoma, etc. To reduce the immunogenicity and enhance

the stability of ADI while administered on patients, the ADI conjugated with polyethylene glycol (PEG 20,000), ADI-PEG 20, was developed by Polaris Pharmaceuticals for selectively degrading L-arginine and inhibiting tumor cell growth [21, 22]. In these phase I and phase II clinical trials, the effectiveness of ADI-PEG 20 treatment on patients with hepatocellular carcinoma and melanoma has been shown. Weekly intramuscular administration of ADI-PEG 20 was sufficient to lower plasma L-arginine level from 130 μmol/L to below the level of detection in 9 (2 complete and 7 partial responses) out of 19 hepatocellular cancer patients for more than 7 days [21]. In the clinical trial of ADI-PEG 20 treatment on melanoma, 25 % response rate and prolonged survival were shown in 24 patients [22]. More clinical trials investigating the antitumor effects of ADI-PEG 20 on patients with different cancers, such as mesothelioma, have been continuously ongoing lately.

L-Arginine and Immune Responses in Cancer

L-Arginine has been demonstrated to enhance wound healing and T-cell-mediated immune functions [23]. The effects of L-arginine on accelerating wound healing may be owing to the increasing levels of proline and collagen derived from L-arginine, and it was also suggested that the rapid depletion of L-arginine in plasma was accompanied by markedly decreased T-cell function in patients with transplantation and trauma. Two specific enzymes in L-arginine metabolism, arginase I and NOS2, have been reported to play important roles in the immune responses associated with tumor growth [24, 25]. Arginase I is one of the two isoforms of the mammalian arginase which functions in the urea cycle, primarily located in cytoplasm of liver, whereas the arginase II regulates L-arginine/ornithine conversion in mitochondria. NOS2 (iNOS) is the cytokine inducible NOS involved in immune responses and produces NO for immune defense due to the cell toxicity of high NO level. The inhibition of arginase I leads to an overexpression of NOS2 and consequently increases the production of NO.

It has been reported that the Jurkat T cells cultured in L-arginine-free medium showed a rapid decrease in the expression of the T-cell antigen receptor epsilon chain (CD3ζ), a principal element in the receptor complex that plays an important role in coupling antigen recognition to intracellular signal transduction, and a significant decrease in cell proliferation [26]. Furthermore, this phenomenon can only be reversed by the replenishment of L-arginine but not the other substitutes. Besides the loss of CD3ζ, T cells in L-arginine-free environment developed alterations in the immune responses, such as inability to upregulate Janus kinase 3 (Jak-3), decreased translocation of NF-KB p65, and failure of interferon-γ (IFN-γ) production [24]. These results suggest that L-arginine depletion has a potential effect on the induction of T-cell dysfunction and leads to inhibition on tumor growth.

L-Arginine Applied in Cancer Therapy

L-Arginine-Containing Peptides

In cancer therapy, targeting the receptor protein on cell membrane and penetrating through cell membrane to effectively deliver therapeutic agents such as nucleotides, peptides, and chemotherapeutic into the target cancer cells are important and still challenging to date. Typically, cancer cell membrane is negatively charged due to its higher expression of anionic molecules. Thus, incorporation of L-arginine or L-arginine-rich peptides, usually <20-mer peptide, into antitumor agents or systems has been widely studied due to their high binding affinity to the receptors on cancer cell membrane or high cell membrane penetration [27]. The possible intracellular uptake mechanisms of cell-penetrating

peptides (CPPs) can be majorly classified into two types: the energy-independent pathways and the endocytic pathways. The energy-independent cell entry pathways, such as inverted micelle, pore formation, membrane thinning and carpet models, are passive, nonspecific pathways which are not sensitive to endocytosis inhibitors and entering cell membrane majorly via physical interactions. On the other hand, the endocytic pathways are endocytosis including phagocytosis and pinocytosis which are dependent on the specific properties of the CPPs and their conjugates.

L-Arginine-containing cell-penetrating peptides with different sizes, such as RGD (Arg-Gly-Asp), TAT peptide (derived from HIV-1 virus), penetratin, PEGA, pVEC, and octaarginine (r8), have gained enormous interest for their efficient in vivo targeting ability on tumors without causing significant toxicity [28]. CPPs have been conjugated on small biomolecules, such as therapeutic peptides and proteins, small nucleotides (small interference RNA, siRNA), and chemotherapeutic drugs, as well as drug delivery carriers, including polymers, nanoparticles, and liposomes, for targeted delivery of these therapeutic agents to tumor tissues. It has been shown that L-arginine-rich peptides accumulated in high level in tumor tissues while administered intravenously and the conjugate of L-arginine-rich peptides with chemotherapeutic drugs can effectively carry the drugs into the tumor tissues for enhanced anticancer activity [29]. Furthermore, peptides such as RGD sequence showed not only targeting ability on tumors but also inhibiting effects on tumor growth, which is similar to the effects of ligand protein targeting on membrane receptors of cancer cells [30]. The potential antitumor effects of CPPs make the targeted delivery systems more efficient on cancer treatments.

Polyarginine

Using polyaminoacids as biomaterials for the development of drug delivery carriers or medical devices has been attracting interests because the polyaminoacids are expected to show stable physical properties, high biocompatibility, and controllable biodegradation rate. Polyarginine, polypeptide of L-arginine, is polymerized L-arginine having higher molecular weight than the short CPPs. Polyarginine (MW 5–50K) is expected to be widely applied in drug delivery systems as a highly positively charged polymer. It has been demonstrated that polyarginine showed controllable biodegradation rate depending on the molecular weight, and as a penetration enhancer, a 35KD polyarginine enhanced the penetration of hydrophilic molecules through cornea tissue [31, 32]. Based on these properties, polyarginine has been applied as a biomaterial for composing drug nanocarriers, surface-modifying metal nanoparticles, and forming stable complex with DNA or RNA molecules [33–35]. In an in vitro study, the polyarginine nanocapsules showed rapid and massive accumulation in non-small cell lung cancer cells and enhanced the antitumor effects of loaded drug [33]. However, the effects of L-arginine-rich peptides, polyarginine, and their degraded forms on L-arginine metabolic pathways were not yet defined.

L-Arginine-Incorporated Drug Delivery System

Albumin-Based Particle Delivery System for Cancer Therapy

The therapeutic effect of L-arginine on cancer treatment is paradoxical. However, it has been reported recently that the dose concentration and delivery environment are crucial to determine if L-arginine, especially L-arginine, can be an efficient anticancer agent [36]. Depending upon the concentration and composition in a solution environment, L-arginine may assemble into molecular clusters that display a hydrophobic surface by the alignment of its methylene groups [37, 38]. The hydrophobic surface of L-arginine clusters may induce necrosis on malignant cells by disrupting their membrane integrity.

Unlike the metabolism of L-arginine, if high concentration of L-arginine is present at the local milieu, this non-metabolic process avoids the development of tumor resistance and can be more efficient on killing different types of malignant cells. Therefore, to make the L-arginine an effective anticancer agent, the delivery method is extremely important to provide an ideal local environment of L-arginine.

Endobronchial intratumoral chemotherapy (EITC), a direct intratumoral injection using a bronchoscopic needle-catheter, has been explored clinically in recent years to improve the treatment of lung cancer [39, 40]. This is achieved without the complications caused by systemic drug toxicity normally associated with conventional chemotherapy. To further improve the effectiveness and safety of intratumoral chemotherapy, various nano-meso-microsphere compositions loaded with drugs or biomolecules have been designed to prolong high intratumoral drug concentrations and to further minimize any risk of systemic toxicity [41, 42]. Microspheres made by different synthetic and biopolymers have been widely studied for their application of drug delivery in cancer treatment [43, 44]. Among the various biodegradable particulate carriers available for consideration, considering the similar nature of amino acid and peptide, we regard the most abundant natural plasma protein, serum albumin, as a most appealing biocompatible carrier for the effective localized delivery of L-arginine [45]. Furthermore, the abundant functional groups on surface of human serum albumin (HAS) or bovine serum albumin (BSA) facilitate physiological absorption and covalent conjugation to L-arginine and other biomolecules.

Generally, all water-soluble chemotherapeutic drugs and biomolecules including protein, polysaccharides, and nucleic acids can be effectively loaded into AMS. The drugs and biomolecules can be loaded with AMS through the in situ loading method, that is, during the formation of microspheres, and post-loading method after the formation of microspheres. While dispersed in aqueous solution or high polar organic solvent, AMS can quickly swell and absorb the solutes in solution. The in-bulk loading efficiency of drugs or biomolecules in AMS can be influenced by the factors including swelling degree of AMS in the solvent, the solubility of the loaded molecules in solvent, and most importantly, the molecular interaction between loaded molecules and polypeptide domains in AMS.

The drugs or bioactive molecules which are incorporated into AMS can be active on the microsphere surface via conjugation and, at the same time, can be released from the MS by diffusion and by degradation of microsphere matrix. From the molecular point of view, the release behavior of loaded molecules from the AMS can be influenced by three kinds of molecular interactions: loaded molecules to environmental solution, AMS to environmental solution, and loaded molecules to AMS. The release of loaded or conjugated bioactive molecules from the AMS is controllable as a function of particle size, matrix cross-link density, and degradation rate of microspheres.

In previous studies, we have demonstrated the low cytotoxicity of AMS and its effectiveness of drug-loaded AMS for the treatment of lung cancers [46, 47]. In view of this, drug- or biomolecule-loaded albumin-based microspheres (AMS) are now being considered for bronchoscopic intratumoral treatment of lung cancer to provide localized, continuous, and prolonged high drug concentration at target tumor sites. The goal is to improve the effectiveness of therapy, to minimize the undesired diffusion through systemic circulation, and to reduce the tumor burden. In our L-arginine/albumin microsphere system, to locally inhibit the tumor growth, the L-arginine molecules incorporated into microspheres may be active by forming L-arginine-rich domains on the surface and, at the same time, free L-arginine can be released from the microspheres by diffusion and by degradation of AMS matrix.

Synthesis and Characterizations of L-Arginine/BSA Microspheres (AAMS)

The albumin-based microspheres developed in our previous study were synthesized through a water-in-organic solvent emulsion system, which is similar to the traditional water-in-oil (W/O) emulsion system except that oil is replaced by organic solvent with low polarity [48]. In this emulsion system as shown in Fig. 41.2, the water phase (discontinuous phase) containing BSA and L-arginine is dispersed into the organic solvent phase (continuous phase) containing polymer stabilizers or surfactants to produce a

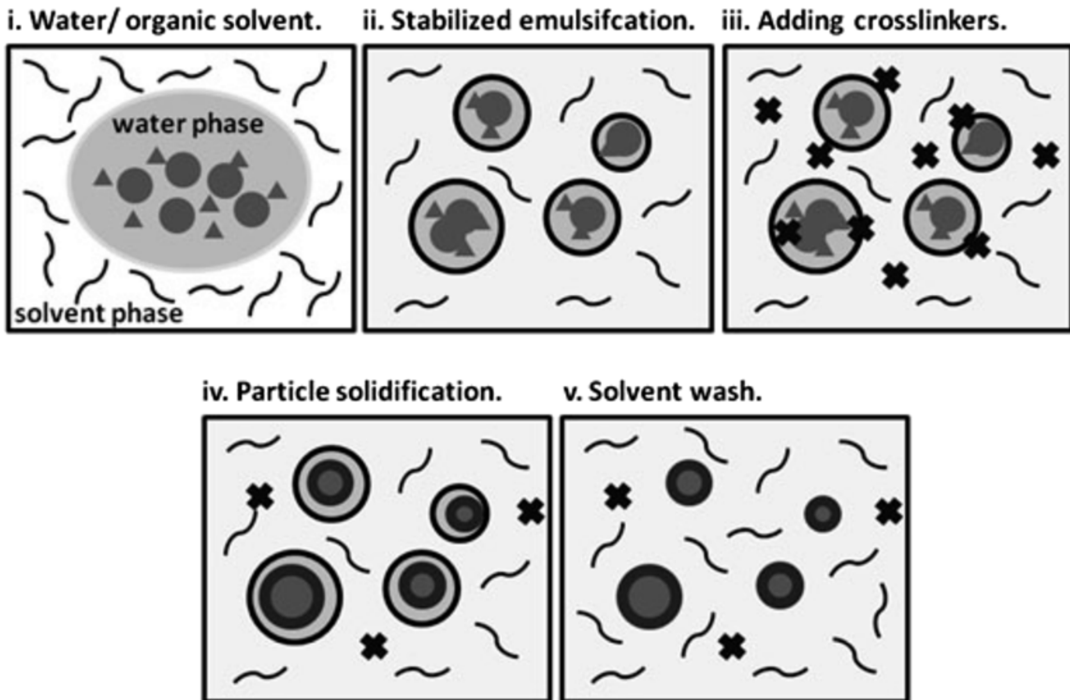


Fig. 41.2 Model of engineered AAMS. Synthesis of L-arginine-incorporated particles. Imaginary microsphere formation in molecular level. The cartoon shows the emulsification process in AAMS synthesis in molecular level: (**i**) Aqueous phase containing matrix molecules are dispersed in organic solvent containing stabilizers. (**ii**) Stabilizers precipitate at the interface to stabilize the emulsification system after mechanical turbulence. (**iii**) Cross-linkers diffuse into water phase through solvent phase. (**iv**) Cross-linkers solidify particle surface. (**v**) Homogenous liquid phase is formed to wash out the solid particles

stable emulsification. After emulsification, the microspheres are solidified by chemical or ionic cross-linkers to maintain the stability of AMS in aqueous solution at body temperature. When glutaraldehyde is introduced as a cross-linker into the emulsion through the organic solvent phase, it diffuses through the interface between water and organic solvent to react with BSA and L-arginine molecules and enables the particle surface to be more reactive for further modification. After solidification, the AMS can be washed out by the addition of wash solvent into the emulsion system. Usually solvents with low polarity are used as wash solvent to prevent the agglomeration among AMS during washing out. Figure 41.2 shows the imagination of AMS synthesis through emulsification in molecular level.

In the volume percentage size distribution, AAMS showed a mean particle size of about 5 μm with a standard deviation of 6.6. The mode of the distribution lied around 4 and about 95 % of AAMS were synthesized in the size range between 1 and 10 μm (Fig. 41.3A). The surface morphology of AAMS was determined in brief, the acetone-washed and then air-dried AAMS showed a rough surface under SEM. The SEM images of AAMS were shown in Fig. 41.3B From the SEM images, it was observed that most of the AAMS appeared as spherical particles without agglomeration during the preparation. The particle size of AAMS was confirmed to be distributed within the range of 1–10 μm under SEM. The zeta potentials of AAMS and AMS were measured while dispersed in water. AMS had a negatively charged surface and the mean zeta potential around −40 mv. For the AAMS which had been incorporated with L-arginine, the particle surfaces are less negatively charged with a higher mean zeta potential value in the range of −20 to −30 mv.

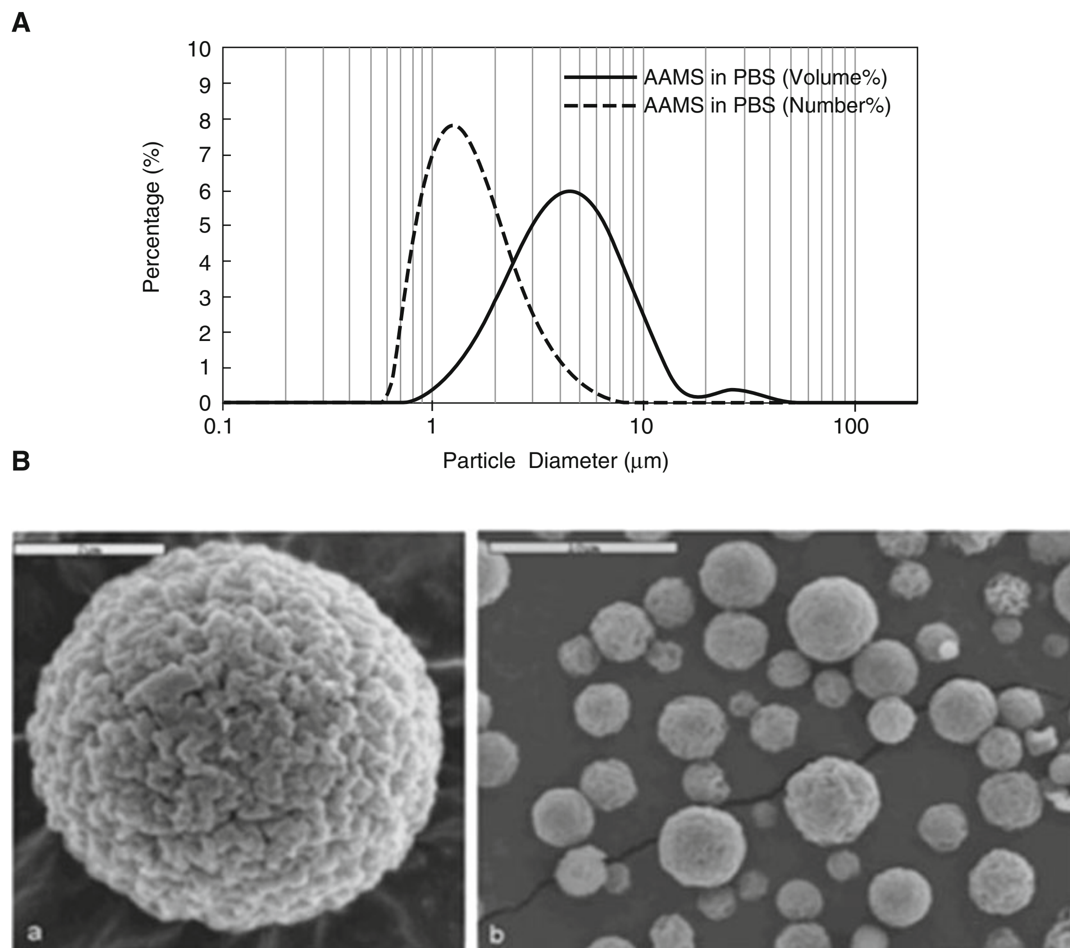


Fig. 41.3 Characterization of AAMS. (**A**) Particle size distribution of L-arginine/albumin microspheres (AAMS) in PBS. *Solid line* represents the size distribution in volume percentage and *dash line* represents number percentage. The particle diameter (*x*-axis) is shown using a logarithmic scale based on 10. (**B**) SEM images of L-arginine/albumin microspheres (AAMS). (**a**) Surface morphology of air-dried AAMS after acetone wash. (**b**) Dry AAMS at low magnitude [48]

In Vitro Effectiveness of AAMS on Inhibiting Lung Cancers

According to the Cancer Facts & Figures 2014 published by American Cancer Society, lung cancer is the leading cause of cancer-related mortality (~159,000 estimated deaths, 27 % of deaths by all cancers), and the 5-year survival rate of lung cancer patients is only 17 %. The plasma L-arginine concentration has been demonstrated to be decreased in lung cancer patients; however, the cause of the depletion has not been determined yet [49]. It has also been reported in vitro that L-arginine inhibits or stimulates the proliferation of lung cancer cells [36, 50]. Whether inhibition or stimulation of lung cancer cell proliferation is dependent on the delivery media of L-arginine is not clear [36].

L-Arginine has been reported to inhibit or facilitate the proliferation of cancer cells based on the metabolism of L-arginine [7, 36, 50]. The mechanism of the cell death through the metabolic process of L-arginine has been shown to induce apoptosis [9]. In a previous study, L-arginine has been shown to inhibit the proliferation of gastric cancer cells and also shown to induce apoptosis [8]. In addition, the apoptosis induction was dependent on the level of NO generated through the metabolism of

L-arginine. Generally, the induction of apoptosis requires high concentration of NO and low NO concentration can lead to resistance to the NO-induced apoptosis [15]. However, a recent study by Shukla et al. pointed out another possible mechanism of L-arginine-mediated inhibition of tumor cell proliferation [36]. The mechanism of cell death by L-arginine was due to the damage of cell membrane that leads to necrosis, as shown in Fig. 41.4a, b. In in vitro studies in various malignant cell types, damage to the cell membrane, deficiency of NO in supernatant, and the absence of apoptotic gene expression had been observed with L-arginine-induced cell death. This indicates that necrosis, instead of the metabolically driven cell death apoptosis, is the most likely mechanism by which L-arginine inhibits the proliferation of cancer cells.

Disruption of cell membrane is the most likely mechanism that L-arginine effectively kills cancer cells by using the AAMS. L-Arginine molecules in a concentrated L-arginine solution have a high tendency to aggregate and assemble into a molecular cluster due to the positively charged amphiphilic molecular structure [37, 38]. With the highly positively charged and hydrophobic surface, the L-arginine clusters disrupt the negatively charged cell membrane and become an effective antitumor reagent. However, the local concentration and environment is a crucial factor to influence the cytotoxicity of L-arginine to cancer cells. The clinical use of L-arginine in cancer treatment is still questionable because the inhibiting effect of L-arginine on cancer cell proliferation is paradoxical. High concentration of L-arginine inhibits cancer cell proliferation, while on the contrary, weak dosage showed stimulation on proliferation. In previous study, it is observed that L-arginine tends to aggregate and induce tumor death at least at a concentration of 10 mM. To achieve this, the delivery method of L-arginine to tumor sites is crucial to create a local environment with high concentration of L-arginine and make L-arginine an effective antitumor agent.

In our previous study, we synthesized the microspheres with 50 % L-arginine and 50 % BSA (w/w). The L-arginine was incorporated with AAMS in the matrix of microspheres and on the surface as well. The surface of AAMS is positively charged and strong interaction between AAMS and cancer cells was observed in the in vitro studies. The synthesized AAMS were expected to act as an antitumor agent by providing an L-arginine-rich surface on microspheres, similar to the L-arginine cluster

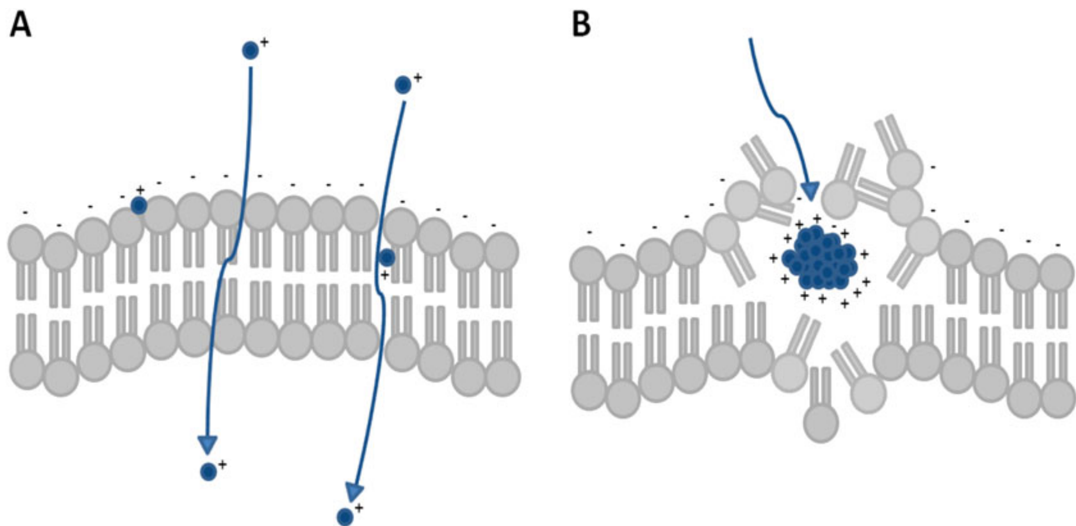
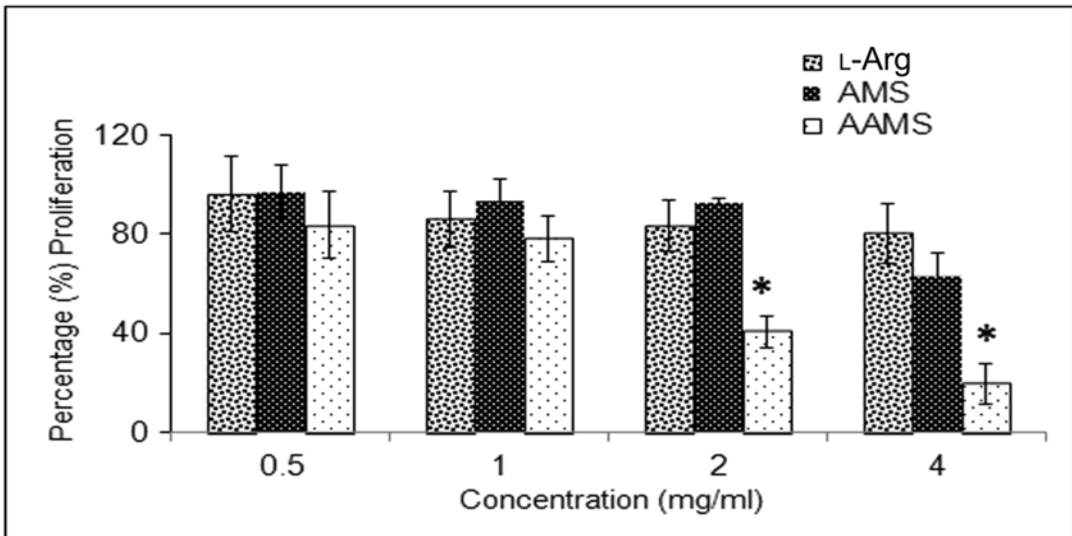


Fig. 41.4 Physical interactions between cationic molecules and cell membrane. Schematic presentation. (**a**) Cationic molecules diffuse through cancer cell membrane which is generally negatively charged. Small cationic molecules can adhere on membrane surface as well as penetrate into or through the membrane to enhance the permeability. (**b**) Clusters of cationic molecules or larger cationic molecules interrupt cell membrane integrity and cause irreversible damage on cell membrane. Necrosis is the main form of cell death induced by cell membrane leakage

surface, and also locally releasing L-arginine to the tumor sites. In the in vitro study, which is shown in Fig. 41.5a, b, the AAMS showed a more effective inhibition on lung cancer cell proliferation than the freely released L-arginine. AAMS inhibited the proliferation of non-small lung cancer cells and malignant mesothelioma cells with a relatively low concentration; moreover, the efficacy has been prolonged since the L-arginine molecules incorporated within AAMS are less likely to be involved into metabolism. This indicates that the L-arginine incorporated on microspheres can be more effective to inhibit the proliferation of cancer cells than the free L-arginine in the environment.



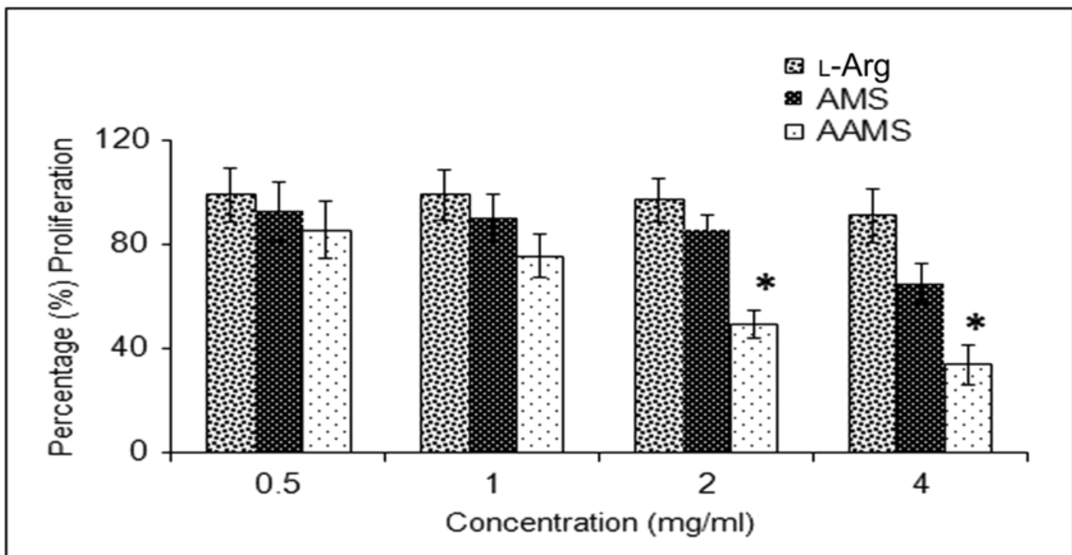


Fig. 41.5 AAMS inhibits lung cancer cell proliferation. The AAMS showed effective in vitro inhibiting effect on cell proliferation in (**A**) non-small cell lung cancer (NSCLC) and (**B**) malignant pleural mesothelioma cells [48]

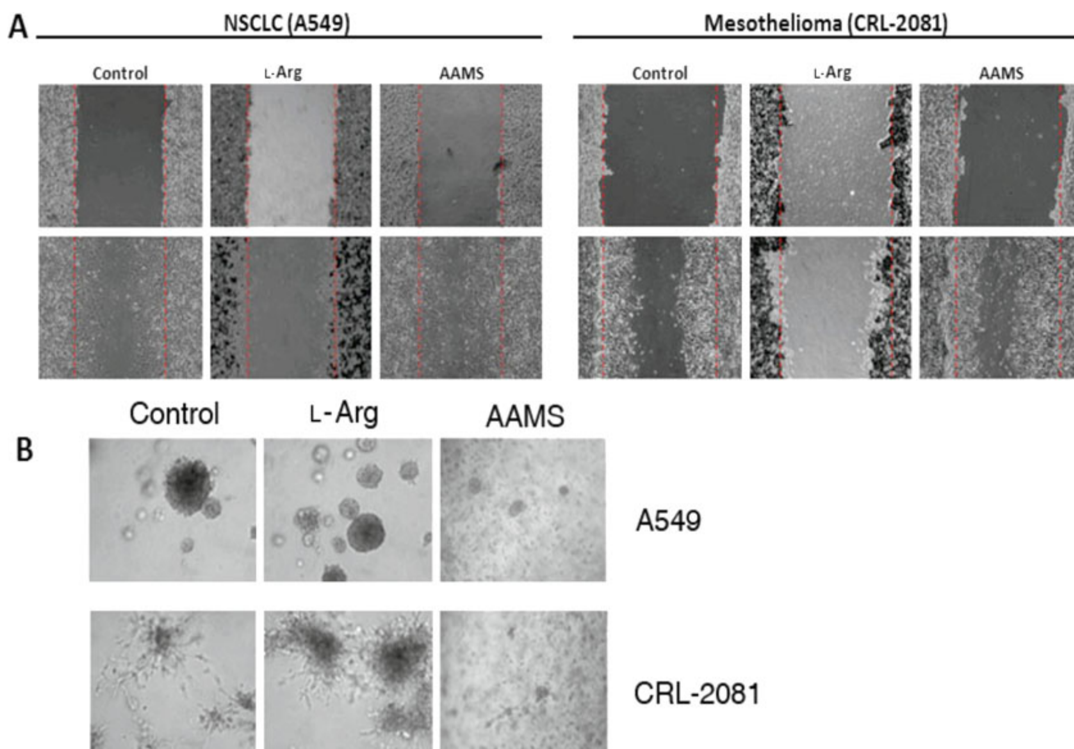


Fig. 41.6 AAMS inhibits wound healing and tumor growth in lung cancer cells. (**A**) In the wound healing assay, the cell invasion rate was inhibited by AAMS, while free L-arginine promoted the invasion rate at the same concentration. (**B**) The tumor growth in Matrigel was inhibited by AAMS but accelerated by L-arginine [48]

The inhibition of tumor growth by AAMS was demonstrated by using the three-dimensional tumor growth assay in Matrigel. Our results showed that the AAMS effectively reduced the size and number of the tumor colonies, while the lung cancer cells treated with free L-arginine showed a prompt tumor growth (Fig. 41.6b). It is noteworthy that in some tumor cell lines, the AAMS in the environment not only reduced the number and size in tumor colonies but also had a significant influence on tumor morphology. In the untreated tumor cells and tumor cells treated with free L-arginine, the cells spread out from the tumor colonies and connected to other cells. However, the cell spreading was inhibited in the cultures treated with AAMS. It has been previously shown that L-arginine inhibited proliferation of gastric cancer cells with increased apoptosis, but there was no effect on tumor cell invasion [8]. Our study showed that the AAMS that we synthesized has a promising inhibiting effect on the cell migration of lung cancer cells, whereas the free L-arginine prompted the cell migration at similar concentration (Fig. 41.6a).

Future Strategies of Developing Antitumor Agents Targeting L-Arginine Metabolism

L-Arginine has been indicated to be possibly the most crucial dietary supplement that influences tumor growth and progress in various malignancies. Tumor growth generally demands higher level of L-arginine, thus L-arginine depletion is considered to be the potential therapeutic method for cancer patients. Achieving L-arginine depletion by introducing exogenous catalytic enzymes to regulate L-arginine

level in the metabolic pathways has been considered as a direct therapeutic strategy to inhibit tumor growth. However, varying effects of L-arginine on cell growth in different cancer cell lines have been observed and the mechanism of action of L-arginine on cancers is not fully elucidated. The efficient control of L-arginine level and metabolism in human body can be difficult and that can be influenced by complicated factors, for example, further enhanced tumor growth may be caused by unexpected bounce back of L-arginine concentration after depletion. Besides, as described previously, high concentration of L-arginine in the microenvironment may result in molecular clusters of L-arginine which may be followed by local depletion of L-arginine because the free L-arginine molecules in the environment could assemble onto the clusters and escape from the metabolic pathways. The AAMS drug delivery system we previously developed might also show the same mechanism as the clusters to inhibit the cell proliferation, migration, and tumor growth by reducing the L-arginine-derived metabolic products essential for tumor development, as well as increasing cell death via necrosis caused by cell membrane interruption. Therefore, due to its physical properties and molecular role in cancer biology, L-arginine has a high potential to be applied into the drug delivery systems providing combination therapy with other therapeutic agents for cancer treatment. In addition, through appropriate design, biomaterials incorporated with L-arginine, such as drug released particles, hydrogels, and specific targeting peptide sequences, are expected to provide multifunctional properties when applied as an antitumor agent in cancer therapy.

Conclusion

L-Arginine and its metabolic products play a critical role in regulating tumor growth in various types of cancers. The environmental L-arginine level significantly influences the immune responses in T-cells which regulate the development and progress of cancer tumor. Besides its function in regulating tumor growth via metabolic and immune pathways, the properties of L-arginine enable it to be a potential agent applied in cancer therapy. The L-arginine incorporated biomolecules such as L-arginine-rich peptides and polyarginine have also been widely applied in various kinds of novel strategies for cancer treatment. Although the mechanism of action of L-arginine is not fully clear, L-arginine has a high potential to be applied in the combination therapy with other therapeutic agents. The L-arginine/albumin microsphere showed significant effectiveness on inhibiting proliferation and migration of the lung cancer cells including non-small cell lung cancer and malignant mesothelioma cells. AAMS acted as a more effective antitumor agent on the lung cancer cells than L-arginine. The AAMS showed constant inhibiting effects on the proliferation and migration of lung cancer cells; whereas the freely released L-arginine showed stimulation on cell proliferation and migration at lower concentrations. This implies that creating a microenvironment with high concentration of L-arginine is a more effective approach to kill cancer cells. In addition, AAMS has a high potential to be applied into intratumoral therapy for different types of cancers due to its antitumor ability, strong interaction with cells, and most importantly, the high safety. The mechanisms of AAMS inhibiting lung cancer cell proliferation is not clear and needs to be further investigated. The present data provide a promising foundation for future in vivo studies and offer a different point of view to explain how L-arginine inhibits growth and migration in lung cancer cells.

References

1. Appleton J. L-Arginine: clinical potential of a semi-essential amino acid. Altern Med Rev. 2002;7:512–22.
2. Closs EI, Simon A, Vekony N, et al. Plasma membrane transporters for L-arginine. J Nutr. 2004;134:2752S–9.
3. Castillo L, Sanchez M, Vogt J, et al. Plasma L-arginine, citrulline, and ornithine kinetics in adults, with observations on nitric oxide synthesis. Am J Physiol. 1995;268:E360–7.

4. Wheatley DN. Controlling cancer by restricting L-arginine availability—L-arginine-catabolizing enzymes as anticancer. Anticancer Drugs. 2004;15:825–33.
5. Lind DS. L-Arginine and cancer. J Nutr. 2004;134:2837S–41.
6. Caso G, McNurlan MA, McMillan ND, et al. Tumour cell growth in culture: dependence on L-arginine. Clin Sci. 2004;107:371–9.
7. Chochung YS, Clair T, Bodwin JS, et al. Growth arrest and morphological change of human-breast cancer-cells by dibutyryl-cyclic-AMP and L-arginine. Science. 1981;214:77–9.
8. Nanthakumaran S, Brown I, Heys SD, et al. Inhibition of gastric cancer cell growth by L-arginine: molecular mechanisms of action. Clin Nutr. 2009;28:65–70.
9. Wolf C, Bruss M, Hanisch B, et al. Molecular basis for the antiproliferative effect of agmatine in tumor cells of colonic, hepatic, and neuronal origin. Mol Pharmacol. 2007;71:276–83.
10. Xu W, Liu LZ, Loizidou M, et al. The role of nitric oxide in cancer. Cell Res. 2002;12:311–20.
11. Torok NJ, Higuchi H, Bronk S, et al. Nitric oxide inhibits apoptosis downstream of cytochrome c release by nitrosylating caspase 9. Cancer Res. 2002;62:1648–53.
12. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol. 2001;2:149–56.
13. Morbidelli L, Donnini S, Ziche M. Role of nitric oxide in tumor angiogenesis. Cancer Treat Res. 2004; 117:155–67.
14. Ma QY, Williamson KE, O'Rourke D, et al. The effects of L-arginine on crypt cell hyperproliferation in colorectal cancer. J Surg Res. 1999;81:181–8.
15. Xie KP, Huang S. Contribution of nitric oxide-mediated apoptosis to cancer metastasis inefficiency. Free Radic Biol Med. 2003;34:969–86.
16. Reynolds JV, Thom AK, Zhang SM, et al. L-Arginine, protein-malnutrition, and cancer. J Surg Res. 1988;45:513–22.
17. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64: 5839–49.
18. Feun L, You M, Wu CJ, et al. L-Arginine deprivation as a targeted therapy for cancer. Curr Pharm Des. 2008;14:1049–57.
19. Delage B, Fennell DA, Nicholson L, et al. L-Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. Int J Cancer. 2010;126:2762–72.
20. Phillips MM, Sheaff MT, Szlosarek PW. Targeting L-arginine-dependent cancers with L-arginine-degrading enzymes: opportunities and challenges. Cancer Res Treat. 2013;45:251–62.
21. Izzo F, Marra P, Beneduce G, et al. Pegylated L-arginine deiminase treatment of patients with unresectable hepatocellular carcinoma: results from phase I/II studies. J Clin Oncol. 2004;22:1815–22.
22. Ascierto PA, Scala S, Castello G, et al. Pegylated L-arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies. J Clin Oncol. 2005;23:7660–8.
23. Barbul A, Lazarou SA, Efron DT, et al. L-Arginine enhances wound healing and lymphocyte immune responses in humans. Surgery. 1990;108:336–7.
24. Rodriguez PC, Ochoa AC. L-Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev. 2008;222:180–91.
25. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005; 5:641–54.
26. Rodriguez PC, Zea AH, Culotta KS, et al. Regulation of T cell receptor CD3ζ chain expression by L-arginine. J Biol Chem. 2002;277:21123–9.
27. Tung CH, Weissleder R. L-Arginine containing peptides as delivery vectors. Adv Drug Deliv Rev. 2003;55:281–94.
28. Shin MC, Zhang J, Min KA, et al. Cell-penetrating peptides: achievements and challenges in application for cancer treatment. J Biomed Mater Res A. 2013;102:575–87.
29. Nakase I, Konishi Y, Ueda M, et al. Accumulation of L-arginine-rich cell-penetrating peptides in tumors and the potential for anticancer drug delivery in vivo. J Control Release. 2012;159:181–8.
30. Lee HY, Mohammed KA, Kaye F, et al. Targeted delivery of let-7a microRNA encapsulated ephrin-A1 conjugated liposomal nanoparticles inhibit tumor growth in lung cancer. Int J Nanomedicine. 2013;8:4481–94.
31. Ohtake E, Natsume H, Ueda H, et al. Analysis of transient and reversible effects of poly-L-arginine on the in vivo nasal absorption of FITC-dextran in rats. J Control Release. 2002;82:263–75.
32. Nemoto E, Ueda H, Akimoto M, et al. Ability of poly-L-arginine to enhance drug absorption into aqueous humor and vitreous body after installation in rabbits. Biol Pharm Bull. 2007;30:1768–72.
33. Lozano MV, Lollo G, Alonso-Nocelo M, et al. Polyarginine nanocapsules: a new platform for intracellular drug delivery. J Nanopart Res. 2009;15:14.
34. Lai BH, Yeh CC, Chen DH. Surface modification of iron oxide nanoparticles with polyarginine as a highly positively charged magnetic nano-adsorbent for fast and effective recovery of acid proteins. Process Biochem. 2012;47:799–805.

35. Oyarzun-Ampuero FA, Goycoolea FM, Torres D, et al. A new drug nanocarrier consisting of polyarginine and hyaluronic acid. Eur J Pharm Biopharm. 2011;79:54–7.
36. Shukla J, Thakur VS, Poduval TB. L-Arginine: appropriate dose and delivery environment makes it an anticancer molecule, through nitric oxide independent pathway. Nitric Oxide. 2010;22:S83–S83.
37. Das U, Hariprasad G, Ethayathulla AS, et al. Inhibition of protein aggregation: supramolecular assemblies of L-arginine hold the key. PLoS One. 2007;2:e1176.
38. Toyama N, Kohno JY, Mafune F, et al. Solvation structure of L-arginine in aqueous solution studied by liquid beam technique. Chem Phys Lett. 2006;419:369–73.
39. Goldberg EP, Hadba AR, Almond BA, et al. Intratumoral cancer chemotherapy and immunotherapy: opportunities for nonsystemic preoperative drug delivery. J Pharm Pharmacol. 2002;54:159–80.
40. Celikoglu F, Celikoglu SI, Goldberg EP. Bronchoscopic intratumoral chemotherapy of lung cancer. Lung Cancer. 2008;61:1–12.
41. Cheung RY, Rauth AW, Wu XY. In vivo efficacy and toxicity of intratumorally delivered mitomycin C and its combination with doxorubicin using microsphere formulations. Anticancer Drugs. 2005;16:423–33.
42. Lammers T, Peschke P, Kuehnlein R, et al. Effect of intratumoral injection on the biodistribution and the therapeutic potential of HPMA copolymer-based drug delivery systems. Neoplasia. 2006;8:788–95.
43. Okada H, Toguchi H. Biodegradable microspheres in drug-delivery. Crit Rev Ther Drug Carrier Syst. 1995;12:1–99.
44. Kawaguchi H. Functional polymer microspheres. Prog Polym Sci. 2000;25:1171–210.
45. Longo WE, Goldberg EP. Hydrophilic albumin microspheres. Methods Enzymol. 1985;112:18–26.
46. Almond BA, Hadba AR, Freeman ST, et al. Efficacy of mitoxantrone-loaded albumin microspheres for intratumoral chemotherapy of breast cancer. J Control Release. 2003;91:147–55.
47. Lee HY, Mohammed KA, Peruvemba S, et al. Targeted lung cancer therapy using ephrinA1-loaded albumin microspheres. J Pharm Pharmacol. 2011;63:1401–10.
48. Lee HY, Mohammed KA, Goldberg EP, et al. L-Arginine-conjugated albumin microspheres inhibits proliferation and migration in lung cancer cells. Am J Cancer Res. 2013;3:266–77.
49. Naini AB, Dickerson JW, Brown MM. Preoperative and postoperative levels of plasma protein and amino acid in esophageal and lung cancer patients. Cancer. 1988;62:355–60.
50. Park KGM, Heys SD, Eremin O, Garlick PJ. The effect of L-arginine on the growth and metabolism of an experimental lung cancer. J Cancer Res Clin Oncol. 1990;38:1709–15.

Chapter 42
Use of L-Arginine and Glycine Supplementation to Reduce Radiotherapy Damage

Cristina Fajardo Diestel, Nara Limeira Horst, Alessandra da Rocha Pinheiro Mulder, and Ruy Garcia Marques

Key Points

- Radiotherapy is an important treatment for a great number of cancers.
- Normal adjacent tissue radiation toxicity remains the most important dose-limiting factor in radiation therapy.
- Despite efforts being employed in drug development for irradiation protection, no ideal compound has been developed so far.
- It is suggested that L-arginine (Arg) and glycine (Gly) can provide an effective low-risk option to decrease the adverse effects of irradiation.
- Arg plays an important role in wound healing and in the maintenance of tissue integrity due to its action as a mediator of angiogenesis, epithelialization, and collagen formation by hydroxyproline production.
- Arg supplementation is still considered controversial in amelioration of radiation-induced acute inflammation, because excessive NO production can generate structural disorders by inhibition of smooth muscle cell proliferation and apoptosis promotion.
- Gly is a simple non-essential amino acid that is being investigated to provide cytoprotection. Gly may immunomodulate the macrophages of Peyer's patches, decrease the direct local intestinal injury and the resultant "cytokine shower", and prevent reactive oxygen species (ROS) formation, and maybe Gly use can be preventive against irradiation damage.

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Abbreviations

Arg	L-arginine
AST	Aspartate aminotransferase
CINC	Cytokine-induced neutrophil chemoattractant
CO_2	Carbon dioxide
DSS	Dextran sulphate sodium
GI	Gastrointestinal
Gly	Glycine
IL-1	Interleukin-1
LDH	Lactate dehydrogenase
MIP-2	Macrophage inflammatory protein 2
NAD	Nicotinamide adenine dinucleotide
NADH	Hydrogen nicotinamide adenine dinucleotide
NH_4	Ammonia
NO	Nitric oxide
NOS	Nitric oxide synthase
ROS	Reactive oxygen species
TNBS	2,4,6-Trinitrobenzene sulphonic acid
TNF-α	Tumour necrosis factor-alpha

Introduction

Cancer is a multifactorial chronic disease caused by disorganized cell growth and is one of the most important causes of death in the world. Ionizing radiation, as surgery and chemotherapy, is an important treatment for a number of malignancies. Radiotherapy is commonly administered to the abdomen and pelvis of patients with gastrointestinal (GI), urological, and gynaecological malignant neoplasms [1].

Radiotherapy involves the use of X-rays by linear accelerators and gamma rays generated by high-energy radioactive isotopes to destroy malignant cells by local action. The goal of radiation therapy is to administer a tumoricidal dose, saving adjacent normal tissues, in which regeneration of the irradiated area begins. The new, more targeted radiotherapy techniques over the last two decades diminished the toxic rates, but several early and late side effects are still observed [1, 2]. Nevertheless, normal tissue radiation toxicity remains the single most important dose-limiting factor in radiation therapy and a major obstacle to uncomplicated cancer cures [3].

Accentuated cell renewal tissues, which require rapid and continuous cell proliferation, such as skin, bone marrow, and GI mucosa, are more vulnerable to the acute toxic effects [4]. The late toxic effects, such as fibrosis, necrosis, and ulcerations, are determined by the total irradiation dose and the size of fractions employed, and not by the proliferative potential of the affected tissue [5]. Although the risk of severe chronic enterocolitis by irradiation appears to correlate with the intensity of the acute syndrome, the absence of severe acute manifestation does not prevent the eventual development of chronic severe actinic effects [4].

Radiotherapeutic injury is complex and healing varies from normal wound healing as a result of repetitive injuries [6]. Ionizing radiation causes several typical changes in tissues in the bowel. Early actinic enteropathy occurs during or shortly after therapy [3] and is characterized by inflammation or cell death including mucosal cell loss, acute inflammation in the lamina propria, and epithelial barrier breakdown that can result in bacterial translocation despite intestinal increased permeability and bacterial overgrowth [3] (Figs. 42.1 and 42.2). These may resolve but can develop into a more chronic

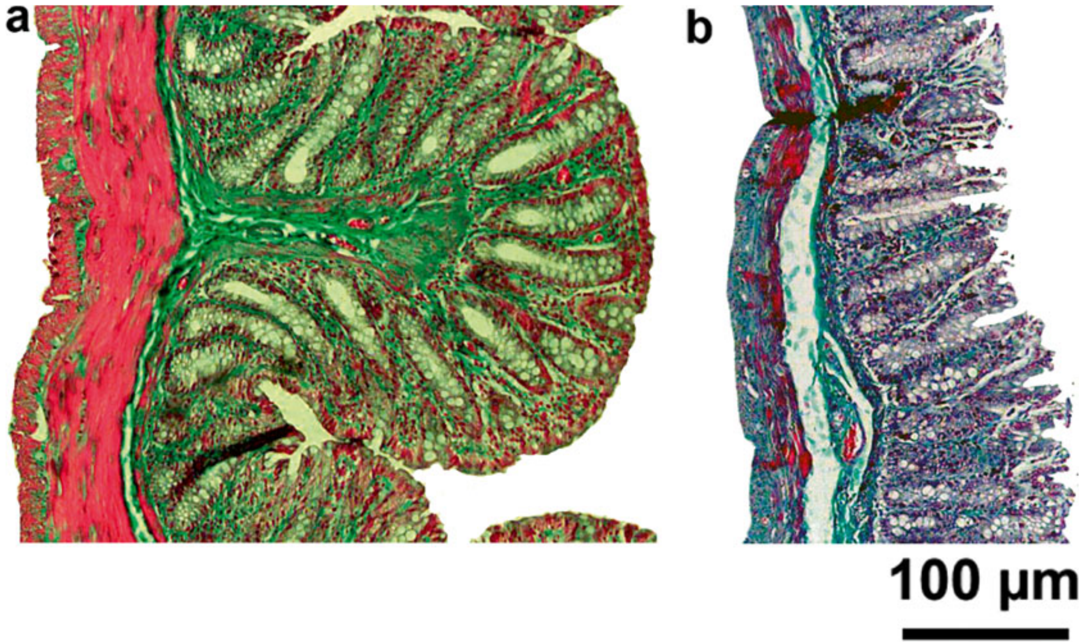

Fig. 42.1 Photomicrography of colonic wall in healthy rats (**a**) and rats submitted to a 1100 cGy single dose of abdominal radiation (**b**), showing a decrease in the total volume of the colonic wall following irradiation

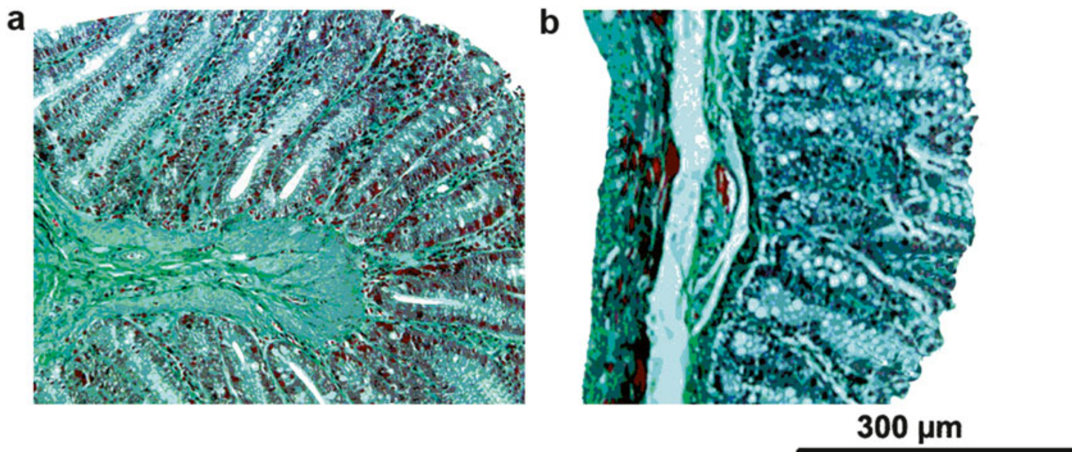

Fig. 42.2 Photomicrography of mucosal epithelial surface in healthy rats (**a**) and rats submitted to a 1100 cGy single dose of abdominal radiation (**b**), showing a decrease in the mucosal epithelial surface of the colonic wall in irradiated rats

change (delayed radiation enteropathy) with persistent cytokine activation in the mucosa and in the submucosa, and fibrosis of connective tissue [7]. These changes may result in intestinal dysfunction (e.g. dysmotility or malabsorption) and structural injury (e.g. stricture formation, fistulas, or perforation) [3, 8]. Clinical presentation will depend on the degree and the extent of tissue damage together with the site of injury.

Almost all patients receiving pelvic or abdominal irradiation experience some form of GI symptoms, such as pain, bloating, nausea, faecal urgency, diarrhoea, and rectal bleeding which can have a significant impact on patient's quality of life [9]. Patients usually notice these symptoms during the second week of treatment (when tissue damage and inflammation is probably at a maximum), and they usually peak by the fourth to fifth week (when histological changes are stable or improving) [10].

Despite efforts being employed in drug development for irradiation protection, no ideal compound has been developed so far. However, there is evidence that special diets or dietary supplementation with isolated nutrients, such as L-arginine (Arg) and glycine (Gly), can provide an effective low-risk option to decrease the adverse effects of irradiation [11].

L-Arginine

L-arginine (Arg) was classified as nutritionally non-essential (dispensable) amino acid for healthy adults [12] and essential (indispensable) for newborns [13] (Fig. 42.3). However, as in certain metabolic conditions, in which the amount of Arg synthesized by the body does not meet the increased organic demand [14], this amino acid was classified as conditionally essential in trauma and stress and has been investigated in some clinical conditions [15–17]. This can occur despite several factors such as increased Arg degradation, reduction of ingestion, and decreased intestinal absorption [18].

Arg presents multiple roles in animal physiology. The many functions of Arg are caused by the action of molecules derived from its metabolism and not by the isolated amino acid action. In animal cells, it regulates the synthesis of nitric oxide (NO), polyamines and creatine [19], urea, proline, glutamate, and agmatine [20], and, by different metabolic pathways, acts in mechanisms of inflammation, wound healing, fibrosis, and protein synthesis. In addition, Arg acts on intestinal motility and detoxification of ammonia [21]. Additionally, Arg is a potent stimulator of endocrine system, increasing the growth hormone, prolactin, insulin, and glucagon secretion [20].

Thus, Arg supplementation seems to prevent or repair damage caused by cardiovascular, pulmonary, hepatic, renal, and gastrointestinal diseases, but it also plays an important role in wound healing and maintenance of tissue integrity due to its action as a mediator of angiogenesis, epithelialization, and collagen formation by hydroxyproline production [12, 22].

However, Arg supplementation is still considered controversial in some clinical conditions, such as in amelioration of radiation-induced acute inflammation. The first question concerns the supplementation dose. Gurbuz et al. [16], for example, used doses of 2 and 4 % of the total ingested energy for 7 days in Sprague-Dawley rats subjected to a single dose of 1100 rads of abdominal X-radiation [16]. Hwang et al. [23] studied animals supplemented with diet and water containing 2 % of Arg for 3 days prior to radiation, but the radiation dose was similar to that used by Gurbuz et al. [16]. In general, Arg

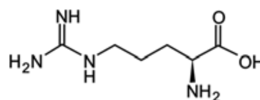

Fig. 42.3 L-arginine structure

has been shown to be remarkably free of side effects for the vast majority of people who have taken it, and the daily dosage recommended for humans ranges from 30 to 60 g in patients with normal liver and kidney functions [24].

Some studies have shown that diets enriched with Arg after irradiation protected the intestinal mucosa by accelerating the healing and prevention of bacterial translocation and body weight loss in rats [16, 17]. However, Hwang et al. [23] questioned the beneficial effect of diets enriched with Arg and L-glutamine on the harm caused by abdominal radiation in rats [23]. The serum levels of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in the L-arginine group were markedly higher when compared with control animals that received L-glutamine. Furthermore, on histological examination, radiation caused more serious damage to various segments of intestine in the L-arginine-fed rats compared to rats on other feeding regimens [23]. De Aguiar Picanço et al. [11] studied the effect of dietary supplementation with Arg and Gly in the structure of the colon wall of rats subjected to abdominal irradiation. They observed that Gly supplementation showed superior effects of Arg supplementation into the colonic wall structure, considering that it was able to maintain the wall thickness and epithelial mucosal surface, while Arg was able to maintain the partial volume of the epithelium and the epithelial surface, but not the total volume of the intestinal wall [12] (Table 42.1).

The reason for this controversy seems to be related to the excessive production of NO, leading to negative consequences. The metabolism of Arg is determined by the expression of two groups of enzymes, arginase and nitric oxide synthase (NOS). The two isoforms of arginase (I and II) are induced by cytokines produced by helper T-II cells. Arginase is important for the production of ornithine, a precursor of proline and polyamines, both necessary for cell proliferation and wound healing [24]. Competing with arginase for the same substrate, there are three isoforms of NOS, with different

Table 42.1 Characteristics of experimental studies with L-arginine in radiotherapy (all of them using radiation dose of 1100 cGy)

Authors	Animals	Groups	Results
Ersin et al. [15]	Sprague-Dawley rats	Groups I and II—glutamine- and L-arginine-enriched diet pre- and post-radiation Groups III and IV—glutamine and L-arginine-enriched diet only post-radiation Group V—control	Both L-arginine- and glutamine-enriched diets have protective effects on gut mucosa
Gurbuz et al. [16]	Sprague-Dawley rats	Group I—diet enriched with 2 % L-arginine Group II—diet enriched with 4 % L-arginine Group III—isonitrogenous—4 % glycine	Enhanced bacterial clearance from mesenteric lymph nodes and improvement in intestinal mucosal recovery in group II
Hwang et al. [23]	Sprague-Dawley rats	Group I—standard diet Group II—diet with 2 % L-arginine Group III—diet with 2 % glutamine	Diarrhoea, enhance of serum levels of AST and LDH, and more serious damage to various segments of intestine in the L-arginine-fed rats
de Aguiar Picanço et al. [11]	Wistar rats	Group I—healthy Group II—irradiated with no supplementation Group III—irradiated and supplemented with L-arginine Group IV—irradiated and supplemented with glycine	Glycine supplementation had a superior effect on the irradiated colon wall compared to L-arginine supplementation. Glycine and L-arginine maintained the epithelial surface of colonic mucosa compared to controls (group II)

cellular distribution and regulatory mechanisms: neuronal NOS or NOS 1, inducible NOS or NOS 2, and endothelial NOS or NOS 3. All isoforms of NOS use Arg and oxygen as substrates to NO and L-citrulline production [25]. Under normal conditions, a small amount of NO is synthesized by the NOS 1 and 3 isoforms, giving a beneficial effect on tissue oxygenation and immune function [26]. However, uncontrolled production of NO by NOS 2 may lead to excessive inflammation, impaired cellular respiration, non-specific cytotoxicity, coagulation abnormalities, and haemodynamic deterioration, furthermore reducing the availability of arginase substrate [24, 27]. The excessive NO production also generates structural disorders by inhibition of smooth muscle cells proliferation [28] and apoptosis promotion [29].

Thus, additional studies of benefits and the potential adverse effects of Arg supplementation in abdominal radiation are necessary, both in animal and human trials.

Glycine

Glycine (Gly), a non-essential amino acid, is known to possess various physiologic functions (Fig. 42.4). Gly is the simplest and smallest amino acid and consists of a single carbon molecule attached to an amino and a carboxyl group. Its small size helps it to function as a flexible link in proteins and allows for the formation of helices, an extracellular signalling molecule, recognition sites on cell membranes and enzymes, a modifier of molecular activity via conjugation and Gly extension of hormone precursors, and an osmoprotectant [30]. Gly is often considered biologically neutral and is used as an isonitrogenous control in studies of supplementation with other amino acids. However, much evidence has accumulated that Gly is an effective anti-inflammatory, immunomodulatory, and cytoprotective agent [31].

Gly is taken up by cells via a variety of Gly transporters, typically by a secondary active transport coupled to hydrogen, sodium, and/or chloride ion uptake [32]. Due to its small size, Gly is extremely adaptable and the preferred candidate for an interior position in proteins [33]. Besides being a decisive building block in many proteins, Gly is also a component of the tripeptide glutathione and of the bile exclude glycocholic acid. In addition, it is an essential substrate for the synthesis of a variety of biomolecules such as creatine, porphyrins, and purine nucleotides. Within cells, Gly is oxidatively (NAD^+-dependently) degraded by the mitochondrial Gly cleavage system to CO_2, NH_4^+, NADH, and a methylene group, which is accepted by tetrahydrofolate, thus forming N^5,N^{10}-methylenetetrahydrofolate [34].

Via methylenetetrahydrofolate, Gly is decisively involved in the metabolism of 1-carbon units and by that in further synthetic pathways. Using methylenetetrahydrofolate, mitochondrial and cytosolic serine hydroxymethyltransferase catalyses the formation of serine from Gly [34]. The reactions catalysed by the Gly cleavage system and by serine hydroxymethyltransferase are reversible and thus can also be used for the synthesis of Gly. Gly is also formed from glyoxylate, a reaction catalysed by alanine:glyoxylate aminotransferase, an enzyme which is predominantly located in peroxisomes [35].

Blood plasma Gly concentration has been reported to vary between 170 and 330 μM both in humans and in experimental animals [36]. Due to active uptake, the intracellular Gly concentrations are significantly higher than the extracellular levels [37]. The Gly concentrations in blood plasma and the extracellular space equilibrate within minutes. The half-life of Gly in the blood depends on the

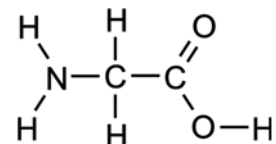

Fig. 42.4 Glycine structure

dose administered and may vary between half an hour and several hours. The majority of Gly administered is taken up by cells and metabolized, primarily in the liver; only a minor amount is excreted in the urine [38].

Since its first description as a cytoprotective [38] agent in 1987, Gly has been shown to protect against ischaemia–reperfusion injury [including related injuries due to anoxia, hypoxia, reactive oxygen species (ROS), chemically induced energy depletion, and mere resupply of oxygen (reoxygenation)] in a great variety of experimental models [31, 36, 39]. In addition to protection from ischaemia–reperfusion injury, substantial experimental evidence has been presented that Gly may also protect against other injurious processes such as liver fibrosis and alcoholic liver disease, gastric ulcer, cyclosporine A-induced nephrotoxicity, and arthritis [30, 31]. Even inhibition of tumour growth in rats and mice has been reported [40].

Gly most likely inhibits both production of inflammatory cytokines/chemokines in macrophages and accumulation/activation of neutrophils, thereby ameliorating colonic injury. It is notable that induction of pro-inflammatory cytokines (IL-1 and TNF-α) and chemokines (CINC and MIP-2) in the colonic tissue was decreased dramatically by dietary Gly, indicating that Gly ameliorates colonic injury by preventing production of TNF-α from tissue macrophages in the colon [41]. Indeed, it has been reported that Gly blunts increases in serum TNF-α levels after lipopolysaccharide injection in the rat [42], and Gly inhibits TNF-α production from isolated Kupffer cells, hepatic resident macrophages, in a direct manner [43].

It has been shown that Kupffer cells contain a Gly-gated chloride channel through which Gly causes an influx of chloride ions into cytoplasm, leading to hyperpolarization of the plasma membrane, thereby preventing activation of these cells [43, 44]. The similar effect of Gly has been observed in alveolar and splenic macrophages [45], suggesting a possibility that Gly inhibits TNF-α production in macrophages in general. It is postulated, therefore, that the mechanisms by which Gly prevents TNBS colitis involve inhibition of TNF-α production from tissue macrophages in the colon [41].

Moreover, Gly is capable of inhibiting activation of neutrophils in a direct manner; Gly reduces the production of superoxide anions from neutrophils caused by formyl-methionine-leucine-phenylalanine in vitro [44]. From another point of view, there are other putative calcium-dependent processes involved in colitis that Gly also may blunt. One possibility is that mast cells are involved in part in the mechanisms of protective effects of Gly. Increasing lines of evidence indicate that mast cells play a certain role in the pathogenesis of experimental colitis [42] and human intestinal bowel diseases [41].

Mast cells release a variety of chemical mediators and cytokines and regulate permeability of the gut [46]. On the other hand, activation of mast cells (i.e. the release of histamine) is regulated by increases in intracellular calcium. Therefore, it is speculated that Gly may blunt calcium-dependent activation of mast cells, thereby modulating mucosal barrier function and inflammatory responses in the gut [47, 48]. This activation may be initiated by the mesenteric injury that results in further secondary organ damage especially on the intestinal wall [36]. By blocking this positive feedback loop, it is possible that Gly may provide cytoprotection [49]. A further hypothesis along these lines may be that Gly may immunomodulate the macrophages of Peyer's patches and decrease the direct local intestinal injury and the resultant "cytokine shower" [47]. Regarding the mechanisms, Gly inhibits the activation of macrophages and neutrophils via a receptor-mediated reaction [31, 43]. These profound phenomena lead to the hypothesis that Gly also is preventive against irradiated tissues [50].

Diestel et al. [50] observed that glycine supplementation for 7 days before and 7 days after a single dose of 1100 cGy of abdominal irradiation in rats was able to significantly increase the total volume of the colonic wall when compared to rats that did not receive any supplementation, remaining similar to the control group (no irradiation) (Fig. 42.5). De Aguiar Picanço et al. [11] observed also in rats that glycine supplementation presented a superior effect on the irradiated colonic wall when compared to L-arginine supplementation. However, the effect of Gly on inflammation/cicatrization in the gut has not been elucidated yet.

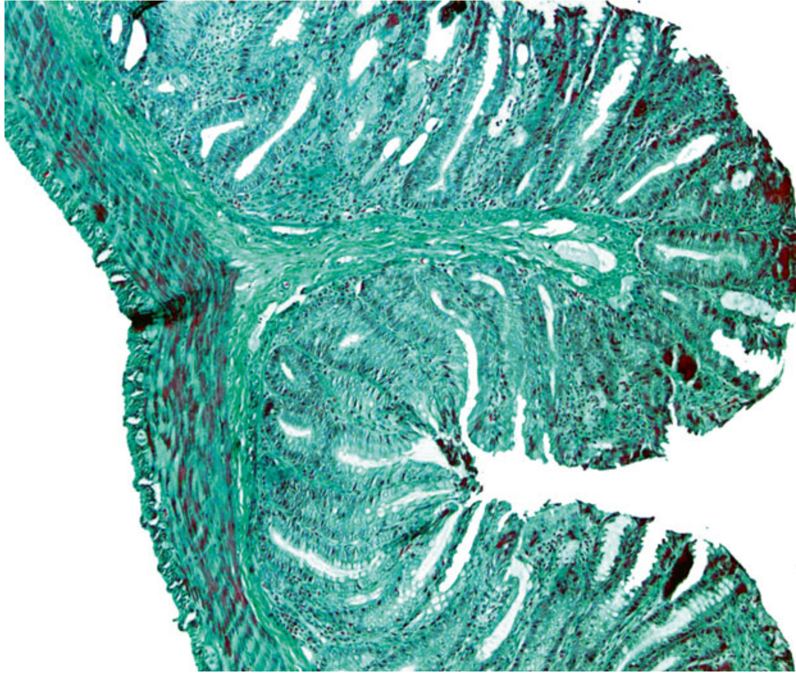

Fig. 42.5 Photomicrography of the colonic wall of rats submitted to a 1100 cGy single dose of abdominal radiation with 14 days of glycine supplementation, showing that the total volume of the colonic wall was similar to the control group (no radiation—Fig. 42.1a)

Gly may also exert different effects to prevent reactive oxygen species (ROS) formation. It is well known for its role in inhibiting free-radical formation that could possibly prevent damage due to radiotherapy [50]. Gly suppresses ROS formation by inhibiting activation of macrophages. This minimizes subsequent transcription factor activation and cytokine production [50]. For example, dietary Gly prevents haemorrhagic shock-induced activation of Kupffer cells, ameliorates oxidative stress, and minimizes the impairment of the activity of antioxidant enzymes (manganese and copper–zinc superoxide dismutase, glutathione peroxidase, and catalase) [30].

This impressive catalogue of functions makes an interesting contrast with Gly's perceived metabolic role as a non-essential amino acid [30]. Importantly, Gly is one of many general nutrients in a variety of foods and has just been viewed as a convenient source of nitrogen to add to solutions of nutrients. The safety of high-dose administration in humans has been established. Although there is no standardization of Gly dose in human or experimental trials, it has been continuously used at different doses and many times without being the main study subject. Jacob et al. and Kallakuri et al. used this amino acid at a dose of 0.5–1.0 g/kg weight in a study about damage caused by ischaemia and reperfusion [49]. Gly was also used in another study on the same subject at a dose of 5 % of the diet offered [39].

The feasibility of a therapeutic approach for irradiated guts using a Gly-rich elementary diet is promising. Although this may have unexpected benefits when such solutions are used in clinical practice, it does raise the spectre of a possible confounding effect in experiments when Gly is added to control solutions to make them isonitrogenous.

Conclusions

Arg supplementation is still considered controversial in amelioration of radiation-induced acute inflammation because excessive NO production can generate structural disorders by inhibition of smooth muscle cell proliferation and apoptosis promotion. Thus, additional studies of benefits and the potential adverse effects of Arg supplementation in abdominal radiation are necessary.

Gly may immunomodulate the macrophages of Peyer's patches, decrease the direct local intestinal injury and the resultant "cytokine shower", and prevent reactive oxygen species (ROS) formation. Additionally, Gly is part of collagen structure and this can increase the collagen production after irradiation. The use of Gly-rich elementary diet is promising in radiotherapy, but the elucidation of its function in addition to increased protein intake is necessary.

References

1. Stacey R, Green JT. Radiation-induced small bowel disease: latest developments and clinical guidance. Ther Adv Chron Dis. 2014;5:15–29.
2. Al-Mamgani A, Heemsbergen WD, Peeters ST, et al. Role of intensity-modulated radiotherapy in reducing toxicity in dose escalation for localized prostate cancer. Int J Radiat Oncol Biol Phys. 2009;73:685–91.
3. Wang J, Boerma M, Fu Q, et al. Significance of endothelial dysfunction in the pathogenesis of early and delayed radiation enteropathy. World J Gastroenterol. 2007;13:3047–55.
4. Pia de la Maza M, Gotteland M, Ramirez C, et al. Acute nutrition and intestinal changes after pelvic radiation. J Am Coll Nutr. 2001;20:637–42.
5. Breiter N, Trott KR. The pathogenesis of the chronic radiation ulcer of the large bowel in rats. Br J Cancer Suppl. 1986;7:29–30.
6. Denham J, Hauer-Jensen M. The radiotherapeutic injury—a complex 'wound'. Radiother Oncol. 2002;63:129–45.
7. Wong MT, Lim JF, Ho KS, et al. Radiation proctitis: a decade's experience. Singapore Med J. 2010;51(4):315–9.
8. Lange MM, Marijnen CA, Maas CP, et al. Risk factors for sexual dysfunction after rectal cancer treatment. Eur J Cancer. 2009;45:1578–88.
9. Andreyev H. Gastrointestinal symptoms after pelvic radiotherapy: a new understanding to improve management of symptomatic patients. Lancet Oncol. 2007;8:1007–17.
10. Khalid U, McGouch C, Hackett C, et al. A modified inflammatory bowel disease questionnaire and the Vaizey Incontinence questionnaire are more sensitive measures of acute gastrointestinal toxicity during pelvic radiotherapy than RTOG grading. Int J Radiat Oncol Biol Phys. 2006;64:1432–41.
11. de Aguiar Picanço E, Lopes-Paulo F, Marques RG, et al. L-arginine and glycine supplementation in the repair of the irradiated colonic wall of rats. Int J Colorectal Dis. 2011;26:561–8.
12. Rose WC, Haines WJ, Warner DT. The amino acid requirements of man. V. The role of lysine, L-arginine, and tryptophan. J Biol Chem. 1954;206:421–30.
13. Wu G, Bazer FW, Davis TA, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
14. Vissers YL, Dejong CH, Luiking YC, et al. Plasma L-arginine concentrations are reduced in cancer patients: evidence for L-arginine deficiency? Am J Clin Nutr. 2005;81:1142–6.
15. Ersin S, Tuncyurek P, Esassolak M, et al. The prophylactic and therapeutic effects of glutamine- and L-arginine-enriched diets on radiation-induced enteritis in rats. J Surg Res. 2000;89:121–5.
16. Gurbuz AT, Kunzelman J, Ratzer EE. Supplemental dietary L-arginine accelerates intestinal mucosal regeneration and enhances bacterial clearance following radiation enteritis in rats. J Surg Res. 1998;74:149–54.
17. Morris Jr SM. Enzymes of L-arginine metabolism. J Nutr. 2004;134(Suppl):2743S–7S.
18. Barbul A. L-Arginine: biochemistry, physiology and therapeutic implications. J Parenter Enteral Nutr. 1986;10:227–38.
19. Flynn NE, Meininger CJ, Haynes TE, et al. The metabolic basis of L-arginine nutrition and pharmacotherapy. Biomed Pharmacother. 2002;56:427–38.
20. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;15(Pt 1):1–17.
21. Mañe J, Fernández-Bañares F, Ojanguren I, et al. Effect of L-arginine on the course of experimental colitis. Clin Nutr. 2001;20:415–22.

22. Debats IB, Wolfs TG, Gotoh T, et al. Role of L-arginine in superficial wound healing in man. Nitric Oxide. 2009;21:175–83.
23. Hwang JM, Chan DC, Chang TM, et al. Effects of oral L-arginine and glutamine on radiation-induced injury in the rat. J Surg Res. 2003;109:149–54.
24. Bansal V, Ochoa JB. L-Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care. 2003;6:223–8.
25. Morris Jr SM, Billiar TR. New insights into the regulation of inducible nitric oxide synthesis. Am J Physiol. 1994;266(Pt 1):E829–839.
26. Muscará MN, Wallace JL. Nitric oxide. V. Therapeutic potential of nitric oxide donors and inhibitors. Am J Physiol. 1999;276(Pt 1):G1313–1316.
27. Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Crit Care Med. 1996;24:1125–8.
28. Albina JE, Mills CD, Henry Jr WL, et al. Temporal expression of different pathways of L-arginine metabolism in healing wounds. J Immunol. 1990;15:3877–80.
29. Liu XM, Chapman GB, Peyton KJ, et al. Carbon monoxide inhibits apoptosis in vascular smooth muscle cells. Cardiovasc Res. 2002;55:396–405.
30. Petrat F, Boengler K, Schuelz R, et al. Glycine, a simple physiological compound protecting by yet puzzling mechanism(s) against ischaemia-reperfusion injury: current knowledge. Br J Pharmacol. 2012;165:2059–72.
31. Wheeler MD, Ikejema K, Enomoto N, et al. Glycine: a new anti-inflammatory immunonutrient. Cell Mol Life Sci. 1999;56:843–56.
32. Zafra F, Gimenez C. Glycine transporters and synaptic function. IUBMB Life. 2008;60:810–7.
33. Eulenburg V, Armsen W, Betz H, et al. Glycine transporters: essential regulators of neurotransmission. Trends Biochem Sci. 2005;30:325–33.
34. Tibbetts AS, Appling DR. Compartmentalization of Mammalian folate-mediated one-carbon metabolism. Annu Rev Nutr. 2010;30:57–81.
35. Ichiyama A. Studies on a unique organelle localization of a liver enzyme, serine:pyruvate (or alanine:glyoxylate) aminotransferase. Proc Jpn Acad Ser B Phys Biol Sci. 2011;87:274–86.
36. Petrat F, Drowatzky J, Boengler K, et al. Protection from glycine at low doses in ischemia-reperfusion injury of the rat small intestine. Eur Surg Res. 2011;46:180–7.
37. Weinberg JM. Glutathione and glycine in acute renal failure. Ren Fail. 1992;14:311–9.
38. Weinberg JM, Davis JA, Abarzua M, et al. Cytoprotective effects of glycine and glutathione against hypoxic injury to renal tubules. J Clin Invest. 1987;80:1446–54.
39. Lee MA, McCauley RD, Kong SE, et al. Influence of glycine on intestinal ischemia-reperfusion injury. JPEN J Parenter Enteral Nutr. 2002;26:130–5.
40. Rose ML, Cattley RC, Dunn C, et al. Dietary glycine prevents the development of liver tumors caused by the peroxisome proliferator WY-14,643. Carcinogenesis. 1999;20:2075–81.
41. Tsune I, Ikejima K, Hirose M, et al. Dietary glycine prevents chemical-induced experimental colitis in the rat. Gastroenterology. 2003;125:775–85.
42. Xu X, Weksler-Zangen S, Pikarsky A, et al. Mast cells involvement in the inflammation and fibrosis development of the TNBS-induced rat model of colitis. Scand J Gastroenterol. 2002;37:330–7.
43. Ikejima K, Qu W, Stachlewitz RF, et al. Kupffer cells contain a glycine-gated chloride channel. Am J Physiol. 1997;272:G1581–6.
44. Stachlewitz RF, Li X, Smith S, et al. Glycine inhibits growth of T lymphocytes by an IL-2-independent mechanism. J Immunol. 2000;164:176–82.
45. Froh M, Thurman RG, Wheeler MD. Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. Am J Physiol Gastrointest Liver Physiol. 2002;283:G856–63.
46. De Winter BY, van den Wijngaard RM, de Jonge WJ. Intestinal mast cells in gut inflammation and motility disturbances. Biochim Biophys Acta. 1822;2012:66–73.
47. Kletke O, Sergeeva OA, Lorenz P, et al. New insights in endogenous modulation of ligand-gated ion channels: histamine is an inverse agonist at strychnine sensitive glycine receptors. Eur J Pharmacol. 2013;710:59–66.
48. Peskin AV, Midwinter RG, Harwood DT, et al. Chlorine transfer between glycine, taurine, and histamine: reaction rates and impact on cellular reactivity. Free Radic Biol Med. 2005;38:397–405.
49. Kallakuri S, Ascher E, Pagala M, et al. Protective effect of glycine in mesenteric ischemia and reperfusion injury in a rat model. J Vasc Surg. 2003;38:1113–20.
50. Diestel CF, Marques RG, Lopes-Paulo F, et al. Role of L-glutamine and glycine supplementation on irradiated colonic wall. Int J Colorectal Dis. 2007;22:1523–9.

Chapter 43
L-Arginine in Cancer Therapy

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Key Points

- L-Arginine is a nonessential amino acid in adult humans.
- Certain tumors lack the ability to synthetize L-arginine from citrulline and may be sensitive to L-arginine depletion therapy.
- Arginase and L-arginine deiminase, two enzymes used to deplete L-arginine, have shown antitumor activity in vitro and in vivo.
- Preliminary clinical trials in patients using either L-arginine deiminase or arginase have shown responses in a number of tumor types.
- While the drugs have been well tolerated, potential problems and pitfalls include drug resistance due to antibody formation and/or induction of argininosuccinate synthetase (L-arginine deiminase) or risk of normal tissue toxicity due to the lack of ornithine transcarbamylase (arginase).
- Combination drug therapy adding other anticancer agents to L-arginine-depleting treatment offers promise in potentiating antitumor response.

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Keywords L-Arginine • ADI-PEG20 • Argininosuccinate synthetase • Melanoma • Hepatocellular carcinoma

Abbreviations

ADI L-Arginine deiminase
Arg Arginase
ASS Argininosuccinate synthetase
OTC Transcarbamylase
PEG Pegylated

Introduction

One method of treating certain cancers is by amino acid depletion. The best example of this is asparaginase, which has been used to lower blood levels of asparagine, a nonessential amino acid in humans. Asparaginase has been used for years to treat acute lymphoblastic leukemia, the most common form of leukemia in children and young adults. Since most human body cells do not require asparagine for their growth and survival, whereas the leukemic cells are auxotrophic for this amino acid, this represents a targeted approach to treatment.

L-Arginine is another nonessential amino acid in adult humans. It is synthesized from citrulline via two key enzymes, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). Citrulline is converted to argininosuccinate by ASS, and argininosuccinate in turn is converted into L-arginine by ASL (Fig. 43.1). Tumor cells that do not express ASS may be sensitive to L-arginine depletion such as with the L-arginine-depleting enzyme, L-arginine deiminase. Alternatively, L-arginine depletion can occur with arginase. This chapter will review the potential use of L-arginine depletion as a method to treat various malignancies.

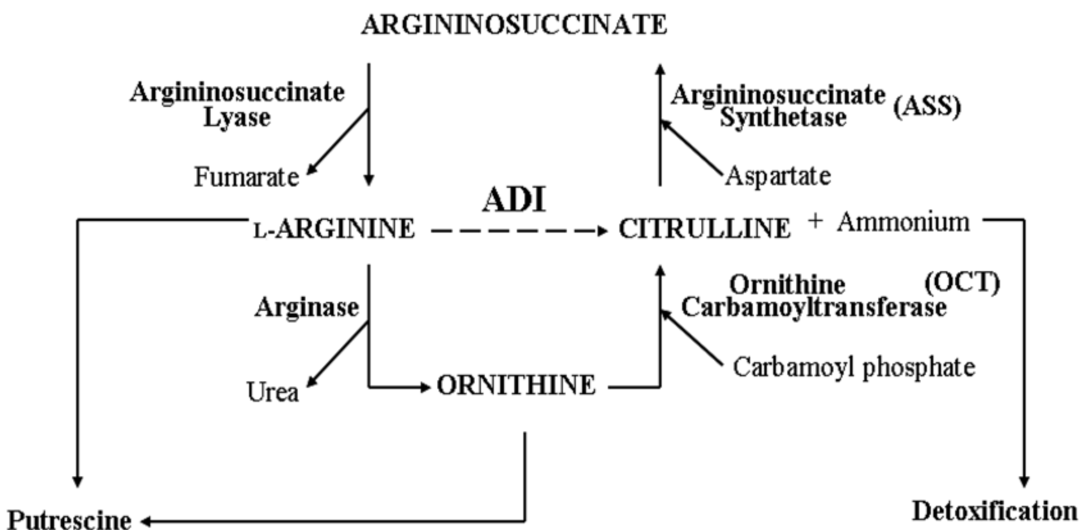

Fig. 43.1 Enzymes involved in the urea cycle

Preclinical Studies

In the 1960s several investigators noted that a number of cell lines could be inhibited by the addition of arginase [1–4]. Subsequently, it was found that irreversible cell destruction was related to the level of L-arginine depleted by arginase [5]. Other investigators found that L-arginine depletion in cell lines with L-arginine deiminase by *Mycoplasma arginini* also produced antitumor activity [6, 7].

In vivo L-arginine depletion was studied with arginase and pegylated arginase [5, 8]. No obvious antitumor activity was observed in either study. The lack of efficacy has been attributed to its physical characteristics including low substrate affinity, short plasma half-life, and low enzymatic activity at normal physiological pH. More recently, recombinant human arginase was shown to inhibit the in vitro and in vivo proliferation of human melanomas [9]. There was dual-phase cell cycle arrest in the S and G2/M phase in vitro and inhibition of growth of melanoma xenografts in vivo.

In another study, cobalt was substituted for manganese metal cofactor in human arginase [10]. This appears to confer more than tenfold higher catalytic activity and fivefold greater stability. Weekly treatment of 8 mg/kg Co-hArgl-PEG had activity against human HepG2 (HCC) and Panc-1 pancreatic cell tumor xenografts. Both cell lines underwent apoptosis in vitro and there was increased expression of activated caspases with evidence of autophagy.

L-Arginine deiminase was also pegylated (ADI-PEG20) and studied in vivo. ADI-PEG20 has shown antitumor activity against human melanomas and hepatocellular carcinomas (HCC) implanted in mice. ADI-PEG20 inhibited the growth of SK-mel2 and SK-mel28 which was implanted in athymic mice. The drug also inhibited the growth of human HCC implanted into severe combined immune-deficient mice with prolongation of survival.

ADI-PEG20 was studied in dogs with oral melanoma. Unlike humans, dogs require L-arginine in their diet so the dog subjects were given sufficient dosage to produce only a partial depletion of L-arginine. In a study conducted at the University of Pennsylvania, ten dogs with oral melanoma were treated with ADI-PEG20. After 2 months the dogs were evaluated for response. Of the ten dogs, three had a partial response, one had stable disease, and two had a complete response.

Arginase

Clinical Trials

In one study, patients with unresectable hepatocellular carcinoma were treated with transhepatic arterial embolization of the liver tumors with lipiodol and gel foam to attempt to induce leakage of hepatic arginase from the liver into the circulation [11]. High-dose insulin was administered to produce a state of hypoaminoacidemia to augment L-arginine depletion. Seven patients with advanced or metastatic hepatocellular carcinoma were treated. Five patients achieved depletion of L-arginine (range 0–20 μM, normal plasma level 100–120 μM). All five patients showed some evidence of tumor response with remission in the primary liver tumor and extrahepatic sites (lymph nodes, lungs, and bones). This suggests that there was a systemic effect from L-arginine depletion and not just a liver response to the embolization. The two nonresponders did not have major reduction in their plasma L-arginine levels. Based on their experience, it was suggested that arginase may be a good drug candidate to treat HCC.

A phase I trial of pegylated recombinant human arginase (peg-rhArg1) was recently reported [12]. In this trial an initial single intravenous (IV) bolus was followed by weekly doses from 500 to 2500 U/kg. Fifteen patients with advanced hepatocellular carcinoma and measurable disease were enrolled. The most common adverse effects were diarrhea, abdominal discomfort, and nausea. The maximum

tolerated dose was 2500 U/kg. Adequate L-arginine depletion was achieved with doses from 1600 to 2500 U/kg and the optimal biological dose was considered to be 1600 U/kg. The best response was stable disease for >8 weeks in 26.7 % of patients.

L-Arginine Deiminase

The tumors that are most likely to respond to L-arginine depletion are those that lack the ASS enzyme. Table 43.1 shows a partial list of human malignancies that can be inhibited by L-arginine deiminase. The incidence of ASS deficiency varied significantly among the different tumor types with melanoma, hepatocellular carcinoma, and prostate carcinoma having the highest incidence of negative ASS expression [13]. Also, renal cell and sarcoma cancer and mesothelioma cancers showed ASS deficiency [14]. Hence, clinical trial have focused on these cancers are ASS deficient.

Hepatocellular Carcinoma

A patient with hepatocellular carcinoma was treated with ADI-PEG20 as a single patient exemption and showed response in tumor size and serum alpha fetoprotein levels [15]. This encouraging report led to a Phase I–II trial of ADI-PEG20 in hepatocellular carcinoma [16]. Patients with unresectable hepatocellular carcinoma were treated at the Pascale Cancer Institute in Naples, Italy. In this part of the study, the weekly intramuscular dose of 160 IU/m^2 was considered to be the optimum biological dose that lowered the plasma L-arginine level to undetectable levels (<2 μM/l) for >7 days. Nineteen patients were accrued onto the study. Fifteen completed all cycles of treatment and two patients stopped due to progressive disease and two due to complication of cirrhosis. The side effects consisted of pain at injection site, elevation of serum uric acid and fibrinogen, and rarely elevation of serum lipase and amylase levels. The response to ADI-PEG20 included two patients (10.5 %) with complete response, seven (36.8 %) with partial response and seven with stable disease and three (15.9 %) with progressive disease. The mean duration of response measured from the date of first treatment was >400 days (range 37–680 days).

The University of Miami experience with ADI-PEG20 in hepatocellular carcinoma is shown in Table 43.2. The drug was well tolerated. Three of nine patients had stable disease, with two patients having prolonged time to tumor progression for 17 and 28 (+) months.

Another phase I/II trial of ADI-PEG20 was done at the MD Anderson Cancer Center using a similar weekly intramuscular schedule [17]. The phase I part consisted of increasing weekly dose up to 160 IU/m^2 which was considered the optimal biologic dose. The phase II part of the study consisted of doses from 160 to 240 IU/m^2 weekly. Thirty-five patients were enrolled onto the clinical trial. Only 1 patient had a partial response and 16 patients had stable disease. Four patients did not complete the

Table 43.1 Malignancies inhibited by L-arginine deiminase in tumor cell culture

T-lymphoma Jurkat	B-cell lymphoma Raji
T-cell leukemia TL-MOR	B-cell lymphoma Manaca
T-cell leukemia MT-2	Histiocytic lymphoma U937
T-leukemia	Myeloid leukemia
T-lymphoblastic leukemia CCRF-CEM	Melanoma
T-lymphoblastoid	Hepatocellular carcinoma
Lymphoblastic leukemia	Mesothelioma
Prostate cancer	Renal cell cancer
Retinoblastoma	Sarcoma

Table 43.2 ADI-PEG20 in hepatocellular carcinoma (HCC)—University of Miami experience

- Nine patients with unresectable HCC were treated with ADI-PEG20
- Median age: 72 prior chemotherapy: 6/9
- Toxicity: mild fatigue, discomfort at injection site. No grade 3 or 4 toxicities noted
- 3 patients (33 %) had prolonged time to tumor progression of 4(+), 17, and 28(+) months
- ADI-PEG20 induced autophagy in cell lines which possess low levels of ASS expression

study due to either allergic reaction to the drug or intercurrent disease. Twenty-eight patients had progressive disease. The mean time to tumor progression was 3.4 months (range 1–13 months). Toxicity consisted of liver function or electrolyte abnormality. Of note, three patients had grade 4 toxicity, consisting of liver function abnormalities in two patients and elevation of serum lipase in one patient. Thus, this trial showed more toxic side effects, particularly hepatic dysfunction and lower response rate than the previous trial. This may be due to patient selection and emphasizes the need to select better performance patients.

A randomized phase II study of ADI-PEG20 was performed in Asian advanced hepatocellular carcinoma patients [18]. Patients were randomized between weekly IM injections of ADI-PEG20 at 160 IU/m^2 versus 320 IU/m^2. Of the 71 accrued patients, 43.6 % had failed prior to systemic therapy. Overall there was no major difference in the disease-control rate and median overall survival between the two arms. Of note, the median overall survival of patients with undetectable circulating L-arginine for more than or equal to and <4 weeks was 10.0 and 5.8 months, respectively ($P=0.25$), log-rank test). The major treatment-related side effects were grades 1–2 local and/or allergic reactions. The authors conclude that ADI-PEG 20 was safe and efficacious in stabilizing the progression of heavily pretreated advanced HCC in an Asian population.

Currently, there is an ongoing multicenter, randomized, placebo-controlled phase III trial of ADi-PEG20 in unresectable hepatocellular carcinoma. This trial allows prior therapy including sorafenib. The endpoints will be overall survival and time to tumor progression. The results of this trial are eagerly awaited. Importantly, tumor tissue analysis for ASS is not required for eligibility requirement, but if available will be studied for correlation to survival. Thus, including patients whose tumor are ASS positive may impact the results of this trial.

Mesothelioma

In mesothelioma, a randomized phase II trial of ADI-PEG20 and best supportive care versus best supportive care alone in patients whose tumor lack ASS has been ongoing. In one report of a single patient who received ADI-PEG20, the repeat FDG-PET scan showed a partial metabolic response with a 40 % reduction in the maximum standardized uptake value [19]. By recist criteria, the patient had stable disease. His tumor was ASS negative secondary to ASS*1* promoter methylation as confirmed by methylation-specific polymerase chain reaction. The patient later progressed and this appeared to correlate with rise in antibody titer to ADI-PEG20 with subsequent rise of serum L-arginine levels to pretreatment values. It was suggested that imaging by FDG-PET scan may be a sensitive indicator of early response to ADI-PEG in ASS-negative patients. Also, in this patient tumor resistance appears to correlate with neutralizing antibody to ADI-PEG20 rather than induction of ASS expression. The final

results of the phase II trial are eagerly awaited as mesothelioma is usually ASS negative and ASS is not often induced and there are few treatment options available for the treatment of unresectable disease. Preliminary analysis suggests a survival advantage for ADI-PEG20 over best supportive care (Szlosarek, personal communication).

Melanoma

ADI-PEG**20** has been evaluated in a phase I–II clinical trial for melanoma [20]. The phase I part of the trial was performed in the USA and in Italy. In the USA, the phase I part enrolled **15** patients. Treatment consisted of weekly IM injection of ADI-PEG**20** in doses ranging from **20** to **160** U/m². In the Italian phase I trial, weekly injections were given in doses from **40** to **640** U/m². The optimal biologic dose (OBD) that reduced plasma levels of ADI-PEG**20** to nondetectable levels (<**2** μM/l) for ≥**7** days was considered. None of the **15** patients enrolled in phase I part of the protocol in the USA had a response to treatment. In the Italian phase I/II trial, there were responders who had received the OBD dose or higher. Altogether, six patients had a response, including one complete response and five partial responses for an overall response rate of **25** %. Six patients had stable disease for **3** months or longer. The drug was generally well tolerated. The most common side effect was discomfort at the injection site. Uncommon side effects include hypotension, elevation of serum uric acid, mild increases in fibrinogen level, and non-symptomatic, mild increase in serum lipase and amylase levels. The maximum tolerated dose was not reached and patients received up to **640** IU/m² I.M. weekly. Since it was difficult to administer doses beyond **640** IU/m due to the amount of fluid that had to be injected, this was consider the "maximum tolerated dose."

A phase II trial in melanoma was performed which was the first to correlate negative argininosuccinate synthetase expression in tumor tissue with clinical benefit in patients receiving L-arginine-depleting therapy [21]. In this study, 27 of 38 patients treated with ADI-PEG20 had melanoma tumors assessable for ASS staining before treatment. Clinical benefit rate (CBR) and longer time to progression were associated with negative expression of tumor ASS. In this trial, only one of ten patients with ASS-positive tumors had stable disease (Table 43.3), whereas 4 of 17 patients (24 %) had partial response and 5 had stable disease, when ASS expression was negative, giving a CBR rate of 52.9 % vs. 10 % ($P=0.041$). Interestingly, two responding patients with negative ASS expression before therapy had re-biopsy after tumor progression and the ASS expression became positive. The survival of ASS-negative patients receiving at least four doses at 320 IU/m² was significantly better than the ASS-positive patients at 26.5 vs. 8.5 months, respectively ($P=0.024$). The serious side effects were uncommon, the most common side effect being discomfort at the injection site. Other side effects include grade 3 allergic reaction, grade 4 neutropenia and thrombocytopenia (one patient), grade 3 neutropenia (three patients), grade 3 anemia (one patient), and grade 3 fatigue (two patients). Four of the five patients who experienced myelosuppression had prior radiation therapy or combined radiation therapy and chemotherapy. It is important to note that only responders have received doses of 320 IU/m²/week. Of ten cutaneous melanoma patients with ASS negative tumors who received 320 IU/m², four (40 %) had partial response and three had stable disease with the CBR of 70 %. In contrast, of seven patients who were ASS negative and had doses <320 IU/m². No partial responses were observed and only two had stable disease/minor response, $P=0.08$ (Fisher's exact test). It is noteworthy that

Table 43.3 ADI-PEG20 in melanoma—University of Miami experience

ASS status	Number of patients	Response	Stable disease
ASS (−)	17	4	5
ASS (+)	10	0	1

ASS argininosuccinate synthetase

two patients who had an initial response at 320 IU/m^2 per week schedule and later progressed were treated at a higher dose and had a second response. Therefore, the data indicate that a higher dose of ADI-PEG20 may be needed to keep L-arginine levels low for response to occur.

Another phase I/II trial of ADI-PEG20 was performed in 31 previously treated melanoma patients [22]. The dose ranged from 40 to 160 IU/m^2 I.M. weekly. The drug was generally well tolerated. The main toxicities were grade 1 and grade 2 side effects, consisting mainly of discomfort at the injection site, rash, and fatigue. No major objectives responses were observed in this trial. Nine patients had stable disease and two of these were >6 months duration. Of the nine stable patients, four had uveal melanoma. ASS expression was negative in 24 patients by immunohistochemical staining. The stable disease rate in uveal melanoma was felt to be encouraging. In this trial, it is important to note that the dose of ADI-PEG20 never exceeded 160 IU/m^2, unlike the previous trial in which the dose of 320 IU/m^2 correlated with response.

Other Malignancies

As of March 21, 2014, clinicaltrials.gov lists several ongoing trials with ADI-PEG20 in various malignancies. These include non-Hodgkins's lymphoma subjects who have failed prior systemic therapy, relapsed/refractory acute myeloid leukemia, and relapsed sensitive or refractory small cell lung cancer and pediatric subjects who are ASS negative. Another trial is ADI-PEG20 with docetaxel in solid tumors with emphasis on prostate cancer and non-small cell lung cancer. Trials that are not yet recruiting include phase I study in patients with tumors requiring L-arginine to assess ADI-PEG20 with pemetrexed and cisplatin, phase II trial of ADI-PEG20 plus concurrent transarterial chemoembolization (TACE) vs. TACE alone in patients with hepatocellular carcinoma, and a phase I trial of ADI-PEG20 plus doxorubicin in patients with HER2-negative metastatic breast cancer. Finally, a phase I trial of ADI-PEG20 combined with cisplatin is recruiting patients with malignant melanoma.

Review of Pitfalls, Problems, and Promise

There are several important potential pitfalls or problems to consider regarding the use of L-arginine-depleting therapy for the treatment of cancer (Table 43.4). For arginase therapy there is a potential for increased toxicity in normal tissues due to lack of the urea cycle enzyme ornithine transcarbamylase (OTC) to rescue L-arginine from ornithine which is the product of arginase. Since the conversion from ornithine to L-arginine requires the presence of OTC and OTC is low in many normal tissue cultures, these normal tissue cells will be susceptible to toxicity from arginase [23]. The human recombinant arginase I cobalt coupled to polyethylene glycol 5000 showed potent in vitro cytotoxicity in many cancer cell lines and hepatocellular carcinoma and pancreatic carcinoma xenografts, but the therapeutic index was narrow.

Table 43.4 Potential pitfalls or problems with L-arginine-depleting therapy

Drug	Potential problems
Arginase	– Activity at physiologic pH – Possible damage to tissues lacking OTC
L-Arginine deiminase	– Immunogenicity – Induction of ASS

OTC ornithine transcarbamylase

Toxicity was observed in normal cells in tissue culture and tissue injury occurred in vivo at doses twice the effective dose. The addition of L-citrulline supplementation was able to rescue most normal cells with selective toxicity to ASS-deficient tumors [24]. Thus, clinical trials with arginase will require L-citrulline supplementation to reduce toxicity and improve the therapeutic index. Table 43.5 lists other potential ways to either reduce toxicity or potentiate activity with L-arginine-depleting therapy.

For therapy with L-arginine deiminase, there are at least two major issues: immunogenicity and induction of ASS. Both of these can lead to drug resistance to this type of L-arginine-depleting therapy. The induction of antibodies to L-arginine deiminase, which is recognized by the human body as a foreign protein, can lead to tumor progression after initial response [19]. It can also lead to possible allergic or anaphylactoid reactions. One method to try to reduce immunogenicity is the use of nanoparticles [25, 26]. Another method is the addition of other agents at the same time. Combination therapy, such as the addition of chemotherapy, may potentially impair antibody formation and thus allow time for the depletion of L-arginine to be effective in promoting tumor cell death. Such combination trials are currently under way, and it would be interesting to correlate tumor response to the antibody levels and blood L-arginine and citrulline levels.

Tumor cells under metabolic stress may undergo autophagy as a survival mechanism. In this respect, we and others have shown that L-arginine deprivation led to autophagy as a mechanism to evade apoptosis [27, 28]. During this period, the cells can turn on ASS transcription to synthesize L-arginine. One method to enhance the cytotoxicity of ADI-PEG20 is the addition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). By combining TRAIL with ADI-PEG20, enhanced apoptosis and accelerated cell death in melanoma cell lines could be achieved [29]. In this study TRAIL could induce the cleavage of two key autophagic proteins, Beclin-1 and Atg5, in the combination treatment compared to ADI-PEG20 used alone. The data indicated that cleavage of Beclin-1 and Atg5 by TRAIL-initiated caspase activation is one of the mechanisms that led to the enhanced cytotoxicity in the combination treatment.

Conversely, L-arginine depletion may potentiate the antitumor effect of DNA damaging agents such as cisplatin, temozolomide, and anti-microtubule agent taxotere in melanoma cells [30]. The addition of ADI-PEG20 is able to sensitize melanoma cells to all three agents, more so with cisplatin and temozolomide than taxotere. Limited array analysis using the cDNA which relates to DNA damage indicates that the addition of ADI-PEG20 decreased ERCC and RAD family which are needed to repair DNA after exposure to cisplatin. The potentiation effect may be related to impairment of DNA repair.

L-Arginine depletion may especially be effective in melanoma cells that have become resistant to BRAF inhibition. The BRAF inhibitors such as vemurafenib and dabrafenib alone, or with MEK inhibitor, have shown to have a high response rate (approximately 60 %) in patients with BRAF-mutated melanomas. Unfortunately, the response duration is only around 8 months, and virtually all patients become resistant to BRAF inhibitors.

Table 43.5 Possible methods to potentiate L-arginine-depleting therapy

Drug	Method
L-Arginine deiminase	– Target BRAF melanomas which become resistant to BRAF inhibitors
	– Nanoparticles to reduce immunogenicity
	– Combine with TRAIL to enhance apoptosis
	– Combine with chemotherapy such as cisplatin which may impair antibody formation
Arginase	– Supplement with citrulline to reduce toxicity
	– Replace manganese with cobalt to increase enzyme activity and add polyethylene glycol to improve stability

Recent data demonstrate that BRAF inhibitor (vemurafenib) resistance confers sensitivity to L-arginine deprivation in melanoma [31]. These cells became ASS non-inducible most likely due to c-Myc degradation. Studies have shown that c-Myc is a positive regulator in ASS transcription and that c-Myc stability is governed by ubiquitination [32]. BRAF inhibitor-resistant cells have lower levels of deubiquitinating enzyme USP28 and therefore cannot prevent c-Myc from ubiquitin-dependent proteasomal degradation. Hence, BRAF inhibitor resistance will suppress c-Myc-mediated ASS transcription and this in turn increases sensitivity to L-arginine depletion by ADI-PEG20. Whether suppressing c-Myc will have other effect or not is not known and is currently under investigation.

Clinical trials with ADI-PEG20 to target such patients will be needed to confirm these preclinical observations. Laboratory studies are currently under way to investigate the mechanism behind this phenomenon. It appears that once melanoma cells develop resistance to BRAF inhibition, they are not able to handle the metabolic stress with L-arginine depletion and thus undergo apoptosis.

Conclusions

Targeting tumor metabolism using L-arginine-depleting therapy is a promising approach to the treatment of certain malignancies which depend on exogenous L-arginine for growth and survival. A number of clinical trials have shown that L-arginine deprivation therapy is generally well tolerated. These clinical trials have shown activity in several malignancies including malignant melanoma, hepatocellular carcinoma, and mesothelioma. A randomized phase III trial is currently under way in hepatocellular carcinoma. Treatment of other tumor types is also being explored and combination therapy adding other anticancer agents to L-arginine-depleting therapy offers promise. Further trials will be needed to better define the overall clinical benefit from this type of targeted therapy.

References

1. Lieberman I, Ove P. Biochim Biophys Acta. 1960;38:153.
2. Freed JJ, Sorof S. Biochem Biophys Res Commun. 1966;22(1):1.
3. Freed JJ, Schatz SA. Exp Cell Res. 1969;55(3):393.
4. Holley RW. Biochim Biophys Acta. 1967;145(2):525.
5. Storr JM, Burton AF. Br J Cancer. 1974;30(1):50.
6. Takaku H, Takase M, Abe S, et al. Int J Cancer. 1992;51(2):244.
7. Sugimura K, Fukuda S, Wada Y, et al. Infect Immun. 1990;58(8):2510.
8. Savoca KV, Davis FF, van Es T, et al. Cancer Biochem Biophys. 1984;7(3):261.
9. Lam TL, Wong GK, Chow HY, et al. Pigment Cell Melanoma Res. 2011;24(2):366.
10. Glazer ES, Stone EM, Zhu C, et al. Transl Oncol. 2011;4(3):138.
11. Cheng PN, Leung YC, Lo WH, et al. Cancer Lett. 2005;224(1):67.
12. Yau T, Cheng PN, Chan P, et al. Invest New Drugs. 2013;31(1):99.
13. Dillon BJ, Prieto VG, Curley SA, et al. Cancer. 2004;100(4):826.
14. Tang SW, Chang WH, Chao YW, et al. J Biomed Sci. 2006;13(2):233.
15. Curley SA, Bomalaski JS, Ensor CM, et al. Hepato-gastroenterology. 2003;50(53):1214.
16. Izzo F, Marra P, Beneduce G, et al. J Clin Oncol. 2004;22(10):1815.
17. Delman KA, Brown TD, Thomas M, et al. J Clin Oncol ASCO Annu Meet Proc. 2005;23:16S.
18. Yang TS, Lu SN, Chao Y, et al. Br J Cancer. 2010;103(7):954.
19. Szlosarek PW, Luong P, Phillips MM, et al. J Clin Oncol. 2013;31(7):e111.
20. Ascierto PA, Scala S, Castello G, et al. J Clin Oncol. 2005;23(30):7660.
21. Feun LG, Marini A, Walker G, et al. Br J Cancer. 2012;106(9):1481.
22. Ott PA, Carvajal RD, Pandit-Taskar N, et al. Invest New Drugs. 2013;31(2):425.
23. Mauldin JP, Zeinali I, Kleypas K, et al. Transl Oncol. 2012;5(1):26.
24. Agrawal V, Woo JH, Mauldin JP, et al. Anticancer Drugs. 2012;23(1):51.

25. Groneberg DA, Giersig M, Welte T, et al. Curr Drug Targets. 2006;7(6):643.
26. Farokhzad OC, Langer R. Adv Drug Deliv Rev. 2006;58(14):1456.
27. Savaraj N, You M, Wu C, et al. Curr Mol Med. 2010;10(4):405.
28. Kim RH, Bold RJ, Kung HJ. Autophagy. 2009;5(4):567.
29. You M, Savaraj N, Kuo MT, et al. Mol Cell Biochem. 2013;374(1-2):181.
30. Feun LG, Wu C, Lee S, et al. Proc Am Assoc Cancer Res Los Angeles CA USA. 2007;48:4042.
31. Li YY, Wu CW, Chen SM, et al. Proc Am Assoc Cancer Res San Diego CA USA. 2014;55:892.
32. Tsai WB, Aiba I, Lee SY, et al. Mol Cancer Ther. 2009;8(12):3223.

Chapter 44
Mechanisms of L-Arginine-Auxotrophic Response and Their Cancer Therapeutic Implications

Wen-Bin Tsai, Yan Long, Niramol Savaraj, Lynn G. Feun, and Macus Tien Kuo

Key Points

- Many human malignancies do not express argininosuccinate synthetase 1 (ASS1), the key enzyme for the biosynthesis of L-arginine. These tumors acquire L-arginine from the circulation for survival. L-Arginine deprivation strategies have been undergoing clinical trials for treating L-arginine-auxotrophic tumors.
- Important mechanism of L-arginine-auxotrophic response includes downregulation of HIF-1α to de-repress ASS1 expression and upregulation of c-Myc to induce ASS1 expression.
- Upregulation of c-Myc under L-arginine-auxotrophic conditions is mediated by the activation of the Ras/PI3K/Akt/ERK growth signal pathway.
- Upregulated c-Myc plays important roles in metabolic reprogramming in L-arginine-auxotrophic response.
- Epigenetic control by DNA methylation is also involved in ASS1 silencing.

Keywords L-Arginine auxotrophy • ADI-PEG20 • c-Myc • HIF-1α • PI3K/Akt/ERK • Argininosuccinate synthetase

Abbreviations

ADI	L-Arginine deiminase
ALL	Acute lymphocytic leukemia
Arg	L-Arginine

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AS	Argininosuccinate
ASL	Argininosuccinate lyase
Asn	Asparagine
ASNase	Asparaginase
ASNS	Asparagine synthetase
ASS1	Argininosuccinate synthetase 1

Introduction

L-arginine (Arg) is a semi-essential amino acid. Because there is no specific storage system for the cellular L-Arg pool, Arg needs to be de novo synthesized or directly acquired from an extracellular source to meet physiological need. Different organs have different requirements of Arg. For example, sufficient Arg is synthesized in the liver and kidney of an adult but not sufficient to the growing child [1]. Arg is essential for fetuses and neonates.

Arg is an important metabolite in the urea cycle (Fig. 44.1). Two major enzymes in this cycle are involved in the biosynthesis of Arg: argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). ASS catalyzes the biosynthesis of argininosuccinate (AS) from citrulline and aspartate. ASS deficiency causes citrullinemia, a rare autosomal recessive disease [2] associated with low Arg and high citrulline in the plasma. ASL catalyzes the conversion of AS to Arg and fumarate which is an important metabolite of the Krebs cycle. ASL deficiency causes argininosuccinic aciduria, another inborn error of metabolism [3].

Malignant cells require sufficient amounts of essential (indispensable) or semi-essential (conditionally indispensable) amino acids for protein biosynthesis to support their highly proliferative

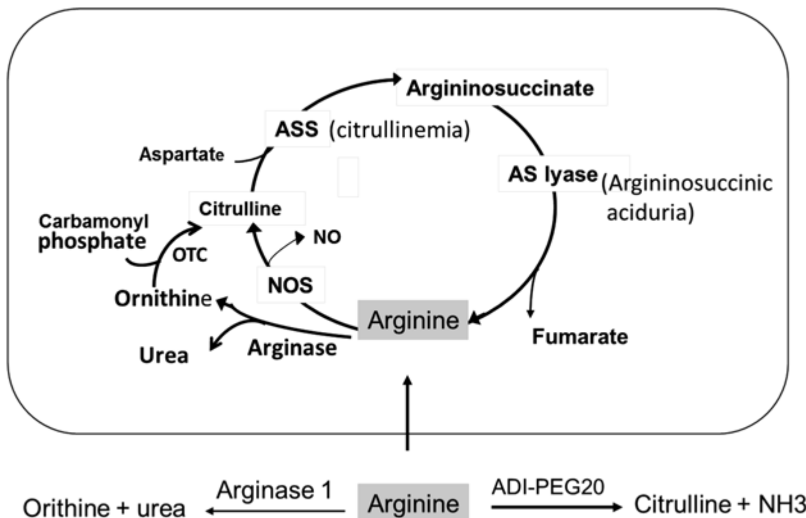

Fig. 44.1 L-Arginine is an important metabolite in the urea cycle. L-Arginine can be synthesized endogenously by two key enzymes, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). Metabolic defects of these enzymes give rise to citrullinemia and argininosuccinic aciduria. Cells can also obtain L-arginine from extracellular source. Deprivation of extracellular L-arginine by ADI-PEG20 or by arginase 1 induces Arg-auxotrophic response in ASS-negative cells

activities. Tumors that do not synthesize sufficient amounts of these amino acids resulting from metabolic defects acquire amino acids from extracellular sources for survival. Depleting extracellular amino acid supplies often leads to cell death. This amino acid-auxotrophic property has led to the development of targeted cancer therapy. Childhood acute lymphocytic leukemia (ALL) acquires asparagine (Asn) from extracellular sources because this tumor expresses very low amount of asparagine synthetase (ASNS), the rate-limiting enzyme for the biosynthesis of Asn from aspartate [4]. This tumor is very sensitive to recombinant L-asparaginase (ASNase) treatment, which hydrolyzes Asn in the circulation. Recombinant ASNases (Elspar and Oncaspar) have been used as single agent for treating childhood ALL for more than 40 years with 20–68 % response rates [5].

The majority of human solid tumors, including melanoma, hepatocellular carcinoma, and prostate cancer [6], bladder cancer [7], and small cell lung cancer [8], require Arg from the circulation for survival because these tumors express reduced amount of ASS1. Tumors with negative ASS1 expression are associated with aggressive phenotypes and exhibited poor disease-free and metastasis-free survival prognosis, which suggests that ASS1 may have a tumor-suppressive property [7, 9] .

The requirement of extracellular Arg supply for tumor growth in these solid tumors provides an opportunity for targeted therapy of these Arg-auxotrophic tumors. Pegylated recombinant L-arginine deiminase (ADI-PEG20) which digests Arg into citrulline and ammonia has been under clinical investigations for treating various lineages of Arg-auxotrophic tumors [10–13]. Another Arg-depleting recombinant protein, human arginase 1, which digests L-arginine into ornithine and urea in the urea cycle (Fig. 44.1), has also been developed for treating Arg-auxotrophic tumors [14–17].

Deprivation of extracellular Arg from ASS1-negative cells induces a wide spectrum of stresses leading to cell death. Cells have developed diverse survival mechanisms to deal with these stresses, which we collectively call Arg-auxotrophic response. Although the entire spectrum of response mechanisms remains to be elucidated in detail, in this chapter, we focus on the early events which occur within minutes of Arg-auxotrophic response and result in the re-expression of ASS1 several hours later. We also discuss the late events involving adaptive response, including metabolic reprograming. We believe that a better understanding of the molecular bases of Arg-auxotrophic response has important implications for developing effective therapeutic strategies for targeting Arg-auxotrophic cancers.

DNA Methylation as an Epigenetic ASS1 Silencing Mechanism

It has been well documented that DNA methylation is an important epigenetic regulation mechanism of gene silencing in eukaryotes. DNA methylation, particularly at CpG sites located around promoter regions, is often associated with inactive genes in eukaryotic genomes. Previous studies have demonstrated that methylation of CpG sites located at −373 and +7 base pairs in reference to the transcription start site (TSS) of *ASS1* was found to associate with *ASS1* silencing in many cancer cell lines and in human tumor biopsy specimens [18, 19]. In two lymphoid cell lines (Myla and SeAx cells) treatment with the demethylating agent 5-Aza-2′-deoxycytidine (Aza-dC), which induces partial demethylation of a 143-bp fragment located −265 bp downstream of the TSS of *ASS1* resulted in re-expression of ASS1 [18]. These studies suggest that DNA methylation is associated with ASS1 silencing. In the same study, cells treated with ADI-PEG20 (0.5 μg/ml, 4 days) showed moderately increased ASS1 mRNA levels as compared with that of cells treated with Aza-dC. However, whether ADI-PEG20-induced ASS1 expression is mediated by demethylation of the *ASS1* locus remains to be investigated.

Transcriptional Regulation of ASS1 Expression in Response to L-Arginine-Auxotrophic Stress

Arg auxotrophy-induced transcriptional regulation of *ASS1* expression either by treating with ADI-PEG20 or by culturing cells under Arg-free medium was investigated in melanoma cells (A2058, SK-MEL-2, and A375 lines) and in breast cancer cells (MDA-MB-231 line). This study was initiated by a transient transfection assay using *ASS1* promoter sequence linked to a luciferase reporter. This study revealed that sequence between −87 and −37 from the TSS of *ASS1* is involved in ADI-PEG20-induced *ASS1* upregulation [20]. Further analysis revealed that an E-box (5′-CACGTG) and GC-box sequences are important for the *ASS1* induction as determined by the site-directed mutagenesis approach. The E-box is recognized by two transcriptional regulators, c-Myc and HIF-1α, and GC-box is recognized by Sp family. Chromatin immunoprecipitation assays demonstrated that under non-stressed conditions the E-box is occupied by HIF-1α. Upon ADI-PEG20 exposure, HIF-1α is rapidly downregulated within minutes but c-Myc expression is upregulated. The upregulated c-Myc then binds to the E-box of the *ASS1* promoter and turns on the expression of *ASS1*. In the presence of $CoCl_2$, a hypoxia mimic which stabilizes HIF-1α and protects it from VHL-mediated proteasomal degradation machinery, resulting in the suppression of ADI-PEG20-induction. These results demonstrated that HIF-1α is a transcriptional repressor that silences *ASS1* expression in many Arg-auxotrophic cancer cells.

Transcriptional induction of *ASS1* expression in response to Arg-auxotrophic stress is mediated by a switch of promoter interactions from HIF-1α to c-Myc which functions as a positive transcription factor. Sp4 was found to be constitutively bound to the GC box located at the *ASS1* promoter for its expression. However, Sp4 expression is not sensitive by Arg-auxotrophic stress (Fig. 44.2).

Signal Transduction Mechanism Associated with L-Arginine-Auxotrophic Stress

c-Myc is an unstable protein. Under non-stressed conditions, the half-life (t½) of c-Myc is **20–30** min [**21**]. In the presence of ADI-PEG**20**, c-Myc is stabilized. Stabilization of c-Myc by ADI-PEG**20** is mediated by the inhibition of ubiquitin-mediated protein degradation machinery [**22**]. Although it has been reported that at least eight protein factors (Fbw**7**, USP**28**, Fbx**29**, Skp**2**, β-TRCP, Truss, Hect-**19**, and Trim**32**) control c-Myc stability through ubiquitination [**23**], we found that FbW**7**α and USP**28** are involved in ADI-PEG**20**-induced c-Myc protein accumulation. FbW**7**α is a subunit of the E**3** ubiquitin ligase complex SCF^{Fbw}**7** that recognizes c-Myc in response to specific stimuli which lead to ubiquitination and subsequent proteasome degradation [**24**]. In contrast, USP**28** forms a complex with Fbw**7**α and counteracts the degradation of c-Myc by removing ubiquitin conjugated by Fbw**7**α[**25**]. Our results demonstrated that ADI-PEG**20** treatment enhanced the formation of the USP**28**, c-Myc, and Fbw**7**α complex, thereby preventing from Fbw**7**α-mediated degradation [**22**].

The signal transduction targeting c-Myc protein for ubiquitin proteasomal degradation is mediated by phosphorylation at two specific amino acid residues at the N-terminus, serine 62 (S62) and threonine 58 (T58). S62 is targeted by ERK and T58 is targeted by GSK-3β [26, 27]. ERK-mediated phosphorylation of S62 prevents c-Myc degradation, whereas GSK-3β-phosphorylated T58 promotes c-Myc degradation [26, 27]. We found that ADI-PEG20 treatment activates ERK and enhances c-Myc phosphorylation at S62. Phosphorylation of c-Myc at T58 is mediated by GSK-3β, a serine/threonine kinase. Phosphorylated c-Myc(T58) is recognized by Fbw7α in the proteasomal protein degradation signaling pathway [27]. It has been demonstrated that GSK-3β itself is a target of PI3K/AKT-mediated

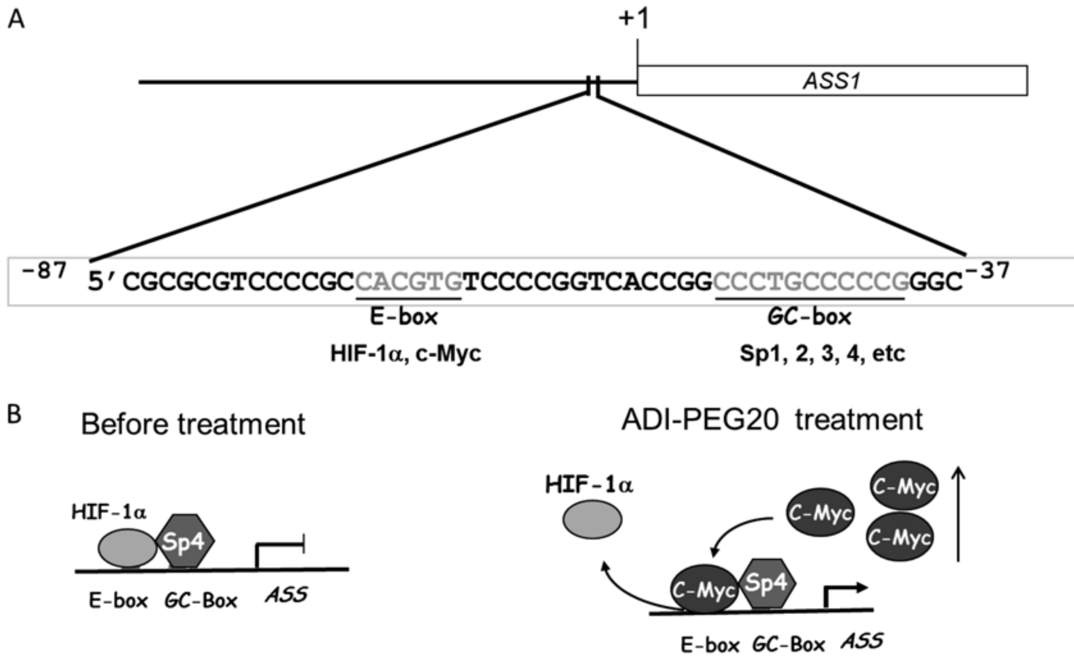

Fig. 44.2 Regulation of ASS1 expression in Arg-auxotrophic cells. (**a**) Promoter sequence involved in transcriptional regulation of ASS1 expression. The locations of two *cis*-acting elements, the E-box and GC-box and their respective transcriptional factors are indicated. (**b**) Schematic diagram showing switch of promoter binding from HIF-1α in non-stressed conditions to c-Myc binding under stressed conditions

phosphorylation at Ser9 position. GSK-3β phosphorylation inactivates its ability of phosphorylating c-Myc(T58), resulting in the stabilization of c-Myc. Although PI3K/AKT also phosphorylates GSK-3α(Ser21), we found that ADI-PEG-20 induces phosphorylation of GSK-3β but not GSK-3α, demonstrating that GSK-3β plays an inhibitory role in ADI-PEG20-mediated c-Myc stabilization (Fig. 44.3).

One upstream signal of GSK-3β is PI3K/AKT and we observed that activation of PI3K/AKT occurs within 15 min after ADI-PEG20 treatment. Inhibition of PI3K suppresses ADI-PEG20-induced AKT(T308) and GSK-3β phosphorylation and c-Myc expression. Although PTEN is a negative regulator of PI3K, it was demonstrated that activation of PI3K by ADI-PEG20 is independent of PTEN.

One important upstream activator of the PI3K/AKT pathway is Ras, which is also an upstream signal of ERK, mediated by the RAF intermediate [28]. Indeed, we found that ADI-PEG20 treatment activates Ras within 5 min, and inhibition of Ras activity by a dominant-negative recombinant attenuates ADI-PEG20 induction of PI3K-AKT. These results demonstrated that upregulation of c-Myc is through the inhibition of GSK-3β activity which is mediated by the activation of Ras/PI3K/AKT/ERK in response to ADI-PEG20 (Fig. 44.3). A melanoma cell line (A375) that is defective in this pathway does not respond to Arg-auxotrophic induction of *ASS1* expression.

L-Arginine-Auxotrophic Response Induces Metabolic Reprogramming

The observations that Arg deprivation induces HIF-1α downregulation and c-Myc upregulation have important implications in cancer metabolism. c-Myc and HIF-1α are among the most important oncoproteins that are critically involved in many aspects of cancer cell behaviors, including growth, proliferation, invasion, and responsiveness to therapies. c-Myc plays a central role in a transcriptional

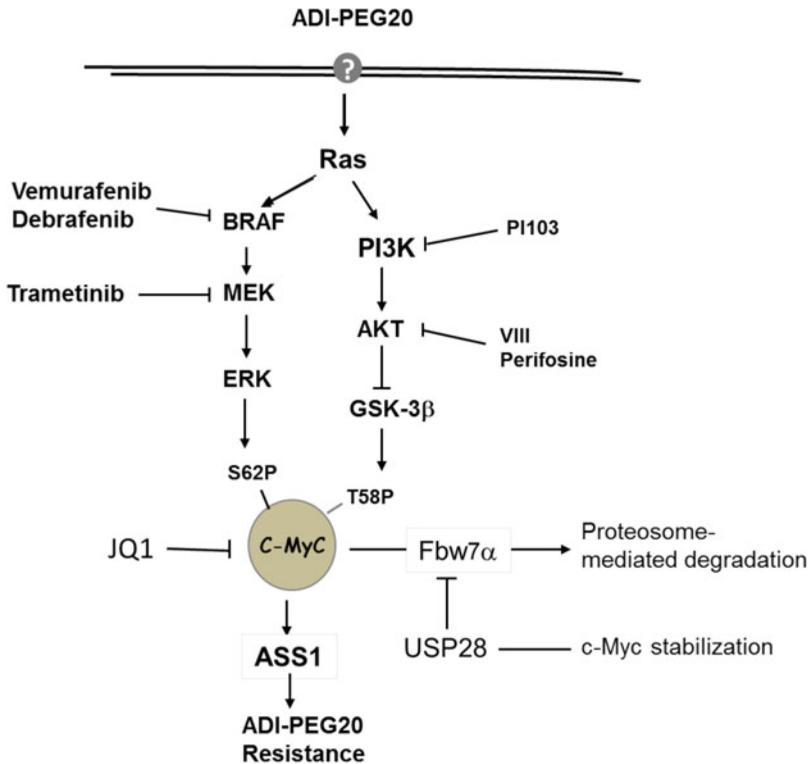

Fig. 44.3 Schematic diagram showing signal transduction involved in c-Myc stabilization and upregulation of ASS1 expression

regulatory network that regulates cell growth, differentiation, apoptosis, and metabolic signaling [29]. Likewise, HIF-1α plays a critical role for tumor angiogenesis, glycolysis, metastasis, redox signaling, and response to radiation and chemotherapy [30]. Previous studies have demonstrated that c-Myc regulates genes involved in the glycolytic pathway by directly binding to the E-boxes located at the promoters of these genes [31].We found that ADI-PEG20 induces expression of glucose transporter 1 and lactate dehydrogenase A but downregulates the expression of pyruvate dehydrogenase. Stable ADI-PEG20-resistant cell lines exhibit altered expression of these glycolytic enzymes (Fig. 44.4). These findings indicate a metabolic rewiring of Arg-auxotrophic cells toward glycolytic pathway (the Warburg effect) [32]. ADI-PEG20 also upregulates glutaminase and glutamyl dehydrogenase, two key glutaminolytic enzymes that catalyze the conversion of glutamine to α-ketoglutarate, an important metabolite in the TCA cycle (Fig. 44.3). Likewise, elevated expression of these enzymes was found in ADI-PEG20-resistant variants [32]. Because glucose and glutamine are the two major energy sources that fuel cancer cell growth, these results suggest that Arg-auxotrophic cells demand more glucose and glutamine supplies to meet the need of energy loss under Arg starvation condition.

Upregulation of glycolytic and glutaminolytic enzymes by ADI-PEG20 is mediated by c-Myc, because knockdown of c-Myc by shRNA strategy reduced the expression of these enzymes. However, once the expression of these enzymes is upregulated, elevated c-Myc is no longer needed to maintain the altered metabolic program. These results demonstrate that altered cancer cell metabolism in response to arg-auxotrophic challenge is associated with their glucose dependence and glutamine addiction.

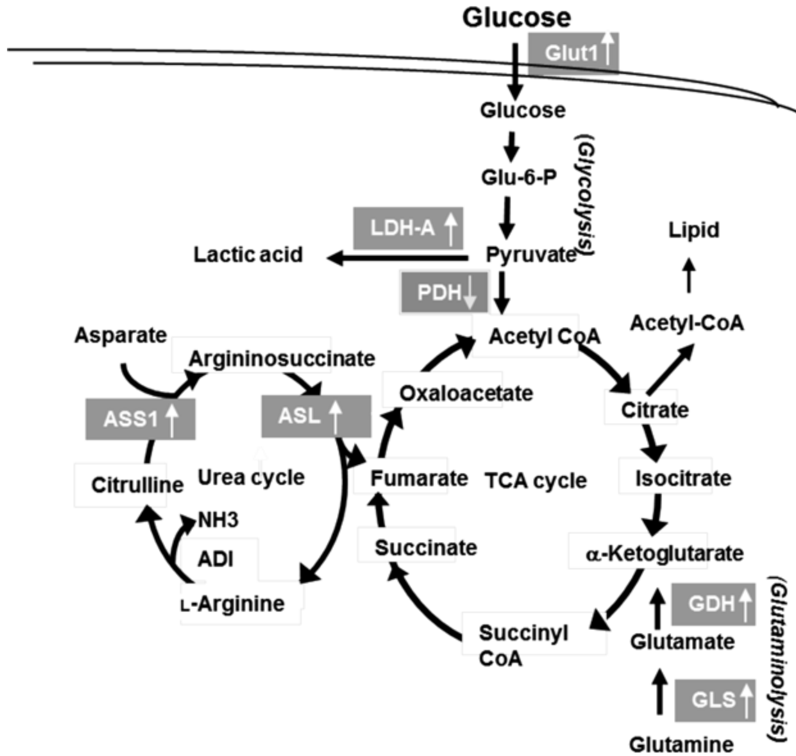

Fig. 44.4 Regulation of cancer metabolism in response to Arg deprivation in Arg-auxotrophic cells. The *upward arrows* denote increases of enzyme levels in ADI-PEG20-resistant cells

We have found that both ASS1 and its downstream enzyme ASL are elevated in response to Arg-auxotrophic stress. These results suggest that robust ASS1/ASL activities in the urea cycle are required to overcome ADI-PEG20-induced metabolic stress [22, 33]. These findings may not be surprising, because recent studies have demonstrated that several enzymes in the urea cycle, including ASS1, ASL, nitric oxide synthetase, and cationic amino acid transporter 1 are co-regulated [34], by forming a supermolecular complex to perform important physiological function [35].

Comparison of Signal Transduction Mechanisms Between Arg-Auxotrophic and Other Amino Acid-Limitation Responses

The signal transduction pathways leading to transcriptional upregulation of ASS1 expression in response to Arg-auxotrophic stress described here differ from those involved in other amino acid deprivation states. Regulation of gene expression in response to amino acid deficiency can be at the transcriptional, posttranscriptional, translational, and posttranslational levels, depending upon the context of genes and the types of amino acid deficiencies [1]. Here, we only focus on transcriptional upregulation of ASNS in response to Asn limitation in leukemia therapy [4]. ASNS catalyzes the synthesis of Asn from aspartate in conjunction with the conversion of glutamine into glutamate.

Upregulation of ASNS is an Asn-auxotrophic response mechanism to replenish Asn and constitutive upregulation of ASNS confers resistance to ASNase treatment. Conversely, inhibition of ASNase re-sensitizes these resistant cells to ASNase [36], demonstrating the importance of ASNS in ASNase sensitivities.

Deprivation of Asn and other amino acids is sensed by the general control non-derepressible 2 (GCN2), which monitors the availability of uncharged tRNA and phosphorylates its targeted gene product, the translational initiation factor elF-2α, resulting in the inhibition of the global translational initiation. However, translation of a fraction of pre-existing mRNA, notably activating transcription factor 4 (ATF4) mRNA, is not suppressed but rather upregulated under amino acid-depleted conditions. ATF4 mRNA contains two upstream open reading frames (uORF1 and uORF2). The 5′ uORF1 has only 3 codons and the 3′ ORF2 encompasses the ATF4 coding region. The uORF1 is a positive translational regulatory element for ATF4 translation, but uORF2 has an inhibitory property. It has been demonstrated that under non-stressed conditions, translation initiated from uORF1 quickly acquires reinitiation through the negative-acting uORF2, resulting in the dissociation of the active translation complex and reduction of ATF4 production. Under amino acid starvation conditions, translation initiated from uORF1 can proceed through the negative effect of uORF2 and reinitiate translation from the AUG codon by ribosomal scanning mechanism, which enhances ATF4 protein synthesis (Fig. 44.5) [37, 38].

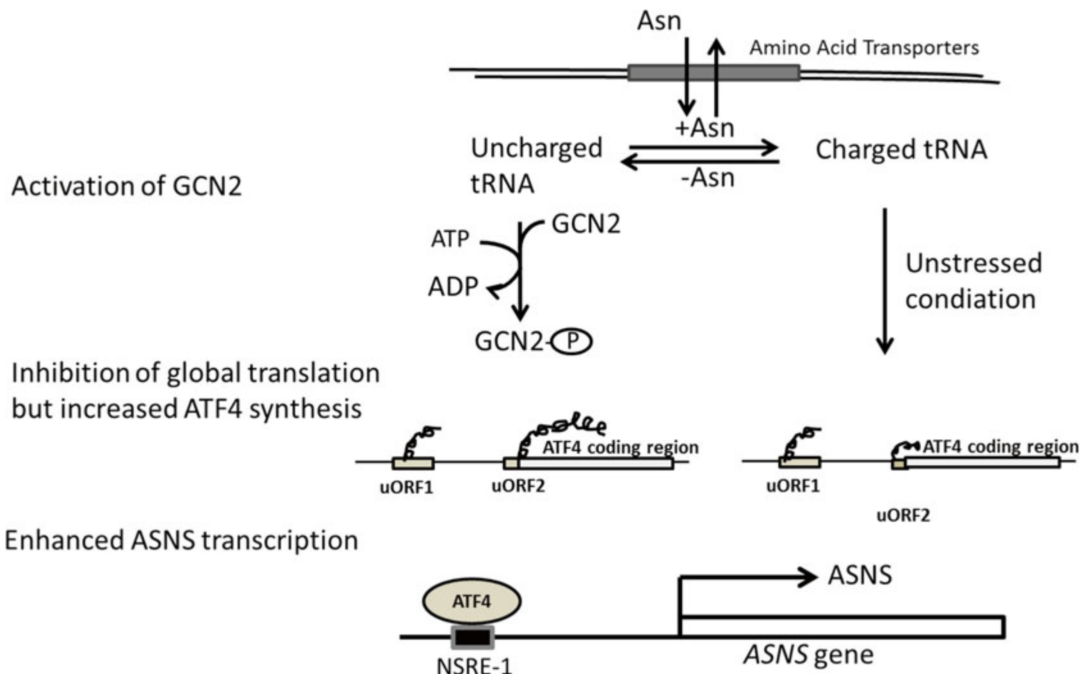

Fig. 44.5 Schematic diagram showing mechanism of ASNS induction under Asn deprivation. Asn deprivation (−Asn) produces uncharged tRNA, resulting in the activation of GCN2 by phosphorylation and the subsequent inhibition of global translation, but increased translation of ATF4. ATF4 is a transcriptional factor that upregulates ASNS expression by interacting with the nutritional sense response element-1 (NSRE-1) located at the promoter of ASNS gene

Arg-Auxotrophic Response Mechanisms and Therapeutic Implications in Cancer Treatment

Elucidation of signal transduction mechanisms of Arg-auxotrophic response leading to ASS1 upregulation, which is a biomarker of drug resistance in Arg-deprivation therapy, should provide mechanistic basis for the development of novel strategies for treating Arg-auxotrophic cancers.

The observations that Ras→PI3K→AKT and Ras→RAF→MAPK→ERK signals are involved in the upregulation of c-Myc leading to ASS1 upregulation suggest that intervention of these pathways may have therapeutic advantages for improving the efficacy of ADI-PEG20. As a proof of principle, we tested two PI3K inhibitors, Ly294002 and PI103; both are pan-PI3K inhibitors for class I PI3K (PI3Kα/β/γ/δ) [39], and two Akt inhibitors, VIII which is an AKT1 and AKT2 isozyme-selective inhibitor [40] and perifosine which targets the pleckstrin homology (PH) domain of Akt, preventing its translocation to the plasma membrane [40, 41]. All these inhibitors displayed suppressive effect on the induction of pT^{308}-AKT, c-Myc, and ASS1 expression by ADI-PEG20. These results demonstrated that the inhibition of PI3K/AKT pathway enhances the cell killing capacity of ADI-PEG20.

Ras genes, consisting of K-ras, H-ras, and N-ras, were the first oncogenes identified in cancer cells. These Ras genes, especially K-ras, and their downstream signals are the most frequently mutated oncogenes in human cancers [28]. In addition to the PI3K and RAF pathways, Ras can also activate the RelGDS→RelA/RelB pathway. Glycine 12 in K-ras is the most frequent mutated residue, the G12C mutation is frequently detected in lung cancer, and the G12D and G12V mutations are frequently detected in pancreatic cancers [28]. Extensive efforts have been devoted in developing effective inhibitors targeting these pathways. The progress has been slow because of the complexity of the network, such as cross-talk and feedback regulations. However, promising outcomes may eventually emerge. For example, although Ras has long been considered as untargetable, however, small chemicals such as Compound 12 [42] and SML-8-73-1 [43] that preferentially inhibit G12C over wild-type Ras have recently been identified.

PI3K and RAF also consist of multiple enzymes, i.e., eight isoenzymes in three classes for mammalian PI3K enzymes and three for RAF (BRAF, N-RAF, and H-RAF). Many pan-PI3K inhibitors have been undergoing clinical evaluations; however, doses needed for fully blocking all the isoforms may exceed tolerable toxicities [42]. About 50–60 % of malignant melanomas harbor BRAF mutations ($BRAF^{V600E}$ and $BRAF^{V600K}$). The inhibitors vemurafenib and dabrafenib have shown remarkable response rates (65–85 %) in treating BRAF mutations-bearing melanoma patients. However, the responses were short-lived (6–8 months) and patients inevitably became resistant to these drugs, with overall progression-free duration of 6–12 months [44]. Similarly, trametinib is an inhibitor to MEK that has been developed for treating BRAF inhibitor-resistant tumor [45]. Combination of ADI-PEG20 with these inhibitors may have additive, if not synergistic, therapeutic advantages. Likewise, while c-Myc has also been considered undruggable, recent study suggested that anti-BET bromodomain compound (JQ1) has potential for future anti-Myc drug development [43].

Since HIF-1α is a repressor of ASS1 expression and derepression of ASS1 by HIF-1α downregulation is a critical early event in Arg-auxotrophic response. Stabilization of HIF-1α can be achieved by the hypoxia mimics $CoCl_2$ and deferoxamine which block hydroxylation of HIF-1α for proteasomal degradation. We demonstrated that stabilization of HIF-1α by $CoCl_2$ or deferoxamine can suppress ADI-PEG20-induced ASS1 expression [20]. These results suggest that improving ADI-PEG20's antitumor effect against Arg-auxotrophic tumors may be achieved by using inhibitors to HIF-1α degradation. In this regard, two FDA approved anti-proteasomal cancer therapeutics bortezomib [46] and carfilzomib (Kyprolis) have been developed for treating relapsed and refractory multiple myeloma [47]. These clinical proteasomal inhibitors may be also applicable for improving the therapeutic efficacy of ADI-PEG20.

The findings that Arg-auxotrophic response upregulates many enzymes involved in the glycolytic and glutaminolytic pathways suggest that interventions of these pathways may also have therapeutic implications. As proof of principle, we demonstrated that ADI-PEG20-resistant melanoma cells are preferentially sensitive to glycolysis inhibitors (2-deoxy-D-glucose and 3-bromopyruvate) and kidney-type glutaminase inhibitors (6-diazo-5-oxo-L-norleucin (Don), azaserine, and Bis-2-(5-phenylacetamindo-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES)) [32]. However, the clinical applications of these compounds have not been demonstrated. Development of novel effective inhibitors is needed.

Conclusions and Future Research

Substantial progress in understanding the molecular basis of Arg-auxotrophic tumors has been made since the discovery that a wide spectrum of human malignancies require Arg for their growth. Moreover, Arg deprivation strategies have been under various stages of clinical investigations for targeted therapy of human Arg-auxotrophic cancers.

Arg auxotrophy is a metabolic aberration in a subpopulation of human cancers that is manifested when extracellular Arg is depleted. Since Arg is a building block of proteins which are essential for a variety of survival signaling pathways, response to Arg-auxotrophic stress in essence can be considered as cellular survival response mechanism. While some of these mechanisms may share those found in general nutritional stresses resulting from global amino acid deprivation, others may be specifically relevant to Arg (Fig. 44.6). Activation of the Ras/PI3K/AKT/ERK growth signal as described here represents an example of Arg-auxotrophic response. Other cell survival pathway such as autophagy which is a catabolic response to nutritional starvation has been documented in response to Arg deprivation [48, 49]. Because these pathways also play important roles in cancer cell growth, proliferation, and invasion, targeting these pathways in combination with the Arg-auxotrophic drug such as ADI-PEG20 is likely to be promising for treating Arg-auxotrophic tumors. Similarly, the findings that increased glycolytic and glutaminolytic pathways which represent adaptive metabolic rewiring to overcome Arg-auxotrophic stresses may also provide additional targets for drug development.

Induction of the Ras signal [22] occurs very rapidly (within 5 min) upon Arg deprivation in ASS1-negative cells. It is presently unknown how Arg deprivation induces Ras activation, although speculations can be offered. Because Ras activation is intimately associated with extracellular mitogen stimulations, it is likely that, immediately after Arg is removed from the extracellular environment, a host of membrane-bound growth factor receptors is mobilized to activate Ras and elicit its downstream growth signaling [50]. This scenario raises an important but challenging question as how do Arg-auxotrophic cells sense Arg deprivation. A relevant question is whether the sensing mechanism resides in plasma membrane (for example, Arg transport) or intracellular Arg contents? Future studies are needed to address these issues.

Downregulation of HIF-1α also occurs within minutes of Arg deprivation in ASS1-negative cells. Currently, we do not know how HIF-1α downregulation is regulated by Arg bioavailability in ASS1-negative cells. Because HIF-1α plays a suppressive function of ASS1 expression that confers Arg auxotrophy in many tumor types, and because ASS1 re-expression is an escape mechanism from Arg auxotrophy and responsible for the development of resistance to Arg-auxotrophic treatments, elucidating mechanisms of HIF-1α de-repression is of vital importance for the development of effective strategies to combat drug resistance in Arg-auxotrophic tumors.

In conclusion, this chapter presents many molecular aspects of survival mechanisms regarding how cells deal with Arg deficiency. Many unknowns remain to be investigated. We believe that as more detailed mechanisms of Arg-auxotrophic response have been learned, we will be in a better position to develop effective strategies to treat Arg-auxotrophic cancers.

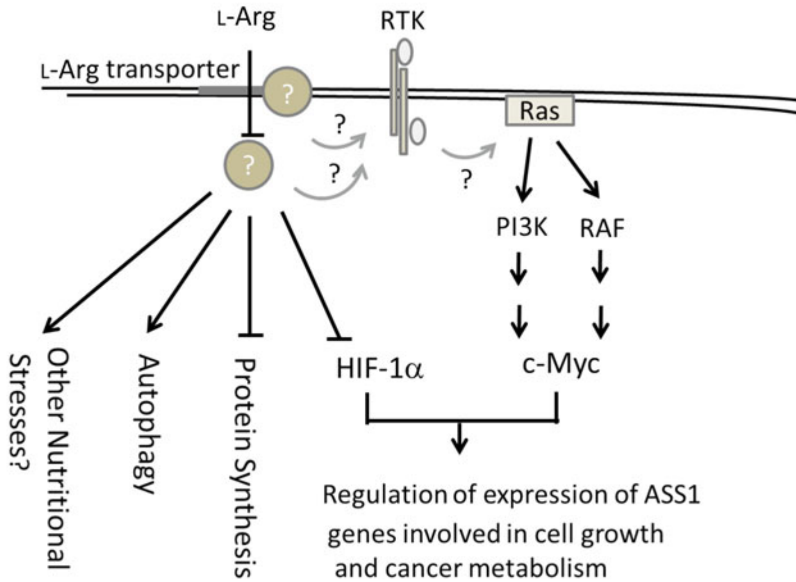

Fig. 44.6 Schematic diagram depicting multiple pathways involved in Arg-auxotrophic response. "?" associated with an *arrow* denotes uncertain elements that require investigations. The Arg deprivation sensing mechanism may be associated with the plasma membrane or reside inside the cells (*filled circles* with "?" inside). The Arg deprivation signal leads to Ras activation which may involve upstream signal like receptor tyrosine kinase (RTK) receptors. The Ras signal stabilizes c-Myc resulting in c-Myc accumulation. Arg deprivation suppresses general protein synthesis but may induce autophagy flux and other nutritional stresses, and downregulates HIF-1α. Downregulated HIF-1α together with upregulated c-Myc induces ASS1 expression. These transcriptional factors also regulate a host of genes involved in cell growth, proliferation, and cancer metabolism

References

1. Chaveroux C, Lambert-Langlais S, Cherasse Y, et al. Molecular mechanisms involved in the adaptation to amino acid limitation in mammals. Biochimie. 2010;92:736–45.
2. Beaud et al, O'Brien WE, Bock HG, et al. The human argininosuccinate synthetase locus and citrullinemia. Adv Hum Genet. 1986;15:161–96, 291–2.
3. Erez A. Argininosuccinic aciduria: from a monogenic to a complex disorder. Genet Med. 2013;15:251–7.
4. Kilberg MS, Balasubramanian M, Fu L, Shan J. The transcription factor network associated with the amino acid response in mammalian cells. Adv Nutr. 2012;3:295–306.
5. Salzer W, Seibel N, Smith M. Erwinia asparaginase in pediatric acute lymphoblastic leukemia. Expert Opin Biol Ther. 2012;12:1407–14.
6. Dillon BJ, Prieto VG, Curley SA, et al. Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to L-arginine deprivation. Cancer. 2004;100:826–33.
7. Allen MD, Luong P, Hudson C, et al. Prognostic and therapeutic impact of argininosuccinate synthetase 1 control in bladder cancer as monitored longitudinally by PET imaging. Cancer Res. 2014;74:896–907.
8. Kelly MP, Jungbluth AA, Wu BW, et al. L-Arginine deiminase PEG20 inhibits growth of small cell lung cancers lacking expression of argininosuccinate synthetase. Br J Cancer. 2012;106:324–32.
9. Huang HY, Wu WR, Wang YH, et al. ASS1 as a novel tumor suppressor gene in myxofibrosarcomas: aberrant loss via epigenetic DNA methylation confers aggressive phenotypes, negative prognostic impact, and therapeutic relevance. Clin Cancer Res. 2013;19:2861–72.
10. Feun LG, Marini A, Walker G, et al. Negative argininosuccinate synthetase expression in melanoma tumours may predict clinical benefit from L-arginine-depleting therapy with pegylated L-arginine deiminase. Br J Cancer. 2012;106:1481–5.
11. Yang TS, Lu SN, Chao Y, et al. A randomised phase II study of pegylated L-arginine deiminase (ADI-PEG20) in Asian advanced hepatocellular carcinoma patients. Br J Cancer. 2010;103:954–60.

12. Glazer ES, Piccirillo M, Albino V, et al. Phase II study of pegylated L-arginine deiminase for nonresectable and metastatic hepatocellular carcinoma. J Clin Oncol. 2010;28:2220–6.
13. Kuo MT, Savaraj N, Feun LG. Targeted cellular metabolism for cancer chemotherapy with recombinant L-arginine-degrading enzymes. Oncotarget. 2010;1:246–51.
14. Tanios R, Bekdash A, Kassab E, et al. Human recombinant arginase I(Co)-PEG5000 [HuArgI(Co)-PEG5000]-induced L-arginine depletion is selectively cytotoxic to human acute myeloid leukemia cells. Leuk Res. 2013;37:1565–71.
15. Stone EM, Glazer ES, Chantranupong L, et al. Replacing Mn(2+) with Co(2+) in human arginase i enhances cytotoxicity toward L-arginine auxotrophic cancer cell lines. ACS Chem Biol. 2010;5:333–42.
16. Tsui SM, Lam WM, Lam TL, et al. Pegylated derivatives of recombinant human arginase (rhArg1) for sustained in vivo activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics in vivo and in vitro and action upon hepatocellular carcinoma cell (HCC). Cancer Cell Int. 2009;9:9.
17. Lam TL, Wong GK, Chong HC, et al. Recombinant human arginase inhibits proliferation of human hepatocellular carcinoma by inducing cell cycle arrest. Cancer Lett. 2009;277:91–100.
18. Delage B, Luong P, Maharaj L, et al. Promoter methylation of argininosuccinate synthetase-1 sensitises lymphomas to L-arginine deiminase treatment, autophagy and caspase-dependent apoptosis. Cell Death Dis. 2012;3:e342.
19. Szlosarek PW, Luong P, Phillips MM, et al. Metabolic response to pegylated L-arginine deiminase in mesothelioma with promoter methylation of argininosuccinate synthetase. J Clin Oncol. 2013;31:e111–3.
20. Tsai WB, Aiba I, Lee SY, et al. Resistance to L-arginine deiminase treatment in melanoma cells is associated with induced argininosuccinate synthetase expression involving c-Myc/HIF-1alpha/Sp4. Mol Cancer Ther. 2009; 8:3223–33.
21. Hann SR, Eisenman RN. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. Mol Cell Biol. 1984;4:2486–97.
22. Tsai WB, Aiba I, Long Y, et al. Activation of Ras/PI3K/ERK pathway induces c-Myc stabilization to upregulate argininosuccinate synthetase, leading to L-arginine deiminase resistance in melanoma cells. Cancer Res. 2012; 72:2622–33.
23. Thomas LR, Tansey WP. Proteolytic control of the oncoprotein transcription factor Myc. Adv Cancer Res. 2011;110:77–106.
24. Yada M, Hatakeyama S, Kamura T, et al. Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J. 2004;23:2116–25.
25. Popov N, Wanzel M, Madiredjo M, et al. The ubiquitin-specific protease USP28 is required for MYC stability. Nat Cell Biol. 2007;9:765–74.
26. Sears R, Nuckolls F, Haura E, et al. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev. 2000;14:2501–14.
27. Sears RC. The life cycle of C-myc: from synthesis to degradation. Cell Cycle. 2004;3:1133–7.
28. Stephen AG, Esposito D, Bagni RK, et al. Dragging Ras Back in the Ring. Cancer Cell. 2014;25:272–81.
29. Grandori C, Cowley SM, James LP, et al. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol. 2000;16:653–99.
30. Semenza GL. Hypoxia and cancer. Cancer Metastasis Rev. 2007;26:223–4.
31. Dang CV, O'Donnell KA, Zeller KI, et al. The c-Myc target gene network. Semin Cancer Biol. 2006;16:253–64.
32. Long Y, Tsai WB, Wangpaichitr M, et al. L-Arginine deiminase resistance in melanoma cells is associated with metabolic reprogramming, glucose dependence and glutamine addiction. Mol Cancer Ther. 2013;12:2581–90.
33. Kim RH, Coates JM, Bowles TL, et al. L-Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. Cancer Res. 2009;69:700–8.
34. Oyadomari S, Gotoh T, Aoyagi K, et al. Coinduction of endothelial nitric oxide synthase and L-arginine recycling enzymes in aorta of diabetic rats. Nitric Oxide. 2001;5:252–60.
35. Erez A, Nagamani SC, Shchelochkov OA, et al. Requirement of argininosuccinate lyase for systemic nitric oxide production. Nat Med. 2011;17:1619–26.
36. Richards NG, Kilberg MS. Asparagine synthetase chemotherapy. Annu Rev Biochem. 2006;75:629–54.
37. Lu PD, Harding HP, Ron D. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J Cell Biol. 2004;167:27–33.
38. Vattem KM, Wek RC. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc Natl Acad Sci U S A. 2004;101:11269–74.
39. Workman P, Clarke PA, Raynaud FI, van Montfort RL. Drugging the PI3 kinome: from chemical tools to drugs in the clinic. Cancer Res. 2010;70:2146–57.
40. Calleja V, Laguerre M, Parker PJ, et al. Role of a novel PH-kinase domain interface in PKB/Akt regulation: structural mechanism for allosteric inhibition. PLoS Biol. 2009;7:e17.
41. Gills JJ, Dennis PA. Perifosine: update on a novel Akt inhibitor. Curr Oncol Rep. 2009;11:102–10.
42. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov. 2014;13:140–56.

43. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell. 2011;146:904–17.
44. Sullivan RJ, Flaherty KT. Resistance to BRAF-targeted therapy in melanoma. Eur J Cancer. 2013;49:1297–304.
45. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N Engl J Med. 2012;367:1694–703.
46. Crawford LJ, Irvine AE. Targeting the ubiquitin proteasome system in haematological malignancies. Blood Rev. 2013;27:297–304.
47. Redic K. Carfilzomib: a novel agent for multiple myeloma. J Pharm Pharmacol. 2013;65:1095–106.
48. Qiu F, Chen YR, Liu X, et al. L-Arginine starvation impairs mitochondrial respiratory function in ASS1-deficient breast cancer cells. Sci Signal. 2014;7:ra31.
49. You M, Savaraj N, Kuo MT, et al. TRAIL induces autophagic protein cleavage through caspase activation in melanoma cell lines under L-arginine deprivation. Mol Cell Biochem. 2013;374:181–90.
50. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 2008;9:517–31.

Chapter 45
The Role of L-Arginine in Wound Healing

Alan N. Gould and Geoffrey P. Candy

Key Points

- Wound healing occurs in a sequence of regulated and overlapping phases.
- Pathways involving L-arginine and the urea cycle are critical in healing.
- Infiltrating macrophages secrete either pro-inflammatory cytokines with inducible nitric oxide synthase (iNOS) activity or anti-inflammatory with increased arginase 1 activity and appear critical in regulating subsequent phases.
- iNOS is important in early phases producing NO to clean the wound.
- NO is important throughout the healing process stimulating fibroblasts to produce collagen, promoting matrix deposition, remodelling and angiogenesis.
- Under- and over-production of NO adversely affects wound healing.
- Arginase 1 provides proline and ornithine from L-arginine for collagen deposition and polyamine production respectively.
- The products of the active iNOS and arginase 1 pathways provide feedback regulation of each other.
- L-Arginine, ornithine and proline supplementation all improve wound healing.

Keywords Wound healing • L-Arginine • Arginase • Nitric oxide • Inducible nitric oxide synthase • Urea cycle

Abbreviations

bFGF	Basic fibroblast growth factor
COX	Cyclooxygenase
EGF	Epidermal growth factor
eNOS/NOS3	Endothelial or constitutive nitric oxide synthase

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FGF	Fibroblast growth factor
H2O2	Hydrogen peroxide
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MMP	Matrix metalloproteinase
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E-2
TGF-β	Transforming growth factor-beta (β)
Th	T cell helper
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor-alpha (α)
VEGF	Vascular endothelial growth factor

Introduction

Wound healing is required to effectively stabilise and repair injury [1, 2]. In an optimal system, the process of wound healing occurs in an orderly and controlled series of phases: homeostasis, inflammation, proliferation and remodelling [3, 4]. However, complications including infection and inappropriate activation of the immune system can impair this process and result in delayed healing and in the development of chronic wounds. Such complications impact significantly on health-care resources, and in 2010, the cost of treating and caring for wounds in the United States alone was estimated at $25 billion [2]. It is therefore important to determine factors that influence the wound healing process to optimise and facilitate appropriate healing.

As early as 1936, Robinson [5] described the use of urea for healing chronic purulent wounds noting the effects as "…a cleansing of the wound by removal of necrotic material and pyrogenic bacteria present, and a promotion of the growth of granulation tissue". To determine the key biochemical pathways and factors affecting the wound healing process, the concentrations of reactants and products within the wound have been collected on implanted polyvinyl alcohol sponges. The recovery of L-arginine, ornithine and proline, as well as urea and nitrite, the breakdown product of nitric oxide (NO), has identified pathways involving L-arginine and the urea cycle as being critical in the healing process [6] (Fig. 45.1).

The semi-essential dibasic amino acid, L-arginine, is essential for growth in rodents [7], and although not required for growth in adult humans, it becomes an essential amino acid in certain conditions, such as wound healing [8, 9]. Within the wound environment, L-arginine concentrations may decrease to undetectable levels [6, 10], and in such cases, the availability of L-arginine become essential in order for the wound to heal [11]. It has also been shown that products of the L-arginine pathways, urea, proline, polyamines and NO, affect the different phases of the wound healing process [12–14].

L-Arginine has a host of functions: as a structural amino acid residue in proteins, as a key intermediate of the ornithine cycle and therefore nitrogen balance and as the precursor of creatinine and urea in the reaction catalysed by the enzyme arginase. It is a secretagogue for insulin, prolactin, insulin and IGF-1 and is the precursor of polyamines and proline. Of particular interest in wound healing, L-arginine is the precursor of NO, a mediator and messenger in a host of biological, immunological and physiological processes (Fig. 45.1) [8, 15].

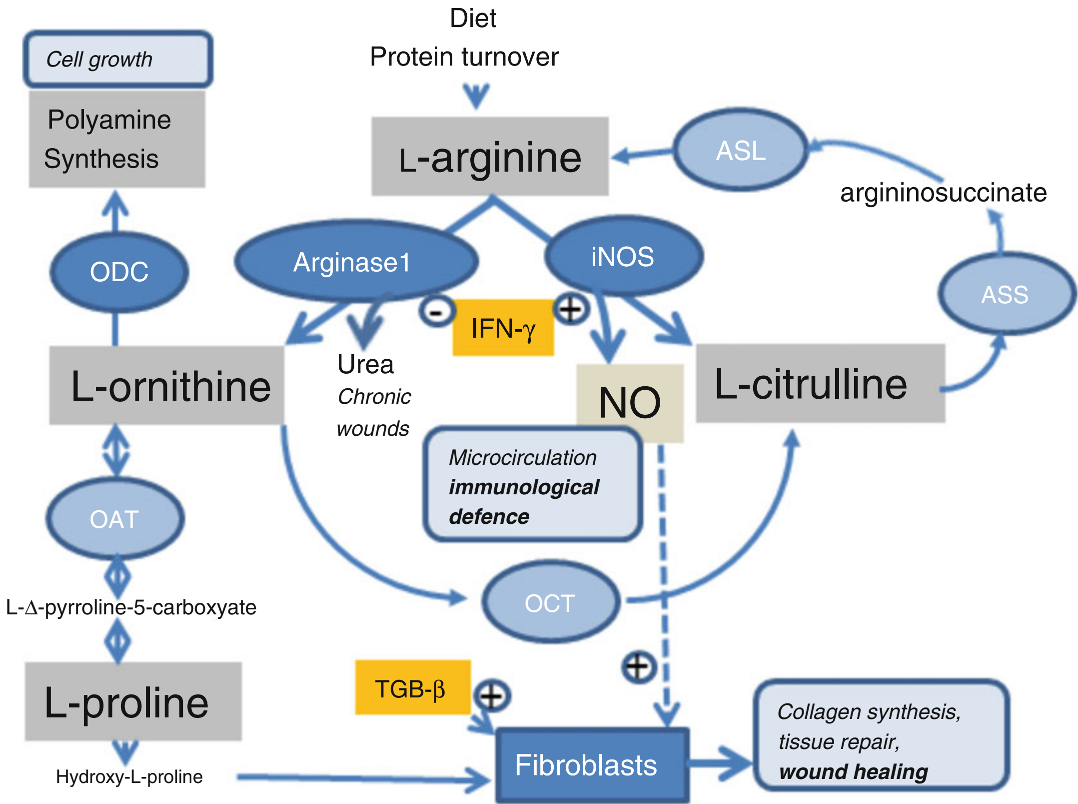

Fig. 45.1 The urea cycle with the metabolites shown that have an effect on wound healing. Abbreviations: *iNOS* inducible nitric oxide synthase, *OCT* ornithine carbonyl transferase, *ASS* argininosuccinate synthase, *ASL* L-arginine-succinate lyase, *ODC* ornithine decarboxylase, *OAT* ornithine amino transferase, *IFN-γ* interferon-gamma, *TGF-β* transforming growth factor-beta, *(+)* upregulation or stimulatory, *(−)* downregulation or inhibitory

Nitric Oxide

NO is produced from L-arginine and oxygen in a reaction catalysed by the enzyme nitric oxide synthase (NOS). Three forms of NOS occur: two constitutive Ca^{2+}/calmodulin-dependent enzymes, the endothelial NOS (eNOS/NOS3) and the neuronal NOS (nNOS/NOS1) [8], and the inducible Ca^{2+}/calmodulin independent form, iNOS. iNOS is induced in various cells types during inflammation in response to cytokines and other mediators [16, 17], with the NO produced by this form of NOS playing an essential role in the wound healing process [16, 18, 19]. NO is involved during the inflammatory and proliferation phases of wound healing to promote matrix deposition, remodelling and angiogenesis [20, 21]. Further, as a radical, NO has antimicrobial and cytotoxic properties that are essential for maintaining sterility in the wound space [22].

One of the key regulators of NO production is the concentration of the precursor L-arginine. Low L-arginine concentrations within the wound environment limit NO synthesis and will affect the entire wound healing process [11]. Equally, excessive production of NO, for example in sepsis, can adversely affect and delay healing [17]. The requirement for L-arginine in wound healing is inferred from concentrations of L-arginine itself and particularly from measured concentrations of the metabolites of the arginase (ornithine) and NO pathways (citrulline or NO and the nitrite degradation products) [6].

The production of NO by cellular components depends on the stage of the wound healing process, with macrophages producing the greatest amount of NO by iNOS, in the early inflammatory phase. In wounds created in a rat model, NOS activity, as measured by citrulline and nitrite production, was highest during the first 3 days post-wounding [6]. This activation is dependent on cytokines, inflammatory mediators, growth factors and hypoxia, and is limited by cofactors and substrate availability. At later stages, increased ornithine concentrations indicate increased arginase activity [6], with NO production by iNOS inhibited by constituents of the wound fluid [16, 23].

Arginase Pathway

The conversion of L-arginine by the arginase I and II enzymes to ornithine is the major route of L-arginine metabolism. Arginase 1 activity is required to promote wound healing, and if this activity is inhibited, as demonstrated in a mouse model, inflammation in the wound increases to impair matrix formation and delay wound healing [24].

Ornithine is the precursor to the polyamines and proline, which are both directly involved in cell proliferation and collagen synthesis, respectively [23]. Collagen is synthesised by fibroblasts and determines the strength of the repaired wound. Proline is a major component of collagen, and the overall wound strength is decreased if the availability of proline is limited [6, 10, 25].

Wound Healing

The phases and physiology of wound healing have been extensively reviewed [3, 26, 27]. Important cellular, non-cellular, cytokines and growth regulators involved in wound healing have been summarised in Table 45.1.

The process of wound healing is accompanied by the rapid activation of the immune system, with neutrophils, macrophages and lymphocytes infiltrating into the wound space (Fig. 45.2). These cells release both innate and adaptive immune system cellular mediators, cytokines and messengers, critical in facilitating and controlling the wound healing process [30, 31]. Re-establishing homeostasis and the subsequent wound healing process is carefully regulated as immune dysregulation can delay wound healing [32].

1. *Haemostatic phase*: Re-establishing homeostasis directly after the initial insult from trauma or surgical intervention is the shortest phase of wound healing and occurs within minutes. Blood vessels contract and the clotting cascade is initiated with the fibrin plug forming a temporary repair of the wound, with aggregating platelets forming a matrix with adhesion motifs for anchoring infiltrating cells [2, 3]. The early infiltration of cells and their production of appropriate cytokines, regulates the subsequent phases of wound healing. Furthermore, NO production by iNOS is essential to counteract potential infection [3, 15].
2. *Inflammatory phase*: The inflammatory phase lasts 1–5 days and is characterised by infiltration of inflammatory cells, initially polymorphonuclear leukocytes or neutrophils and subsequently macrophages into the wound space [3]. These macrophages phagocytose dead cells, and bacteria, and remove such cell debris with proteolytic elastases and collagenases. These enzymes are different from the matrix metalloproteinases (MMPs) produced later in the healing process [15]. This debriding and cleansing of the wound space appears essential for the subsequent orderly progression of wound healing. The infiltrating cells produce a variety of important regulatory cytokines, chemokines and interleukin messengers (Table 45.2), with the expression of several of these mediators, including IL-1, IL-6, IL-8 and TGF-β, directly affected by NO [28].

Table 45.1 Components active in the various phases of wound healing

Phase	Time scale	Cellular components	Non-cellular components	Main enzymes	Regulators
Haemostasis	Injury to 15–30 min	Endothelial cells Platelets	Fibrin Fibronectin	Clotting cascade COX-2	Prostaglandins, thromboxanes Thrombin
Inflammatory	15–30 min to 1 week	Endothelial cells Neutrophils Macrophages Lymphocytes	Fibrin provisional matrix	iNOS COX-2	Cytokines: IL-8 Growth factors: PDGF Others: L-arginine, histamine, leukotrienes, complement, **NO**, H_2O_2 iNOS: TNF-α, IL-1α and β, IFNα, IFNβ, IFNγ Arginase 1: IL4, IL-6, IL-10, IL13, TGF-β, PGE1; PGE2
Proliferative	2 days to a few weeks	Endothelial cells Macrophages Fibroblasts Keratinocytes	Provisional matrix Collagen Proteoglycans	Arginase 1	Growth factors: FGF-2, 7 & 10, VEGF, EGF, TGF-β, PDGF, polyamines Others: MMP/TIMP, NO, angiopoietin, ornithine, urea
Remodelling	Weeks to months	Myofibroblasts Fibroblasts Macrophages Lymphocytes	Collagen Proteoglycans	Arginase 1	Growth factors: TGF-β, PDGF Cytokines: IL-1, IL-10 Others: MMP/TIMP, L-arginine, proline

Adapted from Popovic et al. [13], Werner and Grose [28] and Baum and Arpey [29]

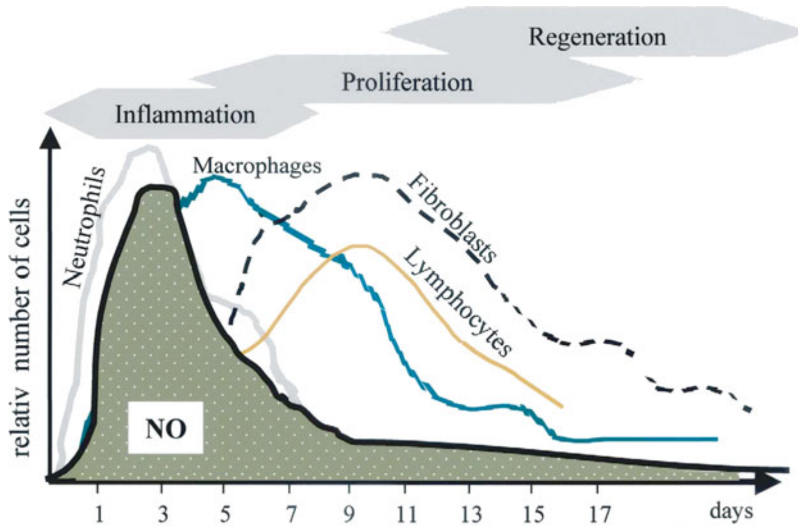

Fig. 45.2 Phases of wound healing, associated cellular components and nitric oxide production. Reproduced from Witte and Barbul [50] with permission from Elsevier Press

Recently it has been recognised that the infiltrating macrophages are of two distinct types characterised by different modes of activation (Fig. 45.3). Classically activated macrophages (CAMs) are activated by interferon-γ and tumour necrosis-α (Th1 response), upregulate iNOS activity against microbes and intercellular pathogens, and secrete pro-inflammatory cytokines [24, 33]. IL-4 and IL-13 induce the alternatively activated macrophages (AAMs; Th2 response) that exhibit upregulated arginase type-1 activity, with anti-inflammatory functions. The AAMs produce collagen and remodel tissues to facilitate tissue repair [24, 34]. These macrophages appear critical regulators of the wound healing process.

The production of high levels of NO during the inflammatory phase is dependent on macrophage-derived iNOS. In subcutaneous wounds NOS activity increases within 24 h of injury, with iNOS expression and NO production reaching their highest levels within 24–72 h [24, 35]. In a mouse model of cutaneous wound healing, arginase activity remains high up to 7 days post-wounding. A reduction in arginase activity is associated with significant delay in wound healing [24].

3. *Proliferative phase*: In response to TGF-β, bFGF and PDGF and other signals, granulation occurs as fibroblasts and undifferentiated mesenchymal cells migrate into the recovering wound area to proliferate and produce collagen. Re-epithelialisation of the wound occurs, as keratinocytes migrate in, proliferate and differentiate and overlay the formed granulation layer [29].

   In the later stages of the "proliferative phase", the arginase pathway forms metabolites and intermediates, proline and polyamines, which are directly involved in collagen synthesis and cell proliferation, respectively [22, 23]. Proline is incorporated into collagen by fibroblasts, with the availability of proline determining the overall wound strength [6, 8, 25]. Interestingly collagen production by fibroblasts is stimulated by NO, suggesting a close relationship between the pathways [36].
4. *Remodelling phase*: Extracellular matrix synthesis and remodelling occurs over several months after wound closure [2]. Key to this phase is the synthesis and breakdown of collagen fibres [37] by MMPs. MMPs are directly involved in cell migration, cytokine and growth factor release from the cells producing them as well as the degradation of these factors [26].

L-Arginine does appear to be required for appropriate cross-linking of collagen since it attenuates methylglyoxal and high glucose induced formation of Nε-carboxyethyl lysine and attenuates arginase expression in a manner similar to aminoguanidine [38]. However, few studies have been conducted during this phase to determine the effects of supplemental L-arginine or ornithine on the healing of excisional wounds.

Table 45.2 Wound concentrations (top of each panel: decreased [▼], increase [▲] or marked increase [▲▲]) and the effect of supplemental amino acids and NO (bottom of each panel: + positive benefit or enhanced healing) at the different phase of wound healing

	Wound healing phase or sepsis					
Molecule	Homeostasis	Inflammation	Proliferation	Remodeling	Sepsis	Note and references
L-Arginine	N/A	Essential [▼] +	Essential [▼] +	Limited data	N/A	+ benefit if added during both inflammation & proliferative phases [10, 11, 14, 16, 47–49]
NO	[▲] N/A	[▲▲] +	[▲] +	[▲] +	[▲▲] N/A	+ benefit if added throughout the healing process [11, 14, 16, 21, 41–43]
Ornithine & proline	N/A N/A	Important [▼] +	Important [▼] +	Limited data	N/A	Data for proline: + benefit if supplemented orally or topically
Urea	N/A	N/A	N/A		N/A	Benefit in chronic wounds [5]

N/A data not available

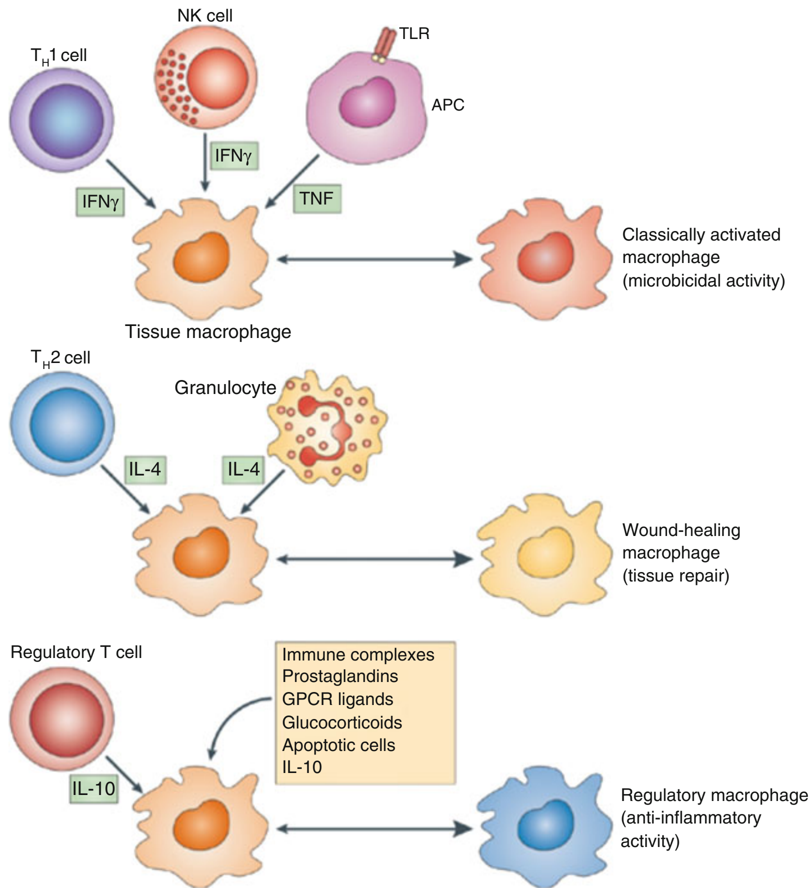

Fig. 45.3 Cytokines produced by immune cells can give rise to macrophages with distinct physiologies. Classically activated macrophages arise in response to interferon-γ (IFNγ), which can be produced during an adaptive immune response by T helper 1 (TH1) cells or CD8+ T cells (not shown) or during an innate immune response by natural killer (NK) cells, and tumour necrosis factor (TNF), which is produced by antigen-presenting cells (APCs). Wound healing (alternatively activated) macrophages arise in response to interleukin-4 (IL-4), which can be produced during an adaptive immune response by TH2 cells or during an innate immune response by granulocytes. Regulatory macrophages are generated in response to various stimuli, including immune complexes, prostaglandins, G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells or IL-10. Each of these three populations has a distinct physiology. Classically activated macrophages have microbicidal activity, whereas regulatory macrophages produce high levels of IL-10 to suppress immune responses. Wound healing macrophages are similar to the previously described alternatively activated macrophages and have a role in tissue repair. *TLR* toll-like receptor. Reproduced with permission from Mosser DM, Edwards JP. Nature Reviews Immunology. 2008;8:958–969. Fig. 3. Additional websites: http://jcs.biologists.org/content/122/18/3209/F1.large.jpg

Controlling Biochemical Pathways in Wound Healing

The early infiltrations of macrophages into the wound space appear to be critical regulators of the wound healing process. Induced CAMs have increased iNOS activity and produce pro-inflammatory cytokines [24, 33]. Interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS) increase the activity of the iNOS and, together with the products of the iNOS pathway (NO and citrulline), decrease the activity of arginase [22]. In contrast AAMs have upregulated arginase type-1 activity, have anti-inflammatory functions and appear key in facilitating tissue repair [24, 34] (Fig. 45.3).

Both the iNOS pathway and the arginase pathway of L-arginine metabolism are important in wound healing and have increased activities during different phases of the wound healing process [6, 23] (Fig. 45.1). However, the iNOS and arginase pathways are intimately linked through the respective products and through effector molecules. The products of each pathway provide regulatory feedback: urea produced from the arginase pathway acts post-transcriptionally to decrease NOS activity in a macrophage cell line [39]. Conversely, arginase activity is reduced by hydroxy-L-arginine, the intermediate metabolite in the conversion of L-arginine to NO [40].

It would seem logical that the arginase pathway should be favoured over the iNOS pathway in order to promote collagen production in the proliferative phase of wound healing. However,

(i) Collagen production in wounds was increased with either supplemental L-arginine or ornithine [11, 14].
(ii) Continuous infusion of L-arginine into a wound produced sustained NO production, reduced VEGF content and decreased the wound vessel number, vascular surface area and granulation thickness, all leading to an overall improvement of the state of the wound [41].
(iii) Decreasing the activity or inhibiting the iNOS pathway and NO production decreased collagen synthesis and wound healing [16].
(iv) In excisional wounds in rats, the application of NO donor *S*-nitrosoglutathione, accelerated wound contracture and re-epithelialisation when applied during both the inflammatory and proliferative phases when compared to application to either phase alone [21].
(v) NO donors incorporated into hydrogel dressing were beneficial in a chronic mouse wound model [42].
(vi) Chronic wounds producing higher amounts of NO healed faster compared to those that had lower levels of NO [43].

These data suggest that the iNOS and arginase pathways are not independent of each other and both pathways are essential for optimal wound healing.

Preferential utilisation of L-arginine by either the iNOS or the arginase pathway would have different results, as iNOS is associated with inflammation and arginase with proliferation and differentiation. The balance between the pathways does not appear to be critical in the acute uncomplicated wound setting as, in the absence of complications such as infection, these wounds will heal. However, a mismatch between relative iNOS and arginase activities may allow chronic wounds to develop [24]. Furthermore, excessive NO synthesis has been shown to have similar effects to depleted NO by decreasing collagen production [43] to impair wound healing [18] and may contribute to the formation of keloid scars [36].

In addition to iNOS, the formation of prostacyclins by cyclooxygenase-2 (COX-2) is another important pathway in inflammatory processes. Interestingly, both iNOS and COX-2 enzymes were active within the same time frame during the inflammatory phase of wound healing. It has been shown that iNOS bound, then S-nitrosylated and activated COX-2 to increase PGE2 production and iNOS inhibitors decreased the formation of prostaglandins [44]. These data suggested a close relationship between these two pathways that may be manipulated to modify the inflammatory response and so enhance wound healing. In foetal tissues, scarless healing was characterised by a lack of significant inflammation [45]. Therefore, targeting the inflammatory phase with drugs such as the COX-2

pathway inhibitors may reduce scar formation and improve outcome in wounds [4]. Although COX-2 inhibitors are widely used as anti-inflammatory drugs, iNOS inhibitory drugs are not available commercially to reduce inflammation. The possibility of using combined inhibition of the pathways outcome has not been investigated.

Finally, although polyamines regulate gene expression, cell signalling and cell receptor activities and are essential for cell proliferation, differentiation and function as well as anti-oxidant properties [46], their role in wound healing appears not to have been studied.

Supplementary Feeding and Wound Healing

Several studies have demonstrated the beneficial effects of supplemental L-arginine and other urea cycle intermediates on wound healing. Supplementing L-arginine in the drinking water improved incisional wound breaking strength, collagen deposition and overall wound healing in normal mice but, not iNOS knockout mice [11]. Using similar methodology, L-arginine increase wound-breaking strength, procollagen mRNA levels and collagen deposition and improved wound healing in rats subjected to trauma/haemorrhagic shock prior to creating a dorsal skin incision [14]. In diabetic rats L-arginine supplementation appeared to normalise the NO pathway by increased wound strength and nitrate/nitrite concentrations but was unable to restore the reduced arginase activity and ornithine concentrations [10]. In human studies, L-arginine improved wound healing in diabetic patients by reversing the impaired healing conditions present in these patients and, in contrast to the animal study [10], maintained the arginase pathway [16].

Similarly, supplemental L-arginine improved wound healing and immune function in elderly people [47], and oral supplements of L-arginine in combination with zinc and vitamin C were beneficial in reducing wound area, exudate, and the incidence of necrotic tissue pressure ulcers in open label studies [48, 49].

Supplementation with ornithine bypasses the arginase and iNOS steps but produces similar healing results to feeding supplemental L-arginine. Furthermore, ornithine increased collagen production and wound-breaking strength without affecting NO production in either iNOS knock-out or wild type mice [47]. L-Proline administered either topically or orally improved a number of biochemical parameters and markers of oxidative stress in full thickness excision wounds created in a rat model. Proline improved wound contraction, strength and re-epithelialisation as well as increased the number of macrophages and fibroblasts, with associated collagen accumulation in the wound space [25].

There appears to be few studies determining the effects of L-arginine and other urea cycle intermediates on cytokine production in the context of wound healing. Such studies would determine which cytokines are affected by L-arginine supplementation.

Conclusions

Wound healing is a complex and regulated process with sequential phases involving multiple substrates, products and regulatory messenger cytokines. L-Arginine and L-arginine metabolic pathways have been identified as playing a critical role in wound healing. Early in the wound process, infiltrating CAMs with increased iNOS activity, and AAMs with upregulated arginase type-1 activity, are important regulators of the subsequent healing phases. Both iNOS and arginase have increased activities at different phases of the wound healing process. These pathways are interdependent with L-arginine metabolites, ornithine, proline, urea and NO regulating the wound healing process through

feedback mechanisms. Throughout the wound healing process, topical NO donors appear to be of benefit in the healing of chronic wounds.

iNOS and NO production is increased during the earlier inflammatory and proliferative phases, whereas arginase and ornithine production appears important in the latter part of the proliferative phase. Ornithine forms polyamines that are chemotactic and proliferation mediators of endothelial, fibroblast and keratinocyte cells. During the remodelling phase, proline is an important structural amino acid in collagen formation with L-arginine required for appropriate cross-linking of the collagens.

Interventional studies using L-arginine and urea cycle intermediates have focused on wound healing outcome. Studies determining enzyme activities and cytokine profiles would elucidate mechanisms and provide further insight into these interventions.

References

1. Sen C, Gordillo G, Roy S, et al. Human skin wounds: a major and snowballing threat to public health and economy. Wound Repair Regen. 2009;17:763–71.
2. Enoch S, Leaper D. Basic science of wound healing. Surgery. 2005;23:37–42.
3. Singer A, Clark R. Cutaneous wound healing. N Engl J Med. 1999;341:738–46.
4. Eming SA, Krieg T, Davidson JM. inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol. 2007;127:514–25.
5. Robinson W. Use of urea to stimulate healing in chronic purulent wounds. Am J Surg. 1936;33:192–7.
6. Albina J, Mills C, Henry W, Caldwell M. Temporal expression of different pathways of L-arginine metabolism in healing wounds. J Immunol. 1990;144:3877–80.
7. Seifter E, Rettura G, Barbul A, Levenson SM. L-Arginine: an essential amino acid for injured rats. Surgery. 1978;84:224–30.
8. Wu G, Morris SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336:1–17.
9. Appleton J. L-Arginine: clinical potential of a semi-essential amino acid. Altern Med Rev. 2002;7:512–22.
10. Witte M, Thornton F, Tantry U, Barbul A. L-Arginine supplementation enhances diabetic wound healing: involvement of nitric oxide synthase and arginase pathways. Metabolism. 2002;51:1269–73.
11. Shi H, Efron D, Most D, et al. Supplemental dietary L-arginine enhances wound healing in normal but not inducible nitric oxide synthase knockout mice. Surgery. 2000;128:374–8.
12. Tong BC, Barbul A. Cellular and physiological effects of L-arginine. Mini Rev Med Chem. 2004;4:823–32.
13. Popovic PJ, Zeh HJ, Ochoa JB. L-Arginine and immunity. J Nutr. 2007;137:S1681–6.
14. Shi H, Wang S, Zhang G, Zhang Y, Barbul A. Supplemental L-arginine enhances wound following trauma/hemorrhagic shock. Wound Repair Regen. 2007;15:66–70.
15. Stechmiller JK, Childress B, Cowan L. L-Arginine supplementation and wound healing. Nutr Clin Pract. 2005;20:52–61.
16. Witte M, Barbul A. Role of nitric oxide in wound repair. Am J Surg. 2002;183:404–12.
17. Park JE, Abrams MJ, Efron PA, Barbul A. Excessive nitric oxide impairs wound collagen accumulation. J Surg Res. 2013;183:487–92.
18. Rizk M, Witte M, Barbul A. Nitric oxide and wound healing. World J Surg. 2004;28:301–6.
19. Kapoor M, Appleton I. Wound healing: abnormalities and future therapeutic targets. Curr Anaesth Crit Care. 2005;16:88–93.
20. Luo J, Chen A. Nitric oxide: a newly discovered function on wound healing. Acta Pharmacol Sin. 2005;26:259–64.
21. Amadeu TP, Seabra AB, de Oliveira MG, Monte-Alto-Costa A. Nitric oxide donor improves wound healing if applied on inflammatory and proliferative phase. J Surg Res. 2008;149:84–93.
22. Shearer J, Richards J, Mills C, Caldwell M. Differential regulation of macrophage L-arginine metabolism: a proposed role in wound healing. Am Physiol Soc. 1997;272:181–90.
23. Curran J, Winter D, Bouchier-Hayes D. Biological fate and clinical implications of L-arginine metabolism in tissue healing. Wound Repair Regen. 2006;14:376–86.
24. Campbell L, Saville CR, Murray PJ, et al. Local arginase 1 activity is required for cutaneous wound healing. J Invest Dermatol. 2013;133:2461–70.
25. Ponrasu T, Jamuna S, Mathew A, et al. Efficacy of L-proline administration on the early responses during cutaneous wound healing in rats. Amino Acids. 2013;45:179–89.
26. Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clin Dermatol. 2007;25:9–18.
27. Beldon P. Basic science of wound healing. Surgery. 2010;28:409–12.

28. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev. 2003;83:835–70.
29. Baum C, Arpey C. Normal cutaneous wound healing: clinical correlation with cellular and molecular events. Dermatol Surg. 2005;31:674–86.
30. Park JE, Barbul A. Understanding the role of immune regulation in wound healing. Am J Surg. 2004;187:11S–6.
31. Stoecklein VM, Osuka A, Lederer JA. Trauma equals danger—damage control by the immune system. J Leukoc Biol. 2012;92:539–51.
32. Kirk S, Hurson M, Regan M, Holt D, Wasserkrug H, Barbul A. L-Arginine stimulates wound healing and immune function in elderly human beings. Surgery. 1993;114:155–60.
33. Mosser DM, Zhang X. Activation of murine macrophages. Curr Protoc Immunol. 2008; Chapter 14.2.
34. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. 2010;32:593–604.
35. Lee R, Efron D, Tantry U, Barbul A. Nitric Oxide in the healing wound: a time course study. J Surg Res. 2001; 101:104–8.
36. Cobbold C, Sherratt L. Mathematical modeling of nitric oxide activity in wound healing can explain keloid and hypertrophic scarring. J Theor Biol. 2000;204:257–88.
37. Young A, McNaught C. The physiology of wound healing. Surgery. 2011;29:475–9.
38. Dhar I, Dhar A, Wu L, Desai K. L-Arginine attenuates methylglyoxal- and high glucose-induced endothelial dysfunction and oxidative stress by endothelial nitric-oxide synthase-independent mechanism. J Pharmacol Exp Ther. 2012;342:196–204.
39. Prabhakar SS, Zeballos GA, Montova-Zavala M, Leonard C. Urea inhibits inducible nitric oxide synthase in macrophage cell line. Am J Physiol. 1997;273:1882–8.
40. Boucher JL, Custot J, Vadon S, et al. N omega-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. Biochem Biophys Res Commun. 1994;203:1614–21.
41. Heffernan D, Dudley B, McNeil PL, Howdieshell TR. Local L-arginine supplementation results in sustained wound nitric oxide production and reductions in vascular endothelial growth factor expression and granulation tissue formation. J Surg Res. 2006;133:46–54.
42. Bohl K, Leibovich J, Belem P, et al. Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice. Wound Repair Regen. 2002;10:286–94.
43. Luk P, Sinha S, Lord R. Upregulation of inducible nitric oxide synthase (iNOS) expression in faster-healing chronic leg ulcers. J Wound Care. 2005;14:373–81.
44. Kim S, Huri D, Snyder S. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science. 2005;23:1966–70.
45. Wilgus TA, Bergdall VK, Tober KL, et al. The impact of cyclooxygenase-2 mediated inflammation on scarless fetal wound healing. Am J Pathol. 2004;165:753–61.
46. Igarashi K, Kashiwagi K. Polyamines: mysterious modulators of cellular functions. Biochem Biophys Res Commun. 2000;271:559–64.
47. Shi H, Fishel R, Efron D, et al. Effect of supplemental ornithine on wound healing. J Surg Res. 2002;106: 299–302.
48. Frias Soriano L, Lage Vazquez MA, Maristany CP, et al. The effectiveness of oral nutritional supplementation in the healing of pressure ulcers. J Wound Care. 2004;13:319–22.
49. Heyman H, Van de Looverbosch DE, Meijer EP, Schols JM. Benefits of an oral nutritional supplement on pressure ulcer healing in long-term care residents. J Wound Care. 2008;17:476–88.
50. Witte MB, Barbul A. General principles of wound healing. Surg Clin North Am. 1997;77:509.

Chapter 46
L-Arginine and Bacterial Translocation: Implications for Health

Mirelle Lomar Viana, Simone de Vasconcelos Generoso, Rosana das Graças Carvalho dos Santos, Valbert Nascimento Cardoso, and Maria Isabel Toulson Davisson Correia

Key Points

- Intestinal barrier dysfunction may allow the penetration of luminal antigens such as bacteria and their toxins, event known as bacterial translocation (BT).
- L-Arginine is an important and versatile amino acid with several immunological and trophic properties under stressful situations.
- It is believed that the main effects of L-arginine on bacterial translocation are due to nitric oxide synthase (NO) and arginase pathways.
- The L-arginine can prevent bacterial translocation due its effects by intestinal mucosa preservation and enhancement of immune response.
- L-Arginine supplementation is interesting for a number of critical clinical situations; however, in cases of sepsis, the use of L-arginine should be carefully evaluated, because an overproduction of NO can be deleterious to the patient.

Keywords Bacterial translocation • L-Arginine • Arginase • Nitric oxide synthase • Immune response • Intestinal barrier

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Abbreviations

^{99m}Tc-DTPA	99m-technetium diethylene triamine pentaacetic acid
^{99m}Tc-EDTA	99m-technetium ethylenediaminetetraacetic acid
AIDS	Acquired immune deficiency syndrome
ARG	L-Arginine
BT	Bacterial translocation
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
eNOS	Endothelial NOS
IL	Interleukin
INF	Interferon
iNOS	Inducible nitric oxide synthase
IO	Intestinal obstruction
L-NAME	NG- nitro-L-arginine methyl ester
L-NMMA	NG-monomethyl-L-arginine
L-NNA	NG-nitro-L-arginine
LPS	Lipopolysaccharide
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
ODC	Ornithine decarboxylase
SD	Standard deviation
sIgA	Secretory immunoglobulin A
SMCs	Suppressor myeloid cells
TCV	Total caloric value
TGI	Gastrointestinal tract
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TNF-α	Tumor necrosis factor

Introduction

The gastrointestinal tract (TGI) has a multitude of functions in addition to digestion. One important function is the ability to serve as a barrier against living organisms and antigens within the lumen, the so-called intestinal barrier function. The breakdown of this barrier may result in the crossing of viable bacteria and their products to mesenteric lymph nodes and more distant sites, a process known as bacterial translocation (BT) [1].

Localized and systemic disorders, such as ischemia, intestinal obstruction, shock, or sepsis, can damage the intestinal barrier, increasing mucosa permeability and allowing for BT. These disorders worsen the primary pathological event and may induce multiple organ failure and death [2, 3].

In order to avoid bacterial translocation, supplementation with immunomodulatory substrates seems essential [4]. In this context, L-arginine has been extensively studied.

L-Arginine or L-amino-5-guanidinovaleric acid (Fig. 46.1) is a basic conditionally essential amino acid with four nitrogen atoms that plays an important role in the transport, storage, and excretion of nitrogen and in the disposal of ammonia via the urea cycle. In catabolic states, L-arginine may become essential because of alterations in the overall metabolism [5]. L-Arginine provided by diet proteins is metabolized by the enterocytes and is responsible for various functions in the gut under stressful situations. It is well established that L-arginine can enhance morphometric aspects, such as the stimulation of enterocyte proliferation, the number of villi, and their height under such adverse conditions [6–9].

L-Arginine also plays a central role in the immune system, and it is especially important for macrophage and T-lymphocyte metabolism [10, 11]. Dietary L-arginine increases the activity of macrophages and enhances the CD4:CD8 ratio, the number of lymphocytes in Peyer's patches, as well as the levels of secretory immunoglobulin A (sIgA). It also increases the expression of the messenger ribonucleic acid (RNA) for the production of Th1 cytokines and Th2 cytokines, suggesting that L-arginine acts both in the cellular and humoral immune response [7, 10, 12].

L-Arginine is also a precursor for the synthesis of molecules with enormous biological importance including urea, ornithine, polyamines, nitric oxide, creatine, agmatine, and many others, besides being a major nitrogen carrier and a component of proteins [13]. It is believed that the main effects of L-arginine on bacterial translocation are due to nitric oxide (NO) and polyamines.

In this chapter we discuss bacterial translocation and the L-arginine mechanisms on this condition, emphasizing the study of the metabolites NO and polyamines.

Bacterial Translocation

Bacterial translocation involves the initial contact of bacteria with the intestinal wall, leading to cytokine production and subsequent inflammatory response. Once the bacteria enter the mucosa, they can be transported to distant organs through the circulation [1].

There are several evidences that BT is associated with the increased incidence of septic complications. Macfie et al. [13] showed 14 % of prevalence of BT, in 927 surgical patients, and a relationship with increased postoperative sepsis. In addition, Nieves et al. [14] observed a prevalence of 33 % of BT when evaluating lymph nodes of patient victims of abdominal trauma.

On the other hand, BT also occurs in healthy individuals. Low levels of bacterial translocation can be an important physiological event to prepare and alert the immune system of the host. Salzedas-Netto et al. [15] showed significant reduction of BT in animals previously challenged with the same bacteria used for BT induction. Therefore, it is possible that BT can occur to present lumen antigens to TGI, generating immunocompetent cells, a process known as oral tolerance [1].

There are three mechanisms involved in BT (Fig. 46.2): modified gut microbiota, reduced intestinal barrier function, and inadequate response of the host immune system [16].

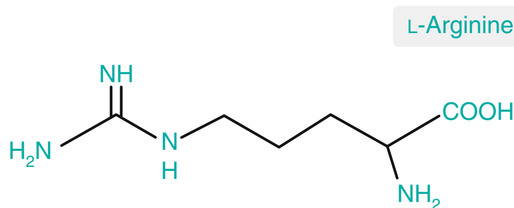

Fig. 46.1 Chemical structure of L-arginine. L-Arginine chemical structure, formed by two amino groups and one carboxyl group

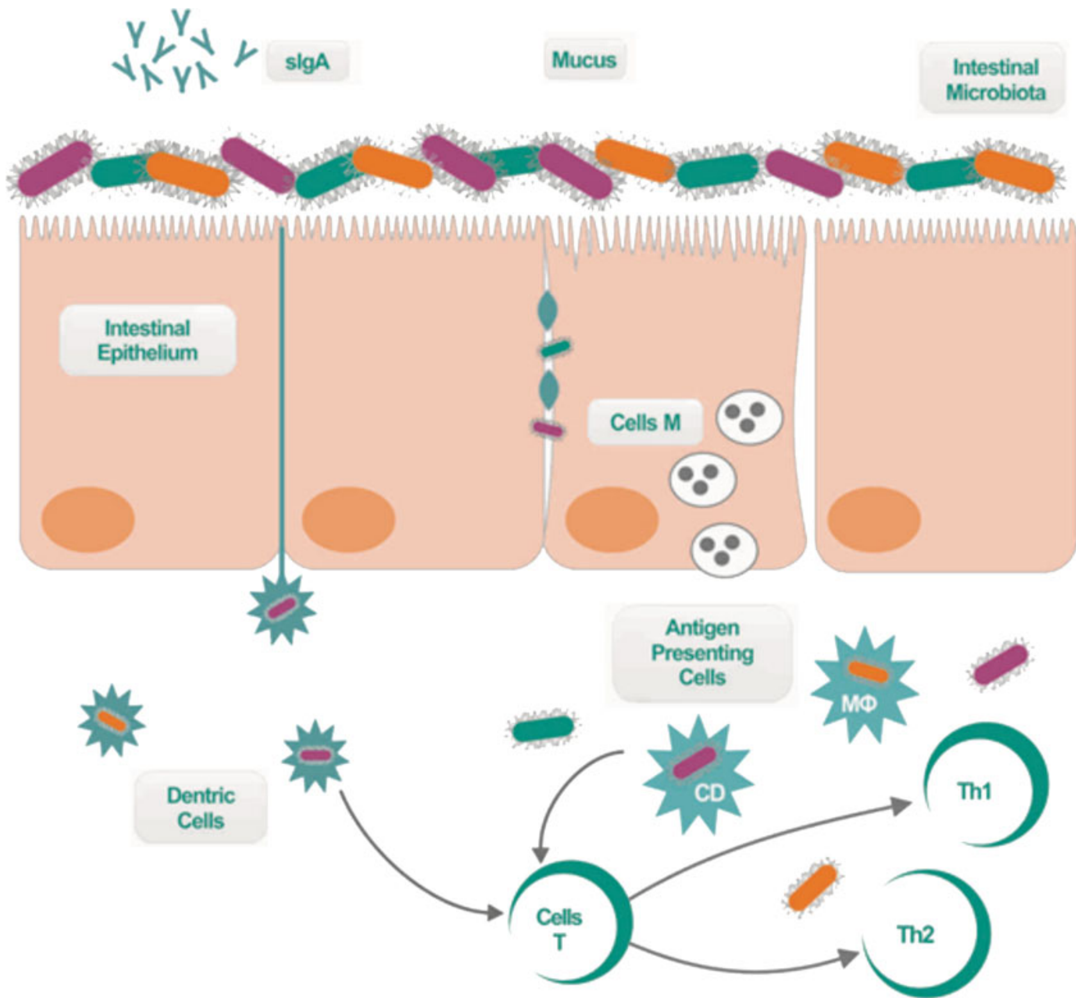

Fig. 46.2 Mechanisms of bacterial translocation. The main mechanisms of bacterial translocation. In cases of bacterial overgrowth, increase of the colonization leading to BT can occur; increases of paracellular permeability induce the opening of the tight junctions with the passage of bacteria, which may lead to the excessive inflammatory response. Lastly, the T cells induce the production of Th1 and Th2 cytokines with activation of B lymphocytes, thereby regulating the production of antibodies. When the system fails, BT can occur

Gut Microbiota

TGI is a dynamic organ with direct or indirect influences in the translocation of intestinal particles [16]. When the ecological balance is affected due to changes in the intestinal microbiota (e.g., use of antibiotics, decreased in gastric acidity and mucus production, obstructive jaundice, and changes in bowel motility), bacterial overgrowth is favored, increasing the colonization and leading to BT [16, 17]. Only a few strains of intestinal bacteria are able to translocate to the mesenteric lymph nodes, these include *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, enterococci, and some streptococci [16].

Parenteral nutrition with disuse of the TGI, malnutrition, diabetes, cirrhosis, and endotoxic shock also induce bacterial overgrowth, promoting subsequent translocation [17]. A recent study showed that 10 of 32

cirrhotic patients had bacterial DNA-positive in blood when intestinal bacterial overgrowth was positive. In a multivariate analysis, only the existence of intestinal bacterial overgrowth was the independent risk factor for bacterial DNA. The authors concluded that the increases in plasma endotoxin and bacterial DNA were directly associated with intestinal bacterial overgrowth in these patients [18].

Barrier Function

The normal intestinal epithelium acts as a selective barrier between the environment of the intestinal lumen and the lamina propria. This barrier consists of a single layer of epithelial cells, which are connected by firm junctions (*tight* junctions) [19]. The epithelium balance is also influenced by local factors such as mucus, stomach acid, pancreatic enzymes, bile, and the intestinal motility [17]. These factors together acting prevents the invasion of bacteria to the epithelium.

The tight junctions allow the selective paracellular permeability, which excludes passive movement of uncharged hydrophilic compounds, such as bacteria and macromolecules. The increase in paracellular permeability induces the opening of the tight junctions with the passage of bacteria, which may induce the excessive inflammatory response. Furthermore, damaged epithelia also allow the access of microorganisms to the bloodstream or lymph nodes through the transcellular pathway [20, 21].

These effects can be observed in critically ill patients and are associated with the high incidence of bacterial and toxin translocation from the intestinal lumen to the systemic circulation, causing infectious complications [22]. In a recent study, Founts et al. [23] showed increased intestinal permeability and bacteria translocation in mice with liver injury. These alterations were accompanied by decreased intestinal expression of the tight junctions and the protein occludin.

The evaluation of changes in intestinal permeability can be performed using specific tests designed to measure the intestinal barrier function, such as large molecules (i.e., sugars and drugs) to determine the paracellular passage from the intestine into the plasma and therefore into the urine [24]. Currently, substances labeled with radioactive isotopes, such as ^{99m}Tc-EDTA e ^{99m}Tc-DTPA, have also been used as an alternative, with good results [24]. In a recent murine study, intestinal permeability of mice undergoing intestinal obstruction was assessed by the determination of the percentage of 99mTc-DTPA found in the blood of the animals. There was good sensitivity of the method to evaluate changes in cell permeability [6].

Immune System

The intestinal tract is an active immune organ, containing several factors involved in the immune response [17]. The gut-associated lymphoid tissue (GALT) is the largest immune organ in the body containing 25 % of the total mucosal immune cell.

The GALT covers the epithelium, inside of the lamina propria and submucosa, including more than a half of lymphoid cells in Peyer's patches, follicle-associated epithelium (consisting of M cells), intra-epithelial lymphocytes, macrophages, neutrophils, and dendritic cells [16]. The M cells are a major component of the GALT and often constitute the first defense line against the passage of microorganisms from the intestinal lumen into the epithelium. M cells are an unusual type of epithelial cells, because they don't have on their surface microvilli or glycocalyx. The M cells have very long cytoplasmic extensions into the lamina propria forming a pocket within the antigens which are phagocytized by macrophages and then penetrate into Peyer's patches [25].

When the intestinal immune system acts, the antigens are transported by M cells to the antigen-presenting cells (macrophages and dendritic cells) into the mesenteric lymph nodes. Then, the processing and presentation of antigens to $CD4^+$ T lymphocytes and inactive B cells occur. These cells are the second line of defense against translocation and initiate the production of cytokines [26].

T cells induce the production of TH1 and TH2 cytokines. Th1 cytokines (IL-2, INF, and TNF-α) stimulate cellular immunity, resulting in activation of macrophages, neutrophils, and T lymphocytes, especially $CD8^+$ T lymphocytes. Th2 cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) are responsible for the activation of B lymphocytes, thereby regulating the production of antibodies [26].

It is widely accepted that cytokine levels after an inflammatory insult, such as lipopolysaccharide (LPS), are characterized by an initial increase and subsequent decrease in TNF-α levels, followed by IL-1, IL-6, and IL-10, respectively. However many septic patients exhibit high levels of TNF-α, IL-1, and IL-6 until death [27]. The presence of sIgA immunoglobulin enhances barrier function of the intestine, playing a key role in the formation of the immune response to microbial colonization [28]. Mucosal secretions, rich in sIgA, can bind to bacteria preventing adherence and mucosal colonization [28].

Nutrient-Related Prevention and Therapy Against Bacterial Translocation

A variety of strategies have been investigated for the treatment of bacterial translocation. Most of them are linked with the ability of some compounds and nutrients to act in the immune system modulation or preventing the bacterial overgrowth, thus maintaining the intestinal barrier.

Aydocan et al. [29] showed that enteral diets supplemented with L-arginine, nucleotides, and omega-3 fatty acids reduce bacterial translocation. The investigators concluded that this effect might be related to improvement in the immune function resulting from the use of immunonutrients.

Enteral diets with glutamine resulted in less intestinal lesions and weight loss, improved nitrogen balance, and reduced bacterial translocation in a colitis model [30].

In a recent study, Sánches et al. [31] showed that treatment with probiotics decreases bacterial translocation, the pro-inflammatory state, and the ileal oxidative damage and increased ileal occludin expression in rats with experimental cirrhosis.

Data of our research group showed that immunomodulatory agents such as glutamine, citrulline, and L-arginine were able to reduce bacterial translocation in an animal model of intestinal obstruction. The probable mechanisms involved are related to the maintenance of the intestinal barrier and the regulation of the immune response [32–34].

Implications of L-Arginine Metabolic Pathways in Bacterial Translocation

L-Arginine is an important and versatile amino acid with several immunological and trophic properties under stressful situations. The L-arginine metabolism generates essential nitrogen compounds as creatine, polyamines, agmatine, and NO (Fig. **46.3**). L-Arginine can be converted in the liver into creatine by L-arginine-glycine amidinotransferase. The creatine is transported into muscle tissue serving as a phosphate carrier and adenosine triphosphate regenerator [**13**].

The exact beneficial effects and mechanisms are still not well described. However, there are strong evidences indicating that L-arginine metabolites such as polyamines and NO are related to its role.

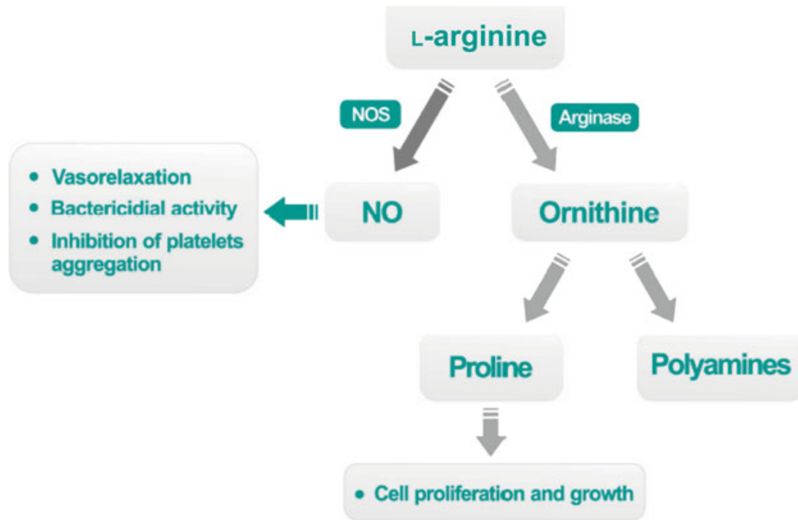

Fig. 46.3 L-Arginine pathways and metabolite. The main pathways of L-arginine metabolism. The nitric oxide synthase (NOS) pathway with nitric oxide (NO) production of nitric oxide (NO) and arginase pathway producing proline and polyamines, both with different actions in organism

L-Arginine and the Nitric Oxide Pathway

Nitric oxide (NO) is a short-live free radical and a very small compound that diffuses freely within cells from its sites of formation to the sites of action. In solution, the NO has a half-life of 0.1–10 s before its transformation into nitrite (NO_2) and nitrate (NO_3). It is an important intracellular signaling molecule and it acts as a biological mediator similar to neurotransmitters in the neuronal system. NO can also regulate the blood vessel tone in vascular systems, and it is an important host defense effector in the immune system since it can act as a cytotoxic agent under pathological processes [35].

The biosynthesis of NO is carried out by L-arginine and the molecular oxygen, utilizing nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) as an electron donor. The overall reaction utilizes $NADPH_2$ and Ca^{2+} as cofactors and consists of two steps. The conversion of L-arginine to NO and L-citrulline via N^w-hydroxy-L-arginine, that is an intermediate which may also function as a substrate for nitric oxide synthase (NOS) [36]. The produced citrulline can be used in the synthesis of L-arginine in the kidney, endothelial cells, macrophages, and cells of the peripheral nervous system [37].

NO is enzymatically produced by three different NO synthases (NOS). Neuronal NOS (nNOS) (NOS1) and endothelial NOS (eNOS) (NOS3) are constitutive enzymes expressed in the plexus myentericus and the vascular endothelium of the gut, respectively. They produce small amounts of NO in response to increases in intracellular calcium. The third enzyme, inducible NOS (iNOS) (NOS2), is normally not expressed, but is produced in larger amounts in macrophages and other tissues in response to pro-inflammatory mediators, such as bacterial membrane lipopolysaccharides, endotoxins, and inflammatory cytokines. It is also calcium-independent and produces NO over prolonged periods of time [38].

NOS enzymes produce NO from L-arginine, and thus competitive L-arginine analogues may prevent them from producing NO. These analogues include NG-monomethyl-L-arginine (L-NMMA), NG-nitro-L-arginine (L-NNA), and NG-nitro-L-arginine methyl ester (L-NAME). These are nonselective inhibitors. Aminoguanidine was the first class of specific iNOS inhibitors; however, it is not a potent inhibitor [39–42].

Nitric oxide is involved in a variety of biological functions throughout the body. It is a potent vasoactive regulator and the main factor of endothelium-derived relaxation. By promoting vasodilation, it increases blood flow to injured tissues [3].

Furthermore, NO plays an important role in the immune response acting on cells of the innate immune response such as monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils. Therefore, during inflammation, it acts mediating cytotoxicity and supporting the nonspecific host defense [43].

Nitric oxide appears to play a dual function in the body, its beneficial or destructive effects depend on the amount produced [43]. The nitric oxide derived by activated macrophages is an important mediator of the inflammatory response; however, when it acts as an oxidant, the excessive production of NO is detrimental to the tissues [44]. Increased expression of iNOS has been demonstrated in disorders such as destruction of the intestinal mucosa, sepsis, and clinical conditions associated with all these disorders [45].

Sepsis and Nitric Oxide

Sepsis is defined as a systemic response to an infection. It is a major health problem because of its significant morbidity and overall mortality rate of 30 % and it generally requires intensive care treatment [36]. Sepsis can be a consequence of bacterial translocation with bacteria after bacteria having penetrated the mucosa and being transported to distant organs through the circulation [20].

The role of nitric oxide on sepsis is controversial. Endotoxins and TH1 cytokines initiate the cascade that causes increased expression of NOS, especially iNOS, in several tissues (lung, liver, intestine) resulting in systemic hypotension. The reversal of hypotension has been the focus of septicemia treatment since it is associated with increased mortality in septic patients. Thus, it is important to evaluate the potential benefits of NOS inhibition in this context [40].

Different explanations may be suggested for the dual personality of NO during sepsis. First of all, there is no doubt about the detrimental effect of excessive NO on vasorelaxation, hypotension, and shock. The NO-mediated hypotension leads to severe hypoxia in peripheral vital organs, resulting in progressive organ failure. Second, increased NO may also provide certain benefits to the patient during sepsis. Increased NO release protects the kidney by causing local vasodilation and by inhibiting platelet aggregation and leukocyte adhesion. In addition, NO may also exert protective effects in other organs via its capacity to counteract oxidative stress, shut off apoptosis, prevent platelet aggregation and leukocyte adhesion, induce anti-inflammatory gene expression, and kill pathogens [46].

Sundrani et al. [39] in a sepsis model demonstrated that nonselective NOS inhibition with L-NMA actually reverses the hypotension but increases leukocyte adhesion and rolling. However, Petersson et al. [41] used the same inhibitor on a colitis model and showed that nonselective NOS inhibition caused a reduction in blood flow during acute inflammation. Thus, the NO was considered crucial for tissue perfusion during the inflammatory process and contributed to the maintenance of microvascular flow, adequate supply of oxygen and nutrients, as well as protection of the endothelium against oxidative stress.

Our group has assessed bacterial translocation (BT) in an intestinal obstruction model and seven day treatment with L-arginine and L-NAME led to absence of L-arginine beneficial effects with increased BT when NO is inhibited. These results point out that L-arginine acts on BT by the NO pathway [38]. Many authors claim that NO synthesis is part of the inflammatory response, to minimize ischemia and exacerbated coagulation while concomitantly fighting bacteremia. Side effects of inhibiting NO synthesis may be more pronounced than those caused by itself and its metabolites, as previously thought [11, 41].

Clinical studies were also performed to evaluate NO action. Avontur et al. [47] observed maintenance of vascular tone and blood pressure in septic patients undergoing nonselective inhibition (using L-NAME). However, there was no clinical improvement or reduction of mortality in these patients compared to the control group. In a randomized, double-blind, placebo-controlled study, Lopez et al. [48]

evaluated the effects of the inhibitor 546C88, a nonselective NOS, and observed that septic patients treated with this inhibitor showed a higher mortality rate than patients in the placebo group. On the other hand, continuous supplementation of L-arginine (the NO precursor) in septic patients did not affect hemodynamic, cardiac, and pulmonary parameters [41]. Latter, in 2009, Luiking et al. [38] observed that septic patients had a reduction in NO synthesis because L-arginine was shifted to the urea synthesis. In this context, it should be considered that increasing NO synthesis is only one of the many factors that contribute to the septicemia process. The isolated inhibition of NO synthesis was not sufficient to stop or reduce the cascade of events that lead to the exacerbated activation of the immune system.

The L-arginine/NO controversy will remain until more studies have consistently confirmed either benefit or detriment of L-arginine supplementation. Zhou and Martindale [49], considering a review from animals and human available data, concluded that L-arginine appears to be safe and potentially beneficial for most all hemodynamically stable ICU populations, at doses delivered in immune modulation formulas.

Arginase-Polyamine Pathway

The enzyme arginase hydrolyzes L-arginine to L-ornithine and urea. There are two types of arginase: arginase-I and arginase-II. The arginase-I is a cytosolic enzyme present in the liver, related to the detoxification of ammonia and urea synthesis. The arginase-II is found in extrahepatic cells' mitochondria, such as macrophages, kidney, intestinal, and endothelial cells, and is involved in the regulation of ornithine, proline, and glutamate cell synthesis [50, 51].

The enzyme ornithine decarboxylase (ODC), responsible for polyamines biosynthesis, is high in the small intestinal mucosa and plays an important role in polyamine metabolism. Polyamines are cationic molecules with low molecular weight. Putrescine, spermidine, and spermine are essential composites for cellular proliferation and differentiation [52].

The usual Western diet daily provides adequate polyamines supply. Meats are rich sources of spermine, while plant foods are high in putrescine and spermidine. Polyamines used by the human body can be also originated from TGI secretions, enterocyte desquamation, or bacterial synthesis [44, 53]. Polyamines, both exogenous as endogenous, are completely absorbed and directed to tissue growth or repair [53].

Polyamines regulate genic expression, signal transduction, ion channel function, DNA and proteins synthesis, and apoptosis. Thus, they are essential in cell proliferation, differentiation, and function. Under cell growth stimulation, the induction of polyamine synthesis is a key factor, preceding DNA replication and protein synthesis [44, 53].

Polyamines also act on fibroblasts, inducing wound healing and extracellular matrix proliferation [53].

There are few studies assessing the clinical effects of polyamines, especially in humans. Several studies have been carried out with animal models, which are presented in Table 46.1. Most of them have shown positive and encouraging results.

The correct L-arginine supply is essential to maintain the adequate immune function, considering the intense activity of arginase in the suppressor myeloid cells (SMCs; immature cells of the myeloid lineage that may differentiate into macrophages, dendritic cells, or granulocytes after stimulation). This reduces the availability of L-arginine, and therefore, it inhibits the proliferation of T cells, in addition to reducing the synthesis of IFN-γ and interleukin-2 (IL-2), growth factors, to T-lymphocyte function. The addition of L-arginine sharply increased the capacity for proliferation and the production of IFN-γ, IL-4, and IL-10 [8, 59, 60].

Thus, it is reasonable that the effects of the arginase-polyamine pathway in bacterial translocation are connected with the ability to maintain the integrity and regeneration of the intestinal barrier rather than the effects on the immune response, assuming that these are the two main mechanisms by which L-arginine helps avoid bacterial translocation [61].

Table 46.1 Summary of studies relating the possible associations between arginase and bacterial translocation prevention

Pathology models	Conclusions	References
Colon anastomosis	Arginase type I activity, protein, and mRNA expression were significantly upregulated at the anastomosis, suggesting metabolism of L-arginine via arginase to polyamines and proline to provide substrate for collagen synthesis and cell proliferation	Witte et al. [54]
Extensive intestinal resection	L-Ornithine decarboxylation was markedly increased. The fact may be related to increased de novo polyamine synthesis in resected animals, suggesting an adaptation mechanism	Lardy et al. [55]
Ischemia/reperfusion	Ornithine alpha-ketoglutarate administration to rats did not prevent ischemic damage of the intestinal mucosa, but it accelerated the repair of the mucosa during reperfusion	Duranton et al. [56]
Small bowel transplantation	The addition of orally supplementation of ornithine alpha-ketoglutarate, significantly reduced bacterial translocation and improved the protein/DNA index as well as the weight gain in rats	De Oca et al. [57]
Colitis	The results indicate arginase protection in colitis by enhancing the generation of polyamines in addition to competitive inhibition of iNOS	Gobert et al. [58]

L-Arginine in Bacterial Translocation

L-Arginine and Intestinal Barrier

The L-arginine effects in maintaining the integrity of the intestinal mucosa have been the focus of several investigations [9, 62]. In the ischemia/reperfusion model, L-arginine improved the weight of duodenal, jejunal, and ileal mucosa. The rate of jejunal cell proliferation in rats reduced or prevented the morphological and functional damage of the intestine and also worked on protecting the lipid peroxidation and maintenance of tissue levels of glutathione, a powerful free radical scavenger [8, 59, 60].

Chang et al. [61] observed that this amino acid supplementation increased the number of villi and reduced the intensity of the intestinal mucosa lesions in an intestinal obstruction model. L-Arginine also presented a protective effect on the intestinal mucosa during endotoxemia caused by LPS, inducing increased proliferation and maintenance of villus enterocytes [8]. In experimental models of radiation-induced enteritis, the number of animals with positive cultures and the number of bacteria present in mesenteric lymph nodes were decreased when the L-arginine was administered after radiation [9, 62].

A study conducted by our group [6] evaluated bacterial translocation and intestinal permeability in mice after treatment with L-arginine. Mice were divided into three groups, treated for seven days before surgical intervention with isocaloric and isoproteic diets. The L-arginine group (ARG) received a diet containing 2 % L-arginine, while animals in the intestinal obstruction (IO) received no supplementation and control groups (sham) received standard chow diet. In order to evaluate the intestinal permeability, after the seven days, the animals were gavaged with radiolabeled diethylenetriamine-pentaacetic acid solution and, after 90 min, they were anesthetized and the ileum ligated. At 4, 8, and 18 h, the blood was collected for radioactivity determination and permeability analysis. In others to evaluate bacterial translocation, another group of animals, also treated for 7 days, was gavaged with 10^8 CFU/mL of 99m-technetium (99mTc) *E. coli*. After intestinal obstruction, BT was determined by the uptake of 99mTc *E. coli* in the mesenteric lymph nodes, blood, liver, spleen, and lungs, 18 h after the operation. The results are shown in Fig. 46.4 and Table 46.2. The data show that L-arginine supplementation reduced intestinal permeability and BT to physiological levels [63].

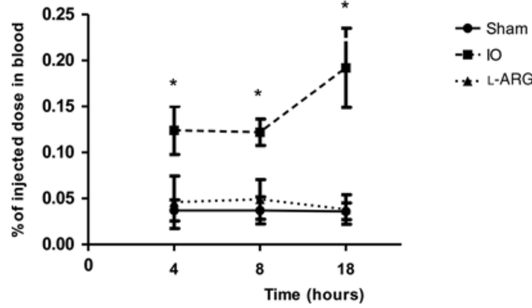

Fig. 46.4 Intestinal permeability. Intestinal permeability after 4, 8, and 18 h of intestinal obstruction (IO). The IO group showed enhancement in intestinal permeability in the times of 4–18 h. The treatment with L-arginine was able to prevent the increase of intestinal permeability. Errors bars show the SD. Data are expressed as mean ± SD ($n = 5$). $^{*}p < 0.05$

Table 46.2 Biodistribution of *Escherichia coli* labeled with 99m-technetium after 18 h of intestinal obstruction

Organ/blood	Sham (cpm/g)	IO (cpm/g)	ARG (cpm/g)
Blood	41.30[a]	175.79[b]	60.60[a]
Liver	204.8[a]	1154.49[b]	556.5v2[a]
Spleen	109.09[a]	1022.22[b]	390.00[a]
Lung	14.29[a]	794.12[b]	110.52[a]
MLN	166.67[a]	660.00[b]	100.00[a]

The results are expressed in SEM of counts per minute/g tissue (cpm/g). Different letters on the same line indicate statistically significant differences ($p < 0.05$). Mesenteric lymph nodes (MLN)

L-Arginine and Immune Response

L-Arginine supplementation increases the immune function in humans and animal models acting in the host defense, inflammation, wound healing, and several other pathophysiological adaptations [8, 11].

L-Arginine has a central role in the immune system and its metabolism is important for macrophages and T-lymphocyte function. L-Arginine participates in the inflammatory response through two principal mechanisms: NO production (via iNOS) in the macrophages and L-arginine utilization for proliferation and activation of T lymphocytes. When TH2 immune response predominates, L-arginine may also follow the arginase pathway. In the latter, the inflammatory modulation is due to the production of ornithine (proline and polyamine precursors) and by the regulation of L-arginine availability, thus modulating NO synthesis and proliferation of T lymphocytes [63].

The exogenous L-arginine supply increases lymphocyte proliferation, especially T-helper cells which induce the appropriate cytokine production, and increases phagocytosis by enhancing the activity of macrophages and natural killer cells [11, 65].

The L-arginine effect on the intestinal mucosal immunity is evident. Shang and colleagues [7], in a study performed with septic rats, found that daily administration of enteral L-arginine, 2 % of the total caloric value (TCV), increased the number of lymphocytes in Peyer's patches, and secretory IgA levels tended to be higher in the groups treated with L-arginine before the induction of sepsis, suggesting the importance of L-arginine on the humoral immune response. The same research group showed, in another study, that L-arginine supplementation increases the expression of mRNA for the production of Th1 cytokines (INF-γ and IL-2) and Th2 cytokines (IL-4 and IL-10) [64].

Kang et al. [65] developed a meta-analysis enrolling data from 11 trials involving 321 patients, with the purpose to evaluate L-arginine effects on immune function in diseases like gastrointestinal

malignancies, pressure ulcers, head and neck cancer, HIV/AIDS, head and neck cancer, unstable angina undergoing angioplasty, older people undergoing vaccination against streptococcus pneumonia, and burns. The data showed that the L-arginine-supplemented group had a significantly greater CD4+ T-cell proliferation; however, the CD4/CD8 ratio was not statistically significant between the L-arginine-supplemented and control groups. Furthermore, incidence of infectious complications was lower in the L-ARG with statistical significance. The group also showed that patients with L-arginine supplementation had a shorter length of hospital stay; however, this was not statistically significant.

Conclusions

Intestinal permeability changes are associated with higher bacterial translocation levels, commonly associated with sepsis. Local immune response and cytokines are involved in modulating intestinal permeability and BT to avoid increased inflammation. The host immune response plays a major role in the overall process. Thus, alternative treatment with immunomodulator agents would be beneficial in this clinical situation.

The L-arginine, due the arginase and NO pathways, can prevent bacterial translocation because of its potential effects mediated by mechanisms of intestinal mucosa preservation, reducing intestinal permeability and promoting tissue integrity and enhancement of immune response, considering its particular importance for macrophage and B lymphocyte production and the ability of L-arginine in modulating the immune response to balance the serum production of pro- and anti-inflammatory cytokines. In addition, L-arginine increases IgA secretion in the intestinal mucosa, contributing also for local immune response.

Furthermore, it is important to consider that one size does not fit all, and L-arginine supplementation should individually be assessed considering the several clinical situations.

In this way, the use of L-arginine is interesting for a number of clinical situations such as surgery, trauma, and burn patients, for example, potentially avoiding bacterial translocation. However, in cases of sepsis, an overproduction NO state, the use of L-arginine should be carefully evaluated, since the excesses of this metabolic intermediate can be deleterious to the patient.

References

1. Gatt M, Reddy BS, MacFie J. Review article: bacterial translocation in the critically ill-evidence and methods of prevention. Aliment Pharmacol Ther. 2007;25:741–57.
2. Ding L, Li J, Li Y, et al. Intestinal barrier damage caused by trauma and lipopolysaccharide. World J Gastroenterol. 2004;10:2373–8.
3. Samel S, Keese M, Laning, et al. Supplementation and inhibition of nitric oxide synthesis influences bacterial transit time during bacterial translocation in rats. Shock. 2003;19:378–82.
4. Tsuei BJ, Bernard AC, Barksdale AR, et al. Supplemental enteral L-arginine is metabolized to ornithine in injured patients. J Surg Res. 2005;123:17–24.
5. Duggan C, Gannon J, Walker WA. Protective nutrients and functional foods for the gastrointestinal tract. Am J Clin Nutr. 2002;75:789–808.
6. Viana ML, Santos RG, Generoso SV, et al. Pretreatment with L-arginine preserves intestinal barrier integrity and reduces bacterial translocation in mice. Nutrition. 2010;26:218–23.
7. Shang HF, Wang YY, Lai YN, et al. Effects of L-arginine supplementation on mucosal immunity in rats with septic peritonitis. Clin Nutr. 2004;23:561–9.
8. Sukhotnik I, Mogilner J, Krausz MM, et al. Oral L-arginine reduces gut mucosal injury caused by lipopolysaccharide in rat. J Surg Res. 2004;122:256–62.
9. Ersin S, Tuncyurek P, Esassolak M, et al. The prophylactic and therapeutic effects of glutamine and L-arginine enriched diets on radiation induced enteritis in rats. J Surg Res. 2000;89:121–5.

10. Stechmiller BC, Childress B, Porter T. L-Arginine immunonutrition in critically ill patients: a clinical dilemma. Am J Crit Care. 2004;13:17–23.
11. Suchner U, Heyland DK, Peter K. Immune-modulatory actions of L-arginine in the critically ill. Br J Nutr. 2002;87:121–32.
12. Yeh CL, Yeh SL, Lin MT, et al. Effects of L-arginine-enriched total parenteral nutrition on inflammatory-related mediator and T-cell population in septic rats. Nutrition. 2002;18:631–5.
13. Macfie J, Reddy BS, Gatt M, et al. Bacterial translocation studied in 927 patients over 13 years. Br J Surg. 2006;93:87–93.
14. Nieves Jr C, Langkamp-Henken B. L-Arginine and immunity: a unique perspective. Biomed Pharmacother. 2002;56:471–82.
15. Salzedas-netto AA, Silva RM, Martins JL, et al. Can bacterial translocation be a beneficial event? Transplant Proc. 2006;38:1836–7.
16. Wiest R, Rath HC. Bacterial translocation in the gut. Best Pract Res Clin Gastroenterol. 2003;17:397–425.
17. Berg RD. Mechanisms promoting bacterial translocation from the gastrointestinal tract [Monograph]. Herborn-Diel: Old Herborn University Seminar, Department of Microbiology and Immunology; 2001.
18. Jun DW, Kim KT, Lee OY, et al. Association between small intestinal bacterial overgrowth and peripheral bacterial DNA in cirrhotic patients. Dig Dis Sci. 2010;55:1465–71.
19. Clavel T, Haller D. Molecular interactions between bacteria, the epithelium, and the mucosal immune system in the intestinal tract: implications for chronic inflammation. Curr Issues Intest Microbiol. 2007;8:25–43.
20. Macfie J. Enteral versus parenteral nutrition: the significance of bacterial translocation and gut-barrier function. Nutrition. 2000;16:606–11.
21. Balzan S, Quadros CA, Cleva R, et al. Bacterial translocation: overview of mechanisms and clinical impact. J Gastroenterol Hepatol. 2007;22:464–71.
22. De-Souza DA, Greene LJ. Intestinal permeability and systemic infections in critically ill patients: effect of glutamine. Crit Care Med. 2005;33:1125–35.
23. Fouts DE, Torralba M, Nelson KE, et al. Bacterial translocation and changes in the intestinal microbiome in mouse models of liver disease. J Hepatol. 2012;56:1283–92.
24. Katouzian F, Sblattero D, Tarcisio N, et al. Dual sugar gut-permeability testing on blood drop in animal models. Clin Chem Acta. 2005;352:191–7.
25. Sawai T, Goldstone N, Drongowski RA, et al. Effect of secretory immunoglobulin A on bacterial translocation in an enterocyte-lymphocyte co-culture model. Pediatr Surg Int. 2001;17:275–9.
26. Kudsk KA. Glutamine: more evidence, more promise. J Parenter Enteral Nutr. 2008;32:492–4.
27. Marchiando AM, Granham WV, Turner JR. Epithelial barriers in homeostasis and disease. Annu Rev Pathol. 2010;5:119–44.
28. Sano T, Ajiki T, Takeyama Y, et al. Internal biliary drainage improves decreased number of gut mucosal T lymphocytes and MAdCAM-1 expression in jaundiced rats. Surgery. 2004;136:693–9.
29. Aydogan A, Kismet K, Kilicoglu B, et al. Effects of various enteral nutrition solutions on bacterial translocation and intestinal morphology during the postoperative period. Adv Ther. 2007;24:41–9.
30. Wang F, Zhao HY, Zhang ST, et al. Effect of enteral nutrition on dextran sulfate sodium induced colitis in rats. J Dig Dis. 2011;12:453–8.
31. Sánchez E, Nieto JC, Boullosa A, et al. VSL#3 probiotic treatment decreases bacterial translocation in rats with carbon tetrachloride-induced cirrhosis. Liver Int. 2014;35(3):735–45.
32. Batista MA, Nicoli JR, Martins FS. Pretreatment with citrulline improves gut barrier after intestinal obstruction in mice. JPEN J Parenter Enteral Nutr. 2012;36:69–76.
33. Viana ML, Dos Santos R, Generoso SV, et al. The role of L-arginine-nitric oxide pathway in bacterial translocation. Amino Acids. 2013;45:1089–96.
34. Quirino IE, Carneiro MB, Cardoso VN, et al. L-Arginine supplementation induces arginase activity and inhibits TNF-α synthesis in mice spleen macrophages after intestinal obstruction. JPEN. 2014.
35. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS international sepsis definitions conference. Crit Care Med. 2003;31:1250–6.
36. Cynober L, Le Boucher J, Vasson MP. L-Arginine metabolism in mammals. Nutr Biochem. 1995;6:402–13.
37. Hallemeesch MM, Lamers WH, Deutz NEP. Reduced L-arginine availability and nitric oxide production. Clin Nutr. 2002;21:273–9.
38. Luiking YC, Poeze M, Ramsay G, et al. Reduced citrulline production in sepsis is related to diminished de novo L-arginine and nitric oxide production. Am J Clin Nutr. 2009;89:142–52.
39. Sundrani R, Easington CR, Mattoo A, Parrillo JE, Hollenberg SM. Nitric oxide synthase inhibition increases venular leukocyte rolling and adhesion in septic rats. Crit Care Med. 2000;28:2898–903.
40. Luiking YC, Poeze M, Ramsay G, et al. The role of L-arginine in infection and septicemia. JPEN J Parenter Enteral Nutr. 2005;29:70–4.

41. Petersson J, Schreiber O, Steege A, et al. eNOS involved in colitis-induced mucosal blood flow increase. Am J Physiol Gastrointest Liver Physiol. 2007;293:1281–7.
42. Wu Y, Kudsk KA, Dewitt RC, et al. Route and type of nutrition influence IgA-mediating intestinal cytokines. Ann Surg. 1999;229:662–8.
43. Bogdan C. Nitric oxide and the immune response. Nat Immunol. 2001;2:907–16.
44. Flynn NE, Meininger CJ, Haynes TE, et al. The metabolic basis of L-arginine nutrition and pharmacotherapy. Biomed Pharmacother. 2002;56:427–38.
45. Luiking YV, Deutz NEP. Isotopic investigation of nitric oxide metabolism in disease. Curr Opin Clin Nutr Metab Care. 2003;6:103–8.
46. Cauwels A. Nitric oxide in shock. Kidney Int. 2007;72:557–65.
47. Avontur J, Nolthenius RPT, Van Bodegom JW, et al. Prolonged inhibition of nitric oxide synthesis in severe shock—a clinical study. Crit Care Med. 1998;26:660–7.
48. López A, Lorente JA, Steingrub J, et al. Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock. Crit Care Med. 2004;32:21–30.
49. Zhou M, Martindale RG. L-Arginine in the critical care setting. J Nutr. 2007;137:1687–92.
50. Zaloga GP, Siddiqui R, Terry C, Marik PE. L-Arginine: mediator or modulator of septicemia? Nutr Clin Pract. 2004;19:201–15.
51. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, et al. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68.
52. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009;158:638–51.
53. Moinard C, Cynober L, De Bandt JP. Polyamines: metabolism and implications in human diseases. Clin Nutr. 2005;24:184–97.
54. Witte MB, Vogt N, Stuelten C, et al. Arginase acts as an alternative pathway of L-arginine metabolism in experimental colon anastomosis. J Gastrointest Surg. 2003;7:378–85.
55. Lardy H, Mouillè B, Thomas M, et al. Enterocyte metabolism during early adaptation after extensive intestinal resection in a rat model. Surgery. 2004;135:649–56.
56. Duranton B, Schleiffer R, Gosse F, et al. Preventive administration of ornithine alpha-ketoglutarate improves mucosal repair after transient ischemia in rats. Crit Care Med. 1998;26:120–5.
57. De Oca J, Millat E, Dominguez MA, et al. Selective bowel decontamination, nutritional therapy and bacterial translocation after burn injury. Clin Nutr. 1993;12:355–9.
58. Gobert AP, Cheng Y, Akhtar M, et al. Protective role of arginase in a mouse model of colitis. J Immunol. 2004;173:2109–17.
59. Cintra AE, Martins JL, Patrício FR, et al. Nitric oxide levels in the intestines of mice submitted to ischemia and reperfusion: L-arginine effects. Transplant Proc. 2008;40:830–5.
60. Sayan H, Ozacmak VH, Altaner S, et al. Protective effects of L-arginine on rat terminal ileum subjected to ischemia/reperfusion. J Pediatr Gastroenterol Nutr. 2008;46:29–35.
61. Chang T, Lu R, Tsai L. Glutamine ameliorates mechanical obstruction-induced intestinal injury. J Surg Res. 2001;95:133–40.
62. Gurbuz AT, Kunzelman J, Ratzer EE. Supplemental dietary L-arginine accelerates intestinal mucosal regeneration and enhances bacterial clearance following radiation enteritis in rats. J Surg Res. 1998;74:149–54.
63. Bansal V, Ochoa JB. L-Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care. 2003;6:223–8.
64. Shang HF, Hsu CS, Yeh CL, Pai M, Yeh S. Effects of L-arginine supplementation on splenocyte cytokine mRNA expression in rats with gut-derived sepsis. World J Gastroenterol. 2005;11:7091–6.
65. Kang K, Shu XL, Zhong JX, Ting-Ting Y, Tao L. Effect of L-arginine on immune function: a meta-analysis. Asia Pac J Clin Nutr. 2014;23:351–9.

Chapter 47
L-Arginine in Pulmonary Tuberculosis

Anna P. Ralph

Key Points

- L-Arginine has important actions in the human immune response to *Mycobacterium tuberculosis*, the bacterium which causes tuberculosis.
- L-Arginine is deficient in patients with tuberculosis.
- The immunological actions of L-arginine in tuberculosis are chiefly indirect, via its metabolite nitric oxide, but also direct, via expression of a T cell receptor (TCR) component.
- L-Arginine is hypothesised to be a beneficial supplementary treatment for people with tuberculosis, but clinical trials to date have shown no benefit.
- Nitric oxide production from L-arginine in lung macrophages can be measured in exhaled breath; in one study, exhaled nitric oxide concentrations were associated with tuberculosis treatment outcomes.
- Further trials of L-arginine supplementary treatment for people with tuberculosis are warranted, using new dosing or administration regimens.

Keywords L-Arginine • Tuberculosis • Nitric oxide • Adjunctive therapy • Exhaled nitric oxide

Abbreviations

ADMA	Asymmetric dimethylarginine
FE_{NO}	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in one second
IFN-γ	Interferon gamma
HIV	Human immunodeficiency virus
M1	Type 1 macrophages
M2	Type 2 macrophages

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M. tuberculosis	*Mycobacterium tuberculosis*
NO	Nitric oxide
NOS	Nitric oxide synthase
TB	Tuberculosis

Introduction

Tuberculosis, a bacterial infection caused by *Mycobacterium tuberculosis*, remains a disease of major global importance. The World Health Organisation (WHO) has estimated that there were 8.6 million infections and 1.3 million deaths from this disease in 2012 [1]. L-Arginine holds promise as an 'adjunctive' (supplementary) treatment for tuberculosis, because of the key role played in human immune responses to *M. tuberculosis* by L-arginine via its metabolite nitric oxide and via direct immune effects of L-arginine itself [2].

Standard treatment of active tuberculosis infection is a complex regimen of four antibiotics administered for a minimum of 6 months. It is also standard for supplementary vitamin B6 (pyridoxine) to be co-administered with this regimen to mitigate the side effects of one of the antibiotics, isoniazid. Treatment of drug-resistant TB is more complex still and requires a longer duration. Efforts to improve TB treatment are thus a major priority; in particular, there is hope of discovery of agents which might accelerate the response to the antibiotic regimen. The field of TB adjunctive therapies is of key interest as a novel way to reduce the duration of antibiotic treatment required and to potentially tackle the growing problem of *M. tuberculosis* resistance to existing agents.

Three studies to date have failed to show any real benefit from supplementary oral L-arginine in tuberculosis. However, studies in cystic fibrosis offer hope that alternative means of administration of adjunctive L-arginine might be of benefit. This review explores the nutritional state in TB; the theoretical rationale for an interest in L-arginine in TB; the specific immunological role of nitric oxide, and L-arginine directly, in human host immune responses to TB; summarises the clinical studies performed to date; and proposes a way forward.

Tuberculosis

Tuberculosis has been a cause of human disease and mortality since antiquity and remains a leading cause of morbidity worldwide [1]. It predominantly infects the lungs, transmitted via airborne particles, although extra-pulmonary sites of disease are also common and increasingly recognised. Children in tuberculosis-endemic areas in particular suffer severe manifestations of tuberculosis, such as tuberculous meningitis, with disabling consequences. A number of factors including the overlap of tuberculosis and human immunodeficiency virus (HIV) pandemics in poorer nations led to a global escalation in tuberculosis rates in the late twentieth century. This culminated in tuberculosis being declared a 'global emergency' by the World Health Assembly in 1993 [3]. Tuberculosis is a leading cause of death in people with HIV, and tuberculosis-HIV co-infection is even more difficult to treat than tuberculosis alone, due to issues such as drug interactions [4]. Large international efforts to improve tuberculosis and HIV control have been met with successes—the TB pandemic does appear to have peaked in the mid-2000s, with case numbers and mortality now reportedly decreasing [1]. However, case numbers remain high, treatment remains complex, morbidity and mortality are substantial, and new approaches to treatment are required.

Tuberculosis is characterised by latent and active phases of infection. *M. tuberculosis* bacilli survive within granulomata, structures which comprise macrophages, T cells and other cells of the

immune system, in organs of infected hosts. The ability of *M. tuberculosis* to lie dormant, evading human immune responses, is a key reason for the difficulty in achieving tuberculosis control. Firstly, latency complicates diagnosis, since tests revealing exposure to TB (tuberculosis skin tests and interferon gamma release assays) cannot distinguish latent from active infection. Secondly, persistence in the host means that infection can become active at a time of immune compromise many years after initial infection; thus people with no recent tuberculosis contact can unexpectedly develop tuberculosis, diagnosis can be delayed, and transmission to others may occur in the interval between onset of active infection and institution of effective treatment. Thirdly, the dormant form of *M. tuberculosis* is metabolically inactive, making it resistant to antibiotics. These factors, added to the absence of an effective vaccine and the complexity of treatment (meaning that many people fail to complete therapy and therefore to have their tuberculosis cured) are all reasons for the exceptional challenges faced in global efforts to control tuberculosis.

Regarding human immunological responses to *M. tuberculosis*, in very simplest terms, this is chiefly via innate and cell-mediated immunity, rather than humoral (antibody-mediated) immunity. A highly complex cascade of events ensues after cells of the immune system come into contact with *M. tuberculosis* antigens. Amongst these responses is the production of interferon gamma (IFNγ) by T cells, leading to activation of macrophages and ultimately to the creation of nitric oxide (NO) from L-arginine, which results in bacterial killing. This particular response is the main reason for an interest in L-arginine in tuberculosis, this being the chief in vivo source of NO.

Nutritional State in Tuberculosis

Recognition of the two-way relationship between malnutrition and increased susceptibility to infection was described in seminal work by Scrimshaw in 1959 [5]. This relationship is of particular importance in tuberculosis, with malnutrition being an important risk factor for tuberculosis, and tuberculosis further aggravating weight loss; cachexia is a hallmark of active tuberculosis [6]. Tuberculosis is also associated with decreased appetite.

Malnutrition in tuberculosis can impact on L-arginine concentrations. L-Arginine is a conditionally essential amino acid, meaning that under usual conditions it can be manufactured de novo rather than being required from dietary sources. However, during hypercatabolic states such as infection, L-arginine synthesis can no longer match catabolism, and an exogenous source becomes essential to maintain the extracellular concentrations required for optimal NO synthesis. Hypoargininemia has been demonstrated in people with pulmonary TB [7] and other pathological states [2]. Preliminary data from 149 outpatients with pulmonary TB also shows L-arginine deficiency relative to healthy local volunteers. Mean serum L-arginine concentration in those with TB was 68.1 μmol/L ± 1.7 (standard error) versus 88.1 μmol/L ± 3.1 in the volunteers, $p < 0.001$ (A. Ralph, unpublished data).

The importance of nutritional status is further illustrated by studies on the impact of nutritional state on tuberculosis progress. Inadequate weight gain during treatment is associated with higher tuberculosis relapse risk [8], and low body mass index (BMI) is associated with an increased risk of early death [9]. Tuberculosis-HIV co-infected people are even more likely to be underweight or have nutritional deficiencies than HIV-negative tuberculosis patients. In recent work in Indonesia's Papua Province, my colleagues and I found that better preservation of BMI, and greater early weight gain, was associated with faster clearance of tuberculosis from sputum [10]. Papuan study participants (Melanesian peoples) were advantaged in this regard, having higher BMI and early weight gain than the non-Papuan (Asian) Indonesians in the study. The association with improved early response to tuberculosis treatment which we showed is not necessarily causative, but is consistent with the known relationship between nutritional status and recovery from tuberculosis.

At the other end of the nutritional spectrum, type 2 diabetes mellitus, usually related to obesity, is also a risk factor for tuberculosis, likely directly due to impaired glycaemic control [11].

Association Between Malnutrition and Immunodeficiency

Protein–calorie malnutrition, which commonly accompanies tuberculosis, causes serious impairment of cell-mediated immunity, attributable in part to atrophy of the thymus and other lymphoid tissues, and a significant fall in CD4 T cell numbers [12]. For instance, malnourished children have severe thymic involution, immaturity of circulating T cells and lack of reactivity to TB antigens [13]. Protein–calorie malnutrition also impairs humoral and innate immune responses [14, 15].

Adjunctive Therapies in Tuberculosis

Adjunctive therapies, also termed adjuvant or supplementary therapies, are those given in addition to standard treatment. Although major gains in tuberculosis diagnostics and treatments have been made in recent years [4], the field of tuberculosis adjunctive therapies, acknowledged to be a priority research area [16], presents important unanswered questions. Current strategies in tuberculosis adjunctive therapy are summarised in Table 47.1. This review, by focusing on the use of L-arginine in tuberculosis, deals with the strategy of using an agent which mediates T cell-macrophage responses as shown in the table.

Requirements of adjunctive therapies in tuberculosis-endemic settings include efficacy, safety and affordability. L-Arginine, which fulfils the requirements of safety and affordability and for which there is a strong theoretical rationale for potential efficacy, is therefore appealing as an investigational adjunctive agent.

L-Arginine and Nitric Oxide in Tuberculosis

L-arginine, a 'conditionally' essential (or semi-essential) amino acid in humans, is derived from exogenous sources (diet) and endogenous sources (whole-body protein degradation plus synthesis from citrulline) Relevant biochemical pathways and mediators in the consideration of tuberculosis are the conversion of L-arginine to L-citrulline plus nitric oxide in macrophages via nitric oxide synthase 2 (NOS2), previously termed inducible NOS (iNOS), or degradation via arginases to L-ornithine. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS.

Table 47.1 Strategies in experimental tuberculosis adjunctive therapies

Strategy	Examples	References
Treatment with agents to reverse the non-replicative, antibiotic-resistant state of *M. tuberculosis* within macrophages	• TNFα antagonist CC-3052 in animal models • Etanercept and infliximab in humans[a] • Corticosteroids[a]	[17–19] [20, 21] [22]
Use of immunosuppressive agents to mitigate inflammatory pathology	• Corticosteroids[a] • Etanercept and infliximab[a] • Vitamin D[a]	[22] [20, 21] [2, 23]
Use of agents which mediate T cell-macrophage responses	• L-arginine/Nitric oxide • Vitamin D[a]	[2, 23]

[a]Experimental agents may have more than one mechanism of action as TB adjunctive therapies

This table summarises the different approaches that are established or being investigated in tuberculosis adjunctive therapy research

Nitric Oxide

The discovery of NO in the late 1980s allowed the identity of the agent previously known as endothelium-derived relaxing factor to be revealed [24]. The vasodilatory effects of NO have been utilised therapeutically for over a century in the form of the nitrate class of cardiac medications.

In addition to its potent endovascular actions, NO plays a key role in the innate immune system and, specifically, in the defence against mycobacteria. It is implicated in both bactericidal and bacteriostatic processes. NOS2 is highly induced by bacterial endotoxin (lipopolysaccharide) and inflammatory cytokines including interferon gamma (IFN-γ). NO is capable of killing tuberculosis bacilli in vitro with a molar potency comparable to that of antibiotics [25], causing death of bacilli through disruption of bacterial DNA, proteins and lipids and by induction of apoptosis of tuberculosis-harbouring macrophages which allows the sequestered *M. tuberculosis* to be exposed to toxic lysozyme.

NO in Mouse Immune Responses to Tuberculosis

Mice and other rodents are susceptible to *M. tuberculosis*; despite some fundamental differences, these animal models nevertheless are used extensively in studying immunological responses to *M. tuberculosis* infection. The main mechanism in activated mouse (murine) macrophages responsible for killing and inhibiting growth of *M. tuberculosis* is L-arginine-dependent production of NO and other reactive nitrogen species [26]. Evidence for the key role played by the L-arginine-NO pathway in murine anti-TB responses includes studies showing that murine cells respond to TB infection by producing NOS2 [26–28], and mice with defective abilities to produce NO are more susceptible to TB, including having higher mortality rates after infection [25, 29].

NO in Human Immune Responses to Tuberculosis

There is firm evidence that human macrophages/monocytes produce NOS2 in response to *M. tuberculosis* infection, and that NO concentrations correlate with *M. tuberculosis* inhibition (see reviews [2, 30]). Some in vitro studies of human macrophage tuberculosis responses were unable to show any importance of NO; thus some researchers conclude that NO is unimportant in human immune responses, in contrast to the situation in mice. However the failure to identify NO activity in those studies was likely due to a number of methodological problems, including the need to add tetrahydrobiopterin to in vitro cultures to allow NO synthase enzymes to homodimerise which is required for enzymatic activity, the use of insensitive methods for detecting NO, or the failure to appreciate different capacities of tissue and peripheral blood macrophages to produce functional NOS2 [27]. Evidence for increased NOS2 expression, increased NO production and subsequent increased capacity for *M. tuberculosis* killing have in fact all been demonstrated in human hosts and human tissue and macrophage studies. Some relevant studies are summarised in Table 47.2.

L-Arginine Can Affect T Cell Function Independently of NO

There is evidence that L-arginine has additional, direct anti-mycobacterial functions independent of its conversion to NO. A number of studies indicate that L-arginine is required for the expression of CD3ζ, a component of the T cell receptor (TCR). Specifically, studies have shown that T cells from the blood

Table 47.2 Evidence from human studies of the importance of nitric oxide as a human antituberculosis immune response

Study type	Results	Selected references
Human macrophage studies	Alveolar macrophages from healthy humans infected ex vivo with *M. tuberculosis* produce NO, and NO production correlates with intracellular growth inhibition of *M. tuberculosis*	[31]
	Blood mononuclear cells from healthy donors infected ex vivo with *M. tuberculosis*, and from people with pre-existing TB infection, produce NO	[32]
	Pulmonary macrophages kill mycobacteria only if they express NOS2; killing is prevented with a NOS inhibitor	[33]
In vivo human studies	In lung resection studies, NOS2 and nitrotyrosine (a tissue marker of NO metabolism) are expressed in macrophages within granulomata and areas of TB pneumonitis	[34]
	NOS2 expression is increased in peripheral blood monocytes from people with TB compared with healthy controls	[35]
	NOS2 is expressed in macrophages from lungs of patients with tuberculosis	[36]
	NOS2 expression is evident in biopsy samples of pleura, lung and lymph nodes from people with active tuberculosis	[37]
	NO production as measured using serum surrogate markers, nitrite and citrulline, is significantly elevated in HIV-positive patients with active tuberculosis and significantly decreases by week 4 of antitubercular treatment	[38]

This table summarises research findings which indicate that nitric oxide is important in human immune responses to tuberculosis

of patients with pulmonary TB treatment [7], or from tissues infected with TB [39], have decreased CD3ζ expression, but this normalises over time with successful TB treatment [7]. In in vitro studies, depletion of L-arginine (but not other amino acids) causes reduced CD3ζ expression, and, consequently, impaired T cell function [40–42]. Addition of L-arginine ex vivo then leads to CD3ζ re-expression and recovery of T cell proliferation [43]. These data suggest that reduced L-arginine, which characterises TB, causes impaired CD3ζ expression and thence, immunosuppression via impairment of T cell function.

Mycobacterial Resistance to NO and Upregulation of Arginase Expression

M. tuberculosis has a range of immune evasion strategies, contributing to its success as a pathogen and its ability to survive in human hosts latently for decades. Two such immune evasion mechanisms are the avoidance of toxic effects of NO and induction of arginase 1 expression in macrophages. An understanding of these mechanisms can aid in the interpretation of findings from clinical studies of L-arginine or NO supplementation in tuberculosis infection.

Like susceptibility to antibiotics, tuberculosis strains differ in their ability to withstand the toxic effects of NO. Susceptibility to NO has been shown to be associated with virulence or with susceptibility to antibiotics in some studies (see review [30]). Genes conferring increased ability to withstand the effects of NO have been identified.

Arginase enzymes degrade L-arginine to ornithine, thus diverting L-arginine substrate away from the citrulline/NO pathway. Production of arginase 1 has been found to be elevated in tuberculosis in a variety of human and animal studies [7, 44, 45]. Increased arginase 1 expression would be expected to have important downstream effects for tuberculosis-infected hosts, including impaired macrophage and T cell anti-TB responses, via impaired NO release and CD3ζ expression, respectively.

There is a spectrum of macrophage responses which include M1 macrophages, previously termed 'classically' activated macrophages, and M2 macrophages, previously termed 'alternatively' activated macrophages. The proposed mechanisms for elevated arginase enzyme concentrations in people with TB are that *M. tuberculosis* can induce arginase 1 production in classically activated (M1) macrophages [45] and can also drive macrophage polarisation towards M2 responses [46]. The M1–M2 paradigm can be helpful in understanding innate anti-*M. tuberculosis* responses in simple terms. A proposed model suggests that in early infection, M1 responses (chiefly characterised by NOS2 expression and NO release) predominate, but as infection becomes advanced, M2 responses predominate, with consequent release of arginase 1 and M2-associated cytokines, leading to a failure of control of TB by the infected host [46].

Asymmetric Dimethylarginine

ADMA is an endogenous inhibitor of NOS2, and higher plasma ADMA concentrations are therefore thought to be associated with lower NO production. In malaria and sepsis, endothelial production of NO is important, with deficiency of NO thought to contribute to endothelial and microvascular dysfunction. In patients with malaria, higher plasma ADMA concentrations are associated with disease severity and mortality and with lower NO (measured as NO in exhaled breath) [47]. Similarly in patients with sepsis, a low L-arginine to ADMA ratio has been identified, this being lower still in more severe sepsis [48]. In this study, higher plasma ADMA concentrations were again associated with higher mortality.

If ADMA is expressed in high concentration in active tuberculosis, then one could hypothesise that NO production from alveolar macrophages could be impaired. Preliminary data indicate a trend towards higher ADMA and a lower L-arginine to ADMA ratio in people with active tuberculosis versus healthy volunteers, but more data are required (A. Ralph unpublished results). Such studies will provide information to help guide clinical studies of L-arginine supplemental therapy, since successful increases in L-arginine bioavailability (e.g. through supplementary treatment with L-arginine) may not translate into increased NO production if NOS2 function is inhibited by ADMA.

Exhaled Nitric Oxide in Pulmonary Tuberculosis

Production of NO from L-arginine can be difficult to measure in vivo due to its short half-life. Measurement of pulmonary NO production can however be achieved through the detection of NO in exhaled breath using validated chemiluminescence analysers. A number of studies have investigated fractional exhaled NO (FE_{NO}) in people with pulmonary tuberculosis. These investigations have provided conflicting results, including both increased [36] or decreased [30, 49] FE_{NO} in TB compared with controls. These differences might be attributable to small sample sizes in some studies, different methodologies for FE_{NO} measurement, severity of TB in the patients assessed and/or different types of controls used. The more recent studies show lower FE_{NO} in TB, and all studies indicate that poorer capacity to generate NO is associated with more severe disease.

In a study of 200 TB patients conducted in eastern Indonesia, FE_{NO} was lower in patients with pulmonary tuberculosis when first diagnosed with TB, prior to commencing treatment, compared with healthy controls (Fig. 47.1) [30]. The average FE_{NO} (geometric mean) in people with TB was 12.7 ppb (95 % confidence interval [CI] 11.6–13.8) compared with 16.6 ppb, 95 % CI 14.2–19.5, in the healthy controls ($p=0.002$). Amongst the TB patients, FE_{NO} has been recovered during treatment (Fig. 47.2). People with more severe TB, such as worse X-ray findings and poorer lung function, had

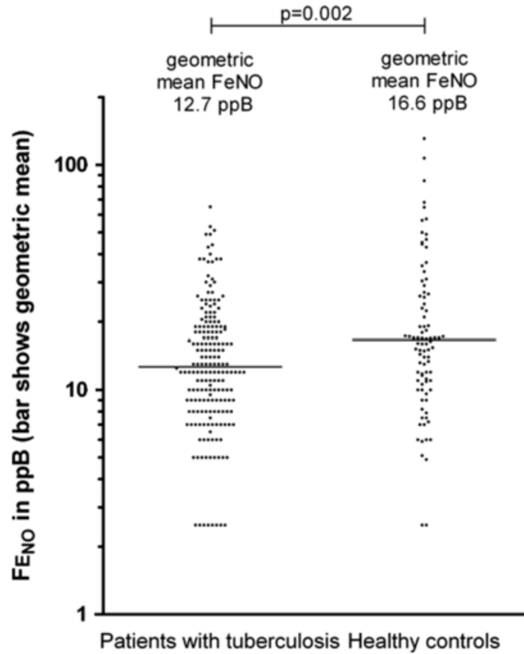

Fig. 47.1 FE_{NO} in people with newly-diagnosed pulmonary tuberculosis compared with healthy controls [30]. This figure shows the broad spread of exhaled nitric oxide results in people with TB and people without pulmonary tuberculosis (healthy controls). It also shows the difference between these groups

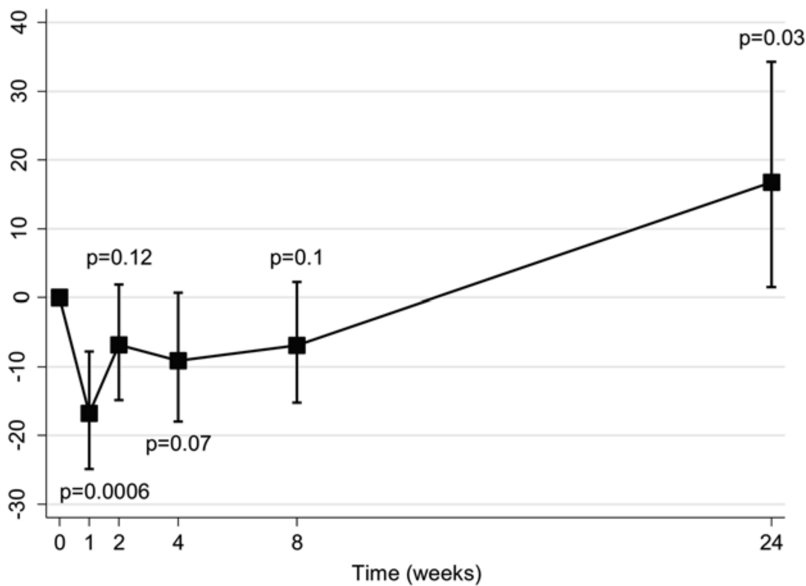

Fig. 47.2 Change in FE_{NO} in tuberculosis patients between diagnosis and treatment completion [30]. This figure shows that amongst people with pulmonary tuberculosis, exhaled nitric oxide concentration falls, then rises during TB treatment. Means (*squares*) and 95 % confidence intervals (*whiskers*) are shown. *p* values relate to differences in FE_{NO} compared with baseline

particularly low FE_{NO} readings. Perhaps most importantly, recovery during the next 2 months of treatment was associated with an incremental change in FE_{NO}. TB patients whose FE_{NO} decreased were more than twice as likely to still have TB present in their sputum after 2 months of treatment, compared with those whose FE_{NO} increased or remained unchanged. The key findings from this study are summarised in Box 47.1.

Box 47.1. Exhaled Nitric Oxide in Tuberculosis: Conclusions from a Study in Indonesia [30]

- Nitric oxide (NO) in exhaled breath appears to be an important marker in human pulmonary TB infection, showing that pulmonary NO in vivo (measured as exhaled NO, FE_{NO}):
  - is low in TB compared with healthy volunteers
  - correlates with disease severity
  - is associated with response to treatment: people who had greater increases in FE_{NO} over time were more likely to achieve sputum culture conversion
  - recovers over time in TB patients (between start and end of 6-month TB treatment)
- An oral 6 g dose of L-arginine was not adequate to increase FE_{NO} or to improve time to sputum culture conversion or other outcomes from TB. Therefore, alternative means by which to improve pulmonary production of NO in pulmonary TB patients need to be investigated.

Legend: This box summarises findings from one study of exhaled nitric oxide concentration in people with pulmonary tuberculosis.

These findings need to be validated in further research. If these findings are able to be replicated, one could conclude that enhancing NO production in people with pulmonary TB, for instance by administering L-arginine as an adjunctive agent in combination with standard antibiotics, could be a novel adjunctive therapeutic strategy. However, oral administration of L-arginine may not be the best strategy, as discussed in the following section.

Clinical Trials of L-Arginine and Nitric Oxide in Tuberculosis and Other Conditions

Many clinical trials of nutritional agents have been undertaken in people with tuberculosis. In some tuberculosis treatment programmes, where finances permit, provision of supplementary meals is routine, in recognition of the malnourished state and low socioeconomic status of many tuberculosis patients. A meta-analysis of nutritional trials in tuberculosis identified 23 trials meeting their criteria [50]. The trials of macronutrients (e.g. supplementary whole food) which the authors reviewed indicated that such an approach can improve weight gain and may be associated with improvements in adherence and some markers of physical function and quality of life. Despite deficiency of various micronutrients being recognised in tuberculosis, especially tuberculosis-HIV co-infection, this meta-analysis also reported disappointingly that micronutrient supplementation in active tuberculosis does not appear generally to be associated with improved outcomes.

Clinical Trials of L*-Arginine and* L*-Arginine-Rich Food*

The hypotheses underlying trials of L-arginine for tuberculosis are summarised in Box 47.2. Three clinical trials have now been conducted in which supplementary L-arginine, or L-arginine-rich food, has been administered for people with pulmonary tuberculosis also receiving treatment with standard antibiotics. These studies have tested 6 g oral L-arginine hydrochloride daily for 8 weeks, 1 g oral L-arginine hydrochloride daily for 4 weeks [51] or L-arginine-rich food (peanuts) [52]. These studies have provided inconsistent results, with the overall conclusion being that oral L-arginine hydrochloride does not appear to be effective in this condition. However for reasons elaborated below, further investigations in this field may yet prove fruitful.

Box 47.2. Hypotheses Underlying Trials of L-Arginine for Tuberculosis

1. Administration of L-arginine will improve nitric oxide production by alveolar macrophages
   – This will be measurable as increased nitric oxide in expired air
2. Increased macrophage nitric oxide production will be associated with improved mycobacterial killing
3. Administration of L-arginine will also lead to improvements in anti-mycobacterial T cell function via enhanced expression of the TCR CD3ζ

Legend: This box summarises the reasons for an interest in researching L-arginine as an adjunctive therapy in pulmonary tuberculosis.

In the first published trial set in Ethiopia, 120 people with pulmonary tuberculosis received 1 g L-arginine hydrochloride daily for 4 weeks, in addition to regular tuberculosis therapy [51]. Faster clearance of TB from sputum and resolution of cough were reported in the arm receiving supplementation, but only amongst HIV-negative participants [51]. In a second study, also performed by the same research group in Ethiopia, an L-arginine-rich food (peanuts) was used instead of L-arginine tablets, while the placebo arm received low-L-arginine wheat crackers [52]. In this study of 180 patients, although no significant benefits were reported overall, higher cure rates were reported in HIV-positive participants receiving the peanut food supplement, compared with those receiving wheat crackers [52].

In a third trial, L-arginine 6 g daily was tested. This was a randomised, double-blind, placebo-controlled trial of oral adjunctive L-arginine and vitamin D in people with pulmonary TB [23]. Findings were negative, in that TB patients who received L-arginine did no better than those who did not, on a variety of measures. Sputum culture conversion was achieved in 48/76 (63 %) participants in the active L-arginine arm after 4 weeks of treatment, versus 48/79 (61 %) in placebo L-arginine arms (no significant difference). Vitamin D was also ineffective. Pharmacodynamic responses to oral L-arginine supplementation in TB patients were also investigated in this study. Despite the dose of supplementary L-arginine being quite large (6 g/day), no increment was seen in FE_{NO} (Fig. 47.3) [30]. It is possible that host factors including NOS expression, arginase concentration and ADMA concentration, might have prevented the supplementary L-arginine from being adequately converted to NO, thus potentially explaining the negative findings in the trial.

While pharmacokinetics (see below) would indicate that greater than once daily dosing would be preferable, the practicality of administering such a regimen for people with tuberculosis is difficult. Tuberculosis treatment is often administered as ‘DOT’ (directly observed therapy), once daily at a clinic. This is to ensure adherence, which is challenging, given the long duration of treatment (6 months minimum). The most practical supplement to give for a patient with tuberculosis should also be once daily.

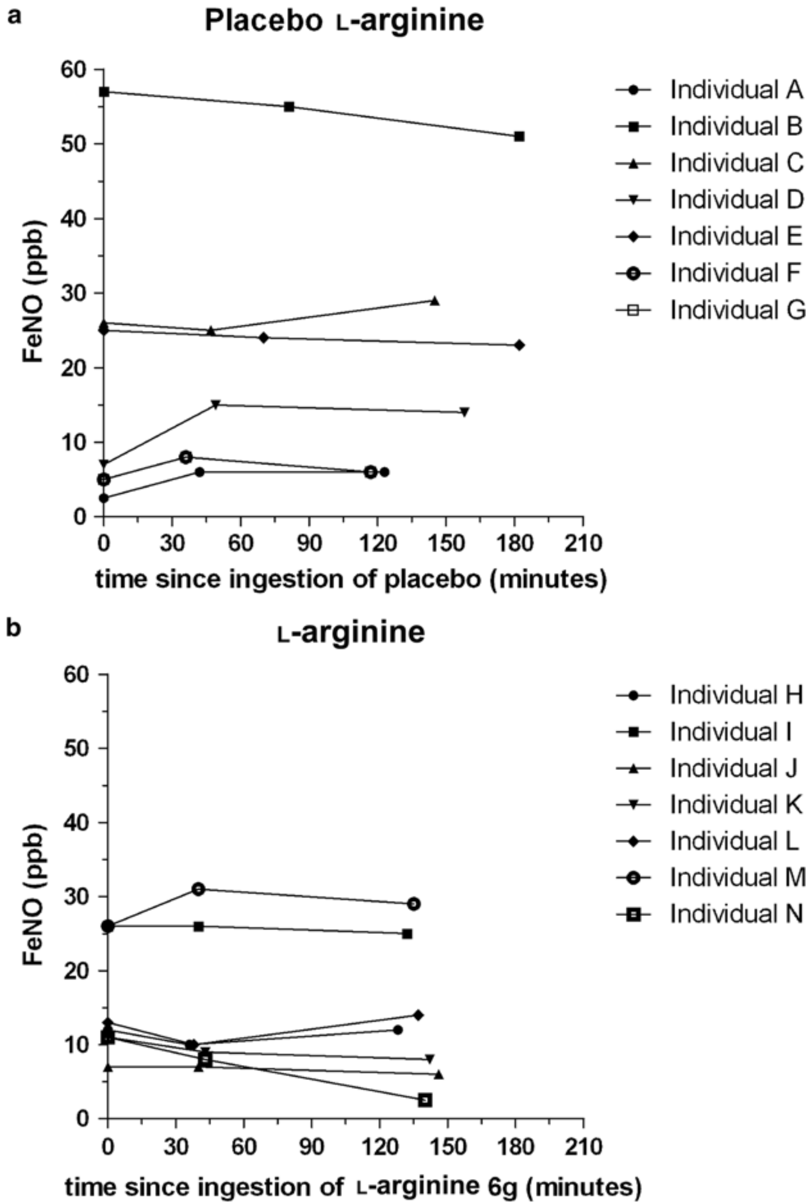

Fig. 47.3 FE_{NO} before and after ingestion of 6.0 g oral L-arginine or placebo [23]. These figures show that a 6 g oral dose of L-arginine did not affect exhaled nitric oxide concentration in seven individuals one study

Limitations of Clinical Studies of L-Arginine/NO

Although the clinical studies performed to date have shown limited if any success in improving tuberculosis outcomes, reasons for this might include that L-arginine doses were too low, or that L-arginine dosing should be administered two or three times daily for best efficacy. At higher doses, orally administered L-arginine might be capable of measurably increasing pulmonary NO bioavailability, thereby improving macrophage killing of tuberculosis bacilli. However, higher oral L-arginine doses have gastrointestinal adverse effects, and dosing (more tablets, more times daily) becomes unwieldy.

Also, an adequate concentration of NO (a level high enough to be bactericidal) may be difficult to achieve. NO concentrations of at least 50 ppm were required in early in vitro experiments to kill TB [53], whereas NO concentrations in exhaled breath are far lower, in the realm of 10–20 ppb. Low-concentration NO may in fact be deleterious, since it can promote TB bacilli to become dormant, a state which is relatively unresponsive to antibiotics [54].

Clinical Trials of Nitric Oxide in Tuberculosis

Nitric oxide can be administered directly via inhalation, but it's extremely short half-life requires continuous, or very frequent, administration. This is entirely impractical in the outpatient setting in which most tuberculosis patients are treated. However, Long and colleagues were able to conduct a small non-blinded trial in 18 tuberculosis patients admitted to hospital [55]. Eight were administered NO via inhalation at 80 ppm for 3 days in addition to their standard tuberculosis therapy, and ten controls received standard treatment only without placebo gas. Although inhaled NO was able to be delivered safely, it did not accelerate response to standard tuberculosis treatment. The small numbers in this study mean that firm conclusions cannot be drawn.

Recognising the impracticality of administration of inhaled NO, other researchers have investigated the effect of inhalable microparticles containing NO donors (10 % diethylenetriamine nitric oxide adduct or sodium nitroprusside), with or without antituberculous antibiotics, on tuberculosis infection in mice. They have concluded from these studies that inhalable NO donors can enhance mycobacterial control in the infected mice [56]. Such studies are still far from potential human application.

L-Arginine in Cystic Fibrosis and Other Pathologies

Another pulmonary disease characterised by impaired NO production is cystic fibrosis, a disease in which repeated pulmonary infections are common. In this disease, NO production is impaired. Although altered and retained pulmonary secretions are of major relevance in the aetiology of repeated infections in cystic fibrosis, impaired NO production could contribute to this predisposition to infection [57]. The reason for low NO production appears to be a multifactorial problem including low NOS2 expression in bronchial epithelial cells [57, 58] and increased pulmonary concentrations of ADMA [59]. FE_{NO} in cystic fibrosis patients appears to be even lower than in tuberculosis patients (5–12 ppb [60, 61] compared with 13–16 ppb in tuberculosis [30, 52]). However, different methods were used to measure FE_{NO} in these studies.

A single small dose of oral L-arginine 200 mg was found by Grasemann et al. to result in a small but statistically significant increase in FE_{NO} amongst cystic fibrosis patients [62]. This is in contrast to the TB study described above, indicating that higher doses may be required in TB than cystic fibrosis. In a later study, a single dose of *inhaled* L-arginine 1.3 g in cystic fibrosis patients increased FE_{NO}, peaking at 3 h after inhalation, with FE_{NO} sustained above baseline out to 6 h, but normalising by 24 h; furthermore this achieved an increase in FEV_1 [63]. A further trial in cystic fibrosis has recently been published by Grasemann et al.'s research group. Nineteen participants were randomised to inhale L-arginine for 14 days followed by placebo for 14 days, or vice versa. Twice daily dosing of inhaled L-arginine at 500 mg per dose for 14 days resulted in a significant increase in FE_{NO} compared with placebo [61]. Additionally, pulmonary function increased in the L-arginine group, and two people cleared their sputum of *Pseudomonas* species; these changes were not statistically significant, but the study was small ($n=19$), designed to test safety and tolerability, not efficacy.

Table 47.3 Safety of inhaled L-arginine in cystic fibrosis

Symptom	L-arginine (number of events, %)	Placebo (number of events, %)
Number of patients	19	19
Increased cough	3 (18 %)	4 (21 %)
Headache	2 (11 %)	2 (11 %)
Cold-like symptoms	2 (11 %)	0
Hoarseness	1 (5 %)	0
Wheeze	1 (5 %)	0
Itchy eye	1 (5 %)	0
Fever	1 (5 %)	1 (5 %)
Rash	1 (5 %)	0
Stomach ache	0	1 (5 %)
Nose bleed	0	1 (5 %)
Hives	0	1 (5 %)
Increased heart rate	0	1 (5 %)

This table shows side effects from inhaled L-arginine in one study. Adapted from Ref. [61]

L-Arginine Dosing and Safety

L-arginine has also been administered for a large number of medical conditions in oral, intravenous and inhaled form (reviewed elsewhere [24, 64]). It is widely acknowledged to be safe and well tolerated. Inhaled L-arginine should not be administered to people with asthma, since increased pulmonary NO can exacerbate wheeze [65]; therefore asthmatic patients would be excluded from studies of inhaled L-arginine. The studies by Grasemann et al. provide important preliminary data to inform potential future studies in tuberculosis; in their studies of inhaled L-arginine [61, 63] there were no important adverse events (Table 47.3) and no increase in airway inflammation, as measured from the white cell count, Interleukin-8 concentration and neutrophil elastase activity in sputum.

Oral L-arginine can be dosed once, twice or thrice daily. Use of 21 g daily in divided doses has been reported as being well tolerated; minor gastrointestinal discomfort has been reported in up to 3 % of patients treated with L-arginine (see review [24]). In the tuberculosis trial of 6 g L-arginine daily described above, there was no increase in reports of diarrhoea, bloating, nausea, vomiting, abdominal pain or other symptoms in those receiving L-arginine compared with placebo [23].

Healthy adult plasma L-arginine levels range from 20 to 180 μmol/L [66]. Orally administered L-arginine-hydrochloride bioavailability ranges from 21 % after a 10 g oral dose in one study [67] to 68 % after a 6 g dose in another [68]. Peak plasma concentrations of 310 ± 152 μmol/L occur at approximately 90 min after a 6 g oral dose, and half-life is reported to be 77.5 ± 9.3 min [68].

Conclusions

Despite the strong theoretical rationale, supplementary L-arginine for people with tuberculosis has not been associated with benefits. Therefore oral L-arginine is not recommended as a supplementary treatment in tuberculosis. While these negative findings may be due to underdosing, administration of higher doses would be limited by potential side effects and the difficulty of adhering to a regimen of tablets several times daily. It is paramount that tuberculosis patients focus on good adherence with their antibiotic regimen; adding in a second complex regimen, such as multiple L-arginine tablets several times per day, would be burdensome and might have the detrimental effect of impairing adherence both to the L-arginine and antituberculosis antibiotics.

However, there is scope for additional research in this field. Inhaled or nebulised L-arginine, a long-acting formulation, or a different formulation allowing higher dosing, or alternative NO donors other than L-arginine, all merit consideration. In low-income tuberculosis-endemic settings, adjunctive therapy must be not only safe and effective but low cost. Thus L-arginine is appealing as an investigational adjunctive agent.

References

1. World Health Organisation. Global tuberculosis report: WHO report 2013. WHO Library Cataloguing-in-Publication Data. WHO/HTM/TB/2013.15; 2013.
2. Ralph AP, Kelly PM, Anstey NM. L-arginine and vitamin D: novel adjunctive immunotherapies in tuberculosis. Trends Microbiol. 2008;16:336–44.
3. Raviglione MC. The TB epidemic from 1992 to 2002. Tuberculosis (Edinb). 2003;83:4–14.
4. Ralph AP, Anstey NM, Kelly PM. Tuberculosis into the 2010s: is the glass half full? Clin Infect Dis. 2009;49:574–83.
5. Scrimshaw NS, Taylor CE, Gordon JE. Interactions of nutrition and infection. Am J Med Sci. 1959;237:367–403.
6. Cegielski JP, McMurray DN. The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. Int J Tuberc Lung Dis. 2004;8:286–98.
7. Zea AH, Culotta KS, Ali J, Mason C, Park HJ, et al. Decreased expression of CD3zeta and nuclear transcription factor kappa B in patients with pulmonary tuberculosis: potential mechanisms and reversibility with treatment. J Infect Dis. 2006;194:1385–93.
8. Khan A, Sterling TR, Reves R, Vernon A, Horsburgh CR. Lack of weight gain and relapse risk in a large tuberculosis treatment trial. Am J Respir Crit Care Med. 2006;174:344–8.
9. Zachariah R, Spielmann MP, Harries AD, Salaniponi FM. Moderate to severe malnutrition in patients with tuberculosis is a risk factor associated with early death. Trans R Soc Trop Med Hyg. 2002;96:291–4.
10. Kenangalem E, Waramori G, Pontororing GJ, Sandjaja, Tjitra E, et al. Tuberculosis outcomes in Papua, Indonesia: the relationship with different body mass index characteristics between papuan and non-Papuan ethnic groups. PLoS One. 2013;8:e76077.
11. Dobler CC, Flack JR, Marks GB. Risk of tuberculosis among people with diabetes mellitus: an Australian nationwide cohort study. BMJ Open. 2012;2:e000666.
12. Delves P, Martin S, Burton D, Roitt I. Control mechanisms in Roitt's essential immunology. 11th ed. Malden, MA: Blackwell; 2006.
13. Parent G, Chevalier P, Zalles L, Sevilla R, Bustos M, et al. In vitro lymphocyte-differentiating effects of thymulin (Zn-FTS) on lymphocyte subpopulations of severely malnourished children. Am J Clin Nutr. 1994;60:274–8.
14. Passwell JH, Steward MW, Soothill JF. The effects of protein malnutrition on macrophage function and the amount and affinity of antibody response. Clin Exp Immunol. 1974;17:491–5.
15. Anstead GM, Chandrasekar B, Zhao W, Yang J, Perez LE, et al. Malnutrition alters the innate immune response and increases early visceralization following Leishmania donovani infection. Infect Immun. 2001;69:4709–18.
16. Onyebujoh P, Rodriguez W, Mwaba P. Priorities in tuberculosis research. Lancet. 2006;367:940–2.
17. Subbian S, Tsenova L, O'Brien P, Yang G, Koo MS, et al. Phosphodiesterase-4 inhibition alters gene expression and improves isoniazid-mediated clearance of *Mycobacterium tuberculosis* in rabbit lungs. PLoS Pathog. 2011;7:e1002262.
18. Koo MS, Manca C, Yang G, O'Brien P, Sung N, et al. Phosphodiesterase 4 inhibition reduces innate immunity and improves isoniazid clearance of *Mycobacterium tuberculosis* in the lungs of infected mice. PLoS One. 2011;6:e17091.
19. Maiga M, Agarwal N, Ammerman NC, Gupta R, Guo H, et al. Successful shortening of tuberculosis treatment using adjuvant host-directed therapy with FDA-approved phosphodiesterase inhibitors in the mouse model. PLoS One. 2012;7:e30749.
20. Wallis RS. Reconsidering adjuvant immunotherapy for tuberculosis. Clin Infect Dis. 2005;41:201–8.
21. Blackmore TK, Manning L, Taylor WJ, Wallis RS. Therapeutic use of infliximab in tuberculosis to control severe paradoxical reaction of the brain and lymph nodes. Clin Infect Dis. 2008;47:e83–5.
22. Thwaites GE, Nguyen DB, Nguyen HD, Hoang TQ, Do TT, et al. Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults. N Engl J Med. 2004;351:1741–51.
23. Ralph AP, Waramori G, Pontororing GJ, Kenangalem E, Wiguna A, et al. L-arginine and vitamin D adjunctive therapies in pulmonary tuberculosis: a randomised, double-blind, placebo-controlled trial. PLoS One. 2013;8:e70032.
24. Ralph A. Pulmonary tuberculosis: towards improved adjunctive therapies [PhD]. Canberra: Australian National University; 2010.

25. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF. The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. Science. 2003;302:1963–6.
26. Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J Exp Med. 1992;175:1111–22.
27. Chan J, Tanaka K, Carroll D, Flynn J, Bloom BR. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. Infect Immun. 1995;63:736–40.
28. Greenberg SS, Xie J, Kolls J, Mason C, Didier P. Rapid induction of mRNA for nitric oxide synthase II in rat alveolar macrophages by intratracheal administration of *Mycobacterium tuberculosis* and *Mycobacterium avium*. Proc Soc Exp Biol Med. 1995;209:46–53.
29. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, et al. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci USA. 1997;94:5243–8.
30. Ralph AP, Yeo TW, Salome CM, Waramori G, Pontororing GJ, et al. Impaired pulmonary nitric oxide bioavailability in pulmonary tuberculosis: association with disease severity and delayed mycobacterial clearance with treatment. J Infect Dis. 2013;204:616–26.
31. Rich EA, Torres M, Sada E, Finegan CK, Hamilton BD, et al. *Mycobacterium tuberculosis* (MTB)-stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB. Tuber Lung Dis. 1997;78:247–55.
32. Kwon OJ. The role of nitric oxide in the immune response of tuberculosis. J Korean Med Sci. 1997;12:481–7.
33. Nozaki Y, Hasegawa Y, Ichiyama S, Nakashima I, Shimokata K. Mechanism of nitric oxide-dependent killing of *Mycobacterium bovis* BCG in human alveolar macrophages. Infect Immun. 1997;65:3644–7.
34. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. Am J Respir Crit Care Med. 2002;166:178–86.
35. Wang CH, Lin HC, Liu CY, Huang KH, Huang TT, et al. Upregulation of inducible nitric oxide synthase and cytokine secretion in peripheral blood monocytes from pulmonary tuberculosis patients. Int J Tuberc Lung Dis. 2001;5:283–91.
36. Wang CH, Liu CY, Lin HC, Yu CT, Chung KF, et al. Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. Eur Respir J. 1998;11:809–15.
37. Schon T, Elmberger G, Negesse Y, Pando RH, Sundqvist T, et al. Local production of nitric oxide in patients with tuberculosis. Int J Tuberc Lung Dis. 2004;8:1134–7.
38. Wanchu A, Bhatnagar A, Khullar M, Sud A, Bambery P, et al. Antitubercular therapy decreases nitric oxide production in HIV/TB coinfected patients. BMC Infect Dis. 2002;2:15.
39. Seitzer U, Kayser K, Hohn H, Entzian P, Wacker HH, et al. Reduced T-cell receptor CD3zeta-chain protein and sustained CD3epsilon expression at the site of mycobacterial infection. Immunology. 2001;104:269–77.
40. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64:5839–49.
41. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, et al. Regulation of T cell receptor CD3zeta chain expression by L-arginine. J Biol Chem. 2002;277:21123–9.
42. Rodriguez PC, Zea AH, DeSalvo J, Culotta KS, Zabaleta J, et al. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. J Immunol. 2003;171:1232–9.
43. Taheri F, Ochoa JB, Faghiri Z, Culotta K, Park HJ, et al. L-Arginine regulates the expression of the T-cell receptor zeta chain (CD3zeta) in Jurkat cells. Clin Cancer Res. 2001;7:958–65S.
44. Mattila JT, Ojo OO, Kepka-Lenhart D, Marino S, Kim JH, et al. Microenvironments in tuberculous granulomas are delineated by distinct populations of macrophage subsets and expression of nitric oxide synthase and arginase isoforms. J Immunol. 2013;191:773–84.
45. El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol. 2008;9:1399–406.
46. Lugo-Villarino G, Verollet C, Maridonneau-Parini I, Neyrolles O. Macrophage polarization: convergence point targeted by *mycobacterium tuberculosis* and HIV. Front Immunol. 2011;2:43.
47. Yeo TW, Lampah DA, Tjitra E, Gitawati R, Darcy CJ, et al. Increased asymmetric dimethylarginine in severe falciparum malaria: association with impaired nitric oxide bioavailability and fatal outcome. PLoS Pathog. 2010;6:e1000868.
48. Davis JS, Darcy CJ, Yeo TW, Jones C, McNeil YR, et al. Asymmetric dimethylarginine, endothelial nitric oxide bioavailability and mortality in sepsis. PLoS One. 2011;6:e17260.
49. Idh J, Westman A, Elias D, Moges F, Getachew A, et al. Nitric oxide production in the exhaled air of patients with pulmonary tuberculosis in relation to HIV co-infection. BMC Infect Dis. 2008;8:146.
50. Sinclair D, Abba K, Grobler L, Sudarsanam TD. Nutritional supplements for people being treated for active tuberculosis. Cochrane Database Syst Rev. 2011;11:CD006086.

51. Schon T, Elias D, Moges F, Melese E, Tessema T, et al. L-Arginine as an adjuvant to chemotherapy improves clinical outcome in active tuberculosis. Eur Respir J. 2003;21:483–8.
52. Schon T, Idh J, Westman A, Elias D, Abate E, et al. Effects of a food supplement rich in L-arginine in patients with smear positive pulmonary tuberculosis—a randomised trial. Tuberculosis (Edinb). 2011;91:370–7.
53. Long R, Light B, Talbot JA. Mycobactericidal action of exogenous nitric oxide. Antimicrob Agents Chemother. 1999;43:403–5.
54. Boon C, Dick T. How *Mycobacterium tuberculosis* goes to sleep: the dormancy survival regulator DosR a decade later. Future Microbiol. 2012;7:513–8.
55. Long R, Jones R, Talbot J, Mayers I, Barrie J, et al. Inhaled nitric oxide treatment of patients with pulmonary tuberculosis evidenced by positive sputum smears. Antimicrob Agents Chemother. 2005;49:1209–12.
56. Verma RK, Agrawal AK, Singh AK, Mohan M, Gupta A, et al. Inhalable microparticles of nitric oxide donors induce phagosome maturation and kill *Mycobacterium tuberculosis*. Tuberculosis (Edinb). 2013;93:412–7.
57. Meng QH, Springall DR, Bishop AE, Morgan K, Evans TJ, et al. Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. J Pathol. 1998;184:323–31.
58. Kelley TJ, Drumm ML. Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. J Clin Invest. 1998;102:1200–7.
59. Grasemann H, Al-Saleh S, Scott JA, Shehnaz D, Mehl A, et al. Asymmetric dimethylarginine contributes to airway nitric oxide deficiency in patients with cystic fibrosis. Am J Respir Crit Care Med. 2011;183:1363–8.
60. Snyder AH, McPherson ME, Hunt JF, Johnson M, Stamler JS, et al. Acute effects of aerosolized S-nitrosoglutathione in cystic fibrosis. Am J Respir Crit Care Med. 2002;165:922–6.
61. Grasemann H, Tullis E, Ratjen F. A randomized controlled trial of inhaled L-Arginine in patients with cystic fibrosis. J Cyst Fibros. 2013;12(5):468–74.
62. Grasemann H, Grasemann C, Kurtz F, Tietze-Schillings G, Vester U, et al. Oral L-arginine supplementation in cystic fibrosis patients: a placebo-controlled study. Eur Respir J. 2005;25:62–8.
63. Grasemann H, Kurtz F, Ratjen F. Inhaled L-arginine improves exhaled nitric oxide and pulmonary function in patients with cystic fibrosis. Am J Respir Crit Care Med. 2006;174:208–12.
64. Boger RH, Bode-Boger SM. The clinical pharmacology of L-arginine. Annu Rev Pharmacol Toxicol. 2001;41:79–99.
65. Sapienza MA, Kharitonov SA, Horvath I, Chung KF, Barnes PJ. Effect of inhaled L-arginine on exhaled nitric oxide in normal and asthmatic subjects. Thorax. 1998;53:172–5.
66. Lerman A, Burnett Jr JC, Higano ST, McKinley LJ, Holmes Jr DR. Long-term L-arginine supplementation improves small-vessel coronary endothelial function in humans. Circulation. 1998;97:2123–8.
67. Tangphao O, Grossmann M, Chalon S, Hoffman BB, Blaschke TF. Pharmacokinetics of intravenous and oral L-arginine in normal volunteers. Br J Clin Pharmacol. 1999;47:261–6.
68. Bode-Boger SM, Boger RH, Galland A, Tsikas D, Frolich JC. L-arginine-induced vasodilation in healthy humans: pharmacokinetic-pharmacodynamic relationship. Br J Clin Pharmacol. 1998;46:489–97.

Chapter 48
L-Arginine in Health and Disease: Recommended Resources and Further Reading

Rajkumar Rajendram, Vinood B. Patel, and Victor R. Preedy

Key Points

- Amino acids such as L-arginine have significant clinical value in modern medicine.
- This chapter lists the most up-to-date resources on the regulatory bodies, journals, books, professional bodies, and websites that are relevant to an evidence-based approach to the use of L-arginine.

Keywords L-Arginine • Amino acids • Evidence • Resources • Books • Journals • Regulatory bodies • Professional societies

Introduction

L-Arginine is a conditionally essential amino acid. L-Arginine is synthesized from citrulline in L-arginine and proline metabolism by argininosuccinate synthetase and argininosuccinate lyase in the cytosol [1].

However, this pathway does not produce sufficient L-arginine, so some is still required in the diet in some circumstances.

L-Arginine is the immediate precursor of nitric oxide (NO), urea, ornithine, and agmatine and has important roles in cell division, wound healing, excretion of ammonia, immune function, and release of hormones [1–3].

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Schultze, a Swiss chemist, first isolated L-arginine in 1886. Since then the knowledge and understanding of L-arginine and its role in metabolism have increased exponentially. It is difficult for experienced scientists to remain up-to-date. For those new to the field it is difficult to know which of the myriad of available sources are reliable. To assist colleagues who are interested in understanding more about L-arginine, we have therefore produced tables containing reliable, up-to-date resources on L-arginine and its role in health and disease in this chapter. The experts who assisted with the compilation of these tables of resources are acknowledged below.

Examples of the applications of the amino acid L-arginine can be found in this book and also via the resources recommended in the tables below.

Tables 48.1, 48.2, 48.3, 48.4, and 48.5 list the most up-to-date information on the organizations and regulatory bodies interested in L-arginine (Table 48.1), professional bodies (Table 48.2), journals (Table 48.3), books (Table 48.4), and websites (Table 48.5) that are relevant to an evidence-based use of L-arginine in health and disease. Of course some organizations, sites, or official bodies are equally placed in several sections.

Table 48.1 Regulatory bodies and other organizations

American Association for Cancer Research www.aacr.org
American Society for Parenteral and Enteral Nutrition (ASPEN) www.nutritioncare.org
American College of Sports Medicine www.acsm.org
British Association for Cancer Research www.bacr.org.uk
Centers for Disease Control and Prevention www.cdc.gov
Department of Health and Human Services www.hhs.gov
European Commission, DG Health and Consumers, Food and Feed Safety ec.europa.eu/food/safety/index_en.htm
European Society for Clinical Nutrition and Metabolism (ESPEN) www.espen.org
European Medicines Agency www.ema.europa.eu/ema
Food and Drug Administration www.fda.gov
Health Canada www.hc-sc.gc.ca
Institute of Cancer Research www.icr.ac.uk
International Life Sciences Institute (ILSI) www.ILSI.org
Japanese Ministry of Health, Labour and Welfare www.mhlw.go.jp/english/index.html (English) www.mhlw.go.jp/ (Japanese)

(continued)

Table 48.1 (continued)

Japanese National Institute of Health and Nutrition
www0.nih.go.jp/eiken/english/index.html (English)
www0.nih.go.jp/eiken/index.html (Japanese)
Japan Food Additives Association
www.jafaa.or.jp/13English/outline.html (English)
www.jafaa.or.jp/index.htm (Japanese)
National Cancer Institute
www.cancer.gov
National Institutes of Health
www.nih.gov
Nutrition
www.nutrition.gov
Sociedade Brasileira de Nutrição Parenteral e Enteral (SBNPE)
www.sbnpe.com.br
US National Library of Medicine and National Institutes of Health
www.ncbi.nlm.nih.gov/pubmed
World Health Organization
www.who.int/en

This table lists the regulatory bodies and other organizations with interests in L-arginine

Table 48.2 Professional societies

American Association for the Study of the Liver Diseases (AASLD)
www.aasld.org
American Diabetes Association
www.diabetes.org
American Heart Association
www.heart.org/heartorg/
American Physiological Society
www.the-aps.org
American Society for Biochemistry and Molecular Biology
www.asbmb.org
American Society for Nutrition
www.nutrition.org
American Thoracic Society
www.thoracic.org
Australasian Society for Inborn Errors of Metabolism
www.hgsa.org.au/asiem
British Nutrition Foundation
www.nutrition.org.uk
Brazilian Physiology Society
www.sbfis.org.br
Brazilian Society for the Study of Obesity and Metabolic Syndrome
www.abeso.org.br
Brazilian Society of Food and Nutrition
www.sban.com.br
Canadian Institutes of Health Research
www.cihr-irsc.gc.ca/e/193.html
Chilean Society and Physiological Sciences
www.cienciasfisiologicas.cl
Endocrine Society
www.endocrine.org
European Association for the Study of Obesity (EASO)
easo.org

(continued)

Table 48.2 (continued)

European Association for the Study of the Liver (EASL)
www.easl.eu
European Federation of Neurological Societies
www.efns.org
European Respiratory Society
www.ersnet.org
European Society of Endocrinology
www.ese-hormones.org
Federation of American Societies for Experimental Biology (FASEB)
www.fasebj.org
Growth Hormone Society
www.ghresearchsociety.org
Group of Research and Innovation in Vascular Health (GRIVAS-Health)
grivashealth.cl
International Council on Amino Acid Science (ICAAS)
www.icaas-org.com
International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN)
www.ishen.org
International Society of Endocrinology
www.endosociety.com
Italian Nitric Oxide Society
www.i-no-s.it/index.php?lang=en
Polskie Towarzystwo Badan nad Otyloscia (PTBO)
ptbo.slam.katowice.pl/
Japanese Society for Amino Acid Sciences
www.asas.or.jp/jsaas/english/index.html
Japan Society of Nutrition and Food Science
www.jsnfs.or.jp/about/about_message.html
Japanese Society of Parenteral and Enteral Nutrition (JSPEN)
www.jspen.jp/top.html
Japanese Society of Internal Medicine
www.naoka.or.jp
Japan Gastroenterological Endoscopy Society
www.jges.or.jp
Japanese Society of Gastroenterology
www.jsge.or.jp
Japan Society of Hepatology
www.jsh.or.jp
Japan Society of Nutrition and Food Science
www.jsnfs.or.jp/english
Japan Society for the Promotion of Science
www.jsps.go.jp/english/index.html
Johns Hopkins Bloomberg School of Public Health
www.jhsph.edu
Korean Association for the Study of the Liver
www.kasl.org
Korean Cancer Association
www.cancer.or.kr
Nitric Oxide Society
nitricoxidesociety.org
Nutrition and Metabolism Society
www.nmsociety.org
Nutrition Society
www.nutritionsociety.org

(continued)

Table 48.2 (continued)

Pituitary Society
www.pituitarysociety.org
Sociedad Española de Nutrición Parenteral y Enteral (SENPE)
www.senpe.com
Societá Italiana di Endocrinologia
www.societaitalianadiendocrinologia.it/
Society for Inherited Metabolic Disorders
www.simd.org
Society for Nutrition Education and Behavior
www.sneb.org
Society for the Study of Inborn Errors of Metabolism
www.ssiem.org
Spanish Society for Biochemistry and Molecular Biology (SEBBM)
www.sebbm.es/EN
Sustainable Energy and Economy Network
www.seen.org

This table lists the professional societies with interests in L-arginine

Table 48.3 Journals that publish articles on L-arginine

Acta Cirúrgica Brasileira
www.scielo.br/scielo.php?script=sci_serial&pid=0102-8650&lng=en&nrm=iso
Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration
informahealthcare.com/journal/afd
American Journal of Clinical Nutrition
ajcn.nutrition.org
American Journal of Gastrointestinal and Liver Physiology
ajpgi.physiology.org
American Journal of Obstetrics and Gynecology
www.ajog.org
American Journal of Physiology: Gastrointestinal and Liver Physiology
ajpgi.physiology.org
American Journal of Physiology, Endocrinology and Metabolism
ajpendo.physiology.org
American Journal of Psychiatry
www.ajp.psychiatryonline.org/journal.aspx?journalid=13
American Journal of Respiratory and Critical Care Medicine
www.atsjournals.org/journal/ajrccm
Amino Acids
www.link.springer.com/journal/726
Biochemical Journal
www.biochemj.org/bj/default.htm
BioFactors
onlinelibrary.wiley.com/journal/10.1002/(ISSN)1872-8081
Biologia
link.springer.com/journal/11756
Biological Trace Element Research
www.springer.com/life+sciences/biochemistry+%26+biophysics/journal/12011
BioMed Central Cancer
www.biomedcentral.com/bmccancer
BioMed Central Gastroenterology
www.biomedcentral.com/bmcgastroenterol
Bioorganic & Medicinal Chemistry Letters
http://www.journals.elsevier.com/bioorganic-and-medicinal-chemistry-letters/
Blood
www.bloodjournal.org

(continued)

Table 48.3 (continued)

British Journal of Clinical Pharmacology
onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291365-2125
Cancer Cell
www.cell.com/cancer-cell
Cancer Immunology, Immunotherapy
www.springer.com/medicine/oncology/journal/262
Cancer Research
cancerres.aacrjournals.org
Cancer Research and Treatment
www.cancerresearchandtreatment.org
Cell Metabolism
www.cell.com/cell-metabolism
ChemMedChem
onlinelibrary.wiley.com/journal/10.1002/(ISSN)1860-7187
Chinese Journal of Physiology
www.cps.org.tw/index.php?action=archives
Circulation Journal
www.j-circ.or.jp/english/circulation_journal/circulation_journal.html
Clinical and Molecular Hepatology
www.e-cmh.org
Clinical Biochemistry
www.journals.elsevier.com/clinical-biochemistry
Clinical Endocrinology
onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-2265
Clinical Hemorheology and Microcirculation
www.iospress.nl/journal/clinical-hemorheology-and-microcirculation
Clinical Nutrition
www.journals.elsevier.com/clinical-nutrition
Diabetes
diabetes.diabetesjournals.org
Diabetes Care
care.diabetesjournals.org
Diabetes, Obesity and Metabolism
onlinelibrary.wiley.com/journal/10.1111/(ISSN)1463-1326
Digestion
www.karger.com/dig
Digestive Diseases and Sciences
link.springer.com/journal/10620
Endocrine Reviews
press.endocrine.org/journal/edrv
European Heart Journal
eurheartj.oxfordjournals.org
European Journal of Clinical Nutrition
www.nature.com/ejcn/index.html
European Journal of Endocrinology
www.eje-online.org
European Journal of Nutrition
link.springer.com/journal/394
European Journal of Pharmacology
sciencedirect.com/science/journal/00142999
European Respiratory Journal
erj.ersjournals.com
European Review for Medical and Pharmacological Sciences
www.europeanreview.org/
European Wound Management Association (EWMA) Journal
ewma.org/english/publications/ewma-journal/latest-issues.html

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Table 48.3 (continued)

Free Radical Research
informahealthcare.com/loi/fra
Frontiers in Nutrition
www.frontiersin.org/Nutrition
Frontiers in Pharmacology
www.frontiersin.org/Pharmacology
Fundamental & Clinical Pharmacology
onlinelibrary.wiley.com/journal/10.1111/(ISSN)1472-8206
Growth Hormone & IGF Research
www.journals.elsevier.com/growth-hormone-and-igf-research/
Gut
gut.bmj.com/
Hepatology
onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291527-3350
Hepatology Research
www.jsh.or.jp
Inflammatory Bowel Diseases
journals.lww.com/ibdjournal/pages/issuelist.aspx
International Journal of Diabetes and Metabolism
ijod.uaeu.ac.ae
International Journal of Surgery
www.sciencedirect.com/science/journal/17439191
Journal of Biological Chemistry
www.jbc.org
Journal of Clinical Biochemistry and Nutrition
www.jstage.jst.go.jp/browse/jcbn/
Journal of Clinical Endocrinology and Metabolism
press.endocrine.org/journal/jcem
Journal of Clinical Investigation
www.jci.org
Journal of Diabetes
onlinelibrary.wiley.com/journal/10.1111/(ISSN)1753-0407
Journal of Endocrinological Investigation
www.jendocrinolinvest.it/jei/it
Journal of Gastrointestinal Surgery: Official Journal of the Society for Surgery of the Alimentary Tract
link.springer.com/journal/11605
Journal of Hepato-Biliary-Pancreatic Sciences
www.editorialmanager.com/jhbp
Journal of Hepatology
www.jhep-elsevier.com
Journal of Immunology
jimmunol.org
Journal of Infectious Diseases
jid.oxfordjournals.org
Journal of Inherited Metabolic Disease
www.springer.com/?SGWID=5-102-0-0-0
Journal of Medicinal Chemistry
pubs.acs.org/journal/jmcmar
Journal of Neurochemistry
onlinelibrary.wiley.com/journal/10.1111/(ISSN)1471-4159
Journal of Neurotrauma
www.liebertpub.com/overview/journal-of-neurotrauma/39
Journal of Nuclear Medicine
jnm.snmjournals.org

(continued)

Table 48.3 (continued)

Journal of Nutrition
jn.nutrition.org
Journal of Nutrition and Metabolism
www.hindawi.com/journals/jnume/2011/617597
Journal of Nutritional Biochemistry
www.jnutbio.com
Journal of Nutritional Science
journals.cambridge.org/action/displayJournal?jid=JNS
Journal of Nutritional Science and Vitaminology
editors.capj.or.jp/~jnsv_web/index.html
Journal of Parenteral and Enteral Nutrition
pen.sagepub.com
Journal of Pediatric Gastroenterology and Nutrition
journals.lww.com/jpgn/pages/default.aspx
Journal of Physiology
onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291469-7793
Journal of Physiology and Pharmacology
www.jpp.krakow.pl
Journal of Sports Sciences
www.tandfonline.com/loi/rjsp20
Journal of Surgical Research
www.sciencedirect.com/science/journal/00224804
Journal of the American College of Nutrition
americancollegeofnutrition.org/content/the-journal
Journal of Transplantation
www.hindawi.com/journals/jtran
Journal of Trauma
journals.lww.com/jtrauma/pages/default.aspx
Langenbeck's Archives of Surgery
link.springer.com/journal/423
Life Sciences
www.sciencedirect.com/science/journal/00243205
Medicine and Science in Sports and Exercise
journals.lww.com/acsm-msse/pages/default.aspx
Mediterranean Journal of Nutrition and Metabolism
www.springer.com/food+science/journal/12349
Metabolic Brain Disease
link.springer.com/journal/11011
Metabolism
www.metabolismjournal.com
Mini-Reviews in Medicinal Chemistry
benthamscience.com/journal/index.php?journalID=mrmc
Molecular and Cellular Biochemistry
link.springer.com/journal/11010
Molecular Genetics and Metabolism
www.journals.elsevier.com/molecular-genetics-and-metabolism
Muscle and Nerve
onlinelibrary.wiley.com/journal/10.1002/(ISSN)1097-4598
Nature Genetics
www.nature.com/ng/index.html

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Table 48.3 (continued)

Neonatology
www.karger.com/neo
NeuroToxicology
www.journals.elsevier.com/neurotoxicology/CachedSimilar
Nitric Oxide: Biology and Chemistry
www.journals.elsevier.com/nitric-oxide-biology-and-chemistry
Nuclear Medicine and Biology
www.sciencedirect.com/science/journal/09698051
Nutrition
www.nutritionjrnl.com
Nutricion Hospitalaria
www.nutricionhospitalaria.com
Orphanet Journal of Rare Diseases
www.ojrd.com
Pediatric Surgery International
link.springer.com/journal/383
Physiological Reviews
physrev.physiology.org
Placenta
www.placentajournal.org
PLOS ONE
www.plosone.org
Proceedings of the National Academy of Sciences of the United States of America (PNAS)
www.pnas.org
Pituitary
www.springer.com/medicine/internal/journal/11102
Seminars in Perinatology
www.sciencedirect.com/science/journal/01460005
Shock journals
lww.com/shockjournal/pages/default.aspx
Sports Medicine
www.springer.com/medicine/journal/40279
Surgery Today
link.springer.com/journal/595
Tohoku Journal of Experimental Medicine
www.jstage.jst.go.jp/browse/tjem
Toxicological Sciences
www.bioxbio.com/if/html/TOXICOL-SCI.html
Transplantation
journals.lww.com/transplantjournal/pages/default.aspx
Transplantation Proceedings
sciencedirect.com/science/journal/00411345
World Journal of Gastroenterology
www.wjgnet.com/1007-9327/index.htm
Zhonghua Shao Shang Za Zhi
zhsszz.periodicals.net.cn/

This table lists the journals publishing original research and review articles related to L-arginine

Table 48.4 Relevant books

Barrett A, Rawlings N, Woessner J. *Handbook of Proteolytic Enzymes*. Academic Press, 1998, UK
Behrman RE, Butler AS. *Preterm Birth: Causes, Consequences, and Prevention*. National Academies Press, 2007, USA
Berg JM, Tymoczko JL, Stryer L. *Biochemistry*, 5th edition. W H Freeman, 2002, USA
Blachier F, Wu G, Yin Y. *Nutritional and Physiological Functions of Amino Acids in Pigs*. Springer, 2013, USA
Chynober LA. *Metabolic and Therapeutic Aspects of Amino Acids in Clinical Nutrition*. CRC Press, 2004, USA
Duggan CP, Watkins JB, Walker WA. *Nutrition in Pediatrics*, 4th edition. People's Medical Publishing House, 2009, USA
Golovinsky EV. *Biochemie der Antimetabolite*. Verlag der Bulgarischen Akademie der Wissenschaften, 2002, Bulgaria
Rosenthal GA, Berenbaum MR. *Herbivores: Their Interactions with Secondary Plant Metabolites*. Academic Press, 1991, USA
Hall JE. *Guyton and Hall Textbook of Medical Physiology*. Saunders, 2011, USA
Knobil E, Sawyer WH. *Handbook of Physiology* (part II), The Pituitary Gland and Its Neuroendocrine Control. American Physiology Society, 1974, USA
Lamprecht M. *Acute Topics in Sport Nutrition*. Karger, 2012, Germany
Mahan LK, Escott-Stump S, Raymond JL. *Krause's Food & the Nutrition Care Process*. Saunders, 2011, USA
Matata BM, Elahi M. *The Molecular Basis for Origin of Fetal Congenital Abnormalities and Maternal Health: An overview of Association with Oxidative Stress*. Bentham, 2011, USA
Mullen KD, Prakash RK. *Hepatic Encephalopathy*. Springer, 2010, USA
Newsholme E, Leech T. *Functional Biochemistry in Health and Disease*. Wiley-Blackwell, 2009, UK
Rao JN, Wang JY. *Regulation of Gastrointestinal Mucosal Growth*. Morgan & Claypool Life Sciences, 2010, USA
Tirapegui J. *Nutrição, Metabolismo e Suplementação na Atividade Física* (Nutrition, Metabolism and Supplementation on Physical Activity). Atheneu, 2012, Brazil
Tirapegui J. *Nutrição - Fundamentos e Aspectos Atuais* (Nutrition Fundamentals and Current Aspects). Atheneu, 2006, Brazil

This table lists books on L-arginine

Table 48.5 Relevant internet resources

Ajinomoto Corporation www.ajinomoto.com/jp
Associative Research Program (CONICYT, Chile) www.conicyt.cl/pia
Canadian Breast Cancer Foundation/Atlantic Chapter www.cbcf.org/Pages/default.aspx
Canadian Clinical Practice Guidelines www.criticalcarenutrition.com
Cardiogenetics cardiogenomics.med.harvard.edu/home
Chemistry of Amino Acids home.nas.net/~dbc/cic_hamilton/amino.html
Complementary and Alternative Medicine Guide www.umm.edu/altmed
Consensus: Growth Hormone Deficiency in Adults www.ghresearchsociety.org/files/2007_Consensus_AGHD.pdf
Consensus: Growth Hormone Deficiency in Children www.ghresearchsociety.org/files/ISS%20consensus.pdf
Critical Care Nutrition at the Clinical Evaluation Research Unit, Kingston General Hospital, Canada www.Criticalcarenutrition.com
Dalhousie University www.dal.ca

(continued)

Table 48.5 (continued)

Diseases
diseases.jensenlab.org
Endocrinology and Clinical Nutrition Research Center
www.ienva.org
European portal for rare diseases and orphan drugs
www.orpha.net
Fondecyt
www.conicyt.cl/fondecyt
Foods Highest in L-Arginine
nutritiondata.self.com/foods-000089000000000000000.html
Genetics Home Reference-MSUD
ghr.nlm.nih.gov/condition/maple-syrup-urine-disease
GeneMANIA
www.genemania.org
HighWire
highwire.stanford.edu
Human Protein Reference Database
www.hprd.org
MITOMAP
www.mitomap.org/MITOMAP
National Institutes of Health grants
www.nih.gov
Nova Scotia Health Research Foundation
www.nshrf.ca
Online Endotext
www.endotext.com
PubChem
pubchem.ncbi.nlm.nih.gov/rest/chemical/homoarginine
UpToDate
www.uptodate.com/home
Vascular Physiology Laboratory
labfisiovascular.blogspot.com

This table lists some internet resources on L-arginine

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References

1. Wijnands KA, Castermans TM, Hommen MP, Meesters DM, Poeze M. L-Arginine and citrulline and the immune response in sepsis. Nutrients. 2015;7(3):1426–63.
2. Wu G, Morris Jr SM. L-Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336:1–17.
3. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc RJ, Carey SM, Smith SB, Spencer TE, Yin Y. L-Arginine metabolism and nutrition in growth, health and disease. Amino Acids. 2009;37:153–68. doi:10.1007/s00726-008-0210-y.

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