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Bohuslav Ostadal
Naranjan S. Dhalla *Editors*

Cardiac Adaptations

Molecular Mechanisms

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Cardiac Adaptations-Molecular Mechanisms

Bohuslav Ostadal · Naranjan S. Dhalla
Editors

Cardiac Adaptations

Molecular Mechanisms

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Editors
Bohuslav Ostadal
Institute of Physiology
Academy of Sciences of the Czech Republic
Prague
Czech Republic

Naranjan S. Dhalla
Department of Physiology
St. Boniface Hospital Research Centre
Institute of Cardiovascular Sciences
University of Manitoba
Winnipeg, MB
Canada

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Dedication

This book is dedicated to Prof. Dr. Makoto Nagano for his exceptional leadership and extraordinary efforts in promoting cardiovascular science, translational medicine, and young investigators throughout the world.

Makoto Nagano, M.D.
Chairman of the Board of Directors,
International Academy of Cardiovascular Sciences;
President,
IACS-Japan;
Professor Emeritus, Jikei Univesity,
Tokyo, Japan

Preface

It is a fundamental property of all forms of life to adapt to changes in the environment. Such adaptations are commonly seen when individuals are exposed to high altitude, cold and hot conditions, or become apparent during ontogenetic development, exercise, and different diseases. These are, however, not limited—as is often done—to modifications that seem favorable to the individual. It is also becoming clear that these adaptations do not take place only at the whole body level, but all organs including the heart are also involved in this process. Cardiac adaptation to a wide variety of stressful situations such as pressure and volume overload, loss of functional tissue, or oxygen deprivation is usually associated with dramatic alterations in the circulating level of different hormones and growth factors providing signals to genetic apparatus in the cell. Different intracellular pathways are thus activated for remodeling of subcellular organelles, including extracellular matrix, sarcolemma, sarcoplasmic reticulum, mitochondria, and myofibrils. Particularly, changes in gene expression and protease activation play a critical role in inducing subcellular remodeling in the heart. In fact, remodeling of subcellular organelles is invariably associated with alterations in their function, and may serve as a compensatory mechanism for adaptation of cells and organs.

When the heart is confronted with an increased workload over a prolonged period, it usually tends to cope with the situation by increasing its muscle mass, a phenomenon referred to as cardiac hypertrophy. Initially, hypertrophy plays a compensatory role since it enables the heart to adapt to excessive hemodynamic load. However, the compensatory nature of hypertrophy if left unattended, deteriorates with time, and eventually ends in heart failure. Although the mechanisms of transition from compensatory cardiac hypertrophy to heart failure are not fully understood, depressed contractility during development of heart failure suggests an adaptive process that conserves the energy level. On the other hand, it can also be argued that contractile failure is a consequence of events associated with maladaptation. It is a real challenge not only to prevent the transition from compensated heart to failure but also to develop ways to manage subcellular and metabolic alterations during the development of contractile dysfunction. It follows that the processes of adaptation and maladaptation play an important role in the pathogeny

of serious cardiovascular diseases, such as hypertension, valvular diseases, congenital heart disease, myocardial infarction, and different cardiomyopathies as well as during adaptation to exercise and high altitude hypoxia.

This book summarizes the present knowledge of different mechanisms involved in the development of positive and negative consequences of cardiac adaptation. Particular attention was paid to the still underestimated adaptive cardiac responses during development, to adaptation to the frequently occurring pressure and volume overload as well as to cardiac changes, induced by enduring exercise and chronic hypoxia. Our effort was to put together the rapidly developing basic and clinically relevant information on adaptive mechanisms and thus contribute to the better understanding of possible prevention and therapy of life-threatening cardiovascular diseases.

The presentation of the subject matter in the form of 24 manuscripts on cardiac adaptations, as developed by several investigators for this book is organized in three parts. Part I dealing with developmental aspects of cardiac adaptation includes seven chapters on comparative and molecular aspects of cardiac development, prenatal and postnatal developments, coronary vascular development, and ontogenetic adaptation to hypoxia as well as cardiac and arterial adaptation during aging. Part II is devoted to cardiac adaptations to overload on the heart and includes eight chapters. Discussion in this part of the book is centered around the mechanisms of cardiac hypertrophy due to pressure overload, volume overload, exercise, gender difference, high altitude, and different pathological situations. Part III of this monograph includes nine articles on molecular and cellular mechanisms of cardiac adaptation. These chapters highlight the roles of sympathetic nervous system with respect to α-adrenoceptor and β-adrenoceptor mechanisms in the development of cardiac hypertrophy. In addition, the modulatory role of mitochondria, autophagy, adenosine, growth factors of different proteins and hormones in cardiac adaptation under several pathophysiological situations is discussed.

We are grateful to Ing. M. Markova from Prague as well as Dr. Vijayan Elimban and Ms Eva Little of Winnipeg for their help in editing the manuscripts. Cordial thanks are also due to Ms. Portia Formento, Springer USA, for her continuous advice and understanding during the editorial process. We hope this book will be of great value to students, fellows, scientists, clinicians, and surgeons. In addition, we believe that cardiovascular investigators will find this book highly useful in their studies for finding solutions to prevent and reverse cardiovascular abnormalities in diverse pathological conditions.

Prague — Bohuslav Ostadal
Winnipeg — Naranjan S. Dhalla

Contents

Contributors

Adriana Adameova Department of Pharmacology and Toxicology, Comenius University, Odbojarov 10, 832 32 Bratislava, Slovak Republic, e-mail: adameova@fpharm.uniba.sk

Veeranjaneyulu Addepalli Department of Pharmacology, SPP School of Pharmacy & Technology Management, SVKM's NMIMS, V. L. Mehta Road, Mumbai, Maharashtra 400056, India, e-mail: veeranjaneyulu.addepalli@nmims.edu

Feriel Azibani INSERM U942, Lariboisière Hospital AP-HP, Université Paris-Diderot, 75475 Paris Cedex 10, France

Aaron L. Baggish Division of Cardiology, Cardiovascular Performance Program, Massachusetts General Hospital, 55 Fruit Street, Yawkey 5, Boston, MA 02114, USA, e-mail: abaggish@partners.org

Michal K. Behulia Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

Slavka Carnicka Centre of Excellence NOREG SAS, Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovak Republic

Paul D. Chantler Division of Exercise Physiology, School of Medicine, West Virginia University, Morgantown, West Virgenia, USA

Zuzana Charvatova Department of Pathophysiology, Charles University, Prague, Czech Republic

Michael P. Czubryt Departments of Physiology, Institute of Cardiovascular Sciences, St. Boniface Research Centre, University of Manitoba, Winnipeg, MB, Canada; Institute of Cardiovascular Sciences, St. Boniface Research Centre, 351 Tache Avenue, RM 4011, Winnipeg, MB R2H 2A6, Canada

Dipak K. Das Cardiovascular Research Center, School of Medicine, University of Connecticut, Farmington, CT 06030-1110, USA, e-mail: ddas054@gmail.com

Elza D. van Deel Division of Experimental Cardiology, Department of Cardiology, Thoraxcenter, Erasmus MC, University Medical Center Rotterdam, 3000 DR, PO Box 1738, Rotterdam, The Netherlands

Claude Delcayre INSERM U942, Lariboisière Hospital AP-HP, Université Paris-Diderot, 75475 Paris Cedex 10, France

Melissa R. Dent St. Boniface Hospital Research Centre, Institute of Cardiovascular Sciences, Winnipeg, MB, R2H 2A6, Canada

Naranjan S Dhalla Departments of Physiology, Institute of Cardiovascular Sciences, St. Boniface Research Centre, University of Manitoba, Winnipeg, MB, Canada, e-mail: nsdhalla@sbrc.ca

Zdenka Dobešová Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

Dirk J. Duncker Division of Experimental Cardiology, Department of Cardiology, Thoraxcenter, Erasmus MC, University Medical Center Rotterdam, 3000 DR, PO Box 1738, Rotterdam, The Netherlands, e-mail: d.duncker@erasmusmc.nl

Vijayan Elimban St. Boniface Hospital Research Centre, Institute of Cardiovascular Sciences, Winnipeg, MB, R2H 2A6, Canada, e-mail: velimban@sbrc.ca

Xiaohong Tracey Gan Department of Physiology and Pharmacology, University of Western Ontario, London, ON N6A 5C1, Canada

Anne Garnier INSERM U-769, Université Paris-Sud, IFR141, 92296 Châtenay-Malabry, France

Dipti Gatne Department of Pharmaceutical Chemistry, SPP School of Pharmacy & Technology Management, SVKM's NMIMS, V. L. Mehta Road, Mumbai 400056, India

Frank N. Gellerich Department for Behavioral Neurology, Leibniz Institute for Neurobiology and Neurological Hospital, Magdeburg, Germany; Department of Neurology, Otto-von-Guericke University of Magdeburg, Magdeburg, Germany

Mahesh P. Gupta Department of Surgery, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637, USA, e-mail: mgupta@surgery.bsd.uchicago.edu

Frédéric Joubert INSERM U-769, Université Paris-Sud, IFR141, 92296 Châtenay-Malabry, France

Michaela Kadlecová Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

Morris Karmazyn Department of Physiology and Pharmacology, University of Western Ontario, London, ON N6A 5C1, Canada, e-mail: mkarm@uwo.ca

Frantisek Kolar Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

Jaroslav Kuneš Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic, e-mail: kunes@biomed.cas.cz

Edward G. Lakatta Laboratory of Cardiovascular Science, Intramural Research Program, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21225, USA, e-mail: lakattae@grc.nia.nih.gov

A. Malik Department of Physiology, St. Boniface Hospital Research Centre, Institute of Cardiovascular Sciences, University of ManitobaWinnipeg, MB, Canada ; Biochemistry and Medical Genetics, St. Boniface Research Centre, University of Manitoba, Winnipeg, MB, Canada

Vojtech Melenovsky Department of Cardiology, Institute for Clinical and Experimental Medicine: IKEM, Videňská 1958/9, Prague 140 21, Czech Republic, e-mail: vojtech.melenovsky@ikem.cz

Alison L. Müller St. Boniface Hospital Research Centre, Institute of Cardiovascular Sciences, Winnipeg, MB, R2H 2A6, Canada

Marta Novotova Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovak Republic

Ehte Orlova Department of Pathophysiology, Institute of General and Molecular Pathology, University of Tartu, 19 Ravila Street, Tartu 50411, Estonia

Bohuslav Ostadal Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic, e-mail: ostadal@biomed.cas.cz

Ivana Ostadalova Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

Zivorad Pesevski Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic ; Institute of Anatomy First Faculty of Medicine, Charles University in Prague, U nemocnice 3, Prague 12800, Czech Republic

Vinodkumar B. Pillai Department of Surgery, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637, USA

Jérôme Piquereau INSERM U-769, Université Paris-Sud, IFR141, 92296 Châtenay-Malabry, France

Tomas Rajtik Department of Pharmacology and Toxicology, Comenius University, Odbojarov 10, 832 32 Bratislava, Slovak Republic

Tanya Ravingerova Centre of Excellence NOREG SAS, Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovak Republic

Jean-Paul Richalet UFR SMBH, Université Paris 13, 74 rue Marcel Cachin, 93017 Bobigny Cedex, France, e-mail: richalet@smbh.univ-paris13.fr

Patricia Roche Department of Pathophysiology, Charles University, Prague, Czech Republic; Department of Intensive Care Medicine, VU University Medical Center, Amsterdam, The Netherlands

Jane-lise Samuel Inserm U942, Hôpital Lariboisière, 41 boulevard de la Chapelle, 75475 Paris Cedex10, France, e-mail: jane-lise.samuel@inserm.fr

Dev D. Santani Department of Pharmacology, L.M. College of Pharmacy, Ahmedabad 380009, India

David Sedmera Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic ; Institute of Anatomy First Faculty of Medicine, Charles University in Prague, U nemocnice 3, Prague 12800, Czech Republic, e-mail: dsedmera@biomed.cas.cz

Teet Seene Laboratory of Functional Morphology, University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia

Enn Seppet Department of Pathophysiology, Institute of General and Molecular Pathology, University of Tartu, 19 Ravila Street, Tartu 50411, Estonia , e-mail: enn.seppet@ut.ee

M. A. Q. Siddiqui Department of Cell Biology, State University of New York Downstate Medical Center, 450 Clarkson Avenue, New York 11203, USA, e-mail: maq.siddiqui@downstate.edu

Adriana Szobi Department of Pharmacology and Toxicology, Comenius University, Odbojarov 10, 832 32 Bratislava, Slovak Republic

Paramjit S. Tappia St. Boniface Hospital Research, Asper Clinical Research Institute, University of Manitoba, Winnipeg, MB, Canada

Robert J. Tomanek Department Anatomy and Cell Biology, Carver College of Medicine, University of Iowa, 1-402 BSB, Iowa, IA 52242, USA, e-mail: robert-tomanek@uiowa.edu

Ivana Vaněčková Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

H. R. Vasanthi Department of Physiology, St. Boniface Hospital Research Centre, Institute of Cardiovascular Sciences, University of ManitobaWinnipeg, MB, Canada; Biochemistry and Medical Genetics, St. Boniface Research Centre, University of Manitoba, Winnipeg, MB, Canada

Vladimir Veksler INSERM U-769, Université Paris-Sud, IFR141, 92296 Châtenay-Malabry, France

Jolanda van der Velden Laboratory for Physiology, VU University Medical Center, Amsterdam, The Netherlands

Renée Ventura-Clapier INSERM U-769, Université Paris-Sud, 5 rue J-B Clément, 92296 Châtenay-Malabry, France, e-mail: Renee.Ventura@u-psud.fr

Monique C. de Waard Division of Experimental Cardiology, Department of Cardiology, Thoraxcenter, Erasmus MC, University Medical Center Rotterdam, 3000 DR, PO Box 1738, Rotterdam, The Netherlands ; Department of Intensive Care Medicine, VU University Medical Center, Amsterdam, The Netherlands

Michael Wagner Department of Cell Biology, State University of New York Downstate Medical Center, 450 Clarkson Avenue, New York 11203, USA

Jeffrey T. Wigle Department of Physiology, St. Boniface Hospital Research Centre, Institute of Cardiovascular Sciences, University of ManitobaWinnipeg, MB, Canada; Biochemistry and Medical Genetics, St. Boniface Research Centre, University of Manitoba, Winnipeg, MB, Canada, e-mail: jwigle@sbrc.ca

Josef Zicha Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 14220, Czech Republic

Part I
Developmental Aspects of Cardiac Adaptation

Chapter 1
Comparative Aspects of Cardiac Adaptation

Bohuslav Ostadal

Abstract Transition from water to land, necessity of thermoregulation, and physical activity essential for survival of individual species represent serious changes in requirements imposed on cardiac function during phylogenetic development. As a result, the heart size in different species of vertebrates, expressed as a ratio of heart weight to body weight, varies considerably. The maximum acceleration of the heart growth during phylogeny occurs when the metabolic activity of animal tissues has substantially increased, i.e., during transition from poikilothermy to homeothermy. Phylogenetic differences in cardiac size, performance, and energy demand are reflected in the construction of an oxygen pathway from blood to mitochondria. The heart of cold-blooded animals is either entirely spongious, supplied by diffusion from ventricular cavity, or the inner avascular layer is covered by an outer compact layer with vascular supply. The compact heart of adult homeotherms is supplied by capillaries from coronary vessels. It was found that the thickness of the compact layer in poikilotherms increases with increasing heart and body weight, suggesting that the compact layer is necessary for the maintenance of the higher blood pressure in the larger hearts. The structural differences between spongious and compact musculature are accompanied by significant metabolic differences: the spongy myocardium is better equipped for aerobic metabolism than the compact tissue. It is obvious that the responses to different types of increased work load in individual species of lower vertebrates differ according to the structural, functional, and metabolic properties of their cardiac muscle. They vary from the isolated enlargement of the individual myocardial layers, i.e., spongious and compact musculature, to the enlargement of the whole heart, predominantly by combination of hypertrophy, and hyperplasia of muscle cells.

B. Ostadal (✉)
Insitute of Physiology, Academy of Sciences of the Czech Republic,
Videnska 1083, 142 20 Prague 4, Czech Republic
e-mail: ostadal@biomed.cas.cz

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Keywords Poikilotherms · Homeotherms · Heart size · Spongious musculature · Compact musculature · Adaptation to cold · Adaptation to chronic hypoxia

1.1 Introduction

The heart is capable of extensive adaptive growth in response to its function, because its performance as a pump is directly determined by the mass of its contractile elements. Cardiac performance, however, must, at the same time, be precisely adapted to oxygen consumption of the total active body mass. As a result, the heart size in different species of vertebrates, expressed as a ratio of heart weight to body weight (i.e., relative heart weight), varies considerably [1–3]. It is obvious that phylogenetic development in cardiac size, performance, and energy demand are reflected in the construction of an oxygen pathway from the blood to mitochondria [3, 4].

Whereas a lot of data are available concerning cardiac adaptation in warm-blooded animals, much less is known about this feature in lower vertebrates. In the present review we tried to discuss, therefore, (1) the relationship between the size of the heart and various factors determining oxygen consumption in different classes of vertebrates, (2) changes of internal myocardial structure and blood supply accompanying adaptive growth with particular attention to cold-blooded animals, (3) adaptation of the poikilothermic heart to the increased work load of different origin.

1.2 Comparative Anatomy

There are several common misconceptions about the cardiac anatomy and physiology of lower vertebrates. The most prevalent one is that all hearts fall more or less on a single continuum, with the heart of homeotherms being perceived as the most complex and efficient. However, some poikilothermic hearts (e.g., of reptiles) have a central cardiovascular complexity that rivals, if not surpasses, that of birds and mammals. Moreover, there is far more intraclass variation among fish, amphibians, and reptiles than there is among birds or mammals [5, 6].

The simplest anatomical form of vertebrate heart is that found in fish. It has four chambers—sinus venosus, atrium, ventricle, and bulbus arteriosus—and is considered to be a “venous heart”, since it pumps only systemic venous blood. Air breathing is associated with various degrees of modification; for example, in lungfish the systemic and pulmonary circulations reach the heart separately: the atrium, ventricle, and bulbus cordis show partial division. The heart of almost all amphibians is characterized by complete atrial division. Separate oxygenated and deoxygenated streams enter the left and right atrium; both atria in turn pump into a

single ventricle, whereas a partial separation of these streams is thought to be aided by deep ventricular trabeculae. Of the five vertebrate classes, reptiles show by far the greatest diversity in cardiac structure and function. The squamate heart has completely separated atria while the ventricle is most appropriately viewed as having three distinct cavities: cavum arteriosus, venosum, and pulmonale. The cavum venosum is the main systemic pump ejecting blood into the left and right aortic arch. Crocodilians have a heart with four separate chambers. At the gross anatomical and functional level, the hearts of adult birds and mammals are identical and constitute a low-pressure pulmonary and a high-pressure systemic circulation.

1.3 Relationship Between Heart and Body Weights in the Animal Kingdom

A basic parameter determining the functional efficiency of the heart in normal animals is cardiac mass. Most authors dealing with this question have attempted to find a relationship between heart and body weight. One of the first to draw conclusion from extensive material was Hesse [1], who collected numerous data after more than 10 years of work on the weight of the poikilothermic and homeothermic hearts. The mean values of the heart ratio (heart weight/body weight $\times$ 100) in individual classes of vertebrates are given in (Fig. 1.1). The relative heart weight is the highest in birds, followed by mammals and by poikilotherms; fish appear to have the lowest heart weight among all vertebrates. The maximum acceleration of the heart growth during phylogeny occurs when the metabolic activity of animal tissues has substantially increased, i.e., during transition from poikilothermy to homeothermy [3, 7]. There are two major factors responsible for this dramatic change: (1) increased energy demand connected with the necessity to maintain the stable body temperature in homeotherms and (2) energy demand induced by anti-gravitation load enabling the movement in birds. Moreover, during transition from water to land, the vertebrate heart has more than tripled in its size (the relative HW in fish 0.081 ± 0.03 and of amphibians 0.299 ± 0.17); when a homeotherm had to fly, the relative HW was almost doubled (mammals 0.643 ± 0.19, birds 1.099 ± 0.32) [8].

It is necessary to stress, that physically active poikilotherms and homeotherms have a greater relative heart weight than the inactive ones [2, 9]. Among 42 species of fish that were classified according to their motility, it appears that the most mobile species have the relatively largest heart. Rays (*Batoidei*) appear to be an exception: although they are not very active, they have a relatively large heart. In these animals, however, a certain proportion of the muscle tissue is transformed into an electric organ whose weight represents a distinct stress. Well marked differences in heart weight/body weight ratio can be observed also in amphibians. Frogs permanently living in water (*Rana esculenta*) have a lower heart ratio than

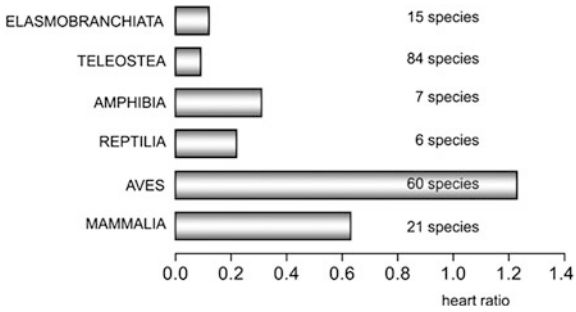

Fig. 1.1 Heart ratio in individual classes of vertebrates. Data from Hesse [1]

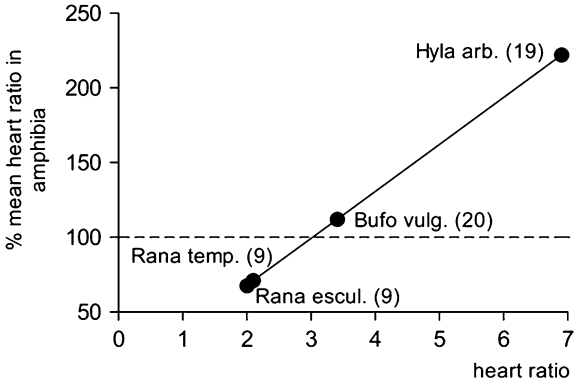

Fig. 1.2 Cardiac adaptations in poikilotherms. Heart ratio in frogs of various mode of life. Data from Hesse [1]

frogs predominantly living out of water (*Bufo vulgaris, Hyla arborea*). Moreover, the heart ratio of climbing tree-frog (*Hyla arborea*) attains even values encountered in homeotherm mammals (Fig. 1.2). A similar trend has been found in reptiles. The heart ratio of slow blind worm (*Anguis fragilis*) is smaller than that of the adder (*Tropidonotus*) or the viper (*Vipera berus*), even though its body weight is considerably smaller.

In homeotherms, the relationship between cardiac mass and physical activity was studied by Clark [2], who introduced the terms "athletic" and "non-athletic" animals. It is again necessary to stress the remarkable role of work against gravity in some homeotherms animals. Typical examples are two species: flying mammal—bat (*Myotis myotis*) and squirrel (*Sciurus*). This becomes especially evident if one compares the high heart ratio of these animals with that of mammals of approximately the same body weight. The role of excessive antigravity work of these mammals is readily noticeable. It is probable that differences in muscle work may also explain differences in heart ratio in domesticated and wild animal species [3].

1.4 Comparative Aspects of the Myocardial Structure/Blood Supply Relationship

The oxygen supply of the heart depends on the proportion between the amount of oxygen consumed by the cardiac cell and that offered to the cardiac cell. The heart is apparently not able to increase its oxygen supply to any appreciable extent by raising the oxygen extraction from the blood. Thus, the dominant factor affecting the supply of oxygen to cardiac structures is governed by the type and overall capacity of the cardiac blood supply. The first coronary vessels appeared in some fish at least 500 million years ago [8]. According to Grant and Regnier [10] a critical role in the development of coronary vessels is played by the pericardial ligaments connecting the heart of lower vertebrates with the remaining organism. These ligaments are well developed in fish and among amphibians in urodels, whereas in anurans and reptiles they are represented only by one or two strands; they disappear completely in mammals. In species where these ligaments were formed, two types of blood supply exist: (1) cephalic—where the coronary arteries arise from the hypobranchial branches and the venous blood flows back into the sinus venosus; (2) caudal—the extracoronary arteries penetrate the pericardium from the systemic circulation and the venous blood returns to systemic veins. Briefly, the blood supply to the heart is either hilar (coronary vessels) or extrahilar (extracoronary vessels) [4].

The development of coronary arteries during phylogeny is closely related to the transformation of the musculature from a spongy avascular myocardium into a compact myocardium supplied from coronary vessels. Whereas the heart of adult homeotherms consists entirely of compact musculature with coronary blood supply, in poikilotherm animals the cardiac musculature consists either entirely of the spongious type, or its spongious musculature is covered by an outer compact layer [3, 4, 10, 11]. The spongy-like musculature is supplied predominantly by diffusion from the intertrabecular spaces. They belong to the chamber cavity and have a continuous endothelial lining. Nevertheless, in some species of fish and reptiles capillaries can also be found in some trabecels of the spongy-like musculature. There are no structural differences neither between the capillaries in compact and spongious musculature or between capillaries in the heart of cold-blooded and warm- blooded animals [12, 13]. It can be summarized that in the animal kingdom generally there exist four types of myocardial blood supply (Fig. 1.3):

(a) spongious musculature only, supplied from the ventricular cavity;
(b) the inner spongious layer is covered by an outer compact musculature with a vascular supply;
(c) as (b) but capillaries are also present in some trabecels of spongious layer;
(d) compact musculature only, which is supplied from coronary vessels.

The trabecular nature of the spongy myocardium increases the surface area and reduces the diffusion distance for oxygen transfer from luminal blood. Presumably, the thickness of the spongy trabeculae is a compromise between minimizing the

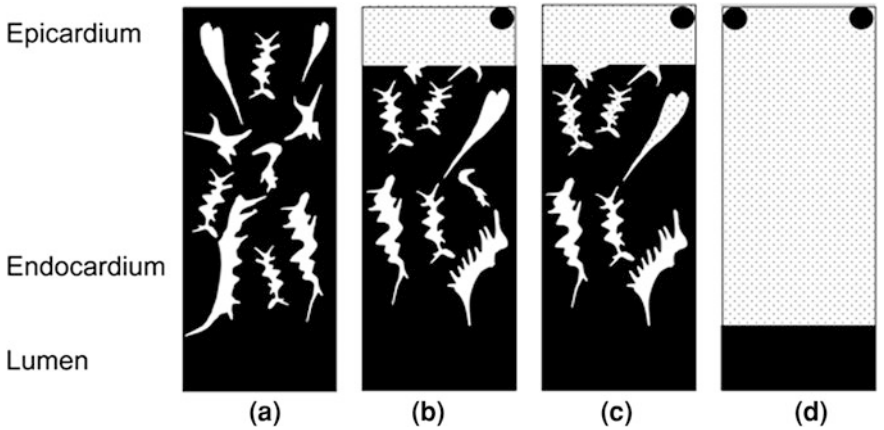

Fig. 1.3 Different types of myocardial blood supply. **a** Spongious musculature supplied from ventricular lumen. **b** Inner spongious layer is covered by an outer compact musculature with vascular supply. **c** As in b, but capillaries are present also in some trabecels of spongious musculature. **d** Compact musculature supplied from coronary vessels. (Adapted from Ostadal et al. [4]

distance for oxygen diffusion and maximizing the cross-sectional area for tension development [14]. Quantitative analysis of the terminal blood bed [4] has revealed that poikilotherms with a low relative heart weight (e.g. fish) have significantly lower lacunar capacity than animals with a higher relative heart weight, such as amphibians and reptiles. Kohmoto et al. [15] demonstrated a high degree of direct myocardial perfusion from the ventricular cavity in the endocardial region of alligator hearts, decreasing, thus, the diffusion distance for oxygen.

The myocardial blood supply of certain lower vertebrates appears to be "primitive and inefficient". However, there may be good reasons for the principles concerned to inform clinicians who treat heart disease [16]. Medical researchers seeking treatment for occluded coronary arteries became interested in the spongy myocardium of fish and reptiles. The information obtained represents a significant step toward achieving transmyocardial blood flow in patients. Surgeons used lasers to punch holes in the left ventricle to permit some oxygenation directly from the luminal blood, a technique known as transmyocardial revascularization [17]. This method may improve myocardial perfusion in patients in whom the standard method of revascularization is counter indicated.

1.5 The Role of Compact Layer in the Adaptive Growth of the Poikilothermic Heart

In this connection, the question arises about the main determinants of the cardiac transformation from the avascular spongy-like into the compact type with vascular supply. The exclusively spongious type can be observed not only in cyclostomes

and some teleost fish but also in some amphibians. On the other hand, the mixed types (compact+spongious) are present in fish [11, 18], amphibians [4], and reptiles [12, 19]. It seems, therefore, that the presence of the compact layer is not related to the phylogenetic position of the poikilothermic heart.

More than 35 years ago, an attempt was made to relate the growth of the compact layer to the physiological factors that determine overall oxygen consumption [3, 9, 11]. These factors are body mass, muscular activity, capacity of oxidative metabolism, and maintenance of body temperature. It was found that the myocardium of fish and amphibians with low body weight is spongious; the thickness of the compact layer increases with increasing heart and body weight. This relation is valid also within the same species investigated, carp and turtle [20]. The suggestion that the total amount of the compact layer might be related more to the physiological demand of the species than to its phylogenetic classification was further supported by an extensive survey by Santer and Greer Walker [21]. The proportion of the cardiac wall occupied by coronary supplied compact myocardium varies considerably, e.g., in fish between 7 and 37 % [22, 23], with the highest proportion in very active species (e.g., tuna, mackerel, sprat, herring, etc.). Moreover, Poupa et al. [24] observed an increase in the thickness of the fish (*Salmonids*) compact myocardium during ontogenetic development. In the turtle, the compact layer composes 55 % of the total cardiac weight [20].

Agnisola and Tota [25] and Totta and Gattuso [26] analyzed the relationship between the ventricular myoarchitecture and hemodynamic conditions in cold-blooded animals. They compared the stroke volume and afterload in different species with various degree of development of the compact musculature with vascular supply. The entirely spongious frog heart moves relatively large volumes against high pressures. A similar relationship can be observed among different species of fish; from the spongious heart of icefish with a "volume" pump chamber to that of tuna with mixed (arterial and lacunar) blood supply, as a prototype of a "pressure" pump. The sedentary icefish living in polar seas are characterized by the evolutionary loss of hemoglobin and the lack of functionally significant amounts of myoglobin. This fact is compensated for by a high blood volume at low rate (approximately 16 beats per minute). On the other hand, athletic tuna living in warm tropical water have a cardiovascular system designed to generate a high pressure in high-resistance systemic circulation. The high values are attained, in contrast with icefish, at a heart rate as high as 120 beats per minute. On the basis of these observations, it may be concluded that generation of higher blood volume requires a higher level of ventricular wall stress, which can be better attained by the development and/or thickening of the compact musculature. These results are in good agreement with those obtained with catecholamine-induced overload in the carp heart [27]. Repeated administration of low doses of isoproterenol did not influence the total weight of the fish heart, but the proportion of the outer compact layer was significantly higher as compared with the spongious one (Fig. 1.4). The response of the poikilothermic heart to catecholamine-induced overload thus differs significantly from that of homeotherms, where isoproterenol induces a significant increase in total heart weight [28]. Whether the observed increase of the

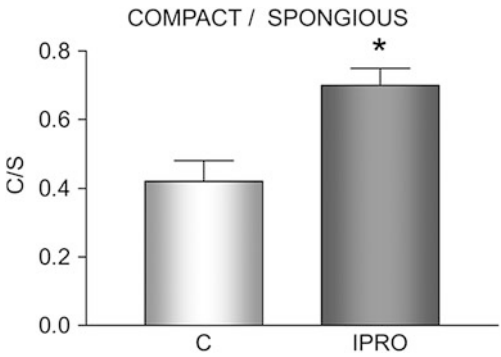

Fig. 1.4 Cardiac adaptations in poikilotherms. Relation between compact and spongious weight in control (*C*) and isoproterenol (*IPRO*) treated (15×5 mg· kg^{-1}) carps (*Cyprinus carpio*). Statistical significance $p < 0.001$. Data from Ostadal et al. [27]

compact/spongious ratio is the first step or the only type of the adaptation to this type of overload remains a matter of speculation.

Clark and Rodnick [29] studied the morphometric characteristics of ventricular enlargement during ontogenetic development of rainbow trout. They have found that increasing ventricle mass during sexual maturation results from an expansion of both myocardial layers, but growth of the compact layer exceeds that of the spongious musculature. They have confirmed the observation of Farell et al. [30] that growth of the trout ventricle occurs through a combination of myocyte hypertrophy and hyperplasia.

All these findings provide additional support for the hypothesis that an increase of the compact layer is necessary for the maintenance of higher blood pressure in the larger hearts (application of the law of Laplace to poikilothermic heart). This hypothesis also means, however, that the primary evolutionary step is not the development of arterial blood supply but the development of the compact layer, necessary for hemodynamic adaptations, i.e., for higher blood pressure generation. Vascularization is thus the consequence of this evolution, since coronary arteries are the only way how to supply the compact musculature with blood.

1.6 Cardiac Energy Metabolism in Poikilotherms

Decisive differences in cardiac energy metabolism in adult vertebrates are the result of changes in metabolic activities of animal tissues, the most obvious results taking place during transition from poikilothermy into homeothermy (for rev. see [31]). Thermoregulating mammals and birds have a relative heart weight and total metabolic capacity on average four times, whereas arterial blood pressure six times higher than in poikilotherms [8]. In terms of anaerobic metabolism, all requisite enzymes of glycolysis are routinely detected in vigorous activities. Hexokinase, which catalyzes the first step in the utilization of exogenously supplied glucose, is about five times more active in hearts of poikilotherms than homeotherms. The mitochondrial enzymes of the poikilotherm and homeotherm heart are similar in their structure and functional properties, but their activity per mg tissue or tissue

protein is considerably lower in cold-blooded hearts [32]. The ratio of creatine kinase to cytochrome-*c* oxidase, a rough estimate of aerobic capacity and cellular energy turnover, is significantly increased in poikilotherms [33]. When challenged by hypoxia, this high level may represent an enhanced efficiency to attenuate the impact of depressed energy liberation. Moreover, poikilothermic heart possesses a high relative glycolytic capacity as indicated by a high pyruvate kinase to cytochrome-*c* oxidase ratio.

The amazing scope of adaptability of the vertebrate heart is seen by comparing two extremes: the primitive cyclostome hagfish and the high energy consuming hummingbird [34]. In the hagfish, ball-like hearts pump almost completely desaturated blood with extremely low oxygen carrying capacity (1 ml O_2/100 ml), and a substantial left shift in the dissociation curve ($P_{50} = 5$). This implies that the hagfish and its heart operate under severe anaerobic conditions. The heart is a low performance pump (cardiac output = 1 ml/min), with a beat frequency of 15–20 per minute, endowed with mitochondria possessing a poor network of cristae, but a high glycolytic capacity, which accounts for its enormous resistance to anoxia. Sugars are its preferred fuel and fatty acids are not used [35]. In contrast, the hummingbird (*Beija flor*) has to fly while sucking nectar from flowers as essential food, consuming 42 ml O_2. g^{-1} h^{-1}. Its blood carrying capacity is 22 vol. % of O_2, which means that cardiac output = 12.6 ml/min, when O_2 utilization is 60 % [36]. It follows that every minute the hummingbird′s heart pumps a weight of blood 12 times greater than the weight of its body at a frequency of more than 1,000 beats per minute. Fat is the preferred fuel and is located in droplets in the vicinity of mitochondria, which are the main constituent of the cardiocytes [34].

Significant metabolic differences exist also between the compact and the spongious layer of the poikilothermic heart [20]. The activities of enzymes that are connected with aerobic oxidation (citrate synthase, malate dehydrogenase) and glucose phosphorylation (hexokinase) are higher in the spongious than in the compact layer; comparable results were obtained from Clark and Rodnick [29]. The spongy myocardium, more likely to receive high levels of metabolites and highly variable oxygen levels, thus seems more equipped for aerobic metabolism than the compact tissue [37]. Similarly, the content of phospholipids is higher in the spongious musculature, the greatest difference being in the content of diphosphatidylglycerol [38]. Furthermore, Maresca et al. [39] and Greco et al. [40] demonstrated that differences in enzyme activities are accompanied by different mitochondrial populations in the two layers. Moreover, the myosin ATPase acivity is significantly higher in the compact musculature as compared with the spongious layer in the carp heart. Since this ATPase activity is known to be related to the speed of contraction, a higher contraction velocity of the compact layer could be expected as well [20].

It may be concluded that structural differences between the compact and spongious myocardium are accompanied by significant metabolic differences between the two layers of the same heart of lower vertebrates. The heterogenous heart of cold-blooded animals, thus, offers a unique opportunity to compare the sensitivity of the two well-defined myocardial layers to increased load [41].

1.7 Cold Adaptation of the Poikilothermic Heart

Many poikilotherms are exposed to large changes in temperature on a seasonal basis. Some teleosts show enhanced swimming performance following acclimation to low temperature which may allow for active foraging and/or escape from predators under winter conditions [42]. A common but not universal response of poikilotherms to low temperature is an increase in the relative heart weight, attaining 10–30 % after a reduction in body temperature of about 10° or greater for about 4 weeks (rainbow trout). The change in heart ratio is due to selective increase in heart mass and to a decrease in body mass. This adaptive growth is presumably due to combination of hypertrophy and hyperplasia but this question is still unresolved [43]. The mechanism underlying the increase in tissue mass appears to be either a more efficient use of the protein synthetic machinery and/or a decrease in protein degradation. An alternative strategy of dealing with low temperature appears to be exhibited in perch. Low temperature acclimatization results in an enhanced ability to maintain high rates of contraction and presumably cardiac output with no increase in heart mass. Acute or chronic decreases in environmental temperature always result in decreases in resting heart rate in fishes. According to Driedzic et al. [43] there are two potential strategies in dealing with a decrease in heart rate which would lead to a decrease in cardiac output. One strategy is to have a larger heart thus a larger stroke volume, the other strategy is to be able to increase maximal heart rate at low temperatures. It is necessary to mention that hearts from cold-acclimated (10°) animals displayed rates of oxygen consumption that were significantly higher than hearts from warm-acclimated (25°) animals when tested at a common temperature of 15°.

Tota et al. [44] studied the heart of highly cold-adapted hemoglobin less Antarctic icefish that exhibit a thermal tolerance range of only about 6° (−1.86–4 °C). The main cardiovascular adaptations in icefish include two to four times higher blood volume as compared with most teleosts, a high cardiac output due to a very large stroke volume (6–15 times greater), low oxygen demand and impressive cardiac enlargement resulting in relative weights similar to that of small mammals. Enlargement of myocytes is due to extraordinary increase in the number of mitochondria, with volume densities the highest reported in any fish [45]. Such remarkable mitochondrial content occurs at the expense of the force generating elements, myofibrils. The high content of mitochondria has been interpreted as a typical response to cold adaptation [46]. In addition, the increased mitochondrial compartment could be part of the compensation for the lack of hemoglobin and myoglobin. The elevation of the heart ratio in icefish is higher than in other fish. In contrast, with the heart ventricle of active teleosts in which the spongiosa is covered by an outer compact layer, in icefish the cardiac enlargement is attained without participation of compact musculature (cardiomegaly of the spongy type) [47]. This type of enlargement realizes a well balanced increase of myocardial trabeculae and intertrabecular spaces in such a way that large intraventricular volumes can be adjusted during diastole and ejected during

systole with relatively low stresses on myofibres. The myocardium of icefish is thus reshaped to deal with the rate reducing effect of very low temperature (absence of myoglobin, mitochondrial proliferation, and reduction of myofibrils) and consequently appears unable to face the cardiac wall stresses associated with higher systemic pressure demands. This vulnerability emphasizes the remarkable cost of cardiac cold adaptation in these unique animals [44].

Tiitu and Vornanen [48] studied adaptation to low temperature in the crucian carp heart. The heart size was not increased, but cold acclimation effectively slowed heart rate and decreased contraction kinetics of the crucian carp heart and thereby preconditioned cardiac muscle for low energy supply of the anoxic winter conditions. Some fish respond to cold temperatures by entering the state of dormancy [49], characterized by locomotor's inactivity and reduction in food intake. In cold-dormant species, adaptive cardiovascular changes would be unnecessary and even maladaptive [48].

1.8 Adaptation of the Poikilothermic Heart to Chronic Hypoxia

Hypoxia is a frequently occurring environmental phenomenon in many fresh water and coastal systems, and can be caused by either anthropogenic input, or naturally occurring biological and physical factors. Recent studies show that hypoxia in marine waters is not restricted to localized areas, but is more extensive and longer lasting (weeks–months) than previously thought [50, 51]. These problems become even more serious if large areas are affected by hypoxia for an extended time, as fish may not be able to leave these areas; avoidance being the predominant reaction to hypoxia [52]. The understanding of how chronic hypoxia affects both swimming performance and cardiovascular function could reveal important information on whether fish will survive and how well they adapt to hypoxic environments. Of the experimental models commonly used for studying the effect of oxygen deprivation, only a limited number are suitable for the cold-blooded heart. The absence of, or partially developed, coronary circulation excludes; for instance, the possibility of using acute or chronic regional ischemia. The most frequently used models are, therefore, systemic and histotoxic hypoxia.

The poikilothermic heart, which is frequently exposed to oxygen deficiency in an aquatic environment, is better equipped biochemically to cope with oxygen deprivation than the mammalian one. Data comparing the sensitivity of the poikilothermic and homeothermic heart to oxygen deficiency are relatively scarce. The anaerobic capacity of cardiac muscle in different chordates from cyclostome (hagfish) to humans has been reviewed by Poupa [8] and Driedzic and Gesser [31]. Force development of isometric cardiac strips in vitro under roughly similar conditions was measured when respiration was blocked by cyanide (histotoxic hypoxia). The highest tolerance to lack of oxygen was observed in the reptilian

(*Monitor lizard*) and hagfish heart; the force development was reduced by no more than 30 % and recovered slowly toward initial values. Large differences were found in fish; contractile force in free swimming cod wad reduced by only 50 % (within 45 min after the onset of hypoxia). The highest sensitivity was observed in homeotherms (humans); a decline of contractile force occurred immediately (within 5 min) and was irreversible. In poikilotherms, the sensitivity to histotoxic hypoxia was increased upon increasing the temperature of the perfusion medium [53]. Myocardial cells of poikilotherms, similar as in homeotherms, may be irreversibly damaged by hypoxia [54]; highly resistant reptilian heart should be noted as a probable exception [55, 56].

Whereas a large amount of information currently exists on the cardiovascular responses of teleosts to acute hypoxia (e.g., Bushnell et al. [57] and Sandblom nad Axelsson [58]), much less is known about the effect of chronic hypoxia on fish cardiovascular function. Marques et al. [59] found that chronic hypoxia (10 % O_2, 3 weeks) leads to a smaller ventricular outflow tract, reduced lacunae, and an increase in the number of cardiac myocyte nuclei per area in the hearts of two teleost species, zebrafish and cichlids. In order to identify the molecular basis for the adaptation to chronic hypoxia, they profiled the gene expression changes in the heart of adult zebrafish. They have analyzed over 15,000 different transcripts and found 376 differentially regulated genes of which 260 genes showed increased and 116 genes decreased expression levels. Two notch receptors (notch-2 and notch-3) as well as regulatory genes linked to cell proliferation were upregulated in hypoxic hearts. They observed simultaneous increase in expression of IGF-2 and IGFbp 1 and upregulation of several genes important for protection against reactive oxygen species. Petersen and Gamperl [60, 61] studied acclimation of Atlantic cod to chronic hypoxia (8 kPa, 6–12 weeks, 10 °C). They have observed that acclimation did not affect body and heart weight or basal in situ cardiac performance under oxygenated conditions. Stroke volume and cardiac output during well oxygenated conditions were significantly reduced in hypoxia acclimated animals as compared with normoxic group. Since the same results were obtained from in vivo and in situ experiments, it may be suggested that in vivo cardiac function in hypoxia-acclimated cod was not lower because of alterations in nervous and/or humoral control, but because of the direct effects of chronic hypoxia on the myocardium. The hearts from hypoxia-acclimated animals maintained maximum performance longer when faced with severe acute hypoxia and recovered better than hearts from normoxia-acclimated fish following an acute hypoxic insult. These results suggest that acclimation to chronic hypoxia increases myocardial hypoxic tolerance and are consistent with the substantial body of research that has been conducted on chronically hypoxic mammals (for rev. see Ostadal and Kolar [62]). There are several mechanisms that have been reported to confer hypoxia tolerance of the mammalian hearts adapted to chronic hypoxia. Amongst these are ATP-sensitive potassium (K_{ATP}) channels, both sarcolemmal and mitochondrial, nitric oxide, HIF-1α, and various protein kinases [62, 63]. However, to answer the question whether the protective mechanisms responsible for increased hypoxic tolerance are the same in poikilothermic and homeothermic animals remains to be clarified in

future experiments. In this connection, the question arises whether the already high tolerance of the poikilothermic heart can be further increased by another protective phenomenon, preconditioning. Overgaard et al. [64] and Gamperl et al. [65] studied this still unanswered question in highly tolerant heart of rainbow trout (*Oncorhynchus mykiss*). They have found that hypoxic preconditioning failed to confer any protection against post-hypoxic myocardial dysfunction; inherent myocardial hypoxic tolerance and preconditioning are not additive. These results resemble the situation in highly tolerant neonatal rat hearts [66]; their already high hypoxic tolerance was impossible to increase by two different protective phenomena, ischemic preconditioning and adaptation to chronic hypoxia. It seems, therefore, that we are dealing with a common biological phenomenon: cardiac tolerance has its threshold.

1.9 Conclusions

What can we gain from studying the heart of lower vertebrates for analysis of mechanisms involved in cardiac adaptation? Although it is impossible to agree fully with the view that ontogenetic development is a recapitulation of phylogeny, comparative studies (more correct than "phylogenetic" since the researcher is comparing not the whole evolutionary range but only some classes—i.e. *pars pro toto* approach) have made significant contribution to our understanding of the function of the cardiovascular system [6, 8, 67]. These model systems will continue to be useful sources of information on developmental processes in mammals; there are increasingly compelling reasons to include studies of a variety of lower vertebrate systems. The reason is the importance of identifying general vertebrate developmental patterns in the cardiovascular system.

Typical example of the broad plasticity of the cardiovascular system is the cardiac ability to adapt to significant changes of environmental factors, which occur during phylogenetic development. Transition from water to land, necessity of thermoregulation, and physical activity essential for survival of individual species represent serious changes in requirements imposed on cardiac function. Whereas the mechanisms of adaptation of homeothermic heart are relatively well described, the possibilities of cardiac adaptation in lower vertebrates are still poorly understood. It is obvious that the responses to different types of increased work load in individual species differ according to the structural, functional, and metabolic properties of their cardiac muscle. They vary from the isolated enlargement of the individual myocardial layers, i.e. spongious and compact musculature, to the enlargement of the whole heart, predominantly by combination of hypertrophy and hyperplasia of muscle cells.

The most valid deployment of comparative methodologies involves more than simply comparing two different species. If the goal is to understand the evolutionary origins of the structure or process, then close attention to the whole evolution is

necessary. Perhaps, more importantly, the value of studying the cardiovascular system in lower vertebrates lies in providing a constant challenge to the notion that simple functions must arise from simple structures [5].

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Chapter 2
Molecular Mechanisms of Cardiac Development

Patricia Roche, Michael P. Czubryt and Jeffrey T. Wigle

Abstract The heart is the first organ to develop in order to supply the ever-increasing metabolic demands of the growing embryo. The heart is a unique structure in the body as it is derived from four distinct pools of progenitors: the first heart field (cardiac crescent), the second heart field (SHF), the proepicardial organ and the cardiac neural crest. These progenitors differentiate into the different cell types that comprise the adult heart: cardiomyocytes, endothelial cells, vascular smooth muscle cells, fibroblasts, and the conduction system. This complex program of differentiation is controlled by different molecular signaling pathways. A key component of the cardiac development program is the exquisitely coordinated expression of various genes in a spatially and temporally controlled fashion. Genes must be activated or repressed within restricted regions at specific times in order for normal cardiac development to proceed. In large part, this regulation of gene expression is controlled by an evolutionarily conserved set of transcription factors and microRNAs (miRNAs). Historically, the study of cardiac transcription factors has been very informative in understanding the early events in cardiogenesis. The rapidly evolving field of cardiac miRNAs promises to further extend our understanding of cardiac development. In this chapter, we will describe essential cardiac transcription factors and miRNAs and their role in controlling cardiac development.

P. Roche · M. P. Czubryt
Departments of Physiology and Biochemistry, University of Manitoba,
St. Boniface Research Centre, Winnipeg, MB, Canada

J. T. Wigle
Biochemistry and Medical Genetics, University of Manitoba,
St. Boniface Research Centre, Winnipeg, MB, Canada

P. Roche · M. P. Czubryt · J. T. Wigle (✉)
Institute of Cardiovascular Sciences, St. Boniface Research Centre,
351 Tache Avenue, RM 4011, Winnipeg, MB R2H 2A6, Canada
e-mail: jwigle@sbrc.ca

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2.1 Introduction to Cardiogenesis

Cardiogenesis begins during embryonic development. In humans, the heart is the first functional organ to develop, at around the third week of gestation. Subsequent embryonic development is dependent on the ability of the primordial heart to meet the embryo's ever-increasing demands for oxygen and nutrients. Heart development requires the precise migration, proliferation, and differentiation of a myriad of different cell types deriving from distinct embryonic origins. For proper cardiac development to occur, these processes need to be tightly controlled by a well-conserved set of molecular pathways. Even minor deviations from the cardiogenesis program can have severe consequences. Abnormalities in cardiac development are the most common form of birth defect, with congenital heart disease (CHD) affecting nearly 1 % of newborns. It is estimated that the number of affected pregnancies is tenfold greater if spontaneous abortions due to heart defects are accounted for [1].

The cardiogenic program involves the expression of evolutionarily-conserved transcription factors, many of which appear to have chamber-specific expression patterns. Cardiac development differs from organogenesis of other major organs, such as skeletal muscle, in that it involves intricate temporal and spatial interplay between a variety of factors. Another unique feature of heart development is evident in adult cardiac disease (e.g. hypertrophy), in which the fetal cardiac gene program is reactivated. In this chapter, we will review the transcriptional control of cardiac development. We will focus on the early pioneering studies on key transcription factors and recent developments in the field including the delineation of the roles of the second heart field (SHF), the proepicardial organ and microRNAs (miRNA).

2.2 Transcriptional Regulators of Cardiogenesis

Early during gastrulation, cardiac progenitor cells arise from the anterior lateral mesoderm and migrate through the primitive node and primitive streak to the cranial and cranio-lateral regions of the embryo. These early progenitors comprise a distinct epithelial cell population called the cardiac crescent that expresses cardiac-specific transcription factors [2] (Fig. 2.1). Positive and negative influences from the underlying endoderm act to induce cardiac specification in these progenitor cells. *Wnt*, the mammalian ortholog of the *Drosophila* gene *Wingless* (required for dorsal vessel formation) [3], is one of the earliest markers of cardiac specification in mesodermal progenitors [4]. Generally, anti-cardiogenic

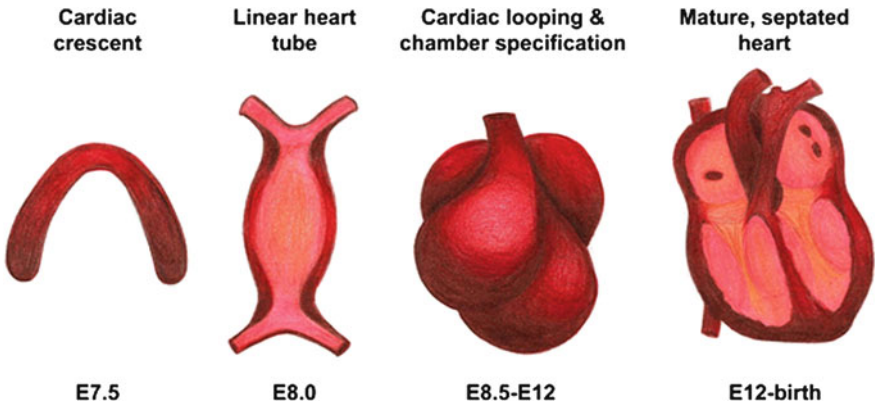

Fig. 2.1 Four major stages in mouse heart development: cardiac crescent formation (E7.5), formation of the linear heart tube (E8.0), cardiac looping (E8.5–E9.5) and specification of chamber identities (E10–E12), and maturation and septation of the heart (E12-birth). Transcription factors critical for modulating these processes are discussed in the text

*Wnt*3*a* and *Wnt*8 signaling appears to exert its effects via the canonical signaling pathway that inhibits cardiac specification, which is relieved by endogenous *Wnt* antagonists (*Crescent*, *Dickkopf*-1) [5–9]. The progenitors comprising the heart fields coalesce to form a parallel pair of vessels which fuse to form the cardiac tube. This tube undergoes rightward looping, followed by a series of septation and fusion events to give rise to the four-chambered heart, which further matures prior to birth (Fig. 2.1). Shortly after birth, cardiomyocytes undergo terminal differentiation, losing their proliferative capacity.

Each of these events relies on specific orchestrated roles of conserved cardiogenic transcription factors. Our focus will be on the most critical transcriptional modulators of heart development, most of which are evolutionarily conserved and implicated in the development of CHD. Here, we discuss some of the major players in the early stages of cardiogenesis (*Nkx*2-5, *MEF*2*C*, *Hand*1, *Hand*2, *Tbx*5 and *GATA*4).

2.2.1 Nkx2-5

Our current understanding of the mechanisms of vertebrate cardiogenesis originates from the study of model organisms, the most notable being the fruit fly *Drosophila*. Vertebrate orthologs of *Drosophila* genes instrumental to the formation of the primitive fly heart are expressed in the earliest stages of cardiac development—the establishment of cardiac lineage in the primary heart field and formation of the cardiac crescent. In some cases, maintained expression of these genes is crucial for the later stages of cardiac morphogenesis. Bodmer demonstrated that fruit flies, which were mutant for the homeobox gene *tinman*, lacked a

heart [10]. The mammalian ortholog, *Nkx*2-5 or *Csx*, unlike *tinman*, is not required for the initial stages of cardiogenesis in higher animals, suggesting functional redundancy with other genes which have not yet been elucidated. Although *Nkx*2-5 null mice have a heart, the heart does not loop properly and there is decreased expression of ventricular markers [11, 12]. In humans, an autosomal dominant form of CHD (atrial septal defects with atrioventricular conduction defects) was shown to be caused by mutations that altered the DNA binding ability of *Nkx*2-5 [13]. Further studies have identified over 40 different mutations in *Nkx*2-5 that give rise to dominant forms of human CHD [14].

2.2.2 MEF2C

Myocyte enhancer binding factor 2C (*MEF*2*C*) is one of the four mammalian orthologs of the *Drosophila MEF*2 gene required for muscle formation in the fruit fly [15]. *MEF*2*C* is expressed at E7.5 in the procardiogenic mesodermal cells of the mouse embryo prior to formation of the linear heart tube. *MEF*2*C*-null mouse embryos fail to properly develop a right ventricle (RV), do not exhibit cardiac looping, and fail to express a subset of cardiomyocyte-specific genes [16, 17]. At the presumed onset of cardiac looping, *MEF*2*C*-null mice also displayed downregulation of *Hand*2 expression and distorted *Hand*1 expression, which supports the role of *MEF*2*C* in ventricular development [17]. A dominant-negative form of *MEF*2*C* causes decreased post-natal myocardial growth in transgenic mice [18]. Targeted deletion of *MEF*2*C* also affects development of the embryonic vasculature, attenuating the expression of endothelial cytokines by the heart, which results in severe vascular anomalies and embryonic lethality by E9.5 [19, 20]. In addition to its role in cardiac myogenesis and right ventricular development, *MEF*2*C* expression is upregulated in response to both physiological and pathological cardiomyocyte hypertrophy [18]. *MEF*2*C* mutations in humans may contribute to non-syndromic congenital heart defects [21]. Recent reports from the Srivastava lab indicate that ectopic expression of *MEF*2*C*, along with the developmentally-important transcription factors *GATA*4 and *Tbx*5, can induce reprogramming of fibroblasts into cardiomyocyte-like cells and improve heart function after myocardial infarction, demonstrating the potency of these factors for governing cell fate [22, 23].

2.2.3 Hand1/Hand2

Proper development of the left and right ventricular myocardium requires expression of two chamber-specific basic helix-loop-helix transcription factors, *eHAND*/*Hand*1 and *dHAND*/*Hand*2. During the linear heart tube stage of murine cardiogenesis, *Hand*2 expression is higher in the RV while *Hand*1 is higher in the left ventricle (LV) [24, 25]. In *Hand*1-null mice, embryonic lethality occurred

between E7.5 and E9.5 due to extraembryonic mesodermal and yolk sac defects, accompanied by an arrest of cardiac development. Rescue of extra-embryonic *Hand*1 expression by aggregation of mutant and tetraploid wild-type embryonic stem (ES) cells extended the survival of null embryos to E10.5, however, the linear heart tube failed to undergo rightward looping [26, 27]. In hypomorphic *Hand*1 mouse lines, embryos are viable to between E10.5 and E12.5 and cardiac looping is rescued, but these mice display a thin LV myocardium and reduced expression of LV-specific markers [28]. Whereas ES cell-derived cardiomyocytes in a *Hand*1-null background display increased differentiation, its overexpression in vivo causes increased cardioblast proliferation resulting in an extended heart tube and extraneous looping [29]. It is also important to note that *Hand*1 expression and cardiac looping are abolished in *Nkx*2.5-null mice [24].

Targeted deletion of *Hand*2 in mice causes embryonic lethality by E10.5 and hypoplasia of the RV [25]. Double-knockout mice lacking *Hand*2 and *Nkx*2.5 develop a single atrial cardiac chamber [30]. The ability of *Hand*1 and *Hand*2 to form both homo- and heterodimers in vivo suggests some degree of functional redundancy during cardiac development [31]. This is confirmed by the recent report that genetic deletion of *Hand*2 specifically from *Hand*1-expressing cells resulted in mutants with impaired formation of epicardial tissues and a failure to develop coronary arteries [32].

Despite the critical roles of *Hand*1 and *Hand*2 in the developing mouse myocardium, the influence of these transcription factors on human congenital disease is far less clear. *Hand*2 deletion from neural crest cells results in impaired outflow tract formation in mice, and *Hand*2 mutations have been associated with right outflow tract stenosis in humans [33, 34]. Mutations of *Hand*1 in Chinese patients were associated with ventricular septal defects, however, *Hand*1 mutations were not associated with Tetralogy of Fallot, one of the most common congenital heart defects [35–37]. The impact of *Hand*1 and *Hand*2 mutations on other cardiac congenital conditions remains to be determined.

2.2.4 Tbx5

Studies in humans and mouse models have shown that the various atrial and ventricular septal defects associated with Holt-Oram syndrome result from loss of function of one allele of the transcription factor *Tbx*5 [38]. The T-box domain via which *Tbx*5 binds its target genes is highly conserved amongst mammals. Early in development, *Tbx*5 is expressed uniformly throughout the cardiac crescent. Temporal and spatial changes in *Tbx*5 expression throughout cardiac development indicate a critical role for this transcription factor in formation of the linear heart tube, cardiac looping, and chamber septation. Upon formation of the linear heart tube, *Tbx*5 expression becomes graded (with higher levels posteriorly). With cardiac looping, its expression is further restricted to the atria and left ventricle, where it marks the boundary at which the ventricular septum is formed [39].

Unlike other transcription factors discussed earlier, *Tbx*5 is not required for the earliest steps of cardiogenesis (i.e. cardiac crescent and heart tube development) though it is instrumental in later processes including chamber specification, septation and cardiomyocyte differentiation [40–42]. For example, *Tbx*5-null mice at E9.5 display arrested cardiac development as demonstrated by the failure of cardiac looping, and hypoplasia of the left ventricle, resulting in embryonic lethality at E10.5 [40]. Studies with embryonic stem cells overexpressing *Tbx*5 suggest that this transcription factor is involved in driving the expression of atrial and conduction system-specific markers [40, 43–46].

2.2.5 *Gata4*

The GATA family of double zinc finger transcription factors have been identified as crucial mediators of vertebrate cardiogenesis, with potential functional redundancy with one another [47–50]. *GATA*4 widely expressed in the developing heart and in adult cardiomyocytes, and plays a critical role in cardiac differentiation and morphogenesis [51–54]. In *GATA*4-null mice, gross cardiac defects (e.g., failure of the linear heart tube to form) result in embryonic lethality around E9.0 [53, 54]. However, for normal heart development, *GATA*4 expression is required only in the underlying endoderm and not within the cardiac mesoderm itself [54–57]. *GATA*4 has been shown to directly regulate the transcriptional activity of numerous cardiac-restricted genes that control cardiac progenitor cell differentiation, including *Mef*2*C*, *Hand*2 and *GATA*6. *GATA*4 associates with *Tbx*5 and *Nkx*2-5 to form multiprotein transcriptional complexes that regulate the expression of a set of downstream genes involved in cardiac development [40, 58, 59]. *Nkx*2-5 and *GATA*4 are in turn themselves regulated by *Nkx*2-5 and *GATA* factors in a positive feedback loop, which amplifies and maintains their expression in cells of the cardiac lineage [60, 61]. Thus, it appears as though the role of GATA factors in cardiogenesis is widespread and involves many other (and more cardiac specific) transcription factors. In humans, heterozygous *GATA*4 mutations result in familial septal defects and are involved in a range of cardiac defects including right ventricular hypoplasia and cardiomyopathy [62].

2.2.6 *Second Heart Field*

Historically, all cardiomyocytes were believed to originate from the cardiac crescent. This view of cardiac development was radically changed upon the discovery of a second source of cardiac cells in 2001, the second heart field (SHF), (Reviewed in [63]). Two studies in chick demonstrated by in vivo labeling the existence of a SHF that contributed to the outflow tract region of the heart [64, 65]. In addition, tissue isolated from this SHF could be differentiated into beating

myocytes. Whereas the first heart field contributes primarily to the left ventricle, the SHF contributes primarily to the RV and the outflow tract. The SHF in mammals was identified by studying a transgenic mouse where *LacZ* had been knocked in upstream of the *Fgf*10 gene [66]. The expression of this integrated transgene recapitulated that of endogenous *Fgf*10 and labeled the RV, outflow tract, and pharyngeal mesoderm. In vivo labeling studies in mice demonstrated that progenitor cells migrated from the pharyngeal mesoderm into the outflow tract during heart development. The SHF was shown to contribute to the RV and the outflow tract of the developing mammalian heart but not to the left ventricle [67, 68]. We will discuss below three transcription factors (*Isl*1, *Tbx*1 and *Hand*2) that have important roles in determining the fate of the SHF derivatives.

2.2.7 *Isl1*

Islet-1 (*Isl*1) is a LIM homeodomain-containing protein that was first isolated through binding to the enhancer region of the insulin gene [69]. More recently, *Isl*1 expression was shown to label the SHF in mice [70]. In vivo *Isl*1 expression is dependent on β-catenin and Forkhead transcription factors [71, 72]. Interestingly, *Isl*1 was shown to directly regulate expression of *Ffg*10, the gene that was first used to label the SHF in mice [73]. However, *Isl*1 is not exclusive to the SHF as it was also recently shown to label cardiac neural crest derivatives [74]. Knockout of *Isl*1 resulted in a heart that lacked expression of right ventricular markers [70]. The migration, proliferation, and survival of the SHF progenitors were negatively affected by loss of *Isl*1 function, indicating the key role of this transcription factor in the fate of the SHF.

*Isl*1 expression labels a pool of cardiac progenitors found in postnatal mouse and human hearts. These progenitor cells could be differentiated into cardiomyocytes with high efficiency [75]. A recent study by Bu et al. [76] established that *Isl*1 positive progenitors could give rise to three different cardiac lineages: cardiomyocytes, vascular smooth muscle cells, and endothelial cells. *Isl*1 expression was required for the differentiation of cardiac progenitors into cardiomyocytes and vascular smooth muscle in vivo but not required for endothelial differentiation [77].

2.2.8 *Tbx1*

T-box transcription factor 1 (*Tbx*1) has been implicated as being a cause of DiGeorge Syndrome and mice heterozygous for *Tbx*1 have cardiac outflow tract abnormalities similar to human patients [78–80]. Tissue-specific deletion of *Tbx*1 demonstrated that loss of *Tbx*1 function in endothelial cells or cardiomyocytes did not affect development of the outflow tract. In contrast, loss of *Tbx*1 function in the SHF (using *Nkx*2.5 Cre) resulted in outflow tract abnormalities that phenocopied

those seen in *Tbx*1-null mice [81]. These defects likely arise from reduced proliferation of progenitors resulting from decreased expression of *Fgf*8 and *Fgf*10 [81, 82]. In addition, the non-canonical *Wnt* family member, *Wnt5a*, has also been shown to be a direct target of *Tbx*1 in the SHF [83]. Gain of *Tbx*1 function in cardiomyocytes resulted in an expansion of the *Ffg*10 domain, a marker of the outflow tract [82]. Loss of *Tbx*1 expression in the mesoderm, but not in the pharyngeal endoderm, was sufficient to result in cardiac outflow tract abnormalities [84].

Fate mapping studies demonstrated that *Tbx*1-expressing cells differentiated into cardiac endothelial cells, vascular smooth muscle cells, and cardiomyocytes [85]. In vitro studies established that a single *Tbx*1-expressing progenitor could be differentiated into all three cell lineages [86]. *Tbx*1 was shown to increase the proliferative capacity of the cardiac progenitor cells but at the same time decrease their differentiation [86].

2.2.9 Hand2

The phenotype of the *Hand*2 knockout mice (above) was suggestive of a potential role for *Hand*2 in the SHF, since the RVs of these mice are hypoplastic [25, 30]. To determine the role of *Hand*2 in different progenitor populations, a conditional allele of *Hand*2 was crossed with a variety of Cre transgenic mice, which were expressed in different cardiac subpopulations [87]. Deletion of *Hand*2 via *Nkx*2-5 Cre, which is expressed in both ventricles but not in early cardiac progenitors, did not dramatically affect the initial formation of the ventricles. However, this deletion did affect the proliferation of cardiomyocytes, resulting in lethality by E12.5. In contrast, deletion of *Hand*2 with *Isl*1-driven Cre expression (deletion in the SHF) resulted in defective RV and outflow tract growth that phenocopied the original *Hand*2 null mice [25, 30]. These elegant experiments clearly demonstrated that *Hand*2 expression is required in the SHF progenitors in order for proper cardiogenesis to occur.

2.3 Proepicardial Organ and Epicardium

In addition to the first and second heart fields, the proepicardial organ (in the embryo) and the epicardium (in the adult) also contribute multipotent cardiac progenitors to the heart. The epicardium is the outer epithelial layer of the heart and is the last region of the heart to form during embryonic development. The epicardium is derived from a transient structure, the proepicardial organ, which forms at the sinus venosus of the developing heart [88, 89]. Cells from the proepicardial organ protrude via an extracellular matrix bridge to cover the developing heart, forming the epicardium [90]. Bone morphogenetic protein (BMP) signalling was

shown to be essential for the proepicardium to protrude and fuse with the heart tube [91]. Early retroviral lineage and chick/quail chimera studies demonstrated that cells of the proepicardium could differentiate into coronary vascular smooth muscle cells and endothelial cells [92, 93]. The transcription factors *Tbx*18, Scleraxis (*Scx*) and Wilms Tumor 1 (*WT*1) are important for the regulation of the differentiation of cells derived from the epicardium, as described below.

2.3.1 *Tbx18*

Analysis of the expression of *Tbx*18 in the mouse demonstrated that it was expressed predominantly in the proepicardial organ and the epicardium [94]. Knockout studies suggested a critical role for *Tbx*18-expressing precursors in generating the venous pole of the developing heart and for the formation of the sinoatrial node [95, 96]. In adult zebrafish, the epicardially-derived cells, marked by *Tbx*18 expression, differentiate into cardiomyocytes [97]. In mice, fate mapping studies showed that *Tbx*18-positive progenitors could differentiate into cardiomyocytes and vascular smooth muscle cells, but not into endothelial cells [98]. This study identified the proepicardial organ/pericardium as being a novel source of cardiac cells. However, a subsequent paper demonstrated that *Tbx*18 is itself expressed in cardiomyocytes and this expression may impact the lineage tracing findings of Cai et al. [99].

2.3.2 *Wilms Tumor 1*

Wilms Tumor 1 (*WT*1) is expressed in the proepicardial organ and the pericardium in the mouse. Loss of *WT*1 function resulted in epicardial defects and a lack of proper intramyocardial blood vessels [100, 101]. Lineage mapping studies demonstrated that *WT*1 was expressed in the mouse epicardium but that these cells could differentiate into vascular smooth muscle cells, cardiomyocytes, and a very small minority of endothelial cells [102]. *WT*1 has been proposed to repress the epithelial nature of the epicardial cells to promote their transition into a more mesenchymal phenotype, which is required for the formation of cardiac progenitors [103]. *WT*1 was also shown to mark a population of resident cardiac progenitor cells in the adult mouse heart that could differentiate into functionally integrated cardiomyocytes [104].

2.3.3 *Epicardially-Derived Cardiac Endothelial Cells*

Unlike the chick model, mouse epicardium progenitors were not observed to efficiently differentiate into endothelial cells [98, 102]. The reasons for this discrepancy were not clear until recently. A study by Katz et al. [105] demonstrated

that a second subpopulation existed in the proepicardial organ that was neither *Tbx*18- nor *WT*1-positive but instead expressed the transcription factor *Scleraxis* and Semaphorin3D receptor. These progenitors differentiate into coronary endothelial cells, sinus venosum, and cardiac endocardium. Thus the proepicardium is an important new source of cardiac endothelial progenitors in both chick and mouse.

2.4 miRNAs and Cardiac Development

2.4.1 Introduction to miRNAs

The field of miRNA biology has recently exploded, with over 15,000 papers published on the subject since the first definitive identification of this class of small regulatory RNAs in *C. elegans* in 2001 [106–108]. Virtually all branches of physiology and pathophysiology have been revolutionized with the increasing recognition of the key role played by miRNAs in health and disease, and cardiovascular science is no exception. This new-found understanding of how miRNAs regulate protein translation has helped to define novel mechanisms governing cardiac development, growth, and dysfunction.

miRNAs are a novel post-transcriptional mechanism of gene regulation, and serve to target specific mRNAs for silencing or degradation, providing an additional layer of control governing protein production beyond mRNA transcription and translation. At the same time, miRNA expression itself is tightly regulated, thus providing for exquisite and fine-grained control over gene transcriptional outputs.

A comprehensive explanation of the mechanism by which miRNAs regulate gene expression is beyond the scope of this chapter, and the reader is directed to recent excellent reviews on the subject [109, 110]. In brief, genes encoding miRNAs are transcribed by RNA polymerase II to generate long primary miRNAs (pri-miRNAs); these initially single-stranded molecules self-hybridize to form complex stem-loop structures, and may encode more than one miRNA in a polycistronic structure. The pri-miRNA is processed in the nucleus by a large complex that includes the RNAse Drosha and numerous cofactors, which cleave off individual stem-loop structures as nascent precursor miRNAs (pre-miRNAs). Another class of miRNAs is encoded by special introns within genes (mirtrons) which produce a pri-miRNA that directly forms a pre-miRNA via splicing, and thus does not require processing by the Drosha complex. Pre-miRNAs are exported from the nucleus via Exportin-5, and once in the cytosol are further cleaved by the Dicer complex to produce mature miRNAs consisting of a guide strand and a passenger strand (designated as miRNA and miRNA*), each approximately 22 nucleotides in length.

The mature miRNA possesses, within its 5′ region (from nucleotides 2 to 8), a seed sequence, which determines the specificity of the miRNA for its target mRNAs. The guide strand forms part of the RNA-induced silencing complex (RISC) and incompletely base pairs with the target mRNA. The result is that the target mRNA is either silenced or is targeted for degradation. While the passenger strand is typically degraded, in some instances this strand can also enter into a RISC complex to regulate other mRNAs. An important consequence of the incomplete base pairing between the miRNA and mRNA strands is that a single miRNA can target multiple target mRNAs. Accurate prediction of the biologically relevant targets of miRNAs remains technically challenging, since it remains unclear exactly how other sequences outside of the seed affect target selection.

The genes encoding miRNAs are themselves highly regulated. miRNA genes frequently exist within the introns of genes, some of which are even targeted by the miRNA. In many cases, cis-acting DNA elements governing expression of the host gene may also control expression of the miRNA. However, miRNA genes, particularly when found outside of mRNA-encoding genes, may also possess their own enhancer elements. For example, expression of the miR-1/miR-133 miRNA cluster is regulated by a variety of muscle-enriched transcription factors such as *Nkx*2-5, *Mef*2*c* and *MyoD* [110].

2.4.2 General Requirement of miRNAs for Cardiogenesis

The pioneering work of the Srivastava lab first demonstrated that specific miRNAs exhibit cardiac-specific expression, and are required for normal cardiac development [111, 112]. While a variety of studies have now implicated specific miRNAs in regulating cardiac specification and development (see below), the broader approach of genetic deletion of components of the miRNA processing pathway has revealed a general requirement for miRNAs at various stages of cardiogenesis. Since Dicer is required for maturation of all pre-miRNAs to miRNAs, deletion of the Dicer gene effectively eliminates miRNA-mediated mRNA silencing and degradation. These studies exploit the Cre-lox conditional gene deletion system by driving Cre expression in mice using cardiac-specific, temporally-activated promoters, and permit the development of a general timeline for when miRNAs are important for particular events or processes.

Zhao et al. [113] deleted Dicer early in cardiogenesis using the *Nkx*2-5-Cre driver mouse line, which resulted in gene excision by embryonic day E8.5. The resulting embryos died by E12.5 with pericardial edema, indicating that heart failure may have occurred. The ventricular myocardium exhibited thin walls, which suggested that the hearts were hypoplastic. While several early markers of cardiac differentiation and patterning showed normal expression, including *Tbx*5 and *Hand*2, the endoderm marker α-fetoprotein was upregulated. This result, combined with the observed hypoplasia, suggests the possibility that cardiac progenitor fate had been altered. This alteration is likely due to the roles of several

key miRNAs in governing progenitor differentiation and proliferation (see below) as well as a demonstrated critical role for Dicer in maintaining stem cell survival during development [114]. In related cell types, Dicer may or may not play a critical role in cell survival. Neural crest cells migrate to form parts of both the cardiac outflow tract as well as craniofacial structures. Deletion of Dicer in these cells using a *Wnt*1-Cre driver demonstrated that Dicer was not required for survival of cells fated to form the outflow tract, although formation of this structure was significantly impaired due to altered neural crest cell migration and patterning [115]. In contrast, neural crest cells that contributed to craniofacial structures failed to survive, resulting in dramatic defects resembling human congenital malformations.

In contrast to early deletion of Dicer, deletion later in development using *αMHC*-Cre resulted in viable pups and normal Mendelian ratios of offspring at birth [116]. However, Dicer-null animals failed to thrive, and all died by four days after birth with signs of dilated cardiomyopathy including reduced sarcomere number and length, and signs of increased apoptosis. Proliferation of cardiomyocytes was normal in the Dicer-null pups, thus later deletion of Dicer in the heart exhibits quite distinct consequences in comparison to early deletion, reflecting a shift in the role of miRNAs at different stages of cardiac development and maturation. However, it is important to remember that deletion of Dicer globally impacts the effects of all miRNAs. Since miRNA expression is dynamically regulated and constantly changing, the true role of miRNAs in cardiogenesis can only be resolved by deletion of specific miRNAs or miRNA clusters.

2.4.3 The Roles of Specific miRNAs in Cardiogenesis

Recent experiments have elegantly delineated the role of individual key miRNAs in cardiogenesis. While a complete description of all miRNAs that have been demonstrated to impact heart development is beyond the scope of this review, we present here an overview of several critical miRNAs in this process.

2.4.4 miR-1 and miR-133

miR-1 is highly expressed in the developing myocardium including the heart tube in the chick and was one of the first miRNAs to be studied for its role in cardiogenesis [113, 117]. miR-1 is bicistronic with mir-133, and there are two copies of this cluster: miR-1-1 and miR-133a-2 on human chromosome 2, and miR-1-2 and miR-133a-1 on chromosome 18. These miRNAs are expressed at relatively high levels in the myocardium, and along with other miRNAs such as miR-208 and miR-499, have been termed “MyomiRs” since they are specific to or enriched in muscle cell types. Another bicistronic cluster on chromosome 1 contains miR-206

and miR-133b, which contain the same seed sequences as miR-1 and miR-133a respectively, but are expressed primarily in skeletal muscle [118].

Cardiac-specific deletion of miR-1-2 (without deletion of the associated miR-133a-1) results in ventricular septal defects and dilated cardiomyopathy, leading to reduced numbers of null animals at birth and the death of approximately 50 % of mouse pups by weaning [113]. This phenotype shows interesting and intermediate similarities to those observed for cardiac-specific Dicer deletion, suggesting that miR-1 may be of particular importance among all miRNAs during cardiac development [113, 116]. Overexpression of an miR-1 transgene under control of the βMHC promoter arrests growth at E13.5 [112]. The hearts of these animals exhibit signs of failure, with hypoplasia and reduced numbers of proliferating cells without increased apoptosis, suggesting that the defects may be due to early exit of cardiomyocytes from the cell cycle. miR-1 is highly upregulated during differentiation of cardiomyocyte progenitor cells, and adenoviral delivery of miR-1 to these progenitors reduced proliferation while inducing differentiation to cardiomyocytes [119]. miR-1 may thus function to drive cardiomyocyte progenitor cells to a cardiac muscle fate. Consistent with this model, one of the gene targets of miR-1 is the Notch ligand *Delta-like* 1, which must be down-regulated to permit muscle differentiation to progress [120]. This result parallels a previous report in Drosophila demonstrating that the fly homolog dmiR-1 targets *Delta* [111].

Genetic deletion of either miR-133a-1 or miR-133a-2 results in offspring with hearts that are largely normal, suggesting that these miRNAs may compensate for each another [121]. Deletion of both miR-133a-1 and miR-133a-2, however, results in dramatic cardiac defects similar to those observed with miR-1-2 deletion. Such defects include ventricular septal defects, right ventricular wall thinning, and dilation and signs of impaired pump function, resulting in significant perinatal lethality. Both proliferation and apoptosis of cardiomyocytes were increased in double knock-out mice. Overexpression of miR-133a under control of the βMHC promoter resulted in a very similar phenotype to that observed in βMHC-miR-1 mice: embryonic lethality, enlarged atria, ventricular septal defects, and reduced proliferation of cardiomyocytes [121].

Superficially, these results suggest that miR-1 and miR-133 may serve similar functions in vivo, including preventing progenitor cells from adopting non-cardiac fates. It is noteworthy that in the miR-133a double knockout mice, increased smooth muscle marker gene expression was observed compared to wild type littermates [121]. However, in contrast to miR-1, miR-133 may maintain the cardiomyocyte progenitor pool and promote progenitor cell proliferation rather than differentiation [112, 120, 122]. Thus, these two miRNAs may serve distinct but overlapping roles in balancing factors that promote progenitor proliferation versus differentiation, such that disruption of either role leads to cardiac dysgenesis.

2.4.5 miR-208 and miR-499

miR-208a and 208b have identical seed sequences, and miR-499 is closely related to both. The genes encoding these miRNAs are found within introns in genes encoding α-myosin heavy chain, β-myosin heavy chain and β-myosin heavy chain 7B, respectively [123–125]. An intricate interplay between these factors and thyroid hormone-mediated cell signaling governs myosin isoform switching during maturation of the heart, as well as re-initiation of fetal β-myosin gene expression during cardiac remodeling. While cardiac-specific overexpression of miR-208a resulted in cardiac hypertrophy in adult transgenic mice, genetic deletion resulted in normal hearts [123]. However, alteration of miR-208a expression resulted in cardiac conduction changes, including prolonged PR intervals in both null and transgenic lines, partially penetrant second degree AV block in transgenics, and possible atrial fibrillation in the nulls. miR-208a may thus be important for proper development of the cardiac conduction system, although it is not critical for survival.

Despite its close relationship to miR-208a and 208b, miR-499 appears to play a significantly different role in the developing myocardium, including defining cardiac cell fate. Like miR-1, miR-499 expression increases during cardiac progenitor differentiation, and overexpression of miR-499 enhances differentiation and reduces proliferation of progenitor cells via regulation of *Sox*6, although the reduction of proliferation was not to the same degree as miR-1 [119, 126]. Fu et al. [127] demonstrated that lentiviral-mediated overexpression of miR-499 in human embryonic stem cells increased the yield of stem cell-derived ventricular cardiomyocytes by nearly 50 % over controls. In an exciting proof-of-concept experiment, Dzau's laboratory has reported evidence of fibroblast-to-cardiomyocyte conversion in situ following direct injection of miRNAs 1, 133, 208 and 499 into the murine myocardium following infarction [128]. Thus, developmentally important miRNAs may be useful for therapeutic strategies to repopulate the damaged myocardium, although the mechanism of this approach remains to be determined.

2.4.6 Other miRNAs

While the above mentioned MyomiRs have attracted significant attention recently, it is likely that numerous other miRNAs remain to be discovered that can exert significant effects on cardiogenesis. For example, the miR-17 ~ 92 cluster comprises six individual miRNAs expressed from a common locus, and is found in the secondary heart field and cardiac outflow tracts [129]. Deletion of miR-17 ~ 92 results in ventricular septal defects, as well as perinatal lethality due to severe lung hypoplasia [130]. miR-17 and miR-20a repress the cardiac progenitor genes *Isl*1 and *Tbx*1 downstream of BMP signaling, suggesting that the miR-17 ~ 92 cluster enhances differentiation of myocardial progenitors to a cardiac fate [129]. MiR-17 ~ 92 and the unrelated miR-130a have both been demonstrated

to target Friend-of-Gata-2 (FOG2), and overexpression of each leads to inhibition of proliferation of mouse embryonic cardiomyocytes in vitro and ventricular hypoplasia with septal defects in vivo, respectively [131, 132].

An important aspect of postnatal cardiac maturation is the withdrawal of cardiomyocytes from the cell cycle. Work from Olson and colleagues demonstrates that the miR-15 family member miR-195 is upregulated from postnatal day 1–10, a time period that overlaps with cell cycle withdrawal [133]. MiR-195 regulates expression of the checkpoint kinase Chek1, and overexpression of miR-195 under control of the βMHC promoter gave rise to ventricular hypoplasia and ventricular septal defects, consistent with early withdrawal of cardiomyocytes from the cell cycle. Conversely, antagonism of the miR-15 family using a locked nucleic acid anti-miR approach caused an increase in cardiomyocyte proliferation.

2.5 Conclusions

Studies of cardiac transcriptional control over the past 15 years have been instrumental in our understanding of the processes of cardiac development. New roles for transcription factors in controlling the differentiation of cardiac cells are still being discovered. The emerging field of cardiac miRNAs has tremendous potential to further extend our knowledge, and has already had a dramatic impact on our understanding of cardiogenesis. The study of these developmental signaling cascades is also clinically relevant as many of them are frequently reutilized in the adult following stresses such as myocardial infarctions and have furthermore been implicated in congenital defects in humans. Despite the remarkable depth of knowledge that we now have in understanding how the heart is specified and forms, a number of outstanding questions remain. For example, the very earliest signals that induce cardiac progenitor specification are unknown. The factors that are redundant with *Nkx*2-5 also remain unclear. A mechanism governing development of the cardiac extracellular matrix is also lacking. Finally, our understanding of the reasons for reactivation of the fetal gene program in response to many forms of cardiac stress remains elusive. Clearly, further research is required to elucidate the complex interplay between cardiogenic factors and the pathways which they activate, during both embryonic development and the onset of cardiovascular disease.

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Chapter 3
Prenatal Adaptations to Overload

Zivorad Pesevski and David Sedmera

Abstract Like every muscle in the body, the heart adapts its structure and mass to functional requirements, which are primarily determined by loading conditions. These can be classified as pressure loading (increased afterload, as in hypertension or banding of an artery), or volume loading (preload, which could be increased by creating an arterio-venous shunt, valve regurgitation, or shifting the blood streams). While numerous models exist for modifying the loading conditions postnatally, this chapter focuses on those few created in the developing heart, chiefly during prenatal development. As the reactions of the early postnatal heart are somewhat similar to the response of the fetal one, discussion of early postnatal models is also included. Principal findings from these studies are considered in the light of clinical scenarios of abnormal loading in congenital heart malformations and their optimal surgical management.

Keywords Chick embryo · Fetal sheep · Hyperplasia · Hypertrophy · Aortic banding · Pulmonary artery banding · Conotruncal banding · Left atrial ligation · Prenatal myocardium

Z. Pesevski · D. Sedmera (✉)
Institute of Physiology, Academy of Sciences of the Czech Republic,
Videnska 1083, 142 20 Prague, Czech Republic
e-mail: dsedmera@biomed.cas.cz

Z. Pesevski · D. Sedmera
Institute of Anatomy First Faculty of Medicine, Charles University in Prague,
U nemocnice 3, 128 00 Prague, Czech Republic

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3.1 Introduction

The human heart starts to beat during the fourth week of gestation, and has to constantly adapt its structure to ever-increasing functional demands of the growing embryo and fetus. Compared to the adult, the function of the developing heart is much more difficult to study, although recent advances in imaging techniques resulting in improved spatial and temporal resolution allow us a glimpse at previously impossible to study stages. Even more difficult task is to create meaningful experimental perturbations; the most popular rodent models (mice and rats) are too small to operate on, and larger animals such as sheep are quite costly. In addition, fetal surgery is not an easy endeavor, so not surprisingly, most experimental perturbation studies were performed in the avian model on the chick embryo. The motivations of investigators are diverse; some want to obtain an insight into mechanics of hearts considerably different from the adult organ, others are trying to create models of human congenital heart disease such as pulmonary stenosis or hypoplastic left heart syndrome, and yet others wish to get a developmental perspective on gene expression regulation in abnormal hemodynamics settings. While it is generally known that response of the prenatal heart to the same stimulus differs from the adult, the details such as differences between embryonic (pre-septation) and fetal heart are less widely appreciated, as is the temporal evolution of the compensatory changes. We organize this chapter primarily by the model system and then chronologically (prenatal versus early postnatal interventions) to minimize the confusion arising from comparing data from widely different models (e.g., pressure overload of pre-septation chick embryonic heart versus pulmonary artery banding in fetal lambs). We are trying to tie the data together in a fashion meaningful to clinicians, both pediatric cardiologists and surgeons, caring for babies with congenital heart disease who often have, in addition to structural defect, remodeled hearts due to long-term hemodynamic alterations.

3.2 Avian Embryonic Models

3.2.1 Increased Afterload Model

Fertilized White Leghorn chicken eggs were incubated blunt end up to Hamburger-Hamilton (HH) stage 21 (3.5 days, [1]). The embryo was exposed via a window in the shell and an incision of the inner shell membrane. A 10–0 nylon suture was passed around the mid-portion of the conotruncus, and tied in an overhand knot snug against its wall, but without any constriction to the blood flow (Fig. 3.1). This procedure was shown to result in an increased pressure load of the embryonic ventricle [2], as there gradually develops a stenosis. Sham controls had the suture passed and removed. Normal embryos were unoperated. The window in the shell was then sealed with parafilm, and the eggs were further incubated to

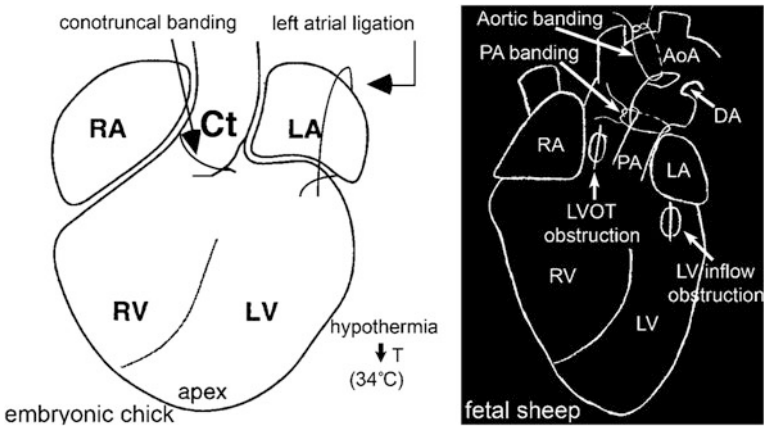

Fig. 3.1 Summary of experimental models affecting hemodynamics in embryonic chick and fetal sheep heart. References to each model are provided in the text

various stages of development. An example of phenotype assessed at HH stage 34 (embryonic day 8) is shown in (Fig. 3.2).

Conotruncal banding has, apart from increasing pressure load, also pronounced effects on morphogenesis, producing cardiovascular defects in survivors (89 % lethality until hatching; [2, 3]). These comprise an invariable occurrence of VSD, with either double outlet right ventricle or persistent truncus arteriosus. This might well be due, apart from mechanical intervention of the suture, to changes in blood flow patterns [4], and hypoplasia of the conotruncal ridges.

The influences of this procedure on myocardial architecture were rapid and pronounced ([5]; Fig. 3.2). Initially, the heart dilated and became more spherical, similar to changes in the adult heart [6]. Trabeculae normally fill the ventricular cavities almost entirely between HH stages 21 and 31 [7], and the precocious reappearance of a trabecula-free lumen is a sign of dilatation and accelerated trabecular compaction. Consistent with this is slightly decreased proportion of intertrabecular spaces. Together with relatively small magnitude of this change, it suggests that there are probably certain limits necessary for adequate myocardial nutrition and efficient blood pumping that cannot be overcome and define survival.

The patterns of trabeculation are modified; the intertrabecular spaces are smaller and more circular in the right ventricle, and trabecular spiralling occurs in the left ventricle. This is an interesting phenomenon, since it mimics the orientation of definitive trabeculation in this location, and is similar to the course of muscle fibers in the compact layer [8, 9]. These are normal features of development that likely reflect adaptation to gradually increasing functional demands [10]. We thus speculate that this spiralling is a mechanism of increasing the pumping efficiency of the pressure-overloaded embryonic heart, as spiralling of myocardial

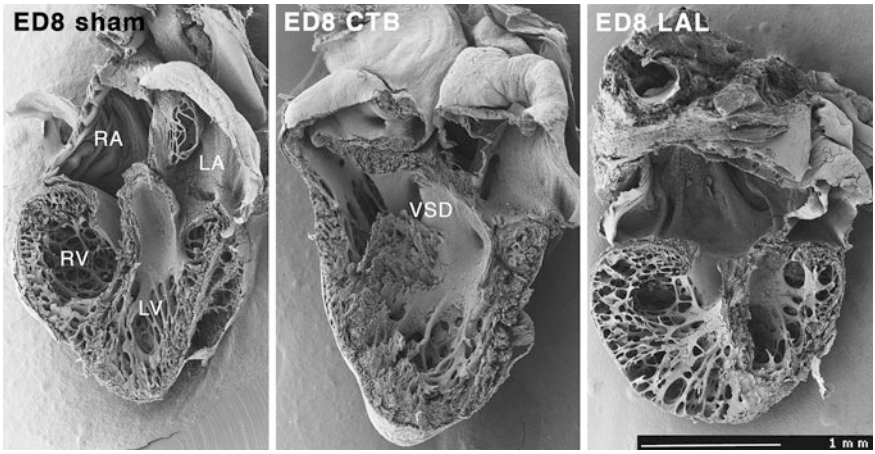

Fig. 3.2 Scanning electron micrographs illustrating early changes in myocardial architecture 4 days after procedure in chick models of hemodynamic interventions. *Left to right* Sham-operated Stage 34 (ED8) heart bisected in frontal plane. *Middle panel* age-matched heart subjected to conotruncal banding at Stage 21. Note an enlargement of the heart, presence of ventricular septal defect (VSD), and a thickening of left ventricular compact zone. *Right panel* heart at the same stage 4 days after left atrial ligation. Note that the apex is formed by distended right ventricle. The heart is smaller than the control and the right ventricle appears dilated with a thinner compact layer

fibers course is a normal developmental adaptation in the compact layer of the fetal heart [11].

Compact layer thickness is a useful parameter in experimental models with altered loading conditions in the embryonic heart. Increase in thickness of the compact myocardium was noted to occur early in this model [5], and together with anomalies in development of the coronary arteries, could contribute to drop in survival occurring around day 8 when the coronary circulation is deployed [12]. Accelerated trabecular compaction with increased compact myocardium proportion and thickness between HH stages 29 and 34 is another phylogenetically well-established mechanism that increases heart performance [13, 14]. In another animal model, namely the carp, the compact myocardium, and its collagenous proteins were found to increase after treatment with isoproterenol [15].

Increased afterload at this stage is a powerful stimulus for active myocardial remodeling based on cell proliferation [16]. Indeed, purely hyperplastic response was reported in this model by Clark et al. [2] biochemically and later confirmed by us using radioactive label dilution approach [17]. This response occurs both in the compact myocardium and trabeculae, as indicated by their increased thickness and relatively minor changes in their ratio [5].

3.2.2 Increased/Decreased Preload

The eggs were incubated to stage 21 as described above. The egg was positioned under the photomacroscope, and the egg shell and its membrane were removed to expose the embryo. The embryo was gently turned to the left side up position, and a slit opening was made in the thoracic wall using a pair of fine forceps. An overhand knot of 10–0 nylon suture loop was placed across the left atrium and tightened, constricting the left atrioventricular orifice and decreasing the effective volume of the left atrium (Fig. 3.1). The embryo was then gently flipped to its original right side up position. The opening in the egg shell was sealed with parafilm, and the eggs were returned to the incubator for reincubation. The phenotype of left atrial ligation (LAL) hearts (Fig. 3.2) shows considerable variability from almost normal to extreme involution of the LV [18]. Relatively high variability of LAL data reflects the different penetrance of the phenotype. This continuous spectrum of cases allows qualitative perception of RV response to a graded overload. Generally, small differences between sham operated and LAL hearts at HH stage 29 could be explained by persistent interventricular communication that allows some blood to pass to the LV. The occurrence of VSD (ca. 25 %) is similar to results obtained recently by Hogers et al. [4] in left vitelline vein clipping model, and might be likewise attributed to changes of intracardiac blood stream patterns. Other associated abnormalities were described in this model [19, 20] and resemble features reported from human pathology [21]. Not surprisingly, there is generally altered atrial morphology with an abnormal development of the interatrial septum. In extreme cases, right atrioventricular valve loses its typical muscular flap-like morphology and looks more like a bicuspid fibrous valve, similar to the left atrioventricular (mitral) valve. This resembles changes observed under increased pressure loading conditions, suggesting that hemodynamic stress is an important determinant of morphology of the developing valvular structures. The ascending aorta is hypoplastic, and the blood is conducted by largely dilated ductus arteriosi. This led Rychter and Lemez to propose it as a model of aortic coarctation. The development of coronary circulation is delayed by approximately two days at day 14 [22]. Lymphatics are also abnormal, being absent in the hypoplastic LV and showing dysplastic valves in the basal portion of the hyperplastic RV [19].

Adaptation of the RV to gradually increasing volume load occurs in three steps. First, dilatation becomes evident as slight thinning and decreased proportion of the compact myocardium. In extreme cases, the trabeculae even change their radial orientation [7, 19] parallel with the outer compact layer, indicating increased circumferential strain. Second, proliferation of trabeculae (starting from HH stage 34) is suggested by their increased number in relative and absolute volumes, but they are finer and their orientation in some cases remains altered. Also, diminished compact layer thickness and increased local porosity (proportion of the intertrabecular spaces within the non-compact myocardium) give evidence of the persisting dilatation. Third, the compact myocardium appears thicker, a finding

that could be interpreted as an acceleration of normal course of development [23] and probably necessary for more efficient force generation (e.g., [24]). Delayed trabecular compaction underscores the more important role of RV trabeculae in contractile function than in the LV, which starts to rely on its compact myocardium sooner [25].

These changes in myocardial architecture and volumes as well as generally decreased proliferative activity could be reversed by subsequent prenatal manipulation. Surgical clipping of the right atrial appendage [26] normalized the hemodynamics and was rapidly translated into increased myocyte proliferation in the left ventricle and a trend toward normalization of abnormal left ventricular myocardial volume. Proliferative activity and diminished differentiation of the cardiomyocytes in the hypoplastic left ventricle was also restored by adenovirally mediated overexpression of fibroblast growth factor-2 [27]. These prenatal interventions demonstrate feasibility and help to justify prenatal interventions aimed at prevention of irreversible changes in selected human cases [28, 29].

In pediatric pathology, coarsening of the trabecular relief and thickening of the RV compact myocardium is a common finding in cases where the right ventricle functions as a systemic pump. This thickening thus, seems to be a secondary effect of increase in the pressure load. Similarly, thickening of the RV wall was observed only at later stages. The relatively slow nature of these changes contrasts with fairly rapid response observed as soon as 24 h after increased pressure loading in the chick embryonic heart (increased cell number, compact layer and trabecular thickening). The results above show that increased pressure is a powerful growth stimulus for hyperplastic cardiomyocyte growth while increased volume load is compensated preferentially by cardiac dilatation.

The changes in myocardial architecture of the underloaded LV are characterized by precocious adherence of trabeculae to the lateral wall and their compaction, perhaps resulting from their more close packing in diminished space. This is illustrated by shift of LV composition at HH stage 34, where the volume proportion of trabeculae is decreased in favor of compact myocardium and lumen. This indicates an acceleration of the normal developmental processes, which shows that growth and morphogenesis are two concomitant but distinct processes.

Tobita et al. [10] studied the details of myocardial architecture in both these models. They found that developmental changes in transmural myofiber angle distribution were significantly delayed following LAL and accelerated following conotruncal banding, suggesting that mechanical load modulates the maturation process of myofiber architecture distribution during myocardial development.

3.2.3 Hypothermia-Induced Hypertrophy

It is well established that heart rate is sensitive to temperature. Hypothermia model is well established, and known to lead to cardiac hypertrophy in the late fetal development [30]. It was demonstrated quite convincingly, as there was a decrease in

nuclear count in histological sections together with an increase in cell diameter on isolated myocytes. Previous studies in this model showed no significant change in DNA content (essentially ruling out a considerable hyperplastic response) together with increased protein content, consistent with hypertrophy [31]. Glycogen content was also increased in the hypothermic hearts. Although the mechanisms are not entirely clear, it was shown that hypothermia leads to the slowing down of heart rate, resulting in an increase of end-diastolic volume. Such increased stretching of the myocytes could stimulate their growth, as seen in previously discussed model of conotruncal banding. Interestingly, while the heart rate and contractility were depressed at low temperature in vivo, in vitro measurements on those isolated hypertrophied hearts showed increased contractility and normal spontaneous rate [32]. Thus, similar to hearts of trained athletes, this hypertrophy is adaptive and could be considered "benign".

3.3 Fetal Lamb Models

3.3.1 Pulmonary Artery Banding

The lamb is the standard model for investigating intrauterine development of the cardiopulmonary system. In the fetal lamb model, it is possible to reproduce anatomical and pathophysiological lesions, the study of which may facilitate development of techniques for their repair. Surgical studies in this fetal model were pioneered and summarized by Rudolph [33]. Early investigations by Vlahakes, Turley and colleagues of simulated cardiac lesions and methods of repair have demonstrated the feasibility of such an approach with this model for examination of both the pathophysiological consequences of cardiac lesions and the technical methods of intrauterine surgery. They studied the use of a fetal lamb model to develop techniques of producing lesions for physiological study as well as a possible method for intrauterine repair [34]. With the timely identification of cardiac lesions in utero, intrauterine cardiothoracic surgery appears possible [35, Toussaint, 1998 359]. While the study comparing the effects of early versus late pulmonary stenosis repair did not show any difference in transverse myocyte diameter even in unrepaired hearts [35], a study comparing the effects of pulmonary stenosis versus atresia [36] did show an increase in cell diameter dependent on level of overload and myocardial weight increase, suggesting a mix of both hypertrophy and hyperplasia. Thus, many factors, apart from methodological approach, influence the nature of myocardial response to prenatal hemodynamic challenge, including species used, timing of surgery, and level of the overload. Even the adult heart, traditionally (and according to the latest evidence, rather incorrectly, as recently reviewed [37]) regarded as a post-mitotic organ, is capable of considerable proliferation in response to a significant and lasting pressure challenge [38].

3.3.2 Aortic Banding

Both pulmonary artery and aorta were banded in study of McAuliffe and Robbins [39]. Invasive hemodynamic measurements confirmed increased pressure gradient as well as changes in LV:RV pressure ratio. As the main focus of this study was documentation of changes in Troponin T expression, only changes in heart to body weight ratio (increased in both cases) were reported. Interestingly, this study revealed that the expression of cardiac troponin is regulated differently and uniquely in this model, with only one dominant isoform present during fetal and adult development with no changes in response to pressure overload. Thus, changes in contractile protein isoform expression known from adult models of heart overload/failure [40, 41] in a sense of shift toward "fetal gene program" could not be documented in this model. Another study in this model [42] showed that left ventricular hypertrophy in the fetus surpassed or slowed down the physiological ontogenic maturation of expression of the SERCA-2a gene. Similar study in this model of preductal aortic coarctation, resulting in a 65 % increase in left ventricular to body weight ratio, was performed by Samson and colleagues [43]. The earliest change detected was at the molecular level—SERCA 2a mRNA levels decreased significantly to less than one-third of the sham group value. At the cellular level, hypertrophy of cardiomyocytes was later followed by hyperplasia and decreased proportion of binucleated cells. This is an interesting observation, as it was normally postulated that prenatal myocardium responds to increase pressure load by hyperplasia, rather than hypertrophy (see above). Unfortunately, the experimental details provided in this (and related) papers do not permit an unbiased re-analysis, but there are many potential pitfalls (contraction state, fixation, direction of sectioning, region selected for analysis) associated with cellular morphometry on sections. For a precise determination of cell volume, one needs to measure both transverse diameter and length, as is routinely done on isolated myocytes. There is an inherent loss of positional information associated with this method—another confounder as there is a known gradient of cell size across the ventricular wall [44].

Flanagan and colleagues [45] investigated the angiogenesis and coronary perfusion in the pressure-overloaded left ventricular myocardium. They found that capillary density was maintained in the overloaded group, and the functional flow parameters and coronary resistance were also in the normal range. This shows that fetal heart adapts its structure in response to pressure challenge (within limits) in a coordinated manner, and the extra myocardium is normal and healthy.

3.3.3 Experimental Left Heart Hypoplasia

Fishman and coworkers [46] created a prenatal sheep model of the hypoplastic left heart syndrome by obstructing the left ventricular inflow or outflow with a catheter (Fig. 3.1). With the inflow obstruction, mean output of the left ventricle decreased

significantly to 30 % of control values. Within a week, the left to right ventricular weight ratio decreased to 70 % of control, and the mean chamber volume ratio decreased to less than 50 %. The outflow obstruction resulted in less pronounced left ventricular output decrease (64 % of control). The average left to right ventricular wall thickness ratio doubled and the mean chamber volume ratio decreased to less than 50 % of control. Increased afterload lead to a rapid increase in LV mass, and morphometric analysis revealed it was due to hyperplasia of ventricular myocytes. Over the long term the mean left to right ventricular weight ratio decreased further and the left ventricular chamber was nearly obliterated, simulating very severe congenital aortic stenosis. These experiments, correlating with the clinical experience, pointed to two different pathways of pathogenesis of hypoplastic left heart syndrome via decreased preload or increased afterload, as seen in mitral or aortic stenosis.

3.4 Other Mammalian Models

3.4.1 Fetal Guinea Pig Model

Increased pressure load is a powerful stimulus for cardiomyocyte proliferation in the developing heart [47]. An elegant fetal surgical study in a small animal models was performed in guinea pigs by Saiki et al. [48]. Fetal guinea pigs of 50–52 days of gestation (65 days gestation period, i.e., last trimester) underwent hysterotomy, and their ascending aorta was narrowed to 50 %. The heart to body weight ratio and left ventricular wall thickness were increased significantly in the banded group. There was also a significant increase in percentage of Ki-67 positive (i.e., proliferating) cells in both ventricles with no changes in apoptosis.

3.4.2 Genetic Mouse Models

Intrauterine demise due to embryonic heart failure is one of the most common results of gene targeting experiments in the mouse. However, the underlying physiological mechanisms responsible for embryonic demise remain elusive. Studies by Collin Phoon and co-workers in a unique mouse model (NFATc1-/-) of missing outflow valves [49], regurgitation into the embryonic ventricle leads to massive volume overload. Using ultrasound biomicroscopy, they showed increasing abnormal regurgitant flow in the aorta extending into the embryonal-placental circulation after E12.5 when outflow valves normally develop. Reduced net volume flow and diastolic dysfunction contributed to heart failure, but contractile function remained unexpectedly normal. Out of 107 NFATc1-/- embryos analyzed, only 2 were in an acute decline with progressive bradycardia, indicating that embryonic heart failure in volume overload settings occurs rather rapidly.

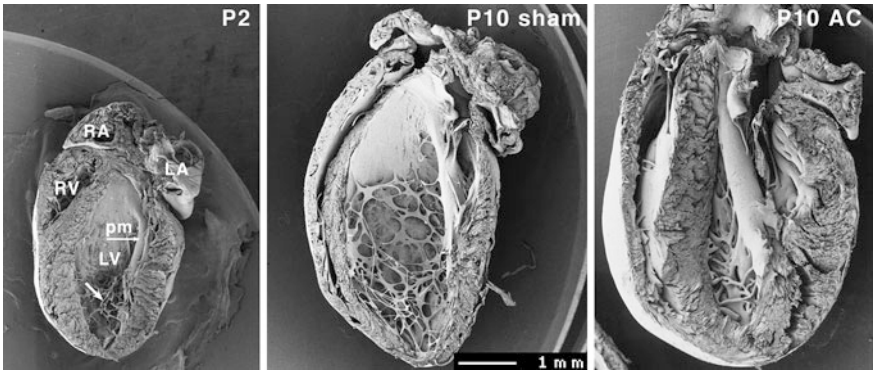

Fig. 3.3 Combination of hypertrophy (and hyperplasia) in rat neonatal model of pressure overload. Note the rapid increase in heart size and ventricular wall thickness 8 days after procedure. *Left panel* neonatal rat heart at day postnatal day 2 (when the aortic constriction was performed). *Middle panel* sham-operated heart at postnatal day 10. *Right panel* heart from animal with suprarenal aortic constriction (*AC*). Scanning electron micrographs, frontal sections, posterior halves. Modified from Ref. [64]

Hyperplasia can also result from genetic perturbation of a regulatory mechanism controlling the size of the heart. In a mouse model where the Salvador gene, a member of the Hippo signaling pathway that controls sizes of organs in Drosophila, was deleted in the myocardium, a considerable cardiomegaly was detected already during embryogenesis [50]. Models such as this can be used to study structure–function relationship and functional studies in these abnormal fetal hearts would certainly yield more interesting data.

3.5 Human Fetal Studies

3.5.1 Hypoplastic Left Heart Syndrome

Szwast and colleagues [51] showed that the functionally single right ventricle in human hypoplastic left heart syndrome fetuses has preserved systolic performance, but there is an impairment of the diastolic function resulting in a less efficient heart and decreased cardiac output.

This abnormal function might lead to problems in other systems, in particular the brain, as was documented by a recent MRI spectroscopic study of the affected fetuses showing metabolic changes suggestive of hypoxia in a significant set of cases [52]. This idea that impaired cardiac performance bears upon the entire fetus gains in importance, and we can expect further studies illustrating this phenomenon. This could be another argument in favor of prenatal correction (or palliation) of hemodynamically significant heart malformations in order to prevent potentially irreversible changes in other organ systems, especially the brain.

3.5.2 Abnormal Hemodynamics in Non-Cardiac Malformations

Changes in hemodynamics leading to right ventricular pressure overload were shown in a rat model of congenital diaphragmatic hernia [53] and is likely present in other situations, such as lung agenesis or ectopia cordis. Longitudinal functional studies of human fetal hearts [54] are useful to establish normal range and allow detection of potential anomalies. This area represents an opportunity for further studies.

3.6 Adaptations in Early Postnatal Period

3.6.1 Physiological Changes After Birth

Profound changes in cardiovascular system occur after birth due to adaptation to breathing of atmospheric oxygen instead of fetoplacental exchange of gases. Smolich [55] summarized changes in myocyte ultrastructure during fetal and postnatal development with a focus on changes induced by hemodynamic alterations after birth. Interestingly, while the right ventricular myocytes in fetal sheep are larger and contain more myofibrils than their left ventricular counterparts, the later are poised for an increased hypertrophic response postnatally.

Rober-Tillig and colleagues [56] have shown that adaptations to postnatal circulatory demand is impaired in growth-retarded neonates with prenatal hemodynamic disturbance, placing them at increased risk of neonatal morbidity. The leading disturbance was reduced stroke volume with compensatory tachycardia, translating into increased cardiac output. Compared with the fetal period, this area is much more amenable to studies even in the clinical settings, and larger mammalian models are well established [57, 58].

3.6.2 Differences in Reaction to Pressure Overload

Abnormal hemodynamic loading is often present in congenital heart disease, both before and after surgical repair. Problems that develop during in utero can exacerbate during postnatal life. However, increased plasticity (compared to later stages) of the neonatal heart might bring improved outcomes of corrective surgical procedures performed during neonatal period [59].

Significant volume and pressure overload due to a left-to-right shunt induce myocardial injury and might eventually cause irreversible myocardial remodeling in children with CHD [60]. The serum cTnI level is a useful biomarker for evaluating myocardial damage associated with pulmonary hypertension in VSD children. In experimental neonatal LV hypertrophy in a rabbit model, myocyte

apoptosis is initiated in the face of normalized wall stress and preserved contractility. The ongoing rate of apoptosis causes a measurable decrease in myocyte number that is coincident with the onset of ventricular dysfunction [61].

In a neonatal sheep model, aortic banding leads to myocyte hypertrophy, which is, in contrast to adult pathological hypertrophy, accompanied by increased capillary growth, resulting in normal capillary density [45]. On the other hand, in a neonatal sheep model of right ventricular pressure overload [38], the hyperplastic component of increased myocardial mass was predominant (only 11 % increase in myocyte volume in face of more than doubled wall thickness). Hyperplastic response was also shown to be responsible in other cases usually extreme cases of increase in myocardial mass [62], suggesting that this phenomenon might correlate with switch to "fetal gene expression program" [63] in such cases. Similarly, we have demonstrated quick and profound changes in heart size (Fig. 3.3) based on a combination of cell hypertrophy and hyperplasia in a neonatal rat model of pressure overload induced by suprarenal constriction of the abdominal aorta [64].

Wang and colleagues [65] followed longitudinally pressure-overloaded left ventricles in young guinea pigs. At 4 weeks, there was compensatory increase in myocardial mass without changes in cell dimensions or volumes, while in the decompensated heart failure phase at 6 months, increased left ventricular myocyte length correlating with chamber dilation and cellular hypertrophy expressed as increased myocyte diameter and length in the other myocardial compartments.

Aoyagi and colleagues [66] showed differences in changes in expression of calcium-handling proteins in young vs. old sheep left ventricular myocardium subjected to pressure overload, which preceded the occurrence of hypertrophy and myocardial dysfunction. The authors suggested that alteration in expression of calcium handling protein genes may be one of the primary responses to pressure overload, rather than a phenomenon secondary to myocardial hypertrophy.

In neonatal (10-days-old) rabbit model, reaction of the right ventricle to increased pressure load was studied by Minegishi and colleagues [67]. Pulmonary artery banding led to hypertrophy accompanied by myocyte apoptosis and fibrosis.

3.6.3 Increased Regenerative Potential

Apart from the above-described rapid reactions to changes in loading, based largely upon cell proliferation, the mammalian neonatal heart shows an increased potential for regeneration. While this phenomenon is well known from studies in amphibians [68] and fish [69], recent study showed that also neonatal mouse could regenerate to a considerable degree amputated part of the ventricle [70]. However, unlike in lower vertebrates where this capacity is present throughout the entire life, in the mouse it is lost within the first week of life. It is supposed that human myocytes do physiologically proliferate for 6 months–2 years after birth [71], which potentially extends this privileged period considerably.

3.6.4 Implications for Neonatal Cardiac Surgery

The available data in general support current practice of performing early anatomical correction (such as arterial switch operation) early in postnatal life, when the growth of myocytes is accompanied by concomitant increase in angiogenesis [72]. Such practice is also advocated in correction of hypoplastic left heart syndrome using biventricular repair technique [59, 73] as the early postnatal left ventricle is capable of remarkable growth, not observed at later stages.

3.7 Conclusions and Perspectives

Developmental cardiology provides sometimes a unique perspective on pathogenetic mechanisms of some cardiovascular diseases, and can help to explain them from developmental perspective. The structure and function of the developing heart are intrinsically linked, so studying both morphology and physiology provides added value and helps with data interpretation. The differences in heart metabolism and function between stages are often more profound than differences between species, so translation of data obtained in animal studies to humans needs to be primarily considered in developmental terms (for example, newborn rodents are more similar to third semester human fetuses than to neonates). Recent advances in our understanding of reactions of the immature heart to experimental hypoxia could help in devising optimal protection strategies for the immature heart, which is subject to increasingly complex surgical procedures aimed at correcting hitherto untractable forms of congenital heart disease.

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Conflict of Interests: None.

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Chapter 4
Hypoxia and Mechanical Factors Drive Coronary Vascular Development

Robert J. Tomanek

Abstract Coronary vascular development requires a variety of signaling molecules expressed spatially and temporally, which are activated by metabolic and mechanical factors. Hypoxia plays the earliest role in this activation via its stimulation of hypoxia inducible factor (HIF–1), and the subsequent activation of VEGF and other growth factors. The early growth of the coronary vasculature consists of a tubular network formed prior to myocardial perfusion. A part of this vascular plexus penetrates the aorta, largely in response to VEGF, to form the coronary ostia, which provides the anatomical substrate for coronary flow. Shear stress then becomes a key regulator of the formation of the coronary hierarchy along with the key growth factors that facilitate the formation of the tunica media of the arterial tree, i.e., FGF, PDGF, and TGF-β. Hypoxia plays a dual role by its activation of HIF-1 and by affecting increases in blood flow and shear stress. Growth of the myocardium involves stretch of both cardiomyocytes and blood vessels, which activates stretch signals in both cell types and causes growth factor paracrine and autocrine signaling and consequently, angiogenesis. Shear stress and stretch are the major mechanical influences that drive coronary vascularization. These mechanical effects are determinants of vessel diameters and composition of their walls. Arterial and venous specification is also affected by blood flow as indicated by endothelial phenotype changes that can occur in response to altered perfusion.

Keywords Angiogenesis · Hypoxia · Shear stress · Coronary development · Arteriogenesis

R. J. Tomanek (✉)
Department Anatomy and Cell Biology, Carver College of Medicine,
University of Iowa, 1-402 BSB, Iowa City, IA 52242, USA
e-mail: robert-tomanek@uiowa.edu

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4.1 Introduction

Hypoxia and mechanical factors are the two primary stimuli for myocardial vascularization and growth during development and in the adult. These stimuli activate signaling events that facilitate a cascade of vasculogenic (formation of vascular tubes from progenitor cells), angiogenic (formation of vascular branches), and arteriogenic (formation of arterioles) events. This stimulus occurs in order to adapt the vascular bed to oxygen demands. During prenatal development, the myocardium is initially avascular because O_2 diffusion is adequate for a myocardial wall, which consists of only a few layers of cardiomyocytes. The expansion of the vascular wall renders the outer myocardial layers relatively hypoxic, an event that triggers molecular responses that initiate vasculogenesis and drive angiogenesis. With formation of the coronary ostia and the onset of coronary circulation, shear stress plays a key role in the formation of the arterial tree. In adults, hypoxia and mechanical factors, e.g., shear stress and stretch, facilitate neovascularization and remodeling by triggering multiple molecular events. The goal of this chapter is to review the roles of hypoxia and mechanical stimuli that are required for or contribute to the formation of the coronary vasculature during development.

4.2 Hypoxia and Neovascularization: General Concepts

Hypoxia is a primary stimulus for vascularization during both prenatal and postnatal growth and remodeling during development and in pathological states, e.g., ischemic heart disease, cardiomyopathy, and cardiac hypertrophy. HIF-1 is triggered by hypoxia and plays a key role in vasculogenesis, angiogenesis, and arteriogenesis [reviewed in [1, 2] When oxygen levels within a tissue fall, HIF-1α proteosome degradation is blocked, allowing HIF-1α and HIF-1β dimerization and translocation to the nucleus where it affects transcription factors (reviewed in [3]). HIF-1α is essential for embryonic survival, as indicated by the finding that its deletion causes degeneration of the developing vascular plexus and fetal death [4]. Hypoxia activates a variety of steps leading to vascularization (reviewed in [5]). These include increased permeability, endothelial sprouting, endothelial cell (EC) proliferation and migration, pericyte and smooth muscle recruitment, and degradation of the ECM.

4.2.1 HIFs Activate Multiple Vascular Growth Factors

Hypoxia-induced HIF-1 transcription upregulates VEGF, stromal-derived factor-1 (SDF-1), angiopoietin-2, placental growth factor (PIGF), platelet derived growth factor B (PDGF-B), and stem cell factor (reviewed in [2, 3]). HIF-1 gene activation

of VEGF, SDF-1, angiopoietin-2, and stem cell factor occurs by direct binding [6]. This experiment certified that HIF-1 increased the expression 245 genes to the same level as hypoxia, that the ECs infected with HIF-1 migrated across basement membranes, similar to ECs cultured under hypoxia, and demonstrated that experiments by Kelly et al. [7] proved that the constitutively active form of HIF-1α is sufficient for the induction of angiogenesis in non-hypoxic tissue [7]. Moreover, in the mouse ischemic limb model, a partial HIF-1α deficiency caused marked declines in VEGF, PIGF, Angiopoietin-1 and -2, SDF-1, and stem cell factor [8]. The effects of hypoxia are not limited to endothelial cells since VEGF expression is increased in cardiomyocytes and pericytes, as well [9, 10].

Vascular development requires HIF-2α, as demonstrated in HIF-2α$^{-/-}$ mouse embryos, which develop severe vascular defects and die before E12.5 [11]. Endothelium-specific HIF-2α expression restores normal vascular development and function and Tie-2 expression. Thus, both HIF-1α and HIF-2α mediate transcriptional response to hypoxia. Moreover, HIF-2α has been shown to be the physiological regulator of erythropoietin production [12].

4.2.2 Hypoxia Upregulates VEGF and VEGFR-1 Signaling

The importance of VEGFR-1 signaling in hypoxia-induced angiogenesis was documented by reporter assay-based work that revealed an upregulation of VEGFR-1 (flt-1), but not VEGFR-2 (flk-1) in the context of hypoxia [13]. The link between VEGF and hypoxia in coronary vessels was provided by Marti and Risau [14], who exposed adult mice to 6 % oxygen for 6 h and demonstrated that both VEGF and VEGFR-1 mRNA were upregulated in the heart, whereas VEGFR-2 mRNA levels were unchanged. Consistent with the role of VEGFR-1 in angiogenesis is the finding that PIGF, which signals only through VEGFR-1, is effective in enhancing EC migration [15]. Moreover, a 12 h exposure of neonatal cardiomyocytes to hypoxia enhanced their PlGF mRNA nearly 4-fold [16].

4.2.3 Hypoxia and Vessel Sprouting

A vascular sprout consists of ECs in a stalk with tip cells with filopodia in the leading front of the branch sensing a hypoxic microenvironment to which they migrate [17]. As already noted in this chapter, the degradation of HIFs under normal oxygen concentrations involves hydroxylation of HIFs by prolyl hydroxylases (PHDs). These proteins are oxygen sensors that are downregulated in the tip cells present in hypoxic tissue regions, thus allowing stabilization of HIF-1α, which facilitates sprouting. There is evidence that loss of HIF-1α, which attenuates VEGF and VEGFR-2 expression in ECs, disrupts hypoxia-induced angiogenesis in tumor cells [18]. Incidentally, HIF-2α levels are not enhanced in the absence of HIF-1α, and HIF-2α are not able to rescue the vascular defects that occur in

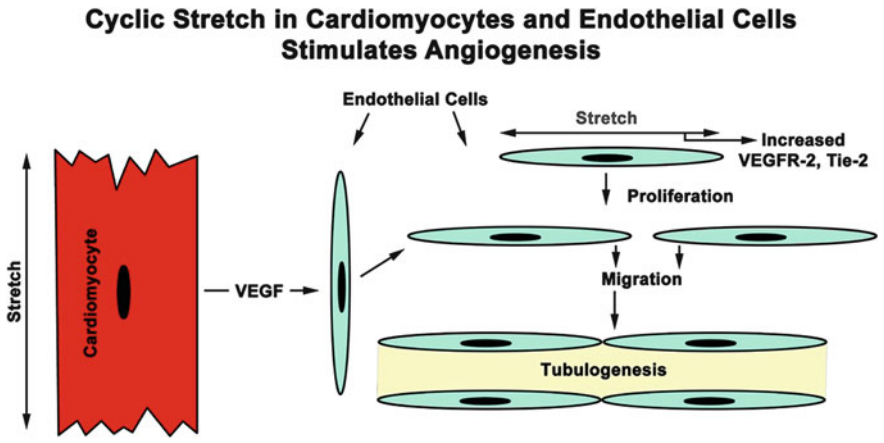

Fig. 4.1 Coronary vessels cell precursors migrate to the heart from the proepicardium, a transient cell cluster on the ventral wall of the sinus venosus in avians and on the pericardial surface of the septum transversum in rodents. The precursor cells migrate via a proepicardial bridge to form the pericardium and then delaminate and undergo EMT. Growth factor signaling from the myocardium facilitates these cells to differentiate into endothelial cells, vascular smooth muscle and fibroblasts, and their migration and vessel formation

embryos that lack HIF-1α PIGF. These findings suggest that HIF-2α plays a role in sprout stabilization, a notion supported by the finding that HIF-1α deficiency in ECs causes an increase in vessel permeability [19].

4.3 Hypoxia Signaling During Coronary Development

4.3.1 Derivation of Coronary Vessel Cells

Prior to the development of the epicardium, the heart lacks coronary vessel cells. As illustrated in Fig. 4.1, the proepicardium (a transient, grape-like cluster of cells) provides all the cells of the epicardium and the coronary vasculature. The proepicardium in rodents is on the pericardial surface of the septum transversum [20], whereas in avian embryos, it forms on the ventral wall of the sinu venosus [21, 22]. These cells migrate to the heart via a proepicardial bridge to form the epicardium [23]. Coronary vascular cells arise from epicardial cells by their delamination and epithelial-mesenchymal transition (EMT) [24] and by cells that migrate directly from the sinus venosus to invade the myocardium and differentiate into capillaries, arteries, and veins [25]. EMT is stimulated by FGFs, VEGFs, and other growth factors from the myocardium [26].

EMT activation in embryonic avian hearts occurs in response to hypoxia and Notch-induced elevation of HIF-1α [27]. The authors noted the Notch 1 intracellular domain in mesothelial cells of the proepicardium during epicardial

formation. Notch 1 intracellular domain was detected in the sulcus epicardial regions, a finding that led the investigators to conclude that a hypoxia/HIF-1-VEGF pathway may promote EMT and coronary progenitor cell differentiation and particularly vasculogenesis in the hypoxic sulcus regions.

Coronary vascularization is a response to a hypoxic environment, which occurs as the myocardium expands. With cardiomyocyte proliferation and hypertrophy, passive diffusion of O_2 and nutrients from the heart chamber lumen are no longer adequate. The cells furthest from the ventricular lumen have the lowest PO_2 and are the site where HIF-1 and VEGF are expressed first and constitute signals for a cascade of events that facilitate vascularization of the tissue regions experiencing inadequate levels of O_2. As we documented in embryonic rat hearts [28], VEGF is expressed as an epicardium to endocardium gradient and this gradient is followed by the pattern of vascularization. VEGF mRNA in the left ventricular free wall was initially highest near the epicardium and then increased toward the endocardium. In the interventricular septum, its highest concentration was in the middle of the septal wall where O_2 was lowest and correlated with the greatest vascularization. As described below, both in vivo and in vitro experiments provide strong evidence for the key role played by hypoxia in prenatal coronary vascularization.

4.3.2 In Vivo Studies

Druyan et al. [29] documented the presence of 3 genes in chick hearts during in ovo development that are stimulated by hypoxia: HIF-1α, heme oxygenase, and hypoxia upregulated protein 1. All 3 genes were expressed at embryonic (E)7 and a significant decline in their expression was noted at E15 and a further decrease at E19. These data, along with some other studies, provide evidence supporting the concept of myocardial hypoxia in the embryonic/fetal heart. HIF signaling in the developing heart is activated as tissue PO_2 values decrease. Lee et al. [30] used the nitroimidazole hypoxic marker, pimonidazole, to locate areas of hypoxia in embryonic mice and discovered hypoxic regions that coincided with VEGF and HIF-1α expression. Moreover, these regions were sites of endothelial cell proliferation and vessel formation. Nanka et al. [31] exposed quail embryos' normoxic conditions and noted that hypoxic regions to the myocardium were characterized by elevated VEGF and HIF-1 expression. When the embryos were exposed to hypoxia, both HIF-1 and VEGF were overexpressed. However, prolonged exposure to hypoxia (16 % ambient O_2) during the embryonic period was lethal by E9, and usually associated with failure of one coronary artery stem to form [32]. Data from embryonic chick hearts exposed to hypoxia reported similar results and also demonstrated that hypoxia, hyperoxia, and overactive HIF-1 resulted in similar types and frequencies of coronary anomalies [33]. Their study underscores the importance of a precise window of O_2 level for the formation and development of coronary arteries.

4.3.3 Experimentally-Induced Hypoxia in Vitro

In order to determine factors that regulate tubulogenesis in the embryonic heart, we have utilized cultures of explanted hearts. In this model, angioblasts delaminate from the epicardium and form vascular tubes in a collagen gel. We studied the influence of oxygen levels on the formation of vascular tubes on the vasculogenic and angiogenic processes by explanting E6 quail ventricular apexes onto collagen gels and culturing them under hypoxic or normoxic conditions [34, 35]. Our data revealed that aggregate tube length was >2-fold higher in hearts cultured in 5 or 10 % O_2 than in those cultured at 20 % O_2. In contrast, tubulogenesis was markedly reduced in explants cultured in a hyperoxic environment (95 % O_2). When neutralizing antibodies to VEGF-A were added to the culture medium, the aggregate tube length was similar to the explants cultured under normoxic conditions. Therefore, the hypoxia-induced tubulogenesis was VEGF-A dependent. Having found that mRNA of three VEGF splice variants 122, 166, and 190 were increased by hypoxia, we exposed the heart explants to each of these splice variants. We found that $VEGF_{165}$ enhanced tubulogenesis in a dose-dependent manner, whereas $VEGF_{121}$ had no effect. These data indicate that $VEGF_{165}$ is the most important VEGF-A splice variant for tubulogenesis in this model. Measurements of the tube diameters revealed that although tubulogenesis was enhanced by either hypoxia (5 or 10 % O_2) or $VEGF_{165}$, the diameters of tubes in cultures containing exogenous VEGF were more than 2 times larger than those from hypoxic or normoxic environments.

Further experiments revealed that VEGFs A, B, and C and VEGFR-1 play a role in coronary tubulogenesis [36]. VEGF-B, VEGF-A, and PlGF are all ligands for VEGFR-1. Because hypoxia upregulates VEGFR-1, but not VEGFR-2, in coronary ECs [14], the increase in this receptor facilitates the binding of both VEGFs A and B. The hypoxic effect on VEGF-B is both organ specific [14] and cell-type specific [37]. In addition to VEGF family members, other growth factors (e.g., FGFs and angiopoietin) contribute to the coronary vascularization process in the embryo. Thus, hypoxia-induced myocardial tubulogenesis involves multiple ligands and signaling pathways.

4.3.4 Hypoxic Induces Embryonic Stem Cells

Hypoxia has been found to accelerate the expression of VEGFR-2 (FLK-1), a marker of hemangioblasts in embryonic stem cell cultures [38]. The same experiments revealed that a documented hypoxic regulation of hemangioblast number is dependent on HIF-1; its deficiency of the latter resulted in fewer $VEGFR\text{-}2^+$ cells under both hypoxic and normoxic conditions. Moreover, hypoxia regulates other mesodermal events by accelerating Brachyury expression, a mesoderm-specific transcription factor. When O_2 levels are decreased in embryonic stem cell cultures, the number of $CD31^+$ ECs increases [39]. Moreover,

when embryoid bodies (3-D structures of embryonic tissues) develop from the embryonic stem cells, a hypoxic environment enhances angiogenic sprouts and an increase in VEGFR-1 and VEGFR-2 occurs in cells derived from the embryoid bodies. These data suggest that embryonic hypoxia may enhance progenitor cell numbers, which in turn accelerate angiogenesis. This conclusion is consistent with the evidence that both VEGF receptors function in embryonic vascular development as demonstrated by embryonic lethality and defects in hematopoietic and angiogenic lineages in VEGFR-$2^{-/-}$ mice [40] and a disorganized vasculature in VEGFR-$1^{-/-}$ mice [41]. However, as noted previously, hypoxia upregulates VEGFR-1, but not VEGFR-2 [13].

4.3.5 Fetal Coronary Development: Responses to Hypoxemia

During fetal life, the coronary circulation is intact and provides nutrition to the compact region of the ventricles (external to the trabecular spongy myocardium). To test the hypothesis that coronary vessels and flow adapt to decreases in O_2 and hematocrit, a number of studies have produced anemia in sheep at late gestational stages of gestation by daily isovolemic hemorrhage; this results in marked declines in hematocrit and O_2, e.g., from 36 to 19 % and from 8 to 2 %, respectively [42]. In this model of chronic fetal hypoxemia, cardiac output is increased, vascular resistance is decreased, and capillary hydrostatic pressure is increased [43]. The loss of blood causes: (1) a reduction in blood O_2 carrying capacity and (2) a compensatory increase in cardiac work necessary to maintain O_2 delivery to tissues. These two effects cause a marked reduction in the myocardial O_2 supply: demand ratio. Maximal coronary conductance was increased 3-fold after 7 days of anemia and increased further in response to adenosine [44]; these findings indicate that coronary vasodilator reserve (the difference between maximal and resting flow) was maintained during anemia, despite a very large increase in resting flow. This increase in vascular capacity suggests that vascular growth can compensate for the defects in the coronary circulation. Hearts of anemic fetuses had greater capillary densities and diameters compared to non-anemic controls [42]. Coronary blood flow increased progressively during the 7-day period; and most importantly, coronary reserve was preserved. HIF-1α protein was elevated after 7 days of anemia, whereas VEGF, VEGFR-1, and LDH (which catalyzes the last step of glycolysis) were elevated by the third day. These data support the earlier findings by [45] that had revealed a relationship between VEGF, HIF-1α, and vascularization in chronically anemic sheep. Moreover, the data are consistent with a study that documented an increase in maximal coronary perfusion in late gestational sheep that were hypoxemic for 5–8 days [46]. That study also verified a preservation of coronary vasodilator reserve despite the doubling of resting (baseline) flow in the hypoxemic lambs.

4.3.6 Hypoxia and Blood Flow Induce Coronary Growth

Vascularization of the fetal myocardium is a response to both metabolic (hypoxia) and mechanical (shear stress and stretch) stimuli. As noted above, hypoxemia in fetal lambs is associated with large increases in maximal myocardial perfusion, indicating an increase in the cross-sectional area of the coronary vasculature. The increases in capillary volume density and diameter and a decrease in intercapillary distance [42, 45] may account, in part, for the enhanced flow. However, a remodeling and expansion of the pre-capillary vessels is the major contributor. To determine if flow independent of hypoxia stimulates the growth of resistance vessels in fetal sheep, flow was increased 2-fold by intracoronary adenosine infusion for 4 days [47]. Although this protocol did not alter mean arterial pressure, heart rate, arterial O_2, or CO_2, the maximal coronary conductance was nearly twice that of the pre-experimental values. Coronary flow reserve was more than doubled by the 4-day treatment and did not increase further under hypoxic conditions. These findings underscore the importance of blood flow in modulating vascular growth in the fetus, which is consistent with its role in adults [48]. Hypoxia may nevertheless exert a role independent of the increases in blood flow because ECs sense low O_2 levels and upregulate genes that are associated with angiogenesis [49, 50]. The independent role of hypoxia is supported by the previously cited in vitro studies on explanted embryonic hearts [34, 35] and by the evidence that a constitutively active form of HIF-1 induces tube formation [6].

4.3.7 Nitric Oxide and High Coronary Blood Flow

Reller et al. [51] established nitric oxide as a determinant of coronary flow both at rest and during hypoxia in fetal lambs. This group demonstrated that the enhanced flow in response to hypoxia was prevented by blocking NOS, despite the fact that L-NNA, a NOS competitor, caused an increase in arterial pressure. Moreover, their data revealed that acute hypoxemia increased coronary blood flow to a level greater than that induced by adenosine. Inhibition of NOS reduced coronary flow to values similar to those attributed to adenosine alone. Consistent with these findings, a role for eNOS, an O_2^-sensitive synthase [52], that is activated during chronic hypoxia, was documented in a study in which fetal guinea pigs were housed in a 10.5 % O_2 environment for 14 days [53]. This exposure led to increases in both eNOS expression at the mRNA and protein levels in coronary arteries; but in the myocardium, both were decreased, indicating that the response to hypoxia was tissue specific. The importance of the enhanced production of NO due to eNOS upregulation is that it contributes to acetylcholine-induced coronary vasodilation, as documented in fetal guinea pig hearts [54]. These findings confirm the observation of Thornburg and Reller [55] that the large production of NO in the fetal heart contributes to the high coronary flows in response to acute hypoxemia.

4.4 Formation of the Coronary Ostia and the Coronary Hierarchy

4.4.1 A Vascular Plexus Penetrates the Aorta

Normally 2 coronary ostia are formed, the left above the left sinus of Valsalva and the right above the right sinus of Valsalva. Numerous strands of the vascular plexus surrounding the root of the aorta penetrate the aortic wall [56] via apoptosis [57], but only 2 channels are formed as the remaining strands disappear. This ingrowth, first documented by Bogers et al. [58] in chick hearts, has been verified by others in avians and rodents [59–61]. The precise signals for this event are not well understood, but require VEGF signaling. My colleagues and I [36] discovered an intense accumulation of VEGFR-2 and VEGFR-3 transcripts at the sites of the coronary ostia; this region also contains cells that stain intensely for VEGF. When we administered a soluble VEGFR-1/VEGFR-2 chimera to embryonic quail, coronary artery stem formation was either prevented or limited to a single coronary stem [62]. We then found that inhibition of VEGF-B had the greatest effect in limiting coronary artery stem formation. The fact that this VEGF signals via VEGFR-1suggests a link between HIF-1α and VEGF regulation of ostial formation since hypoxia signals through VEGFR-1 [13]. Recall that these events occur prior to perfusion through the coronary vascular plexus (Fig. 4.2).

4.4.2 Formation of the Ostia and Recruitment of Vascular Smooth Muscle Cells

Parasympathetic ganglia are associated with the forming ostia [60] and their role in ostial formation is indicated by the evidence that neural crest ablation alters their position in the aorta or limits arterial stem formation [63]. As documented in avians, vascular smooth muscle cells (VSMCs) are recruited to the forming arterial stems from epicardially-derived progenitors [64–67]. This has also been shown in mice, with the exception that a short segment of the proximal coronary artery stem consists of neural crest-derived VSMCs [68]. It is at this point in time that coronary flow is initiated and provides the flow-associated factors that act as mechanical stimuli for vascular growth, i.e., shear stress, stretch, and pressure. The recruitment of VSMCs and their assembly on endothelial-lined channels to form a tunica media is influenced by several growth factors. PDGF-B signaling stimulates VSMC and pericyte proliferation [69] and TGF-β by activating its type I receptor (Alk5), facilitating development of the tunica media in downstream vessels [70]. Experiments in my laboratory demonstrated that FGF-2 and PDGF also influence the temporal regulation of coronary artery stem formation and growth of the coronary tree [71]. When neutralizing antibodies to these growth factors were injected into the vitelline vein of quail embryos, in ovo coronary artery anomalies

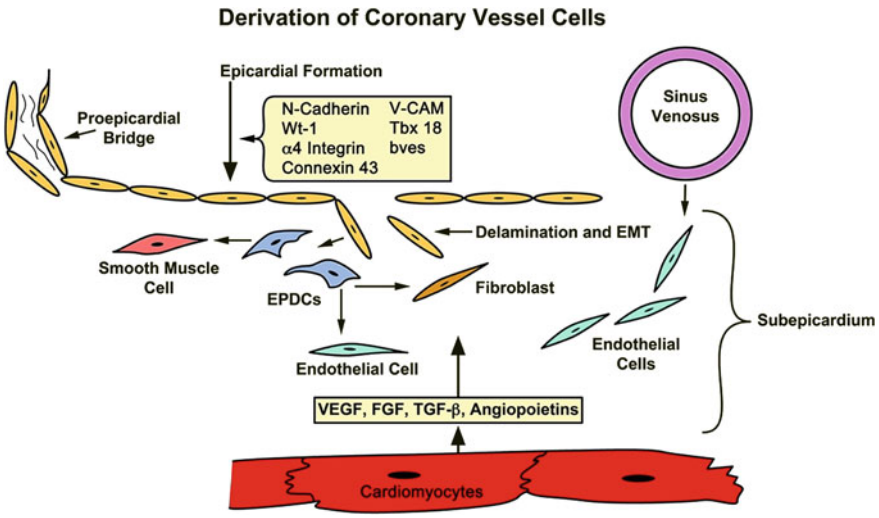

Fig. 4.2 This figure summarizes the effects of hypoxia and shear stress on vascular tube formation and development of the coronary ostia and stems. The vascular plexus is formed prior to myocardial perfusion in response to hypoxia inducible factor-1α (HIF-1α), which affects upregulation of *VEGF*, *FGF*, and angiopoietins. A portion of this plexus penetrates the aortic wall at 2 sites, induces apoptosis and forms the coronary ostia. The onset of blood flow (and the effect of shear stress) through the ostia provides a signal for smooth muscle recruitment and formation of the tunica media or coronary stems and their down-stream branches. Activation *FGF*, *PDGF*, and *TGF* facilitates artery formation

were observed. When the antibodies were introduced prior to the formation of arterial stems, the embryos usually developed only a single coronary artery stem. If the injections were delayed and both coronary stems developed, the arteries usually had smaller diameters and limited VSMCs in the media. Interestingly, we also found that VEGF continued to contribute to media formation after the stems were formed. Apparently, the levels required for this action are low, since high levels of VEGF and retinoic acid, characteristic of an earlier (tube formation) stage have been shown to prevent VSMC differentiation [72].

4.5 Mechanical Factors Stimulate Coronary Angiogenesis and Arteriogenesis

4.5.1 Flow and Shear Stress

The classical studies of Clark (1918) and Clark and Clark (1939; 1940) documented the role of flow in the regulation of vascular growth. Experiments in tadpoles and rabbit ear chambers linked flow with capillary sprouting and

development of arterioles and venules. The data revealed that capillaries with high flow remodel into larger vessels, whereas those with low flow disappear. These observations explain what occurs during the development of the coronary hierarchy. The onset of flow when the coronary ostia are formed causes rapid growth of the arterial system via remodeling and recruitment of VSMCs. At the same time the parts of the vascular plexus that have limited flow are pruned. Vessel diameter is dependent on flow and in coronary arterioles has been shown to prevent inward remodeling [73], thus facilitating increases in diameter that are required during prenatal and postnatal growth.

Effects on endothelial cells. Elevations in blood flow have been shown to stimulate myocardial capillary growth [48, 74, 75]. Endothelial cells sense shear stress and transmit signals to the cell's interior that cause changes in gene expression and EC morphology and function (reviewed in [76–78]). Transduction and signaling in endothelial cells activated by shear stress involves integrins, receptor tyrosine kinases, ion channels PECAM-1, and other molecules, which transmit signals to the nucleus via eNOS. ECs respond to these signals by proliferating and migrating. The proliferative response has been linked to ERK ½ phosphorylation [79]. Formation of vascular tubes by shear stress-activation is facilitated by increases in ICAM-1 mRNA [80]. Shear stress induces an interplay between 2 membrane receptors, VEGFR-2, and integrins [81].

Effects on vascular smooth muscle cells (VSMCs). Like ECs, VSMCs respond to Shear stress by the activation of mechanoreceptors, some of which are similar in both types of cells. It has long been known that arteriogenesis (formation of arterioles, or collateral growth) is regulated by shear stress associated with changes in flow.

4.5.2 Cyclic Stretch

Effects on ECs. A second, equally important mechanical influence on blood vessel formation is cyclic stretch (strain). The cyclic nature of stretch is of particular importance in heart because the myocardium and its blood vessels are stretched during each diastolic phase of the cardiac cycle. Endothelial responses to cyclic stretch also include many components of signaling, some of which are the same as those induced by shear stress. Stretch of endothelial cells activates parameters that influence proliferation and migration; these include, but are not limited to, integrins [82], voltage-gated potassium currents [83], and hyperpolarizing factor synthase [84]. Cyclic stretch of human umbilical vein cells (HUVEC) induced remodeling in these cells, as demonstrated by rearrangement of the cytoskeleton and polarization of the cells; these events were associated with thyrosine phosphorylation [85].

We tested the hypothesis that cyclic stretch of cardiomyocytes produces factors that trigger angiogenic events in microvascular endothelial cells [86]. The conditioned media from cardiomyocytes that had been exposed to cyclic stretch (30

cycles/min) for 0.5–18 h contained higher levels of VEGF mRNA and protein. Addition of the conditioned media to the cultures of microvascular cells increased their migration and tube formation. VEGF mRNA also increased in the endothelial cells when they were subjected to stretch. Additional experiments aimed at determining growth factor and receptor regulation in endothelial cells subjected to cyclic stretch were then conducted [87]. These experiments revealed that thyrosine receptors, VEGFR-2 (Flk-1), Tie-1, and Tie-2 were increased in the endothelial cells from rat microvascular coronary vessels, as well as in HUVEC exposed to cyclic stretch. In cardiomyocytes cyclic stretch caused an upregulation angiopoietins 1 and 2, as well as VEGF. Chang et al. [88] also found that cyclic stretch upregulates Tie-2 protein in HUVEC. Moreover, in vivo experiments that utilized an intraventricular balloon to stretch the ventricle demonstrated a nearly 6-fold increase in VEGF [89]; this finding along with the in vitro data documents myocardial VEGF as a responder to stretch. These experiments demonstrate that both autocrine and paracrine signaling occur in response to cyclic stretch and are summarized in Fig. 4.3.

4.6 Co-Ordinated Growth of the Coronary Vasculature

Following the establishment of coronary flow, growth of the vasculature needs to include: (1) increases the number of vessels; (2) increases in the diameters of some vessels; (3) vascular pruning, and (4) specification of arteries, veins, and lymphatics. These events take place in the context of a rapidly growing myocardium and changing hemodynamics.

4.6.1 Capillary Growth

Capillary growth compensates for the increase in myocardial mass and is related to the increase in hypoxia and the consequential increase in perfusion. During postnatal growth, capillary segment distribution develops so that arteriolar capillaries (those capillary segments closest to the arteriole) and venular capillaries (those capillary segments closest to the venule) are staggered, thereby providing at least one arteriolar segment at any level of cardiomyocyte [61, 90, 91].

4.6.2 Arteriolar and Arterial Growth

Although the major branches of the coronary arterial are already formed soon after birth, e.g., 12 days in the rat [92], these vessels remodel to increase their diameters and to grow in length. For example, the diameter of left main coronary increases

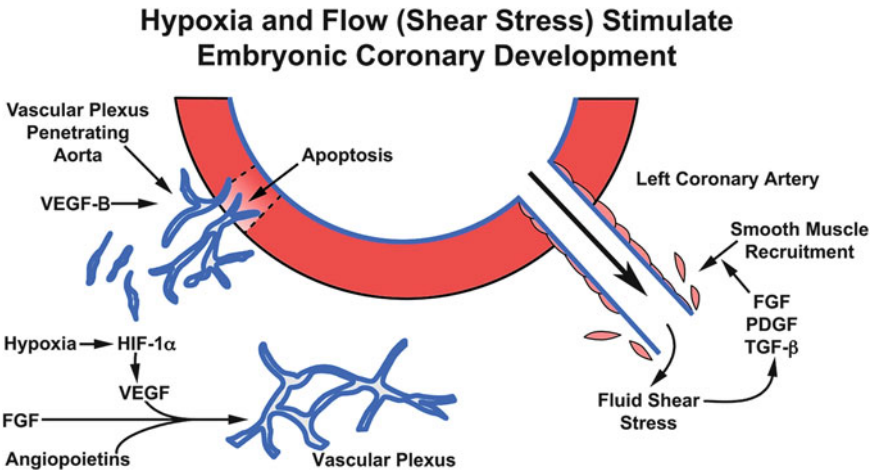

Fig. 4.3 Cyclic stretch of cardiomyocytes upregulates VEGF, which is a ligand for VEGFR-2 on endothelial cells. This signaling enhances affects endothelial cell (EC) proliferation, migration, and tubulogenesis. At the same time myocardial stretch also stretches ECs which respond by upregulation of VEGFR-2, angiopoietin, and its Tie-2 receptor. These changes in the EC independently result in proliferation, migration, and tubulogenesis. Therefore, cyclic stretch of the myocardium causes both paracrine and autocrine signaling

by 62 % between days 2 and 8 [93]. The relationship between blood flow and cross-sectional area of the left anterior descending artery was assessed from echocardiographic measurements in children aged 1 month–16 years [94]. Blood flow per heart beat and blood flow volume per minute increased with age, consistent with the increase in cross-sectional area. These findings suggest that flow increases in response to myocardial growth and thereby causes increased shear stress and vascular remodeling.

Arteriolar growth in humans is characterized by an increase in the number of the smallest arterioles (<20 μm in diameter) and a development of the tunica media [95]. In the rat, total artery an arteriole lengths increase by 47 % during the first 3 weeks of postnatal life [96]. Using 3D microvascular-CT reconstruction, Ohuchi et al. [97] found that increases in arteriolar length and volume of porcine coronary arteriolar trees were most marked during late fetal and the first postnatal month. In the LV, the growth pattern continued up to the fifth postnatal month, consistent with greater growth of the LV compared to the RV; this arteriolar growth in the LV is consistent with the high coronary reserve in that ventricle [98]. Blood flow prior to birth is higher in the RV than in the LV, but is reversed during the early postnatal period because of afterload; and consequently, work load is increased [99]. Accordingly, blood flow increases in response to a higher O_2 demand and provides the mechanical stimulus for vascular growth.

4.6.3 Specification of Arteries and Veins

Arterial and venous identity is regulated by several signaling pathways, e.g. Notch, VEGF, and sonic hedgehog (reviewed in [100]). Angioblast-derived ECs express specific markers for arteries or veins enabling arterial-venous specification. Despite this genetic predetermination, ECs are able to adapt to the magnitude of flow. For example, in the absence of flow, embryonic vessels grow but do not develop as arteries and veins [101–103]. If arterial ECs are transplanted into veins, thus exposing them to a reduced blood flow, they begin to express venous markers [104]. Thus, blood flow is a determinant of EC specification as well as vessel size.

Effects on VSMCs. The molecular basis of stretch on VSMCs has been reviewed in detail [105]. Multiple sensing mechanisms are involved as well as interactions with ECs. Depending on the degree of stretch, proliferation may be inhibited (high levels) or stimulated (low levels). With prolonged stretch, apoptosis is increased.

4.7 Conclusions

Development of the coronary vasculature involves closely regulated temporal and spacial events that involve multiple signaling cascades. Both hypoxia and mechanical influences (shear stress and stretch) play key roles in the activation of these signaling cascades. Hypoxia-induced HIF-1 and VEGF are the major players during the early period of vascular formation, including the penetration of vascular tubes into the aorta to form the coronary ostia. The onset of coronary flow and shear stress then provide a stimulus for the formation of the coronary arteries and arterioles, a process facilitated by a number of growth factors, including FGF, PDGF, and TGFβ. Stretch, another mechanical factor, upregulates VEGF, VEGFR-2, angiopoietin and Tie-1, and evokes endothelial cell proliferation, migration, and tube formation. Shear stress not only regulates vessel formation, but also contributes to vascular remodeling and vessel specification.

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Chapter 5
Cardiac Metabolic Adaptation During Postnatal Development

Jérôme Piquereau, Marta Novotova, Anne Garnier, Frédéric Joubert, Vladimir Veksler and Renée Ventura-Clapier

Abstract The adult heart is the highest energy consumer organ in the body. Energy is produced almost entirely by oxidative metabolism in mitochondria. After birth the heart has to respond to an increase in workload and consequently in energetic demand in face of a profound change in its metabolic environment like an increase in oxygen pressure and a shift in available substrates. These changing conditions induce a cardiac metabolic adaptation consisting in qualitative and quantitative aspects in order to meet the new working conditions. The newborn heart is mainly oxidative and mitochondria produce the main part energy. During the early postnatal phase of cardiac development, following the hyperplasic cardiac growth, the cardiomyocyte undergoes a hypertrophic growth with a profound cytoarchitectural remodeling and new metabolic constraints. The highly differentiated adult cardiac cell increases its efficiency and becomes highly compartmentalized both structurally and functionally. Energy has to be efficiently transferred from sites of energy production to sites of energy utilization for contractile activity, and energy fluxes become highly organized and integrated in the cytoarchitecture.

Keywords Cardiac metabolism · Development · Mitochondria · Creatine kinase · Compartmentation · Cytoarchitecture · Efficiency · Contraction

J. Piquereau · A. Garnier · F. Joubert · V. Veksler
INSERM, U-769, Université Paris-Sud, IFR141, F-92296, F-Châtenay-Malabry, France

M. Novotova
Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovak Republic

R. Ventura-Clapier (✉)
INSERM Faculté de Pharmacie, U-769, Université Paris-Sud,
5 rue J-B Clément, 92296 Châtenay-Malabry, France
e-mail: Renee.Ventura@u-psud.fr

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5.1 Introduction

Evolution has allowed the emergence of species, whose existence depends on the coordinated activity of multiple organs. These entities, among which the heart, are differentiated during the organogenesis stage of prenatal development which is accompanied by cell specialization leading to an organism comprising cellular functional groups, efficiently coordinated. To be adapted to the changing needs of the organism, their maturation is realized all along the prenatal life and continues during postnatal life. The heart, which becomes functional very early [1], is no exception to this rule. Knowing that its role is to ensure a sufficient blood flow to meet the demand in oxygen and nutrients for all of the cells in the organism, the heart is doomed to adapt to all changes that the animal undergoes during development. This adaptation is clearly visible in the morphological changes of the cardiac pump which rapidly evolves from a primitive heart tube to a four-chambered organ during prenatal development (for review see [2, 3])..

At birth, the heart of the newborn appears morphologically similar to the adult one. However, these macroscopic similarities are not indicative of a mature organ just yet. Indeed, the period immediately after birth is a transition phase between the fetal life and adulthood that is marked by numerous metabolic and structural changes of the cardiac muscle cell. The adaptation of the cardiomyocyte is essential for the survival because the demands of the organism in its prenatal life are very different from those encountered during postnatal life. Indeed, at birth the heart must cope with the establishment of a new way of functioning. During the fetal life, the two ventricles work in parallel and both participate in the systemic circulation (for review see [4]). The pulmonary circulation is reduced (about 10 % of total flow) due to the strong resistance of the collapsed lungs and consequently the blood entering the right heart preferentially goes to the systemic circulation via the shunts provided by the *ductus arteriosus* and the *foramen ovale*. At birth, the dramatic drop in pulmonary resistance due to the expansion of the lung alveoli induces an increase in systemic pressure which leads to the closure of shunts [5, 6]. Then, the ventricles turn to the pump in series that is characteristic of the mature cardiac physiology. These significant changes in the cardiovascular system during the first hours of life (or during the first days depending on species (for review see [6]) lead to newly established levels of oxygen in the vessels as well as new pressure and load conditions for the two ventricles which have a considerable impact on cardiac function. The fetal cardiomyocyte and the general organization of the fetal heart are no longer perfectly adapted to their new functions and need to undergo maturation.

Whereas the heart of the newborn is subjected to significant hemodynamic changes, it is also subjected to a constant increase in workload because of the rapid growth of the organism at that time. The increase in body weight and the concurrent increase in irrigation needs cannot be met without more efficient cardiac function which requires cellular adaptive phenomena. The cardiac response to these new physiological conditions consists of an important increase in its muscle mass (initially by hyperplasia, then by cellular hypertrophy [7–10]) accompanied by a

cytoarchitecture remodeling of the contractile apparatus but also a complete overhaul of the metabolic functioning of the cardiac cell. Therefore, during the development, the cardiomyocyte is the scene of the implantation of energy production and energetic transfer systems which will exhibit an increasing efficiency [11–13].

Bioenergetics is an essential component of cardiac physiology. Knowing that the role of the heart is to pump blood to ensure blood flow within the body, this organ is endowed with a contractile ability which requires an adequate supply of adenosine triphosphate (ATP), the archetypal energetic molecule. Consequently, the increase in work carried out by the heart during ontogenic development leads to a significant increase in energy demand that is met by optimizing the production and the transfer of energy within the cardiac cell. The cardiomyocyte development is subjected to metabolic maturation processes which are necessary to adapt to new working conditions. Thus, the metabolism of the cardiac cell is very different depending on the stage of development considered [14, 15].

This review focuses on the metabolic remodeling that accompanies cardiac maturation during development.

5.2 Metabolic Pathways of the Cardiomyocyte

Whereas the mechanisms involved in energy production of the cardiomyocyte may seem complex at first glance, the pattern of energy processes can be simplified by focusing on the two major producing pathways. Indeed, energy production of the cardiac cell relies, for the most part, on lipids and carbohydrates [16–19] which play less or more important roles during organ development [20, 21]. The use of one or the other of these substrates is in no way coincidental. The orientation of the metabolism toward a preferential consumption of fatty acids or carbohydrates (glucose and lactate) brings into play a set of factors that favors lipid metabolism, with high energy efficiency, or the glycolytic metabolism, which is less efficient in terms of ATP production but is also less dependent on oxygen supply. Thus, the emergence of a given cell metabolism results from the energy demand, the oxygen content but also the availability of the substrates themselves, among others things.

The energy production from these molecules is obviously dependent on specific cellular machineries. These are differentially established during development of the heart [22] so that the cardiomyocyte can gradually be armed to adjust its metabolism in the face of a constantly evolving cardiac work. Whereas lipid and carbohydrate utilizations have their specificities, they are however not completely separated and share some common pathways. Thus, the use of lipids or glucose as fuel is very different considering the anaerobic phase of glycolysis, but they are very similar in their mitochondrial phase (Fig. 5.1). Indeed, glycolysis leads to the generation of pyruvate which, if not converted to lactate by cytosolic lactate dehydrogenase (LDH), is taken up by the mitochondria and converted to acetyl-CoA by the mitochondrial pyruvate dehydrogenase (PDH). Acetyl-CoA, which is also the product of mitochondrial fatty acid degradation by the β-oxidation, is then

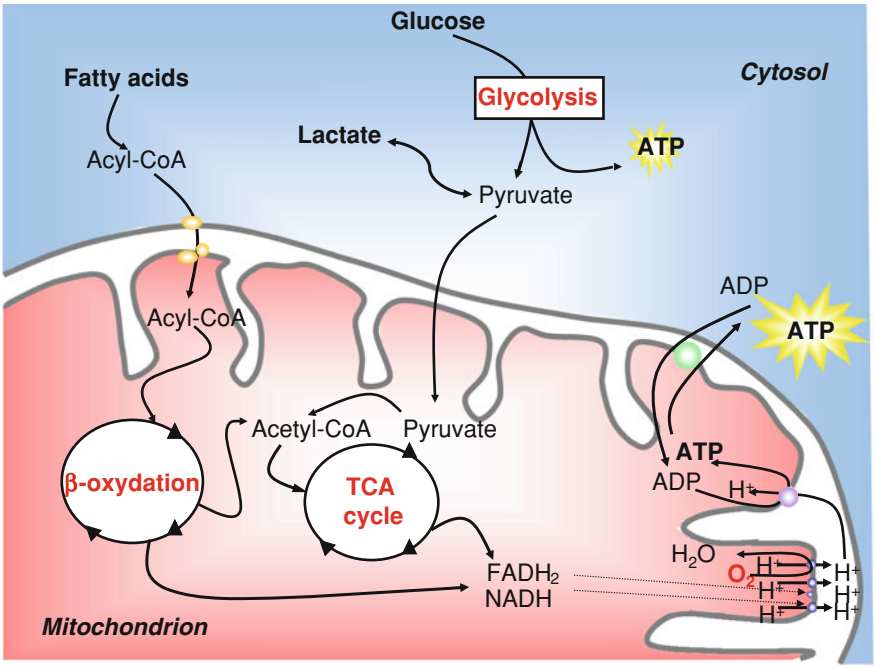

Fig. 5.1 Energetic pathways of the adult cardiomyocyte. Energy production is mainly derived from oxidative metabolism in mitochondria. The main substrate is fatty acids that are imported in the cardiomyocyte through specific transporters. Glucose provides only a minor fraction of ATP through anaerobic glycolysis and pyruvate oxidation. The β-oxidation and the tricarboxylic cycle provide reduced equivalents that are oxidized by the respiratory chain. For the sake of clarity the creatine kinase system has been omitted (see Fig. 5.6)

degraded by the reactions of the tricarboxylic acid (TCA) cycle which lead to the production of reduced equivalents (NADH and FADH2) whose oxidation is responsible for the formation of the electro-chemical gradient necessary for mitochondrial energy production [23, 24] (Fig. 5.1). Energy production within the cardiomyocyte is never performed exclusively by anaerobic glycolysis during development [11, 22]. This is even more true considering that the role of lactate as energy supplier is directly dependent on its oxidation capacity after its conversion into pyruvate [25, 26].

5.3 Fetal Heart and Mature Heart: Two Metabolisms at the Extreme Opposite from One Another

The amount of energy produced by each pathway previously described varies considerably during development of the cardiac pump. The heart shifts from a metabolism which is preferentially glycolytic in the early stages of development to

a metabolism which is almost exclusively oxidative at maturity [11, 27]. The metabolic characteristics at a given age are not only dependent on the presence and the activity of the enzymes and transporters involved in a specific energy pathway, they are also very dependent on the substrate availability. For example, fatty acids are able to inhibit the processes involved in the use of carbohydrates, while on the other hand lactate shows an inhibitory effect on lipid oxidation [28]. Circulating levels of substrates thus play a major role in establishing the glycolytic metabolism encountered in the fetal heart [29, 30] since this stage of development is marked by a low level of fatty acids [31, 32] and a high level of lactate [15, 21, 27] in the blood. Therefore, prenatal metabolism is characterized by a predominant use of carbohydrate and fatty acid oxidation contributes only for about 15 % of total energy production [11]. This metabolic profile is obviously very favorable to the fetal heart because it allows energy production via glycolysis, which is well-suited for a fetal environment characterized by a low oxygen level [33]. The immature heart is thus more resistant to hypoxia than the adult, possibly as a consequence of its greater capability for anaerobic glycolysis [34]. The reliance on glycolysis for energy production is quite notable since this anaerobic mechanism could provide up to 50 % of the total ATP production [20]. This is actually favored by glycolytic enzymes displaying a high activity [30, 35] and by regulations more favorable to anaerobic ATP production than that observed in adults [27, 36]. Whereas the appearance of glycolytic energy production of the fetal heart is undeniable, the fact remains that a part of energy is derived from oxidative processes [28, 37]. Despite a low activity of the respiratory chain and a low mitochondrial mass before birth [38, 39], the involvement of mitochondria in energy supply must not be marginalized. Indeed, fetal heart tissue is endowed with oxidative capacities [40] which allow energy production from various substrates that are available in the blood [25, 28, 41]. However, the energy provided by oxidation is preferentially produced from lactate which is responsible for most of the oxygen consumption of the fetus [41–43], the oxidation of glucose and fatty acids being relatively low [28, 44].

At maturation, the cardiomyocyte metabolism is greatly different from the fetal one. Energy production of the adult cell is opposite to the one encountered in the fetus since the relative contribution of the different substrates is completely reversed. The adult heart is an organ in which oxidative metabolism is involved in 90 % of energy production with a preferential consumption of fatty acids [18, 45–48]. Carbohydrates, for their part, play a lesser role and are responsible for only a minor amount of the acetyl-CoA oxidized by mitochondria [47, 49].

As in the prenatal period, the blood concentration of substrates plays an important role in determining the adult metabolism. In adult mammals, the cardiac environment is characterized by a high concentration of fatty acids in the plasma [14, 48]. Knowing that the adult heart is associated with higher circulating level of oxygen [41], higher mitochondrial mass and optimal activity of enzymes of the TCA cycle and of the respiratory chain [38, 39, 50] than the fetal heart, the conditions obviously favor fatty acid oxidation as a source of energy production. Specialization of the cardiac cell for lipid metabolism is especially marked since the high blood levels of fatty acids directly represses the use of carbohydrates by

limiting the uptake of glucose and lactate in the cardiomyocyte and by inhibiting the mechanisms involved in their oxidation [51, 52]. This negative effect on carbohydrate oxidation is realized through the inhibition of PDH which is phosphorylated by pyruvate dehydrogenase kinase (PDK), whose activity is stimulated by the high cellular concentration of acetyl-CoA and NADH, which are encountered with high lipidemia [43, 47, 53, 54]. One of the direct consequences of the inhibition of pyruvate oxidation is a decrease in glucose consumption which can even lead to glycogen synthesis by the cardiomyocyte [55]. Moreover, this decrease in glucose use is enhanced by the fact that at maturity, ATP more strongly inhibits phosphofructokinase (PFK) which is a key regulator of the glycolytic flux [27, 36].

Finally, the circulating substrates can directly influence the expression of genes involved in the metabolic pathways. For example, fatty acids can directly activate peroxisome proliferator-activated receptors (PPARs) that control the expression of proteins involved in fatty acid metabolism. Long chain fatty acids are the preferential agonists of the nuclear receptor PPARα Lambert[56], thus increasing the expression of proteins involved in the binding, transport, and oxidation of fatty acids as well as mitochondrial biogenesis [57–59].

5.4 From Glycolytic Metabolism to Oxidative Metabolism

The mechanisms involved in energy production at fetal and adult stages are a consequence of the changes that occur during development in order for the cell to adapt to the increasing load and energy demand of the heart.

Birth is characterized by a marked proliferation of mitochondria that is thought to be mediated in part by thyroid hormones [60]. In mice, the oxidative capacity progressively increases during postnatal development showing a 3-fold increase between 3 days and adult [13].

The transition stage, during which the metabolism shifts from use of carbohydrates to fatty acids use, begins at birth and is associated with rapid and profound changes in circulating substrate concentrations. Indeed, at birth the newborn shifts from a condition marked by a high amount of lactate supplied by the placenta to a situation in which the main energy source of nutrients becomes milk which is rich in fatty acids [32]. This results in an almost immediate reversal of the lactate/fatty acid ratio in the blood [11]. This change in substrate concentrations is accompanied by a profound metabolic remodeling. The postnatal development is effectively marked by an increase in cardiac mitochondrial mass associated with an increase in cytochrome c expression, in the activity of enzymes of the TCA cycle and in the activity of complexes of the mitochondrial respiratory chain such as cytochrome c oxidase and NADH dehydrogenase [13, 49, 50, 61]. Moreover, experiments performed by our team on murine permeabilized cardiac fibers have evidenced an earlier maturation of complex II of the mitochondrial respiratory chain which increased from 54 $\pm$ 6 to 76 $\pm$ 11 % of the adult value between 3 and 7 days after birth compared to complex I

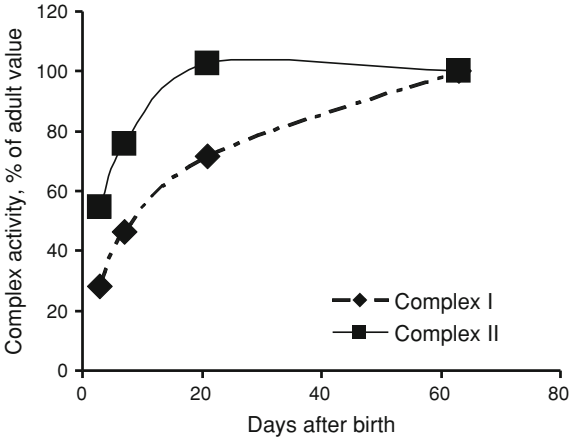

Fig. 5.2 Comparison of the activity of complex I and complex II of the respiratory chain during postnatal development. Activity has been estimated by measuring oxygen consumption rate in permeabilized cardiac fibers with glutamate and malate (complex I) and succinate (complex II). Values are normalized to adult values

(Fig. 5.2). The functional maturation of complex I was delayed and at 3 and 7 days, the activity represents only 28 ± 4 and 46 ± 6 %, respectively of the adult value (Fig. 5.2). This earlier maturation of complex II is in line with the high capacity of energy production from fatty acids of the newborn heart. Indeed, the ability to oxidize palmitoyl-carnitine was already high 3 days after birth and at 7 days, it was not different from that of the adult heart (Fig. 5.3). Thus, within 1 week, the capacity of the cardiomyocyte for oxidative metabolism and fatty acid utilization is relatively advanced in mice.

It is important to keep in mind that the maturation state of the mitochondrial respiratory chain is not the only factor responsible for the emergence of a metabolism based on the use of fatty acids. The heart uses fatty acids mainly from the bloodstream (for review see [62]), meaning that a penetration of these substrates into the cell is required before their transportation to cardiac mitochondria. Although the mechanisms involved in the fatty acid uptake in the cardiac cell are not well understood, it appears that the proteins "plasma membrane associated fatty acid binding protein" (FABPpm) and "fatty acid translocase" (FAT), also known as CD36, play a key role [63–65]. After their internalization, fatty acids are esterified to acyl-CoA by fatty acyl-CoA synthase (FACS) and are taken up by the mitochondria through the carnitine palmitoyl transferase I and II (CPTI and II) [66], where they undergo the β-oxidation cycle. Interestingly, this pathway of lipid transport undergoes a significant maturation during postnatal development which is characterized by a rapid increase in the expression of FABPpm and FAT/CD36 [67]. This induces a much greater influx of fatty acids than in the fetus with the relative cellular volume of lipid droplets reaching a peak at 7 days (Fig. 5.3). As can be observed in electron microscopy (Fig. 5.4), lipid droplets increase in number and size between 3 and 7 days of age suggesting a higher ability to internalize circulating lipids in 7-day-old mice. Interestingly, mitochondria between 3 and 7 days establish close contacts with the lipid droplets that are coated with perilipins.

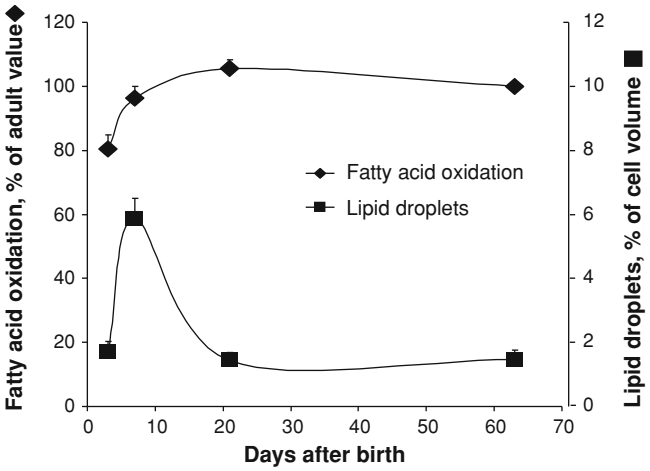

Fig. 5.3 Fatty acid oxidation and lipid droplets during postnatal development. Fatty acid oxidation has been estimated by measuring oxygen consumption of cardiac skinned fibers in the presence of palmitoyl-carnitine. The relative volume of lipid droplets has been measured by a stereological method using electron microscopy

The use of fatty acids after birth is also facilitated by the removal of the inhibition of CPT1 by high cytosolic level of malonyl-CoA in the fetal heart. Malonyl-CoA which is synthesized by the acetyl-CoA carboxylase (ACC) from acetyl-CoA, prevents the entry of acyl-CoA in mitochondria and plays an important role in the orientation of the fetal metabolism [11, 68]. At birth, the level of malonyl-CoA drastically falls due to ACC inhibition by 5-adenosine monophosphate-activated protein kinase (AMPK) whose activity increases after birth [68, 69]. Moreover, this decrease in the intracellular concentration of malonyl-CoA is enhanced by an increase in its degradation by malonyl-CoA decarboxylase (MCD) [70].

These short-term regulations are accompanied by a transcriptional metabolic remodeling. The increased intracellular level of fatty acids is coincidental with an increased expression of the proteins involved in mitochondrial biogenesis and fatty acid metabolism pathway (Fig. 5.5). Down-stream targets of PPARα (MCAD, CPT1, and CD36) and to a lower extent PPARα expression are increased in 3- and 7-day-old animals and then return to adult value after 3 weeks. The expression of the PPAR co-activators (PGC-1α, PGC-1β, and PRC) that are the master regulators of mitochondrial biogenesis [71, 72] and of citrate synthase are also higher in 3- and 7-day-old animals compared to adult. It is tempting to speculate that the increased capacity to oxidize fatty acids is responsible for the decrease in lipid droplets after 7 days. Similarly, proteins involved in mitochondrial fusion (mitofusins 1 and 2 and OPA1) and mitochondrial fission (DRP1) are also more highly expressed in the first weeks after birth.

From what is described above it appears that cardiac metabolism is subjected to a wide array of regulatory factors which undergo a series of changes responsible

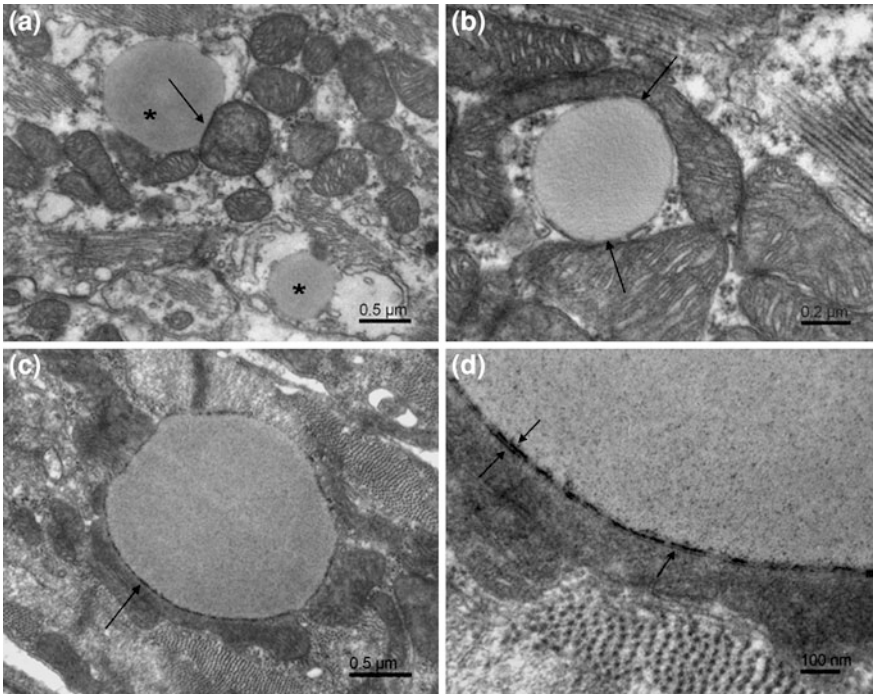

Fig. 5.4 Electron micrographs of lipid drops in cardiomyocytes of 3(A,B)- and 7(C,D)-day-old mice. **a** lipid drops (*) are present in the free cytosol. Their surface is predominantly free, sometimes a small surface area is in tight contact with the mitochondrial surface (*arrow*). **b** Details of a lipid drop partially surrounded by mitochondria. At the contact regions of both mitochondrial and lipid surfaces, the presence of dense material is observed (*arrows*). **c** Mitochondria surrounding a large lipid drop are in close contact with the border of the drop. In the contact area a dense material can be seen (*arrow*). **d** Detail of the surface of a lipid drop. At high magnification two parallel layers of dense material at the contact regions are present (*arrows*) and represent perilipin. The sections were contrasted with uranyl acetate and lead citrate

for the shift of the metabolic profile of cardiomyocytes during the first weeks of postnatal development. The cardiomyocyte adapts its metabolism in order to evolve from a glycolytic metabolism, which favors cell proliferation [73], to an oxidative metabolism, which is able to provide a substantial amount of energy to ensure the contraction of the mature heart.

5.5 From Energy Production to its Utilization

Whereas a sufficient energy production is clearly a prerequisite for the proper functioning of the heart, it is equally important that the ATP generated reaches the cellular sites requiring the energy.

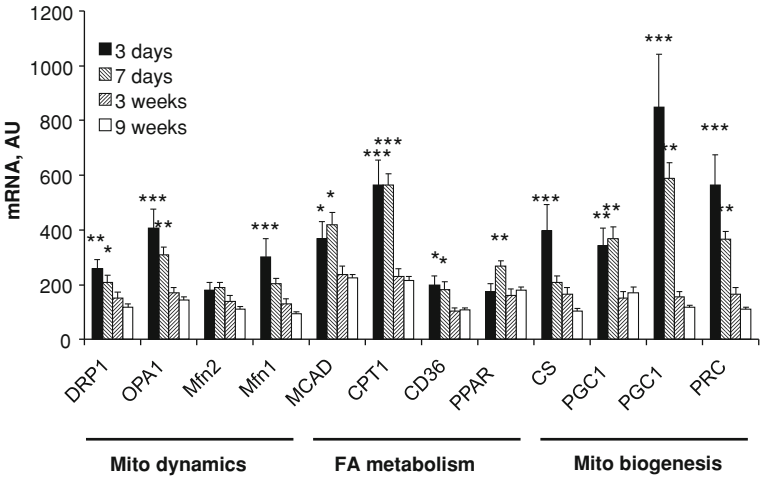

Fig. 5.5 Expression of genes of energy metabolism during postnatal development. Real-time RT-PCR was performed using the SYBR® Green method on a LightCycler rapid thermal cycler (Roche Diagnostics) as previously described [13]. DRP1: dynamin-related protein 1; OPA1: optic atrophy 1 protein; MFN1 and 2: mitofusins 1 and 2; MCAD: medium-chain acyl-CoA dehydrogenase; CPT1: carnitine palmitoyl transferase 1; CD36: fatty acid translocase; PPARα: peroxisome proliferator–activated receptor α; CS: citrate synthase; PGC-1α and β: PPARγ co-activator α and β; PRC: PGC-1-related coactivator. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

In the adult cardiomyocyte, the main energy consumers are the sarcoplasmic reticulum ATPases (SERCA), the ATPases of the myosin myofilament (myosin-ATPases) and the sarcolemmal Na–K ATPase which are involved in the excitation–contraction coupling [74]. Knowing that most of energy production is of mitochondrial origin, the energy must be transferred from the mitochondrial to the ATPase compartments. However, the mature cardiac cell is characterized by a very limited diffusion due to a high density of intracellular components and especially myofilaments and mitochondria embedded in the cytoskeleton. Therefore, the establishment of effective mechanisms for energy transfer is required.

Very early, the scientific community has sought to bring the phenomena of energy transfer within the mature cardiac cell to light. Already in 1981, Samuel Bessman proposed that creatine kinase (CK) could play a role in facilitating ATP transfer [75]. CK catalyzes the reversible transfer of a phosphate moiety between ATP and creatine (Cr) (Cr + ATP↔ADP + phosphocreatine (PCr)). There are four distinct CK isoforms [74, 76] including two cytoplasmic (M and B), which exist as dimers giving three isoenzymes (MM, BB, and MB), and two mitochondrial (the ubiquitous Mi-CKu and the Mi-CKs expressed in striated muscles [77]) which can form both dimeric and octameric structures [78]. The system of energy transfer by CK, commonly called “CK shuttle”, relies on the specific location of the different isoforms. Thus, the cytosolic CK, mainly represented by the MM-CK isoenzyme in the heart [79], is present in the cytosol but also bound to the sarcolemma, to the myofilaments and to the sarcoplasmic reticulum in the

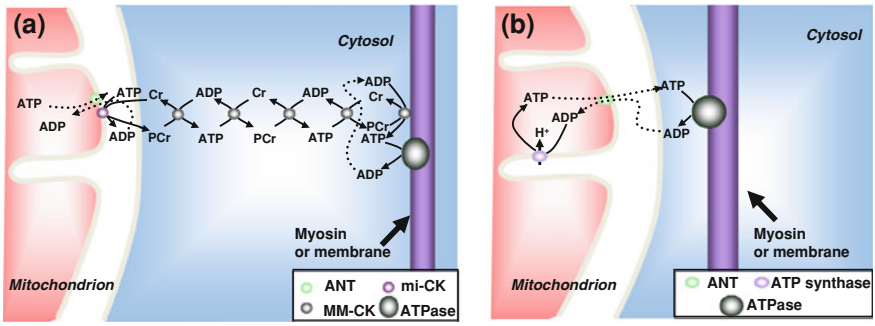

Fig. 5.6 Compartmentation of energy in cardiac cells. **a** Creatine Kinase system in adult cardiomyocyte. The structural and functional coupling between mi-CK and ANT leads to the transfer of phosphate moiety of the newly produced ATP to Creatine. This phosphate moiety is then transferred, through the cytosolic CK, to utilization sites where CK bound in the vicinity of the ATPase rephosphorylates the ADP locally produced. The release of Cr induces a metabolic signal which is transferred, through the cytosolic CK, to the mitochondrion. **b** Direct adenine nucleotide channeling in adult cardiomyocyte. The proximity between mitochondria and ATPases allows a direct transfer of the newly produced ATP from the mitochondrion to the ATPase. The ADP locally produced is immediately taken up by the mitochondria so that it stimulates the energy production and relief the inhibition on the ATPases

vicinity of ATPases [80–82]. The mitochondrial CKs are located close in proximity to the adenine nucleotide translocase (ANT) in the mitochondrial intermembrane space. Thus, the distribution of the different CK isoforms allows the establishment of a system in which energy transfers are governed by CKs and their substrates, PCr and Cr [75, 79, 83]. This system relies on the compartmentalization of the isoforms in the sites of energy production and energy consumption which favors the formation of PCr in the mitochondria and leads to ATP rephosphorylation in the vicinity of the ATPases (Fig. 5.6a). The phosphate moiety of PCr produced by mi-CK is transferred, through the cytosolic CK, to MM-CK bound in the vicinity of ATPases which rephosphorylates the ADP locally produced in order to adjust the ATP/ADP ratio. Similarly, the energy consumption signaling is carried by Cr which is transferred to the mitochondria by the same cytosolic CK in order to stimulate ATP production [74, 84]. Therefore, Cr acts as a feedback mechanism of energy state; this notably allows a perfect balance between production and consumption of energy. Due to the proximity of all the enzymes involved in this process, energy supply and especially the elimination of ADP in the vicinity of ATPases is much more efficient than in a diffusion mechanism [85].

Although the system governed by the CK phosphotransferases is very effective, it is not exclusive and other energy transfer enzymes like adenylate kinase and others also form interconnected energy shuttles [86]. Moreover, the specific arrangement of the cellular components (mitochondria, sarcoplasmic reticulum, and myofilaments especially) in cardiomyocytes leads to a situation in which the transfer of nucleotides is easier between neighboring organelles than through the cytoplasm [87]. This mechanism called direct adenine nucleotide channeling

(DANC) establishes a direct communication between mitochondria and ATPases through the compartmentalization of adenine nucleotides (Fig. 5.6b) depending on cell architecture. Similarly to the kinase system, this DANC allows maintaining a high ATP/ADP ratio in the vicinity of ATPases, and the efficiency of these processes is due to the ability of mitochondria to remove ADP from the microenvironment of ATPases; ADP is a limiting factor for the ATPase activity and its uptake by mitochondria induces a stimulation of energy production. This very simple system which allows overcoming diffusion limitation of energy-rich molecules (especially ADP diffusion [85]) plays a significant role in energy transfer. Indeed, the energy supply for myosin-ATPase and for SERCA by DANC may be as substantial as the energy supply realized by CK system [87].

These two systems of energy transfer are constantly in competition and are able to compensate each other when one or the other becomes deficient [87, 88]. In normal conditions, the CK system is the main one and it provides about two-thirds of the energy when DANC contributes only for one-third [87]. Each system significantly increases its performance when the competing mechanism becomes less effective [89, 90]. Whereas it is well-documented that the adult cardiomyocyte needs these efficient mechanisms of energy transfer [13, 87, 91, 92], it seems that the metabolism and the organization of the fetal cardiac cells do not require this kind of processes. As it has previously been described in this review, the energy is mainly produced from glycolysis in the fetal heart. It is known that the glycolytic enzymes can be organized into supramolecular complexes bound in the vicinity of ATPases [19, 93, 94] so that they directly participate in the regulation of the local ATP/ADP ratio. Therefore, this kind of organization does not require any specific transfer system. Moreover, the energy transfer realized by CK shuttle or by DANC is really favorable in a situation in which the diffusion of molecules is limited by the density of intracellular components; this is not the case in the fetal cardiomyocytes since the cellular architecture is not as dense as the one encountered in adult cardiac cells [95–97]. Thus, the simple diffusion of energy through the cytosol is much more efficient in the fetal cardiac cell than in the adult one. Therefore, the processes of energy transfer do not seem essential in fetal heart; this is confirmed by the fact that several studies showed that these processes do not play a major role in immature cardiac cell [12, 13, 98, 99].

5.6 Establishment of Energy Transfer Mechanisms During Development

Clearly, immature and adult cardiomyocytes widely differ on the mechanisms responsible for the energy supply for ATPases. Thus, the cardiac cell should be subjected to essential maturation processes which allow the establishment of these energy transfer systems.

Regarding the CK shuttle, the system depends on the expression and on the activity of the different isoforms but also on the presence of Cr which has to be imported in the cardiomyocyte through the creatine transporter (CrT) [100, 101]. Results published by several studies realized in rodents and rabbits [12, 98, 101, 102] have shown that the process of phosphotransferases is not efficient in utero due to a very low CK activity during this development stage. However, the role of CK quickly becomes incontestable during maturation of the cardiac cell since its activity and cellular creatine content significantly increase during the first weeks after birth [12, 101, 103]. Moreover, this period is marked by a greater ability of CK to support the work of ATPases [12, 13]. Whereas a lot of studies performed in rodents seem to demonstrate that the critical stage for CK shuttle establishment would be in the first 3 weeks of postnatal life, it appears that the maturation of this system is not triggered by birth. Indeed, it has been shown that CK is able to support the ATPase activity of myofilaments before birth in species like guinea pig whose maturation is more advanced at birth [98]. This thus suggests that a functional CK shuttle is dependent on the general state of maturation of the cardiac cell rather than on the physiological changes induced by birth. It is important to note that, whatever the species, the expression of MM-CK always precedes the expression of mi-CK which physically appears and becomes functional quite late [99, 102]. This phenomenon is not senseless because MM-CK may participate in the supply of energy produced by the cytosolic glycolytic enzymes in the fetal cardiomyocyte which is highly dependent on that energy pathway [98].

Regarding the system of energy transfer by DANC between mitochondria and ATPases, it depends on the specific architecture of the cardiomyocyte. Indeed, the specific arrangement of intracellular components (mitochondria, SR, and myofilaments) allows the establishment of microdomains at the interface of organelles in which the transfer of energy-rich molecules is more efficient than by diffusion through cytosol [13, 91, 104]. Thus, the immature cardiac cell, which does not exhibit the same degree of structural organization as the adult one, undergoes a whole set of maturation processes of the architecture before showing an efficient DANC [13]. The establishment of the final architecture of the cardiomyocyte is a long process which occurs during the prenatal development but also during the early stage of postnatal development. The cardiac cell undergoes morphological changes, evolving from a fetal polygonal shape to an adult elongated shape [10, 96], which are accompanied by considerable changes in intracellular structures including a significant increase in myofibrils and mitochondria contents [13]. Thus, the internal organization of immature cardiomyocytes, characterized by a low density of intracellular structures, becomes more complex during maturation. This period is also marked by a densification of the microtubules [105] and a higher expression of proteins of the cytoskeleton, such as α-actinin, vinculin, and desmin [95]. Knowing that these molecules interact with intracellular organelles, a spatial rearrangement of these intracellular structures occurs so that the organization shifts from a random distribution of mitochondria to an organized mitochondrial network (characteristic of adult cytoarchitecture [106, 107]) which allows the emergence of an efficient DANC [13]. A detailed study of the

maturation of energetic microdomains, realized in our laboratory [13], clearly showed that a significant rearrangement of cytoplasmic components occurs between 3 and 7 days after birth in mice. Within only 4 days, the cell evolves from an anarchic intracellular organization, in which the intermyofibrillar mitochondria are not aligned and are separated from myofilaments by a wide cytoplasmic space, to an organization which closely looks like the adult one. Indeed, 7 days after birth, all of the cellular components are established and mitochondria are aligned along the myofibrils and in direct contact with the myofilaments. In this study, the quantification of cellular components by stereological methods helped to understand the structuration degree of the cells 7 days after birth; indeed, the volume of free cytoplasm, which is characteristic of immature cardiomyocytes [108], dramatically decreased between the ages of 3 and 7 days. A simultaneous measurement of energetic contribution of DANC confirmed a significant higher efficiency of this system over this period of 4 days. Interestingly, it seems that there exists a perfect concordance between DANC establishment and the transition step, between the hyperplasic and the hypertrophic stages of cardiac growth, which begins about 4 days after birth of the mouse [10]. It is noteworthy that beyond the mere establishment of the cytoarchitecture of cardiomyocytes during maturation, this period of 10 days, during which hyperplasia and hypertrophy coexist, is also marked by the emergence of the SR contribution in the contraction of the cardiac cell which mainly depends on trans-sarcolemmal calcium flux in immature cell [109, 110]. This reflects a real acquisition of a complex internal structure by the developing cardiomyocyte; this high degree of organization reaches its paroxysm with the formation of transverse tubules around the 15th day after birth [111] which are therefore required in that restricted cytosol to allow calcium to access all the myofibrils. Overall, the emergence of a mature excitation–contraction coupling is indicative of the organizational requirement demanded by the more and more complex structure in the developing cardiac cell where cytosolic diffusion become limiting when the structure reaches a determined density degree.

5.7 Conclusion

The study of cell metabolism during cardiac development shows that the bioenergetics of the cell is very responsive to organ maturation. The permanent increase in cardiac workload directly leads to an improvement of the contractile capacities of the cardiomyocyte. Thus, this cell adapts to its changing environment by orienting its metabolism toward a more efficient substrate (lipids) but also by establishing highly specialized transfer systems that are better suited to face the density of the emerging cytoarchitecture. Interestingly, metabolic maturation precedes the maturation of excitation–contraction coupling as a prerequisite for efficient energy yield. It would seem that the hyperplasic stage of cardiac growth would be the scene of the acquisition of adult physiological characteristics by the young cardiomyocyte. Therefore, the perfect synchronization of the transition

between hyperplasic and hypertrophic growth with the establishment of cellular architecture and the emergence of oxidative metabolism would become almost obvious. Indeed, whereas it is conceivable that the pseudo-organized cell of neonatal heart is able to increase cardiac muscle mass by proliferating in the early postnatal life, it is, however, more difficult to envisage this process in a cell which has already acquired a cellular architecture with the complexity of the adult cardiomyocyte. Consequently, it is conceivable that, as it has been previously suggested [95, 112], the densification of the internal structures of the cardiomyocyte could be a major component which lead to the end of hyperplasia and to the shift to strictly hypertrophic cardiac growth. Therefore, the major role of this hypertrophic stage would be to increase myofilament density and the amount of mitochondria, without inducing drastic metabolic changes.

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Chapter 6
Ontogenetic Aspects of Cardiac Adaptation to Chronic Hypoxia

Bohuslav Ostadal, Ivana Ostadalova, Zuzana Charvatova and Frantisek Kolar

Abstract One of the most common insults during early stages of postnatal ontogenetic development is hypoxemia due to cyanotic congenital heart defects. The question of the presumed cardiac impact will be, therefore, of considerable importance. Experimental results have clearly shown that the immature heart is significantly more tolerant to acute oxygen deficiency than the adult myocardium. However, the mechanisms of this difference have not yet been satisfactorily clarified; they are likely the result of developmental changes in cardiac mitochondrial function and energy metabolism. Adaptation to chronic hypoxia confers long-lasting protection in both adult and immature heart. However, the already high resistance of the newborn heart cannot be further increased; the effects of protective mechanisms appear only when the ischemic tolerance starts to decrease during development. Early chronic hypoxia, although transient, may have serious sex-dependent late consequences on the adult cardiovascular system. These results support the view that precise knowledge of individual developmental periods that are critical for cardiac ontogeny is crucial for better understanding of the mechanism of cardiac adaptation to oxygen deficiency.

Keywords Ontogeny · Immature heart · Cardiac development · Cardiac tolerance to ischemia · Adaptation to chronic hypoxia · Cardiac protection · Late effects · Sex differences

B. Ostadal (✉) · I. Ostadalova · F. Kolar
Institute of Physiology, Academy of Sciences of the Czech Republic,
Videnska 1083, 142 20 Prague 4, Czech Republic
e-mail: ostadal@biomed.cas.cz

Z. Charvatova
Department of Pathophysiology, Charles University, Prague, Czech Republic

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6.1 Introduction

One of the most common insults during early stages of postnatal ontogenetic development is chronic hypoxemia due to congenital cyanotic heart defects or pulmonary disease secondary to prematurity. Such hypoxemia may persist for several weeks or months until surgical repair of the structural defects or improvement in pulmonary function makes the individual normoxemic [1]. In addition, the immature heart of children who have undergone open-heart surgery is subjected to acute ischemic arrest. It follows that understanding the mechanisms by which cyanotic congenital heart disease modifies the structural and functional properties of the cardiac muscle, including its tolerance to acute oxygen deprivation may provide insight into the therapeutic strategies limiting myocardial damage [2]. Accordingly, the experimental studies of the pathogenetic mechanisms of cardiac adaptation to chronic oxygen deprivation must shift to early ontogenetic periods. It is understandable that these facts have stimulated the interest of experimental cardiologists in developmental approach.

Unfortunately, to date no model is available that adequately mimics chronic perfusion of hearts with hypoxic blood caused by congenital cyanotic defects. Therefore, similar models as in adults, i.e., chronic hypoxia simulated in the normobaric or hypobaric chamber, are being used in experimental studies performed during early stages of postnatal ontogeny. Adaptation to chronic hypoxia is characterized by a variety of functional changes to maintain homeostasis with minimum energy expenditure [3]. Such adjustments may protect the heart under conditions that require enhanced work and consequently increased metabolism. Adaptation thus increases cardiac tolerance to all major deleterious consequences of acute oxygen deprivation. In addition to protective effects, adaptation to chronic hypoxia also induces other adaptive responses including hypoxic pulmonary hypertension and right ventricular hypertrophy, which may under excessive hypoxia result in congestive heart failure [4]. In a short survey we would like to discuss the effects of chronic hypoxia on the developing heart with particular attention to the cardioprotective effects.

6.2 Hypoxic Tolerance of the Immature Heart

Cardiac tolerance of the immature heart to oxygen deficiency is significantly higher as compared with the adult myocardium; myocardial infarction is extremely rare during early ontogenetic development. Riva and Hearse [5] observed that age-dependent changes in resistance to global ischemia in the isolated rat heart showed a biphasic pattern with increasing tolerance from 5 to 23 days of age, followed by a decline to adulthood. Detailed analysis of the tolerance of the isolated rat heart to global ischemia during the first week of life has revealed a significant decrease from day 1 to 7 [6], suggesting a possible triphasic pattern of the ontogenetic

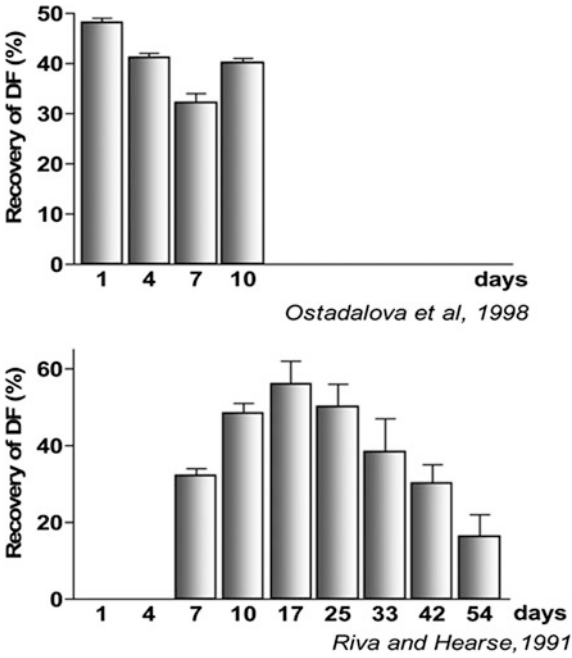

Fig. 6.1 Tolerance of the isolated perfused rat heart to acute ischemia (expressed as the recovery of the developed force—DF). Data for postnatal days 1–10 from [6], data for days 7–54 from [5]

development of cardiac sensitivity to ischemia (Fig. 6.1). The sensitivity of neonatal myocardium may be species dependent; Baker et al. [7] have shown that the neonatal pig heart is more susceptible to ischemia than the neonatal rabbit heart.

The mechanisms of the higher resistance of the immature heart to oxygen deprivation have not yet been satisfactorily clarified (for rev. see [8, 9]). For the explanation of this fact, the physiological alterations during the perinatal period should be taken into consideration. The major changes in oxygen saturation can be observed within delivery: during the short period of time mammalian fetus (and its heart) comes from the hypoxic environment with low PO_2 and low oxygen saturation (18 %) into the normal atmosphere (PO_2 160 mm Hg), arterial saturation increases more than 5 times (to 97 %). The delivery is, furthermore, accompanied by the transition from the amniotic fluid to the air, by the marked decrease of ambient temperature, by the termination of placental nutrition and by oxidative stress. This transition requires appropriate physiological adaptations: onset of pulmonary respiration, transition from fetal to neonatal circulation, switching-on of thermoregulation and increase of basal metabolic rate. As a consequence of the dramatic changes at birth, mammalian hearts meet suddenly and extremely high reactive oxygen species (ROS) concentration. The neonatal heart can probably use ROS for the up-regulation of protein degradation which permits production of amino acids, necessary for the maintenance of energy homeostasis during neonatal starvation [10–12].

The reason of the higher tolerance during further development can be still only hypothetical. It may be speculated that an explanation of the phenomenon lies in

the greater anaerobic glycolytic capacity, higher glycogen reserves of the immature heart [13] or amino acid utilization by transamination [14]. Another factor that may contribute to the increased tolerance of the immature heart is the age-related change in calcium handling [15]: the relative contribution of transsarcolemmal calcium influx and calcium released from the sarcoplasmic reticulum varies significantly during development. The contraction of the immature myocardium, where the sarcoplasmic reticulum is not fully developed, depends to a large extent on the fluxes of calcium across sarcolemma [16]. Calcium overload, common in the adult myocardium, was not described in the immature heart.

Still unclear is the role of mitochondria in the developmental changes of cardiac tolerance to oxygen deprivation, in spite of the fact that mitochondria are responsible for cellular oxygen handling. Mitochondrial oxidative phosphorylation is not completely developed in the rat heart at birth; cardiac maturation during the first postnatal week is characterized by increasing content and specific activity of cytochrome *c* oxidase and enhanced flux of adenine nucleotides across the inner mitochondrial membrane [17, 18]. We have shown previously [19] that the content of cytochromes in the rat cardiac mitochondria increased two-fold between birth and day 30, similarly as the expression of adenine nucleotide translocase 1. Moreover, in newborn animals, a single population of mitochondria with relatively high mitochondrial membrane potential (MMP) was observed. Starting with the weaning period, a second population with significantly lower MMP occurs. The collapse of MMP due to the opening of a high conductance mitochondrial permeability transition pore (MPTP) has been implicated in the molecular mechanisms associated with ischemia/reperfusion (I/R) injury of the adult heart [20, 21]. We have observed, however, significant ontogenetic differences in the role of MPTP in the I/R injury. Whereas the blockade of MPTP by sanglifehrin in perfused rat heart had a protective effect on I/R-induced damage in the adult myocardium as has already been demonstrated [22], it had no effect in the neonatal heart [23]. For the explanation of this difference, a modified amount of cyclophilin receptors in the neonatal heart or lower sensitivity of MPTP in the neonatal heart to pore opening factors have to be taken into consideration. Furthermore, we have found [23] that in cardiac mitochondria isolated from neonatal rats, Ca^{2+}-dependent and cyclosporine-sensitive MPTP is less sensitive to Ca^{2+} ions as compared with adults (Fig. 6.2). We can only speculate that its lower sensitivity to the calcium-induced swelling may be related to the higher ischemic tolerance of the neonatal heart. All these results support the hypothesis that cardiac mitochondria are deeply involved in the regulation of cardiac tolerance to oxygen deprivation during ontogenetic development.

6.3 Adaptation of the Immature Heart to Chronic Hypoxia

In the chronically hypoxic newborn mammal, body growth is blunted [24]. Neonatal growth retardation during moderate (15 % O_2) or severe (10 % O_2) hypoxic exposure can be almost entirely attributed to the effects of hypoxia on the

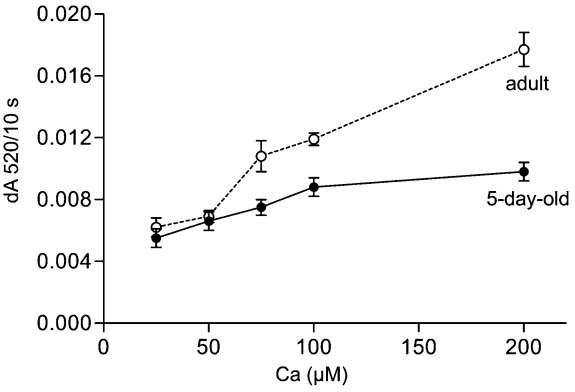

Fig. 6.2 The extent of mitochondrial swelling of heart mitochondria (determined as the decrease in absorbance at 520 nm adult and 5 day-old rats. Data from [23]

newborn, and is not mediated by the maternal response [25]. The faulty maternal lactation and limited food availability to the suckling are not the primary mechanisms of the neonatal growth retardation in chronic hypoxia, as was suggested by the observations that the cellular responses to hypoxia differ from those of experimental starvation [26]. In this connection it is necessary to mention that nutritional status markedly influences cardiac development. Slow-growing pups confer smaller cardiomyocyte length and volume [27], accompanied by qualitative changes of the subcellular structures. The development of membrane binding sites for adrenergic receptor ligands is retarded and the resulting receptor deficit probably contributes to reduced responsiveness to adrenergic stimulation [28, 29]. These changes are connected with alterations of cardiac ornithine decarboxylase activity [30]. Moreover, we have observed [31] that the number of cardiomyocytes in both the right and the left ventricular myocardium was significantly lower in slow-growing weanling rats. Early postnatal nutritional modification altered also protein remodeling in the immature myocardium: concentration of collagenous proteins in slow-growing rats significantly decreased [32]. Furthermore, undernutrition markedly decreased the basal values of left ventricular pressure and contractility in 3 week-old rats [33]; data on younger animals are, unfortunately, lacking.

Mild (19 % O_2) level of 1 week hypoxic exposure significantly increased cardiac weight and DNA synthesis [25]. This suggests that hypoxia can truly stimulate cardiac muscle cell multiplication as it has been demonstrated in neonatal rats exposed to sideropenic anemia [34, 35] or to low oxygen atmosphere [36, 37]. In high altitude-exposed neonatal animals the enlargement and activation of DNA synthesis was significantly more expressed in the right ventricular myocardium. The mechanisms behind the hypoxia-induced cardiac hyperplasia are unclear, but it is possible that they relate to the greater cardiac work caused by the higher cardiac output, blood viscosity, and pulmonary vascular resistance.

As has been mentioned in the Introduction, chronic hypoxia is the main pathophysiological feature of hypoxemic congenital heart disease. Timing of corrective surgery is critically important, with early surgery desirable to promote more normal

development. Many children undergoing cardiac surgery in the first year of life exhibit varying degrees of cyanotic heart disease where the myocardium is chronically perfused with hypoxic blood. We have observed [38] metabolic adaptation to chronic hypoxia in the myocardium of children with cyanotic congenital cardiac malformations. The aerobic capacity of the energy metabolism was significantly reduced in hypoxic hearts as compared with normoxic patients. Understanding the mechanisms by which cyanotic congenital heart disease modifies the myocardium and how the modifications impact on the cardiac tolerance to ischemia may provide insight into the treatments for limiting myocardial damage during cardiac surgery [2]. Unfortunately, clinical data are still missing.

6.4 Cardioprotective Effects

Adaptation of the adult hearts to chronic hypoxia confers long-lasting cardiac protection against I/R injury. This phenomenon has been demonstrated both in human populations living at high altitude (for rev. see [39]) and in many animal studies (for rev. see [4]). Hearts adapted to chronic hypoxia exhibit decreased infarct size [40], increased postischemic recovery of contractile function [41] and decreased severity and number of ischemic and reperfusion arrhythmias [42,43]. Protective mechanisms include mitochondrial potassium channels, oxygen free radicals, NO, different protein kinases, opioids, and erythropoietin; however, other factors cannot be excluded (for rev. see [44]).

Whereas an increasing number of data is available concerning the protective effect of adaptation to chronic hypoxia on the adult myocardium, much less is known about the possible cardioprotective effect on the immature heart. In this connection the question arises whether adaptation to chronic hypoxia can further increase the already high ischemic tolerance of the immature heart. However, only a few authors have compared resistance to oxygen deprivation in chronically hypoxic versus normoxic immature myocardium. We have observed [45] that chronic hypoxia, simulated in the barochamber, results in similarly enhanced cardiac resistance in rats exposed to chronic hypoxia either from the 4th day of postnatal life or in adulthood. Similarly, Baker et al. [7] demonstrated that adaptation to chronic hypoxia increased the tolerance of the developing rabbit heart (day 7 to day 28 of postnatal life). However, it follows from our results [46] that the protective effect of chronic hypoxia is absent in newborn rats; exposure of pregnant rats to simulated intermittent hypobaric hypoxia failed to improve cardiac ischemic tolerance in newborns. Early postnatal exposure to chronic hypoxia increased cardiac tolerance to acute ischemia by the end of the first postnatal week. Decreasing tolerance to ischemic insult during early postnatal life is thus counteracted by the development of endogenous protection. These results suggest that we might be dealing with the more general biological phenomenon: the already high resistance of the cardiac muscle cannot be further increased by different protective mechanisms. A similar situation as in the neonatal mammalian heart can

be observed also in the highly tolerant adult hearts of poikilotherms [47] or in the myocardium of young females [48]. As far as the clinical relevance of this developmental approach is concerned, metabolic adaptation to chronic hypoxia and activation of protective pathways have been observed in the myocardium of children with cyanotic congenital cardiac malformations [38, 49, 50, 51].

For completeness it may be of interest to mention the effect of aging. La Padula and Costa submitted 7 week-old rats to sustained simulated altitude of 5,000 m for their entire lifetime. They have found that whereas cardiac tolerance to acute hypoxia was in adult animals (up to 18 months) significantly increased, it was lost in senescent rats (25 months). Loss of adaptation involving an exaggeration of pulmonary hypertension (chronic mountain sickness) is frequent in aged people living at high altitude in the Andes [39].

Overwhelming majority of studies, analyzing the possible mechanisms of cardiac adaptation to chronic hypoxia deals exclusively with the adult myocardium (for rev. [4, 44]). We have shown previously that blockade of mitochondrial K_{ATP} channels with 5-hydroxydecanoate completely abolished the cardioprotective effect of adaptation to chronic hypoxia also in neonatal rats; a similar effect had the blockade of NO by L-NAME. It seems, therefore, that both mitochondrial K_{ATP} channels and NO may play an important role in the mechanisms of adaptation of the immature heart to chronic hypoxia [52, 53]. Furthermore, it has been shown that also angiotensin II is involved in the mechanisms of adaptation of the immature heart to chronic hypoxia. The chronic blockade of angiotensin II type 1 receptors (AT_1) by irbesartan completely abolished the cardioprotective effect of chronic hypoxia [54]. The involvement of AT_1 receptor pathway in the adaptive responses of the immature hearts to chronic hypoxia should be taken into consideration in the treatment of children suffering from cyanotic congenital heart disease.

6.5 Late Effects of Early Cardiac Adaptation to Chronic Hypoxia

Perinatal hypoxemia, although transient, may have serious late consequences on the adult cardiovascular system. This fact is in accordance with Barker′s concept [55] of fetal and neonatal programming, which is based on epidemiological studies showing that perinatal pathogenetic factors may be linked with the development of adult cardiovascular diseases. In recent years, the Barker′s hypothesis opened the field for extensive research into the fetal origin of adult diseases (for rev. see [9]). Experimental studies of the late effects of chronic hypoxia on cardiac tolerance to hypoxia and ischemia are, unfortunately, not concise; they differ in the critical ontogenetic period studied (prenatal, perinatal, early postnatal), intensity and duration of hypoxia. Nevertheless, animal studies have repeatedly suggested a possible link between early hypoxia and increased risk of cardiovascular disease in

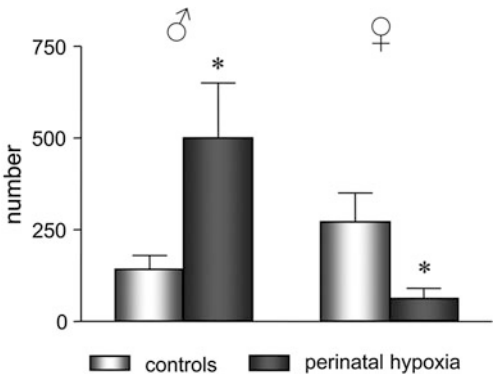

Fig. 6.3 Total number of arrhythmias over 30 min coronary artery occlusion in control and perinatally hypoxic adult male and female rats. Statistically significant difference ($p < 0.01$) between control and perinatally hypoxic group. Data from [57]

the offspring. Li et al. [56] have found that prenatal chronic hypoxia (10 % O_2, from day 15 to 21 of gestation) significantly increases the sensitivity of the adult 6 month-old male rat heart to ischemia–reperfusion injury, as indicated by increased myocardial infarct size and decreased postischemic recovery of left ventricular function. We have observed that alternative end-points—ischemic arrhythmias and LDH release—were able to differentiate the degree of ischemic insult already in 3 month-old rats exposed perinatally to chronic hypoxia [57]. According to Peyronnet et al. [58] perinatal exposure of rats to hypoxia exerts adverse effects on the development of the autonomic nervous system related to cardiovascular events and increased hemodynamic response under stress conditions in adults. Furthermore, Rohlicek et al. [1] observed that adult rats made hypoxemic neonatally showed a markedly greater cardiac output response to acute hypoxia than controls. This may be due to altered myocardial function and/or changes in the autonomic nervous system response to acute hypoxemia. Similarly, Hampl and Herget [59] and Hampl et al. [60] have shown that perinatal hypoxia increases the susceptibility to hypoxic pulmonary hypertension later in life.

The cardiogenic developmental mechanisms of the increased susceptibility of the adult hearts to I/R injury are not known at present. Li et al. [56] have observed that I/R-induced apoptosis was by 44 % higher in the hearts of rats that had experienced early chronic hypoxia compared with control animals. This finding was in agreement with the results of myocardial infarction, which showed a 56 % increase in prenatal hypoxic hearts. In addition, they have found [61] that cardiac Hsp 70 expression was significantly lower in hypoxic hearts than in controls. It has been well documented that Hsp 70 plays an important role in protection against I/R injury and that the degree of postischemic functional recovery correlates with the absolute Hsp 70 tissue content [62]. It is, therefore, likely that decreased Hsp 70 levels may play a key role in the increased susceptibility of the adult heart to I/R injury in animals exposed to chronic hypoxia during early phases of ontogenetic development. We have observed in the rat model that late myocardial effects of hypoxemia, experienced in early life, may be sex-dependent [57]. Perinatal exposure to chronic hypoxia significantly increased cardiac tolerance to acute I/R injury (expressed as lower incidence of ischemic arrhythmias) in adult female rats;

the effect on arrhythmias in males was the opposite (Fig. 6.3). A similar sex-dependent effect of early hypoxia was later confirmed by Xue and Zhang [63]. The question remains of the cause of these sex differences. A large body of evidence indicates that "genomic" and "non-genomic" effects of estrogens are involved. According to Patterson et al. [64] this difference may be in part caused by the greater expression of estrogen receptors in the immature female heart. These results suggest that early chronic hypoxia may significantly influence cardiac tolerance to ischemic injury in adults in a sex-dependent manner,

6.6 Conclusions

Our present focus on the hypoxic immature heart is driven by clinical urgency: cyanotic congenital cardiac malformations remain the single largest cause of mortality from congenital defects and ischemic heart disease is no more the disease of the fifth and older decades but its origin as well as risk factors such as genetic predisposition, hyperlipoproteinemia, smoking, hypertension, obesity, and diabetes are present already during early ontogeny. These results suggest that the developmental approach offers new possibilities in the studies of pathogenesis, prevention and therapy of critical cardiovascular diseases. Moreover, epidemiological studies clearly show that the number of adult patients undergoing surgery for cyanotic congenital heart defects has increased significantly. This group of patients is growing older and is approaching the age characterized by significantly increased risk of serious cardiovascular diseases, such as hypertension and ischemic heart disease. Therefore, it can be expected that more such patients will require catheterization or cardiac surgery. Under these conditions, the question of the presumed cardiovascular impact of early exposure to chronic hypoxia is of considerable clinical importance.

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Chapter 7
Heart and Arterial Aging

Paul D. Chantler and Edward G. Lakatta

Abstract Aging is the dominant risk factor for cardiovascular disease, and is linked to the age-associated changes to the structure and function of the heart and arteries. Age-associated changes occur in everyone but not necessarily at the same rate or to the same extent, and this may account for the difference noted in the development of cardiovascular disease between individuals of the same chronological age. Age-associated changes in cardiovascular physiology must be differentiated from the effects of pathology, such as coronary artery disease, which occur with increasing frequency as age increases to gain an understanding of normative aging. Prominent age-associated changes in the cardiovascular system include arterial remodeling, an increase in arterial stiffness, and an impaired endothelial vasoreactivity. This results in an increased afterload (including an increase in aortic and brachial pressures) on the left ventricle resulting in left ventricular wall thickening, and prolonged relaxation of the left ventricle in diastole. During stress there is also a decreased responsiveness to β-adrenergic receptor stimulation in the context of increased circulating catecholamines. These changes not only ultimately impair the ability of the cardiovascular system to respond to times of stress (exercise, illness, and mental stress) in older individuals, but also set the stage for the development of cardiovascular diseases in the elderly. Those individuals who maintain an physically active lifestyle, or who partake in exercise training later in life, however, can either ameliorate or delay some, but not all, of the cardiovascular alterations that accompany advancing age.

P. D. Chantler
Division of Exercise Physiology, School of Medicine, West Virginia University, Morgantown, WV, USA

E. G. Lakatta (✉)
Laboratory of Cardiovascular Science, Intramural Research Program, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21225, USA
e-mail: lakattae@grc.nia.nih.gov

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7.1 Introduction

Complex and diverse changes occur to the structure and function of the heart and arterial system with advancing age. When attempting to understand the effects of aging on the cardiovascular (CV) system, it is important to take into account the complex interplay between the heart as a pump and the afterload on the heart imposed by the arterial system [1]. With advancing age, silent changes occur within the heart and vasculature that alter (uncouple) their cross talk, affecting the hemodynamic profile and efficiency of the CV system. The heart loses myocytes and becomes slightly hypertrophic (due in part to cardiomyocyte enlargement) and hyporesponsive to sympathetic (but not parasympathetic) stimuli, so that the exercise-induced increases in heart rate and myocardial contractility are blunted in older persons. The arteries become elongated, thicker, and stiffer, due to collagen and calcium deposition and fragmentation of elastic fibers in the medial layer, with evidence of endothelial dysfunction, and biochemical patterns resembling the proinflammatory profile of early atherosclerosis. These arterial changes are accompanied by an increased systolic blood pressure (increasing cardiac afterload) while diastolic blood pressure tends to decrease (compromising cardiac oxygenation).

A fundamental understanding of age-associated changes in CV structure and function is required for effective and efficient prevention and treatment of CV disease in older persons, because although CV adaptations that occur with age are considered to be representative of "normative" aging, they lower the threshold for the development of CV disease, which affects 70 % of individuals 60 years of age. However, not everyone ages at the same rate. There is a continuum of age-associated alterations to cardiac and arterial structure and function in healthy humans that appears to influence the steep age-associated increases in CV disease (i.e., hypertension, atherosclerosis, stroke etc.). Whereby, the age-associated changes in structure and function become "partners" with pathophysiologic disease mechanisms, lifestyle, and genetics in determining the threshold, severity, prognosis, and therapeutic response of CV disease in older persons. Thus, the key paradigm shift in our appreciation of aging of the heart and arterial system is that the components of aging associated with CV disease risk should no longer be simplistically attributed to an increased time of exposure to other established CV risk factors. Instead, the risky components of aging need to be recognized as accelerated or dysregulated age-associated alterations in the CV system, at the molecular, enzymatic, biochemical, cellular, histologic, and organismal levels. This chapter will discuss the changes to the CV system with aging and the role that age-associated changes play in reducing CV reserve to make the heart more susceptible to CV disease.

7.2 Part I: Cardiovascular Aging at Rest

7.2.1 Arterial Aging

The central arteries not only behave as a conduit to deliver blood from the heart to peripheral tissues, but act as a "Windkessel" to cushion the blood flow and pressure oscillations caused by the left ventricular (LV) ejection ensuring a steady flow of blood to the peripheral organs. Aging influences multiple signaling mechanisms that modulate the ability of larger arteries to adapt, repair, and govern their structural and functional properties. Table 7.1 summarizes the age-associated changes in central arterial structure and function in non-human primates and in healthy humans. Some of these features are discussed in the following sections.

7.2.1.1 Arterial Lumen Diameter

Both cross-sectional and longitudinal studies in healthy humans indicate that central elastic arteries dilate with age, leading to an increase in lumen size (Fig. 7.1a) [2, 3]; in the presence of CV disease the dilation of the aorta is further accentuated [3]; the length of the aortic arch also increases with age [4]. Studies have found an inverse and independent association between aortic root diameter and pulse pressure (an index measure of arterial stiffness), suggesting that luminal size may play a role in the pathogenesis of systolic hypertension [5]. However, longitudinal studies are needed to clarify the relationship between aortic root diameter and pulse pressure, particularly given its implications for therapies to delay or to prevent systolic hypertension. Taken together, these findings suggest that the age-associated dilation and lengthening may help to offset wall stiffening and loss of distensibility by augmenting the storage capacity of systolic blood volume.

7.2.1.2 Arterial Wall Thickness

The thickness of the arterial wall, indexed as intimal media thickness (IMT), increases two-to three-fold between 20 and 90 years of age (Fig. 7.1b) [6]. Post-mortem studies in humans indicate that mainly the thickening of the aortic wall occurs in the intimal layer [7]. Although, the increase in IMT with age is often ascribed to "subclinical" atherosclerosis [8], and therefore used as a surrogate measure of atherosclerosis, increased IMT should not be construed as synonymous with "subclinical atherosclerosis," particularly in the absence of plaques because: (1) IMT is usually measured in areas devoid of atherosclerotic plaque, and is only weakly associated with the extent and severity of coronary artery disease [9]; (2) aortic IMT increases with age in populations with a low incidence of atherosclerosis [7]; and (3) animal models (rats, rabbits, and nonhuman primates) of aging that are usually devoid of atherosclerosis exhibit diffuse intimal thickening

Table 7.1 Aging of large arteries

	Humans >65 years	Monkeys 15-20 years	Rats 24-30 months
Endothelial dysfunction	↑	↑	↑
Diffuse intimal thickening	↑	↑	↑
Luminal dilation	↑	↑	↑
Stiffness	↑	↑	↑
Matrix			
Fibronectin/Collagen	↑	↑	↑
Frayed Elastin	↑	↑	↑
VSMC			
Migration	↑	↑	↑
Proliferation	↑	↑	↑
Local ANG-II Signaling	↑	↑	↑
Arterial Wall ANG-II	↑	↑	↑
MMP-2 Activity	↑	↑	↑
Caplain 1 Activity	?	?	↑
MCP-1/CCR2	↑	↑	↑
TGF-β	↑	↑	↑
NADPH oxidase	↑	↑	↑
Nitric oxide bioavailability	↓	↓	↓
TNF-α	?	↑	?
iNOS	?	↑	↑
CAM-1	?	↑	↑
MFGE8	↑	↑	↑
Hypertension	+/-	-	+/-
Atherosclerosis	+/-	-	-

? information unknown, + present with aging, - absent with aging, *VSMC* Vascular Smooth Muscle Cell, *ANG* Angiotensin, *MMP* matrix metalloproteinases, *MCP-1* monocyte chemotactic protein-1, *TGF β* Transforming growth factor *β*, *TNF α* Tumor necrosis factor *α*; *iNOS* inducible isoform of nitric oxide synthases, *CAM-1* cell adhesion molecule; *MFGE8* milk fat globule-EGF factor 8 protein

with age, which is similar to that observed in grossly normal arterial segments in humans [10–12]. These results indicate that factors other than atherosclerosis can influence IMT (i.e., aging and arterial pressure). Nonetheless, an increase in IMT remains a useful marker of subclinical vascular disease, whereby a 0.1 mm increase in carotid artery IMT is associated with an 18 % increase for stroke and 15 % for myocardial infarction [13]. The age-associated arterial thickening, in the absence of plaque formation, may reflect an adaptation to maintain an equilibrium state in which the effects of pressure and flow on the arteries are in balance. Whereas, at more severe arterial thickening (atherosclerosis), IMT may represent an attempt to maintain a constant tensile stress.

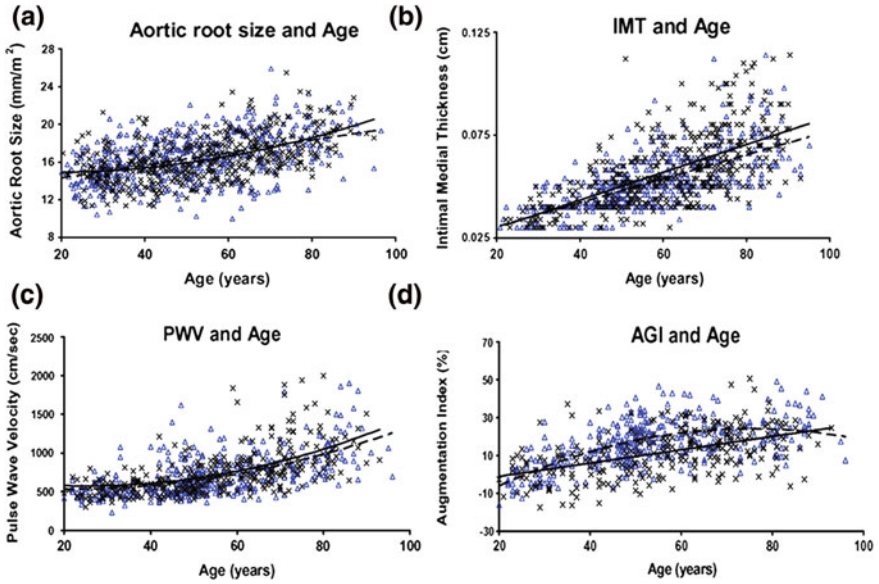

Fig. 7.1 Age-associated changes in arterial structure and function in men (x) and women (Δ). Best fit regression lines (*quadratic* or *linear*) are shown for men (*solid lines*) and women (*dotted lines*). **a** Aortic root size, **b** Common carotid IMT, **c** Carotid–femoral pulse wave velocity (PWV), **d** Carotid arterial augmentation index (AGI). Note that unlike pulse wave velocity (PWV), which increases quadratically with age, the age-associated increase in AGI is linear in men and convex shape in women, suggesting that factors other than stiffness also modulate the origin of reflected waves and the amplitude of AGI. Reproduced with permission from Najjar et al. [11]

Animal models of arterial aging have provided insights to the mechanisms behind arterial thickening, which are due to myriad age-associated biochemical, cellular, and morphologic changes in the arterial wall, and which are modulated by the same factors that have been implicated in the genesis of various CV diseases [11]. For example, the diffusely thickened aging intima contains matrix proteins, collagen, glycosaminoglycans, and also vascular smooth muscle cells (VSMCs) that are thought to have migrated from the media, increased expression of aortic intimal adhesion molecules [14] and increased adherence of monocytes to the endothelial surface [15]. Within the thickened intima, the levels of the inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) and its receptor are also elevated [16]. The expression and activity of transforming growth factor (TGF) -β1, a multifunctional growth factor that regulates cell replication, synthesis of extracellular matrix components, and the response to injury [17], are also increased in the aged intima [18]. Of note, in grossly normal specimens from humans over age 65, aortic intimal cell infiltration and matrix deposition are increased dramatically compared to specimens from younger individuals ($\approx$20 years) [19]. In the medial layer of central arteries, salient age-associated changes also occur with the deposition of extracellular matrix proteins such as

fibronectin and type-2 matrix metalloprotease (MMP-2) [14, 18, 20], which promote not only matrix protein degradation, facilitate VSMC migration [21], but also activate TGF- β. The VSMCs from the aortic media layer also appear larger in size and fewer in number in old versus young rats [22].

7.2.1.3 Arterial Endothelial Function

Endothelial cells (EC) play a pivotal role in regulating several arterial properties, including tone, permeability, angiogenesis, and the response to inflammation. Several features of these arterial properties undergo functional age-associated alterations. In the brachial artery, endothelial function, as assessed by flow-mediated vasoreactivity (examining endothelial- dependent vasodilation), declines after 40 years of age in men [23]. In women, the loss in endothelial-dependent vasodilation occurs later around 50 years of age and coincides with menopause [23, 24]. Further, the loss in endothelial-dependent vasodilation after menopause in women is steeper than that observed in men, suggesting that estrogen plays a protective role in maintain endothelial function [23, 24]. Indeed, estrogen supplementation in postmenopausal women increases endothelial function, likely through improvements in the release of nitric oxide (NO) and prostaglandin, and activation of potassium or calcium channels [25–27]. In contrast to endothelial-*dependent* vasodilation, there is no age-associated loss in endothelial-*independent* vasodilation in healthy men or women (irrespective of menopause status) [24, 28, 29], indicating a preservation of VSMC function but impaired NO-mediated vasodilation with aging. Importantly, a loss in endothelial-*dependent* vasoreactivity is an independent predictor of future CV events [30, 31], and thus should be considered another marker of arterial aging. Reduced production and/or bioavailability of NO is a primary mechanism mediating impaired endothelium-dependent dilation in healthy older humans [32], partly as a result of the increased production of oxidative stress.

With aging the endothelium undergoes considerable remodeling. Morphologically, aortic EC from older donors are flattened and enlarged, and the number of EC with polyploid nuclei increases with advancing age [10, 33]. There is an increased endothelial permeability, alterations in the arrangement and integrity of the cytoskeleton, the appearance of senescence-associated β-galactosidase staining, and the expression of several inhibitors of the cell cycle [10, 33]. Aged ECs secrete more plasminogen activator inhibitor-1 [34], favoring thrombosis formation. Furthermore, with aging, EC production of vasoconstricting growth factors such as angiotensin II and endothelin increases, and that of vasodilatory factors (e.g., NO, prostacyclin, and endothelium-derived hyperpolarizing factor) are reduced [20, 35, 36]. Advancing age also is associated with decreased EC capacity for replication and repair, which has been linked to increased apoptosis [10], telomere shortening [37, 38], proinflammatory state [39], reduced NO bioavailability [40], and decreased number [41] and the function [42] of endothelial progenitor cells. The loss of EC function with age is likely mediated partly by an imbalance between factors promoting growth, migration, survival; and factors enhancing oxidative stress and promoting senescence.

7.2.1.4 Arterial Stiffness

The ability of the large conduit arteries to accommodate the volume of blood ejected by the LV can be described in terms of "stiffness", which can be measured noninvasively as pulse wave velocity (PWV). With healthy aging, central [43], but not brachial [44], PWV increases in a mildly concave manor, with the slope increasing modestly at midlife (Fig. 7.1c). Increased central PWV and its associated increases in arterial pressures leads to an increase in the load (afterload) on the LV, leading to LV hypertrophy, and increased oxygen consumption.

Traditionally, the increase in central PWV with age has been attributed to the repeated cycles of distensions and elastic recoils of the arterial wall, which are thought to accelerate the fragmentation and depletion of elastin, as well as the deposition of collagen [45]. It is now recognized, however, that arterial stiffening can be modulated by several factors including lifestyle considerations (e.g., salt intake, exercise, and weight loss) [46, 47], signaling pathways (e.g., NO) [48], inflammation, and genetics [49]. Further, prominent age-associated increases in central PWV have been demonstrated in populations with little or no atherosclerosis, indicating that arterial stiffening can occur independently of atherosclerosis [50]. However, central PWV is further increased in the context of atherosclerosis and diabetes [51, 52], indicating that the changes are not only governed by structural changes within the matrix but also by endothelial regulation of VSMC tone and of other aspects of arterial wall structure/function.

The age-associated changes to the arterial medial layer in the content and integrity of elastin and collagen, as well as, their linkages to other matrix constituents and endothelial dysfunction are implicated in arterial stiffening. Elastin content decreases with advancing age due, in part, to repression of elastin gene expression by B-Myb; and to degradation of elastin fibers [11], a process that is accelerated by age-associated enzymatic processes, such as MMP-2, the levels and activity of which are increased in the aortic wall with advancing age [18]. There is also excessive synthesis and deposition of collagen types I and III in the media [18], and adjacent collagen fibrils undergo nonenzymatic glycation and oxidation of free amino groups to form advanced glycation end products with advancing age [53]; this further increase the stiffness of the collagen network. In addition to structural properties, arterial stiffness is determined by VSMC contractile tonus at least in vivo, which is controlled, in part, by neurohumoral factors, e.g., catecholamines and angiotensin [54–56].

Changes in arterial stiffness have significant implications on pulse wave reflection and blood pressures (see below). Increased arterial stiffness also has clinical implications, whereby a 1 m/s increase in central PWV, corresponds to an age-, sex-, and risk factor-adjusted risk increase of 15 % in CV and all-cause mortality [57]. Thus, arterial stiffening, like IMT and endothelial function, should be viewed as another marker of aging, which, when accelerated, also becomes a risk factor for CV disease.

7.2.1.5 Aortic Impedance

The relationship between the steady and pulsatile components of flow and the resulting pressure wave in the aorta defines the aortic input impedance. The impedance modulus (the ratio of oscillatory pressure and flow) is usually considered in the frequency domain. Characteristic impedance is the impedance to pulsatile flow in the absence of wave reflections [58] and like central PWV, is directly related to stiffness of the arterial wall, and inversely related to lumen diameter to the power of five [59]. However, the age-associated increase in aortic diameter dilation restrains characteristic impedance prior to 60 years of age, despite an increase in PWV [59, 60]. Attenuation of the rate of increase in aortic diameter with age, along with a further increase in PWV increases characteristic impedance after 60 years of age [59, 60]. Fluctuations of the impedance modulus about this mean level are determined by reflected pulse waves. As the initial forward (incident) wave propagates through the arterial tree, it encounters an impedance mismatch (changes in aortic geometry, local arterial branching, and smaller high resistance arterioles) causing partial reflections of the incident pressure waves traveling back to the aorta (Fig. 7.2). In young individuals, whose arterial wall stiffness is compliant, the reflected wave does not reach the large aorta until diastole. In contrast, the age-associated increase in central arterial stiffness increases the velocity of the reflected wave, which shifts the return of the reflected waves to an earlier time during systole (Fig. 7.2). Increased characteristic impedance and early wave reflection, shift impedance curves to higher frequencies and increase markedly the impedance to LV ejection over the frequency band (1–4 Hz) [61]. These reflected waves play an important role in influencing the central to peripheral pressure augmentation across the arterial tree. The augmentation index (AGI), a measure of wave reflection and arterial stiffness, increases substantially in individuals up to the age of 50 years [8, 43, 62, 63]. However, unlike central PWV, the rate of increase in the AGI slows at older ages, especially in women [64] (Fig. 7.1d). These data suggest that AGI might be a more sensitive marker of arterial aging in younger individuals, whereas aortic PWV is more sensitive in older individuals. The zero frequency impedance modulus (the systemic vascular resistance [SVR]) usually in older persons in whom predominantly systolic hypertension does not occur are spared from an increase in SVR with age [65–67], Unloading the aged heart with a balanced vasodilator (sodium nitroprusside; SNP) reduces aortic impedance through its actions on improving VSMC tone of small arteries, which lowers SVR and extends distally the location of the wave reflection site (lowers AGI) [68]. In contrast, SNP has minimal effects on altering PWV [68].

7.2.1.6 Blood Pressure

Resting brachial systolic pressure (SBP) rises linearly with age [69], whereas brachial diastolic pressure (DBP) rises until 50 years of age, levels off from ages

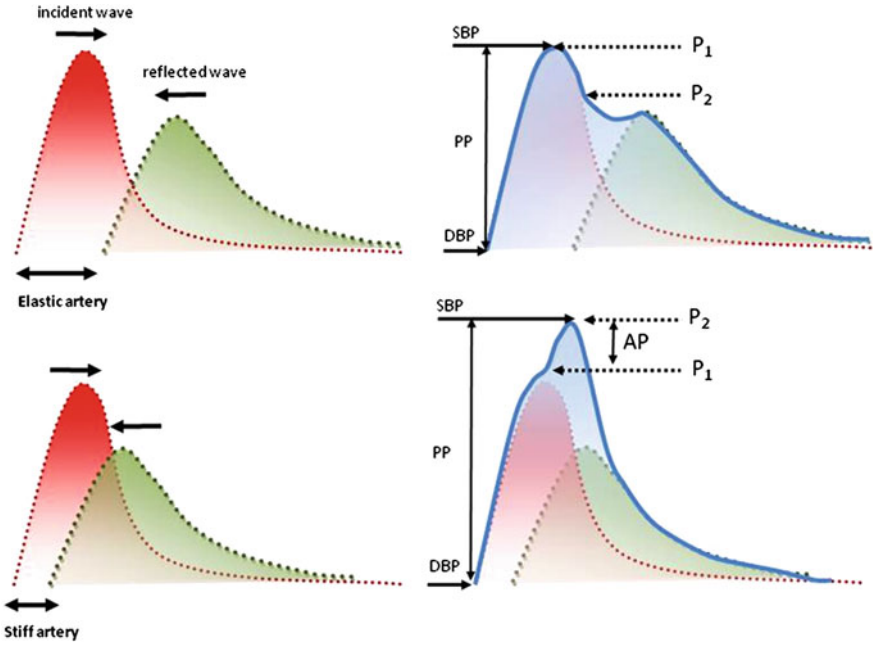

Fig. 7.2 The shape of the pulse waveform is the result of the summation (*solid blue line*) of the incident wave (*dashed red line*) and the reflected wave (*dashed green line*) which propagate along the arterial tree. The reflected wave is created by the wave encountering an impedance mismatch (bifurcations, diameters changes, etc.). The incident and reflected waves change shape as they travel along the arteries depending on vessels characteristics. **a** In healthy young individuals, the aorta is elastic and the reflective wave does not return to the aorta until diastole or late systole, thereby busting diastolic coronary filling. **b** In older individuals, the aorta is stiffer and the reflected wave will arrive sooner and will significantly increase the aortic pulse pressure and myocardial oxygen demand. Stiffness is not the only parameter influencing the shape of the waveform. Vascular tone plays a role on the amplitude of the reflected wave, while heart rate induces changes on the relative duration of direct and reflected waves. P_1 the pressure at the first inflection point; P_2 the pressure at the second inflection point; AP augmentation pressure ($P_1 - P_2$), AGI, augmentation index defined as AGI (%) = 100 AP/PP, where PP is pulse pressure (systolic-diastolic pressure). In young healthy compliant aorta the AP is zero, because the P_2 is embedded in the diastolic phase (see **a**), whereas in a stiff aorta AP is present, because P_2 occurs shortly after P_1 "hits" (see **b**) augmenting the pressure

50 to 60, and declines thereafter [69], and is attributed, in part, to the return of the reflective wave in systole boosting SBP and lowering DBP. Further, in women there is a rightward shift by approximately a decade in the age-associated blood pressure response compared to men. Consequently, brachial pulse pressure (PP) increases steeply over the age of 50 years [63, 69] and, is in part, due to the combination of reduced arterial compliance [69]. The increase in aortic diameter, in young and middle-aged adults, may represent an active adaptation to stiffening of the arterial wall that serves to limit the increase in PP [70].

The blood pressure waveform varies substantially between the peripheral muscular and the central elastic arteries, and there is a gradual increase in SBP as the pressure wave propagates distally through the more muscular vessels [71]. It is the aortic SBP that the LV encounters during contraction, and it is the aortic DBP that determines coronary perfusion. Therefore, central blood pressure is physiologically more relevant than peripheral BP for the pathogenesis of heart disease [61, 72, 73]. The increase in SBP and PP with age is steeper when measured in the central versus peripheral arterial beds [63, 74]. Sex-related differences in the central pressure responses with age have also been reported. Both cross-sectional and longitudinal studies have shown that, central SBP is lower in women compared to men below 30–40 years, but the increase in central SBP with age in women is greater than men [63, 74]. This finding is consistent with known sex differences related to aortic characteristic impedance and wave reflection [75].

7.2.1.7 Arterial Elastance

Arterial elastance (E_A) is a composite measure that incorporates many of the arterial parameters mentioned above (arterial compliance, SVR, characteristic impedance, and systolic and diastolic time intervals), and thus reflects the net arterial load imposed on the LV [76]. As detailed previously, most of the components of E_A (resistance, compliance, and stiffness) change with age, and as such E_A gradual increases with age [77–79]. Importantly, examining the properties of the arterial system in terms of elastance enables a direction comparison with changes in LV function with age, referred to as arterial–ventricular coupling (see Sect. 7.2.3).

7.2.2 Cardiac Aging

The primary function of the heart is to impart energy to blood in order to generate and sustain an arterial pressure necessary to provide adequate perfusion of organs. With aging, the structure and function of the heart undergoes modest changes. Its function involves a complex integration of electrical, biochemical, and mechanical processes, which is also dependent on the preload and afterload imposed on it by the arterial system. For example, blood flow ejected by the heart per beat (cardiac output; CO) is regulated by multiple mechanisms, the macroscopic descriptors of which include the heart rate and factors that affect stroke volume (SV): the quantity of blood that fills the heart prior to excitation *(preload);* the mechanical load encountered following the onset of contraction *(afterload);* the intrinsic myocardial contractile properties *(contractile* or *inotropic* state or level of effectiveness of excitation–contraction coupling); and coronary flow. A unified interpretation of identified cardiac changes that accompany advancing age in otherwise healthy persons suggests that at least in part, these are adaptive, occurring to some extent in response to arterial changes that occur with aging (Fig. 7.3). Table 7.2, summarizes the changes in CV function with age in healthy persons.

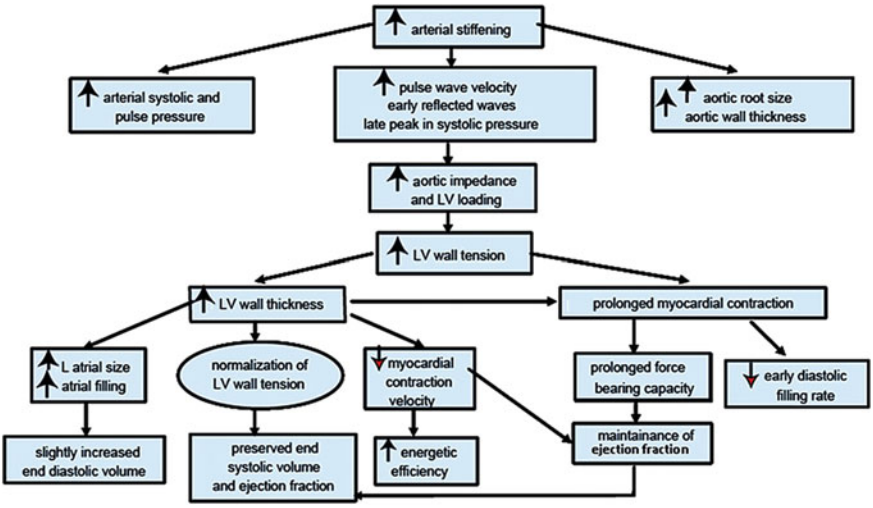

Fig. 7.3 Arterial and cardiac changes that occur with aging in healthy humans. Reproduced with permission from Najjar et al. [163]

7.2.2.1 Preload

Preload is the pre-excitation determinant of myocardial function and pump performance and is influenced, in part, by ventricular filling properties (fiber stretch, end-diastolic blood volume, and filling pressure). LV compliance (inverse of stiffness) is one factor that determines filling pressure. In humans, it is difficult to determine LV compliance as it requires the simultaneous measurement of end-diastolic pressure and volume, which has not been characterized in healthy young and older persons. LV compliance, characterized using mean pulmonary capillary wedge pressure (a surrogate for LV end-diastolic pressure) and calculating end-diastolic volume (EDV) from echocardiography, decreases with increasing age, leading to a diminished diastolic performance (2). The mechanisms for decreased LV compliance with age are multifactorial. The passive stiffness properties affecting LV compliance are likely to involve fewer cardiomyocytes and a dynamic remodeling of the extracellular matrix, resulting in an imbalance between collagen synthesis and degradation, which in turn increases myocardium collagen accumulation and fibrosis [80–83]. However, in large part, the age-associated prolongation of Ca^{2+} contractile myofilaments activation contributes to a reduced LV compliance [81].

There are age-associated changes to LV filling whereby the early diastolic filling rate (E-wave) progressively slows after 20 years of age, with an approximate 80 % reduction by 80 years [84] (Fig. 7.4). This reduction may be in part be due to the cardiac structural remodeling or to residual myofilament Ca^{2+} activation from the preceding systole [85]. Despite a reduction in LV filling in early diastole, more filling occurs in late diastole (A-wave), due in part, to a more vigorous atrial contraction, which is often accompanied by atrial enlargement [86, 87]. As such the ratio of early

Table 7.2 Supine rest and exhaustive upright exercise: changes in arterial and cardiac function between ages of 20 and 80 years in healthy men and women

Oxygen consumption	Peak	↓ (50%)
Arteriovenous O_2 difference	Peak	↓ (25%)
Cardiac index:	Rest	↔
	Peak	↓ (30%)
Heart rate:	Rest	↔/↓
	Peak	↓ (25%)
Preload (EDV):	Rest	↔/↑
	Peak	↑ (30%)
Afterload	--	--
Arterial (SVR):	Rest	↑ (13%)
	Peak	↑ (30%)
Cardiac (ESV):	Rest	↔
	Peak	↑ (275%)
Cardiac (EDV):	Rest	↔/↑
	Peak	↑ (30%)
Net Afterload (E_A):	Rest	↑ (10%)
	Peak	↔/↑ (15%)
Contractility (E_{LV}):	Rest	↑ (15%)
	Peak	↓ (60%)
E_A/E_{LV}:	Rest	↔/↑ (9%)
	Peak	↓ (15%)
Plasma catecholamines	Peak	↑
Cardiac and vascular responses to β-adrenergic stimulation	Peak	↓

EDV end diastolic volume, *SVR* systemic vascular resistance, *ESV* end systolic volume, E_A arterial elastance. E_{LV} left ventricular end systolic elastance, E_A/E_{LV} arterial-ventricular coupling

and late filling velocity decreases from 2.0 to 0.6 between 20 and 70 years of age [88] (Fig. 7.4). There is also a prolongation in the isovolumic relaxation period that occurs in the healthy human heart with aging, which may be in part attributable to such prolonged contractile protein Ca^{2+} activation, which extends the continued ejection of blood from the LV during late systole. This is a beneficial adaptation with respect to enhanced central arterial stiffness and early reflected pulse waves. An adaptive consequence of this altered LV filling pattern is that LV EDV, in the supine position, remains similar or increases slightly with age [89].

7.2.2.2 Left Ventricular Ejection and Contraction

Blood ejected from the heart (SV) is influenced by the preload and afterload imposed on the heart, along with the contractile state of the heart. In the upright resting position, SV increases slightly with age due to the slight increase in preload, as ESV remains unchanged (SV = EDV−ESV) [89] (Table 7.2). The relationship between the pumping properties of the LV (stroke work) to EDV is a measure of LV function. With increasing age, the stroke work-EDV relationship is

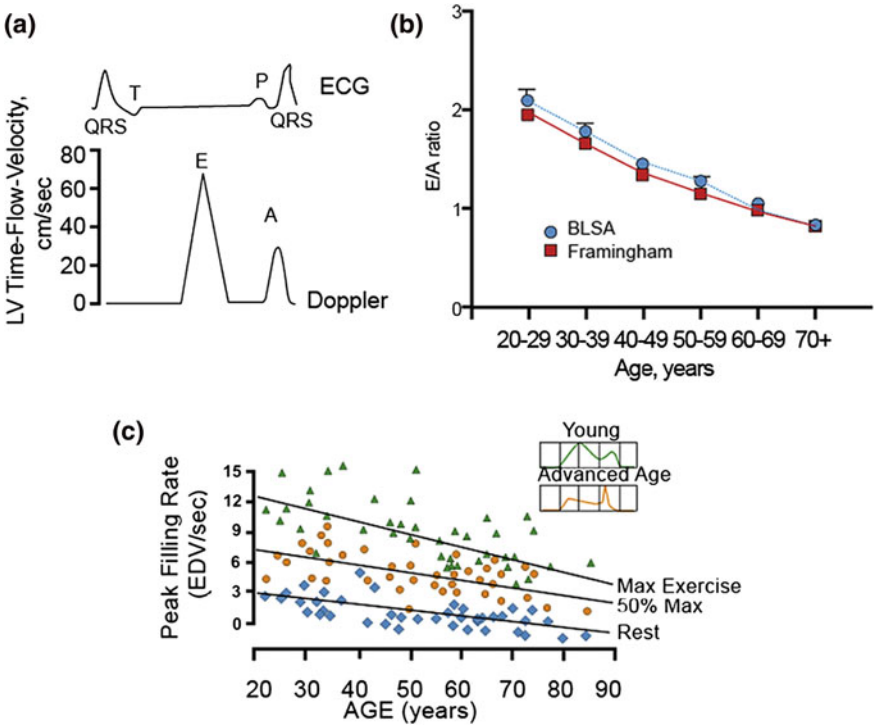

Fig. 7.4 LV filling at rest and with exercise. **a** The Doppler diastolic time-flow-velocity profile, showing the E and A waves from which the indexes of diastolic filling performance are derived. Time is represented on the horizontal axis. A simultaneous ECG is shown as a timing reference to indicate atrial and ventricular activation. LV indicates *left* ventricular. **b** The ratio of early *left* ventricular diastolic filling rate (E) to the atrial filling component (A) declines with aging, and the extent of this E/A decline with aging in healthy BLSA volunteers is identical to that in participants of the Framingham Study. **c** Maximum LV filling rate at rest and during vigorous cycle exercise examined via equilibrium gated blood-pool scans in healthy volunteers from the BLSA. EDV, end-diastolic volume. [**b**, **c**] Reproduced with permission from Strait and Lakatta [164]. Cardiac aging: aging from human to molecules. Muscle

shifted rightward, at least in men [77, 89], indicating that the LV of older persons operates from a greater preload (and increased myocardial oxygen demand). Ejection fraction (EF) is the most commonly used clinical measure of LV systolic performance, and is preserved at rest with aging (Table 7.2). LV end-systolic elastance is a measure of LV load-independent contractility and is slightly increased with age [90], but this increase likely reflects the age-associated increase in LV wall thickness, tension, and stiffness [2, 83, 91]. Of note, the age-associated increase in LV wall thickness is likely an adaptive mechanism to reduce LV wall tension. It is suggested in rat models of aging that the speed and extent of shortening are less in cardiac muscle from senescent versus younger adult rats [92]. These data suggest that LV contractility is impaired with age at rest.

7.2.2.3 Heart Rate and Cardiac Output

In the supine position at rest, heart rate (HR) does not change with age [89], but decreases slightly in the seated position with age. CO, the product of HR and SV, in the supine and seated resting position does not vary with increasing age [89].

7.2.2.4 Calcium Regulation in Cardiac Myocytes

Altered calcium (Ca^{2+}) homeostasis is one of the most important hallmarks of the aged heart. Ca^{2+} homeostasis is involved in cardiac excitation–contraction coupling. During aging, the magnitude of the L-type Ca^{2+} channel current (^{I}Ca, L) becomes significantly increased in parallel with the enlargement of cardiac myocytes, resulting in an unaltered ^{I}Ca, L density. Since the inactivation of ICa, L is slowed, and the action potential duration is prolonged, the net Ca^{2+} influx during each action potential is increased in cells of senescent myocardium relative to cells of adult control. While neither mRNA nor protein levels of the sarcoplasmic reticulum (SR) Ca^{2+} release channel (ryanodine receptor) significantly change with advancing age, the mRNA abundance and the density of SR Ca^{2+} pump decrease with aging and are associated with a diminished SR Ca^{2+} sequestration rate in the aged heart. The multiple changes in Ca^{2+} cycling that occur during aging result in an augmented Ca^{2+} influx, slowed SR Ca^{2+} sequestration, and prolonged durations of the Ca_i transient and contraction. These alterations which prolong electromechanical systole may be construed as an adaptation in that they prolong the forcebearing capacity of the senescent cells following excitation. This is helpful with respect to maintaining the cardiac function in the aged heart. However, they also increase the risk of Ca^{2+} overload and Ca^{2+}-dependent arrhythmias during stress in the senescent heart. Although reduced β-adrenergic receptor (β-AR) responses with aging contribute to diminished contraction reserve, these may be viewed in part, as adaptive, in that they protect against Ca^{2+} overload during stress.

7.2.3 Arterial-Ventricular Coupling

Aging is therefore associated with alterations in both arterial and LV properties. Importantly, the LV and the central arteries have bidirectional constant interactions. This interaction can be examined by depicting the function of the LV in the terms of LV elastance (chamber stiffness), as we did with the arterial system (i.e., E_A) (Fig. 7.5a) as LV end-systolic elastance (E_{LV}), a load-independent measure of LV chamber performance at end systole. With increasing age E_{LV} also increases [77–79] which suggests that the heart undergoes important adaptations resulting in an increase in E_{LV} to compensate for the increase in E_A. Interestingly, their ratio (E_A/E_{LV}) is fairly well maintained with advancing age in healthy persons, an age-associated adaptation to try and maintain CV efficiency [79]. Although E_{LV} is as a

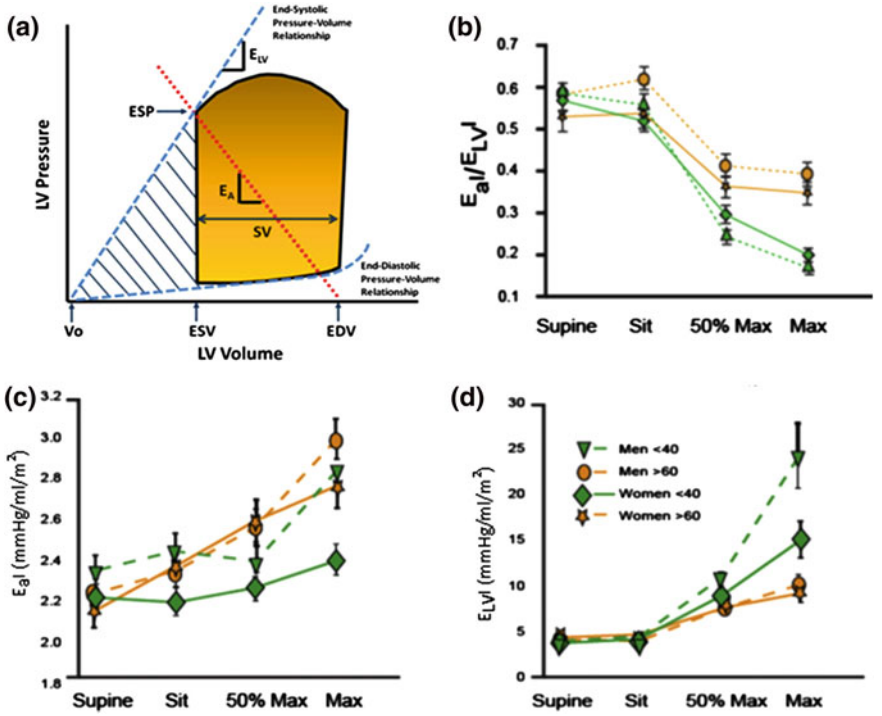

Fig. 7.5 Ventricular pressure–volume diagram (**a**) from which effective arterial elastance (E_A) and *left* ventricular (LV) end-systolic elastance (E_{LV}) are derived. E_A represents the negative slope of the line joining the end-diastolic volume (EDV) and the end-systolic pressure (ESP) points. E_{LV} represents the slope of the end-systolic pressure–volume relationship passing through the volume intercept (V0). The shaded area represents the cardiac stroke work, and the hatched area represents the potential energy (PE). LV ESP is the LV pressure at the end of systole. EDV is the LV volume at the end of diastole. End-systolic volume (ESV) is the LV volume at the end of systole. Stroke volume (SV) is the volume of blood ejected by the LV with each beat and is obtained from subtracting ESV from EDV. $E_AI/E_{LV}I$ (**b**), E_AI, (**c**), and $E_{LV}I$ (**d**) indexed to body surface area in men (*dashed lines*) younger than 40 years (*triangle*) and older than 60 years (*circle*), as well as women (*solid lines*) younger than 40 years (*diamond*) and older than 60 years (*star*) in the supine and seated positions, at 50 % of maximal workload, and at peak exercise. $E_AI/E_{LV}I$ decreases during exercise in both young and older men and women ($P < 0.0001$); however, older men and women have a blunted decline in $E_AI/E_{LV}I$ ($P < 0.001$). E_AI increases during exercise in both young and older men and women ($P < 0.0001$). At maximal exercise, E_AI is greater in older versus younger women ($P < 0.002$). In contrast, E_AI does not differ between young and older men. $E_{LV}I$ increases during exercise in both young and older men and women ($P < 0.0001$). At maximal exercise, $E_{LV}I$ is greater in younger versus older men ($P < 0.001$) and tended to be greater in younger than older women ($P < .07$). Modified from Najjar et al. [163]

load-independent index of LV contractility [93], it is also influenced by the geometric (structural remodeling) and biochemical properties (i.e., stiffness of the myocytes, composition of muscle, fibrosis, collagen etc., in LV wall) that underlie LV end-systolic stiffness [94]. Thus, the increase in E_{LV} with age is likely due to the

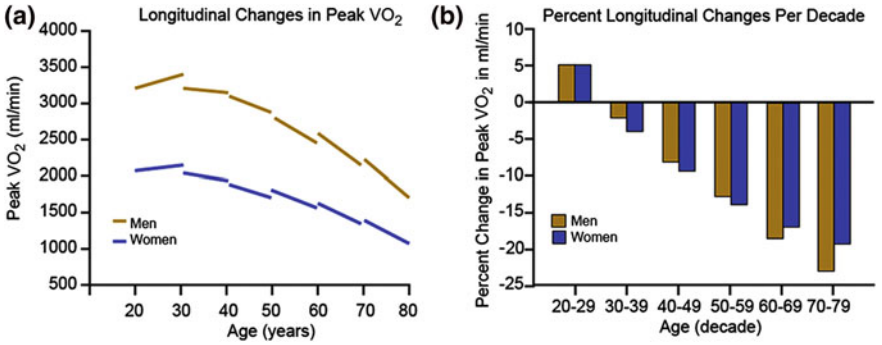

Fig. 7.6 Longitudinal changes in peak aerobic capacity (VO_{2peak}) by gender, predicted from the mixed-effects model, and separated by gender in *panel* **a** with the percent change by decade represented in *panel* **b**. **a** VO_{2peak} declines progressively more steeply with advancing age, with similar declines in men and women. Note that VO_{2peak} is only slightly higher in men than women at younger ages, converging by old age. **b** Per-decade longitudinal changes in VO_{2peak} by gender and age decade, derived from the mixed-effects model. Note that longitudinal declines in VO_{2peak} steepen with age, and that men decompensate at an accelerated, although similar rate after age 60. Modified from Fleg et al. [95]

age-associated changes to LV structure namely a reduction in cardiomyocyte number, increase in LV wall thickness, tension, and stiffness in the heart [2, 83, 91].

7.3 Part II: Cardiovascular Aging Response to Aerobic Exercise

7.3.1 Aerobic Capacity

One of the most important age-associated physiological changes, with regard to quality of life and functional independence, is a decline in peak aerobic capacity (VO_{2peak}). Longitudinal assessments of healthy sedentary individuals from the BLSA suggest that the age-associated decrease in VO_{2peak} is nonlinear, increasing progressively each decade (Fig. 7.6) [95]. For example, whereas the decline in VO_{2peak} was 3–6 % in the third and fourth decades, the decline in VO_{2peak} was $>$ 20 % per decade, after age 70. The accelerated decline in VO_{2peak} persisted even after adjusting for fat-free mass, and for differences in physical activity [95]. These data suggest that the loss in VO_{2peak} is not entirely due to the loss of muscle mass or physical inactivity. By definition, the age-associated reduction in aerobic capacity (VO_{2peak} = CO x arteriovenous O_2 content) can be attributed to changes that alter the delivery of oxygen to exercising muscles (cardiac function) or the ability of muscles to utilize oxygen. In regard to the latter, there is an age-associated decline ($\approx$25 %) in peak arteriovenous O_2 difference consistent with the

marked reductions in capillary density, and mitochondrial enzyme activities with aging [96]. Indeed, aging is associated with an impaired skeletal muscle mitochondrial function, secondary to mitochondrial DNA oxidative damage and loss [97]. In regard to the former, altered arterial afterload, LV contractility, including their coupling (E_A/E_{LV}), and diminished effectiveness of the autonomic modulation of HR, collectively comprise a sizeable component of the age-associated deficit in CV reserve. The subsequent sections will discuss the age-associated alterations in peak CV function.

7.3.1.1 Aortic Impedance

The effect of age on vascular impedance during exercise has not been studied in humans. However, in the canine model, it has been observed that aortic impedance, which does not vary with age at rest [98], increases over a wide range of exercise stress in older but not younger dogs. Although changes in the passive stiffness characteristics of the aorta in both dog [99] and human are an apparent cause of an increased aortic impedance, age differences in autonomic modulation of VSMC might also play a role. Thus, β-AR blockade during exercise increases aortic impedance in younger dogs, and the age-associated differences in impedance seen during exercise in the absence of β-AR blockade are abolished [98]. The zero frequency component of aortic impedance, SVR decreases during exercise. While, the reduction in SVR during exercise is blunted in older than young persons [89, 100, 101], the extent to this reduction depends on the maximum work capacity, which depends, in part, on physical fitness and other non-cardiac neuroendocrine and metabolic factors that are affected by aging.

7.3.1.2 Blood Pressure

During exercise, the control of SBP involves complex interactions between the peripheral vasculature and the heart, and its modulation through the baroreceptors, and the central nervous system, as well as locally by factors that are responsible for autoregulation at an arteriolar level. Further, the type of exercise performed (treadmill versus bicycle), and whether individuals hold onto handle bars for support (increase in isometric force) can influence the change in pressures. Typically, during exercise the rise in SBP is linearly related to the exercise workload, whereas DBP remains relatively stable [102]. In general, peak exercise values of SBP, DBP, and mean BP are greater in older vs. younger individuals [89, 100, 101]. Central pressures also increase during upright bicycle exercise and the magnitude of change in central SBP (and AGI) from rest to low/moderate exercise intensities is similar in young and older men [103, 104]. However, older men have higher absolute values of central SBP (and AGI) compared with younger men [103, 104]. These findings suggest that the elevated central SBP (and AGI) during exercise in older men is due to higher resting values [103, 104]. Thus, it would

seem that the older CV system tolerates the hemodynamic stress of moderate aerobic exercise and there is no evidence of additional central hemodynamic maladaptation during exercise in older healthy men [103].

7.3.1.3 Left Ventricular Performance

Although SV during strenuous aerobic exercise appears to be only minimally affected by increasing age, a substantial change occurs in the means by which a given SV is achieved. In older adults, LV emptying during maximal upright cycle exercise is substantially impaired, as evidenced by an ESV more than double that of younger adults. An apparent adaptive compensation for the impaired systolic emptying is that the older LV becomes more dilated during diastole than its younger counterpart, thereby preserving SV via the Frank–Starling mechanism [89]. In women, although EDV during exhaustive exercise is similar at older and younger ages, the change in EDV from rest to exercise significantly increases with age [89]. Potential mechanisms for this greater use of the Frank–Starling Mechanism during exercise in older individuals could be due: (1) a lower peak HR (30 % decrease between 20 and 85 years of age) in older individuals [89], thereby allowing for a greater filling time; (2) a limited ability of the older heart to empty due (higher end systolic volume; ESV), in part, to a reduced LV contractility and a stiffer heart. As both maximum contractile reserve and the regulation of arterial impedance during exercise [98] depend, in part, on the response to β-AR stimulation (see below), a deficit in the effectiveness of the latter with aging could account for the failure of ESV to decrease, and for LV contractility to increase to the same extent during exercise as noted in older individuals.

7.3.1.4 Beta-Adrenergic Responsiveness

Several features of the hemodynamic profile during intense exercise in older versus younger adults mimic the effects of β-AR blockade: slower HR, blunted systolic emptying, larger EDV, and impaired EF (and E_A/E_{LV}). These changes, coupled with age-associated blunting of CV reflexes mediated by the β-AR system, suggest a deficit in β-AR CV responsiveness. Consistent with this hypothesis, acute β-AR blockade causes nearly twice the attenuation of maximal exercise HR in healthy subjects < 40 year old compared with those > 60 years, while substantially increasing EDV and ESV in the younger but not in the older group possibly due to a stiffer vasculature. Thus, β-AR stimulation makes younger men seem like older ones (Fig. 7.7) [105]. Reduced β-AR responsiveness to exogenous catecholamines is evidenced by the lesser cardioacceleration caused by intravenous boluses of isoproterenol in older versus younger subjects. Further, evidence for an age-associated reduced cardiac response to β-AR stimulation derives from the fact that plasma catecholamine levels during maximal treadmill exercise increase with age [106]. Although clearance of plasma catecholamines is

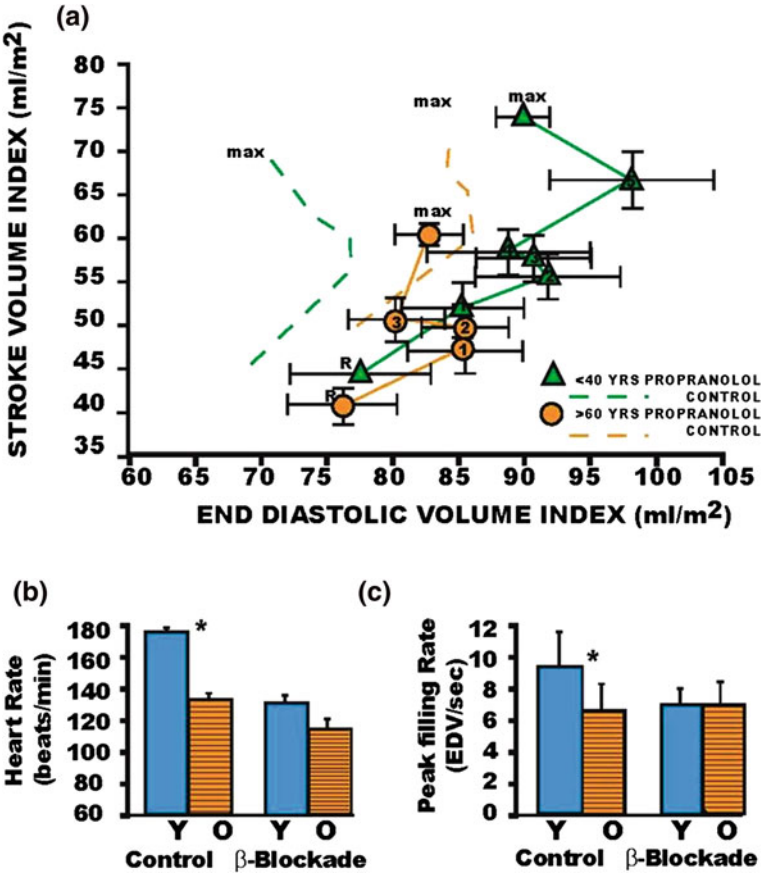

Fig. 7.7 **a** Stroke volume index (SVI) as a function of end-diastolic volume index (EDVI) at rest (R) and during graded cycle workloads in the upright-seated position in healthy Baltimore Longitudinal Study of Aging men in the presence and absence (*dashed lines*) of *β*-adrenergic blockade. R, seated rest; 1–4 or 5, graded sub-maximal workloads on cycle ergometer; max, maximum effort. SVI versus EDVI functions with symbols are those measured in the presence of propranolol; dashed line functions without symbols are those SVI versus EDVI measured in the absence of propranolol. Note that in the absence of propranolol, the SVI versus EDVI relation in older persons (*dashed lines*) is shifted rightward from that in younger persons (*dashed lines* with points). This indicates that the left ventricle (LV) of older persons in the sitting position compared with that of younger persons operates from a greater preload both at rest and during sub-maximal and maximal exercise. Propranolol markedly shifts the SV-EDVI relationship in younger persons (*triangle* without points) rightward, but does not markedly offset the curve in older persons (*circle*). Thus, with respect to this assessment of ventricular function curve, *β*-adrenergic blockade with propranolol makes younger men appear like older men. The abolition of the age-associated differences in the LV function curve after propranolol are accompanied by a reduction in heart rate, which at maximum, is shown in B. **b** Peak exercise heart rate in the same subjects as in **a** in the presence and absence of acute *β*-adrenergic blockade by propranolol. **c** The age-associated reduction in peak LV diastolic filling rate at maximal exercise in healthy subjects is abolished during exercise in the presence of *β*-adrenergic blockade with propranolol. Y, <40 years; O, >60 years [92]. Reproduced with permission from Strait JS, Lakatta EG (2011). Cardiac aging: aging from human to molecules

moderately reduced with age, the greater rise in plasma levels is best explained by increased spillover from the nerve terminal into the plasma with aging. The greater augmentation of plasma catecholamines in older versus younger subjects during acute exercise appears to be a compensatory response to reduced cardiac β-receptor density in the former group [107].

7.3.1.5 Peak Arterial–Ventricular Coupling

During exercise, E_A/E_{LV} declines to prioritize cardiac efficacy over energetic efficiency. In order for E_A/E_{LV} to decrease, E_{LV} must increase to a greater extent than the increase in E_A. With increasing age, however, E_{LV} fails to increase in proportion to the increase in E_A; hence, the E_A/E_{LV} during exercise in older persons decreases to a lesser extent than it does in younger persons [108]. The loss in E_{LV} reserve capacity (max-rest) with increasing age seems to be greater in men than women (Fig. 7.5c). This altered arterial–ventricular load matching in older vs. younger persons during exercise is a mechanism for the deficit in the acute LV EF ($E_A/E_{LV} \approx (1/EF)-1$]) reserve that accompanies advancing age in many individuals [108]. An acute pharmacologic reduction in both cardiac and vascular components of LV afterload by SNP infusions in older, healthy BLSA volunteers augments peak E_A/E_{LV} and EF in these subjects [68, 109]. Because of concomitant reductions in preload and afterload during SNP infusion, the LV of older persons delivers the same SV, stroke work, and CO while working at a smaller size.

During exercise, E_A either increases [108, 110], declines [111], or remains unchanged [112], and its response is dependent on the changes in its components and has important implications on Frank–Starling Mechanism [113]. E_A is linearly related to HR and SVR, and inversely related to compliance [110, 113–115]. Both SVR and compliance usually decrease during exercise (reflecting less resistance to blood flow in the microcirculation, but increased stiffness of the conduit arteries) [110]. With advancing age, the ability to increase HR, and lower SVR during exercise is blunted, in addition, the reduction in compliance (due to a greater increase in PP) is also limited [89, 116]. Despite these adverse age-associated changes, the increase in E_A during exercise in healthy young and older men is similar [108]. Further, the increase in E_A- during exercise in older women is similar to that of men; however, young healthy women demonstrated a minimal increase in E_A during exercise [108]. Perhaps the blunted changes in SVR, compliance, and HR are compensated or by the greater increase in blood pressure during exercise in older versus younger healthy men [101].

7.3.2 Role of Habitual Exercise on CV Function with Age

Regularly performed physical exercise is associated with enhanced arterial and cardiac function, and a reduced risk of CV disease. Determining the effects of

biological aging per se on the CV system is difficult to interpret due to the corresponding reductions in physical activity levels, which impair CV function independent of intrinsic aging processes. Regular physical activity/exercise may exert its beneficial effects on the blood vessels and heart by minimizing or delaying the adverse age-associated changes noted in the previous sections. We will discuss below some of the exercise-related benefits on CV function in older persons by examining data collected from master athletes and healthy individuals who have undergone an exercise intervention.

7.3.2.1 Physical Activity and Arterial Aging

Initial evidence illustrating the beneficial effects of regular physical activity/exercise on arterial aging comes from comparisons between old sedentary and endurance trained/master athletes. It is thought that master athletes represent a relevant model, because changes observed with advancing age are thought not to reflect physiological changes due to a more sedentary lifestyle [117]. Older male and female endurance trained athletes have a lower resting aortic PWV, AGI, brachial SBP and a greater endothelial dependent vasodilation than their sedentary peers [43, 118–120]. These findings provide evidence that being physically activites can limit the increase in arterial stiffness with age. In contrast, older endurance trained athletes have similar carotid SBP and IMT compared to their age-matched sedentary peers [121, 122]. The lack of change in carotid IMT may be due to the failure of long-term exercise to alter carotid distending pressures [122]. Indeed, when local carotid distending pressure is reduced by pharmacological means, there are equivocal reductions in carotid wall hypertrophy [123].

Interventional studies directly examining the beneficial effects of physical activity/exercise training on CV aging have reported beneficial improvements in arterial function. Three-months of aerobic exercise training, primarily walking, is effective in increasing central arterial compliance in middle-aged and older persons [124, 125]. However, in frail octogenarians and adults with chronically elevated arterial blood pressures, exercise training had little effect on central arterial stiffness [126–128]. Regular aerobic exercise in previously sedentary middle-aged and older individuals prevented the age-associated loss in endothelium-dependent vasodilation [129]. Improvements in endothelium-dependent vasodilation are also found in animal models of aging and exercise training [130–132]. These results suggest that impaired endothelium-dependent vasodilation may not be an inevitable consequence of aging, but rather, at least in part, due to age-associated reductions in physical activity. In contrast, exercise training, in middle-aged and older healthy men, do not seem to alter carotid IMT [122, 133], but decreases IMT of the femoral, popliteal, and brachial artery [134, 135]. These results suggest that regular exercise training typically does not alter the large conduit vessels, but may exert beneficial effects on the more muscular vessels, which may in turn exert beneficial functional effects on the larger conduit vessels.

Such changes to the arterial system may be related to the intensity of exercise performed and the duration of the exercise stimulus. Moderate exercise training (~50 % of peak capacity) improved endothelial function in a group of young healthy men, whereas no changes were observed for mild (~25 % peak capacity) or high-intensity (~75 % peak capacity) training for 12 weeks [136]. However, in a healthy older population a simple brisk walk improved endothelial function [129]. It is possible that too high an exercise intensity could negate the effects of exercise due to increased oxidative stress, especially if basal oxidative stress levels are already high. Evidence suggests that exercising over a short period of time, i.e., days rather than weeks, does not result in arterial adaptations. For example, 10 days of training did not alter endothelial function or arterial stiffness in obese individuals, this was despite improvements in aerobic capacity [137]. Also, no change in peripheral arterial IMT was observed with 8 weeks of training in older men [133]. Most studies in healthy individuals, have prescribed at least 10 weeks of training to stimulate improvements in endothelium function [129, 138, 139]. These data suggest weeks rather than days of exercise training is required to stimulated improvements in arterial function.

The mechanisms mediating these favorable changes in arterial function in middle-aged and older persons remain relatively unknown. It is possible that the changes in the arterial expression, architecture, and/or bioactivity of structural proteins that play a major role with arterial aging, are either reversed or ameliorated through exercise. Favorable effects of exercise on improving the balance with pro-and anti inflammatory and oxidative stress markers could improve NO bioavailability, and alters MMP/TIMP activity. MMP activity is greatly affected by mechanical stretch [140]. Thus, exercise training, which acts as a mechanical stressor to the arteries [141], could exert its effects by altering MMP/TIMP activity, directly, and indirectly, via the proposed anti-inflammatory and oxidative stress responses of exercise. In heart failure patients, short-term aerobic training favorably altered MMP/TIMP activity [142]. Exercise training consistently improves NO function, and subsequently endothelial function, in healthy individuals and patients with CV risk factors and disease [143]. It is likely that exercise training improves NO vasodilator function by both direct and indirect means. For example, during exercise the endothelial surface is exposed to increased blood flow (shear stress), this promotes endothelial NO release, smooth muscle cell relaxation, and vasodilation. Within minutes of exercise, eNOS activity is enhanced by increasing the phosphorylation of eNOS at Ser177. In patients, with coronary artery disease, 4 weeks of aerobic exercise caused a threefold increase in AKt kinase-dependent phosphorylation of eNOS at Ser177, which correlated with enhanced eNOS activity, and improved endothelial function [144]. Over the course of hours, exercise increases the protein expression of eNOS [145], and 4 weeks of aerobic training increased eNOS messenger RNA expression with a twofold increase in vascular eNOS protein content [144]. Thus, exercise training may improve arterial function via intermittent increases in shear stress, which stimulates NO bioactivity, and up-regulating eNOS protein expression, and phosphorylation [143]. It is possible that the repeated induction of NOS

activity with exercise training prolongs the half-life of NO by reducing its degradation by free radicals [146] or by directly decreasing free radical production.

7.3.2.2 Physical Activity and Cardiac Aging

Important adaptations occur to the aged heart through exercise training. Unlike age-matched sedentary peers, master athletes have a normal LV filling pattern, and an increased LV wall thickness, which corresponds with an increase in supine resting EDV (and SV) [147–150]. Improvements in peak cardiac function are also evident, whereby peak exercise EDV and SV are greater and ESV is lower; as such peak CO and VO_{2peak} are much greater in master athletes compared to age-matched sedentary peers [147, 148, 150]. Although master athletes express a gradual loss in VO_{2peak} with increasing age, the absolute values are higher irrespective of age than the inactive individuals [95].

Consistent with these cross-sectional observations, exercise interventional studies in older persons have reported physiological eccentric LV remodeling with chronic exercise training [151–154]. However, in terms of resting (supine or seated) cardiac function (SV, EDV, EF or LV contractility), moderate/high intensity exercise training (8–48 weeks) in previously healthy sedentary older persons has limited effects [149, 155, 156]. Further, 1 year of progressive and vigorous endurance training in sedentary healthy older individuals did not alter LV contractility (no change in the slope of the stroke work–EDV relationship) or LV stiffness (no change in LV pressure–volume curves or LV stiffness constants) measured in the supine position [152]. However, the lack of improvement in LV systolic function at rest in response to exercise training is consistent with previous studies in younger subjects showing that training does not generally have a significant effect on resting LV systolic performance [157, 158]. One possible reason for the lack of change in LV stiffness with exercise training is that the age-associated formation of cross-linked advanced glycation end products in the LV wall, along with a loss in the number cardiomyocytes are pathologically irreversible once formed [159].

In older healthy individuals, peak exercise cardiac function is typically improved after chronic exercise training depicted by increases in peak CO, SV, EF, LV contractility, and VO_{2peak}, and a reduction in ESV [149, 152, 155, 160, 161]. The improvement in VO_{2peak} after exercise training is, in part, due to an increase in peak CO but also due to a greater redistribution of CO to the exercising limbs, resulting in a proportional increase in systemic arteriovenous O_2 difference without a change in peripheral oxygen extraction [155]. The greater ability to reduce ESV and to increase EF after exercise training in older individuals is not likely to be due to enhanced myocardial β-AR responses with conditioning, as the diminished β-AR responsiveness with aging is not altered by chronic conditioning [162]. Alternatively, the improved ability of the LV to empty as fitness increases may relate to a reduction in arterial stiffness in the conditioned state [43] and thus to a reduction in afterload.

Although it remains unclear to what extent the age-associated CV deficits are due to the age-associated reduction in physical deconditioning, it is clear that regular exercise training is an effective strategy for combating several adverse cardiac (and arterial) changes associated with aging, at least up to the 7th decade. Despite these favorable effect they are not uniform in nature; for example, there appears to be no beneficial effects of exercise training on LV stiffness and carotid IMT. Further, most of the studies were collected in men and to what extent the same physiological changes occur to the heart and blood vessels in women, both pre and post-menopause, is unclear.

7.3.3 Relevance of CV Aging to Clinical CVD

Our present understanding of the age-associated alterations in cardiac and arterial structure and function provides valuable clues that may assist in the development of effective therapies to prevent, to delay, or to attenuate the CV changes that accompany aging. Many of these age-associated changes are being increasingly recognized as risk factors for CV disease (IMT and arterial stiffness). One way to conceptualize why the clinical manifestations and the prognosis of these diseases worsen with age is that in older individuals, the specific pathophysiologic mechanisms that cause clinical disorders are superimposed on cardiac and arterial substrates that are modified with age (Fig. 7.8). Imagine that age increases as one moves from the lower to the upper part of Fig. 7.8, and that the line bisecting the top and bottom parts represents the clinical practice "threshold" for disease recognition. Thus, entities above the line are presently classified as "diseases" and lead to heart and brain failure. The changes to the CV system presently thought to occur as a result of the "normal aging process" (those addressed in this chapter) are depicted below the line (Fig. 7.8). These age-associated changes in CV properties alter the substrate on which CV disease is superimposed in several ways. First, they lower the extent of disease severity required to cross the threshold that results in clinically significant signs and symptoms. For example, a mild degree of ischemia-induced relaxation abnormalities that may be asymptomatic in a younger individual may cause dyspnea in an older individual, who, by virtue of age alone, has preexisting slowed and delayed early diastolic relaxation. Age-associated changes may also alter the manifestations and presentation of common cardiac disease. This usually occurs in patients with acute infarction, in whom the diagnosis is delayed because of atypical symptoms result in increased time to onset of therapy. Age-associated changes including those in β-AR responsiveness and in arterial stiffness, also influence the response to and, therefore, the selection of different therapeutic interventions in older with CV disease. In one sense, those processes below the line, in Fig. 7.8, should not to be considered to reflect normal aging. Rather they might be construed as specific risk factors for the diseases they relate to, and thus might be targets of interventions designed to decrease the occurrence or manifestations of CV disease at later ages. Such a strategy would thus advocate treatment of normal aging. Additional studies addressing this strategy are required.

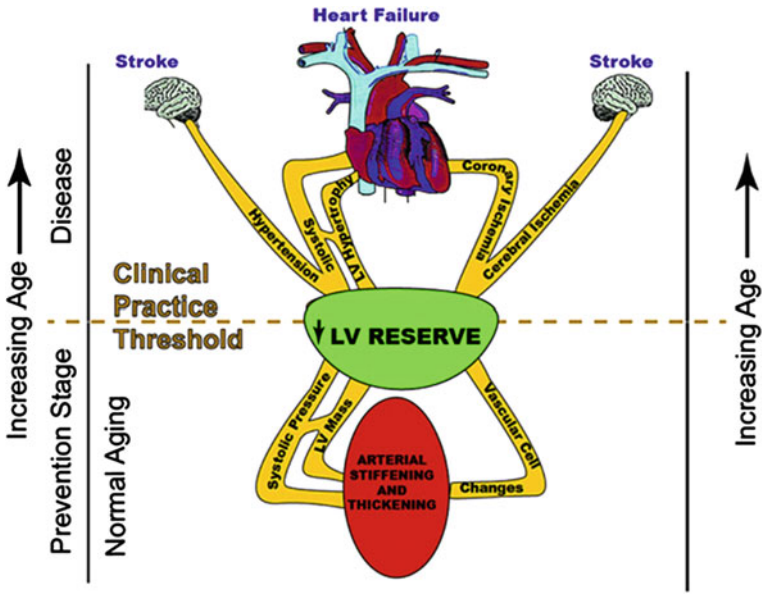

Fig. 7.8 Pathways linking aging to heart failure. (Modified from Lakatta EG. Age-associated cardiovascular changes in health: impact on cardiovascular disease in older persons. Reproduced with permission from Najjar et al. [163]

7.4 Summary

Aging is the dominant risk factor for CV diseases. However, aging should no longer be viewed as an immutable risk factor. A steady stream of incremental knowledge, derived from both animal and human studies, has established that several of the aging-associated changes in the heart and in the walls of the central arteries are themselves potent and independent risk factors for CV diseases. This suggests that these age-associated alterations in CV structure and function could represent the link that explains, at least in part, the risky component of aging. Policy makers, researchers, and clinicians should intensify their efforts toward identification of novel pathways that could be targeted for interventions aiming at retardation or attenuation of these age-associated alterations, particularly in individuals in whom these alterations are accelerated. Future studies would then examine whether these strategies (i.e., those targeting CV aging) can have a salutary impact on the adverse CV effects of accelerated CV aging. As such, CV aging is a promising frontier in preventive cardiology that is ripe for and in dire need of attention.

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Part II
Cardiac Adaptations to Overload

Chapter 8
Differences in Concentric Cardiac Hypertrophy and Eccentric Hypertrophy

Alison L. Müller and Naranjan S. Dhalla

Abstract Cardiac hypertrophy is an adaptive process which occurs as a result of increased stress endured by the heart and this cardiac remodeling serves as a reactive mechanism to compensate for volume overload or pressure overload. An increase in pressure, common in hypertension or resistance training, results in a concentric hypertrophic phenotype whereas an increase in volume, as seen with valvular defects or endurance training, results in an eccentric hypertrophic phenotype. Concentric hypertrophy is associated with increased left ventricular wall thickness whereas eccentric hypertrophy is characterized by dilatation of the left ventricular chamber; however, there occurs a general increase in the overall size of cardiomyocytes under both conditions. Although various hormonal systems are activated during the development of cardiac hypertrophy, differences in the type of ventricular wall stress and strain seem to determine the occurrence of eccentric or concentric remodeling in addition to changes in myocardial structure. There are variations between the eccentric and concentric hypertrophic phenotypes with respect to gene and protein expression, signaling transduction pathways, and local hormone release. Both types of cardiac hypertrophy are known to occur under physiological and pathological situations; the lack of inflammatory response and fibrosis in the heart differentiates physiological from pathological hypertrophy. It is suggested that concentric and eccentric hypertrophy are the result of differences in the effects of increased ventricular wall tension superimposed by the impact of hormones released locally in the heart in response to stress.

A. L. Müller · N. S. Dhalla (✉)
Institute of Cardiovascular Sciences, St. Boniface Hospital Research Centre
and Department of Physiology, Faculty of Medicine, University of Manitoba,
351 Tache Avenue, Winnipeg, MB R2H 2A6, Canada
e-mail: nsdhalla@sbrc.ca

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8.1 Introduction

Cardiac hypertrophy is a phenomenon that manifests into two physiologically unique phenotypes known as concentric hypertrophy and eccentric hypertrophy indicating that the heart responds differently to stressful stimuli. In 1975, Grossman et al. [1] evaluated cardiac hypertrophy variations in patients with left ventricular (LV) pressure overload (PO) and those with LV volume overload (VO). In patients with PO, it was observed that there was an increase in the values of the ratio of LV wall thickness to radius as a response to increased LV peak systolic and end diastolic pressures. On the other hand, patients experiencing VO had consistently increased end-diastolic pressure without a change in the ratio of wall thickness to radius. It was hypothesized that the heart primarily responds to systolic stress which is why significant compensatory remodeling occurs in patients with PO and not in those experiencing VO [1]. In addition to pathological cardiac remodeling, physiological cardiac remodeling is observed in athletes as a response to intensive exercise; both eccentric and concentric hypertrophy can occur and the specific phenotype depends on the type of exercise being performed [2]. In general, it has been observed that athletes who train in more endurance-focused sports, which include running and rowing, exhibit eccentric hypertrophy whereas athletes focusing on resistance training, such as weight-lifting, present with concentric hypertrophy [3, 4]. It is noteworthy that individuals who combine both endurance and resistance training, such as triathletes, show an amalgamation of both forms of hypertrophy [2]. The mechanism by which the hypertrophic remodeling occurs in athletes mirrors that of pathological remodeling in the sense that the remodeling response corresponds to the same type of physiological stresses. Endurance training results in an increase in volume load and is thus comparable to pathological VO where the heart responds accordingly by ventricular cavity dilatation or eccentric hypertrophy [2, 3, 5–8]. Concentric hypertrophy, which occurs in strength athletes, is in response to significant increases of pressure loads on the heart, reminiscent of PO concentric hypertrophy which causes increased LV wall thickness [2, 4, 7, 9]. It is important to point out that the purpose of this chapter is not to center the discussion around the differences between physiological and pathological remodeling, but to focus on the variations and similarities between eccentric and concentric forms of cardiac remodeling in order to better understand the heart's physiological response, both mechanical and biochemical, to different stressors.

In view of the fact that the heart can physiologically alter its structure as a response to changes in stimuli, most notably from the sympathetic nervous system (SNS) and hormonal changes, numerous studies have been carried out to elucidate the

mechanism of this phenomenon and quantify the dimensional changes in order to accurately diagnose forms of hypertrophy. Identifying the type of cardiac remodeling can help determine potential future cardiovascular dysfunction which may be useful in helping to treat patients. It was found that concentric LV hypertrophy appears in 85 % of patients with diastolic heart failure, whereas 84 % of patients exhibiting eccentric hypertrophy had systolic heart failure [10]. These differences are evident at a structural level where patients with diastolic heart failure were found to have cardiomyocytes with increased diameter and a lesser decrease in volume compared to cardiomyocytes measured in patients with systolic heart failure [11]. When evaluating hypertrophy in obese patients, it was observed that eccentric LV hypertrophy can cause diastolic filling pattern abnormalities comparable to concentric hypertrophy [12]. In addition, obese patients with eccentric LV hypertrophy have been shown to have increased dependence on left atrial contraction for LV filling and abnormal relaxation of the LV observed from prolonged LV deceleration time, a higher peak velocity of atrial filling, and a decreased early and late ventricular filling velocity [13, 14]. A study evaluating childhood risk factors, for both eccentric and concentric hypertrophy, found that in subjects who exhibited either form of hypertrophy had a tendency to be obese and/or diabetic. These individuals shared significantly increased waist circumferences, systolic and diastolic blood pressures, glucose, insulin, total to high-density lipoprotein cholesterol ratios, triglycerides, and urinary albumin-creatinine ratios. Furthermore, it was possible to determine eccentric LV hypertrophy from both childhood and adulthood body mass index (BMI) values and concentric LV hypertrophy from the presence of diabetes mellitus in adulthood and diastolic blood pressure in childhood [15]. In a study evaluating hypertrophy in obese rabbits, features of both concentric and eccentric LV hypertrophic remodeling occurred with diastolic filling abnormalities [16]. This correlates with clinical studies evaluating cardiac remodeling in obese individuals as they tend to experience simultaneous increases in both cardiac preload and afterload which can lead to concentric and/or eccentric hypertrophy [17]. There is a general consensus among different studies that have evaluated cardiovascular remodeling using various pathology and histological techniques. To summarize, concentric hypertrophy arises from conditions where the heart is subject to chronically increased afterload, including hypertension and aortic stenosis, and eccentric hypertrophy arises from VO traditionally attributed to mitral regurgitation and systolic dysfunction [1, 10, 18–22]. The process of both physiological and pathological hypertrophic remodeling is significantly influenced by neurohumoral activation and hemodynamic load [23].

8.2 Pathological Concentric and Eccentric Hypertrophic Remodeling

For the most part, in the average patient, cardiac hypertrophic remodeling represents pathological cardiac dysfunction, regardless of whether concentric or eccentric alterations are occurring. However, differentiating between these types of

cardiac remodeling is important in determining appropriate therapy for the patient to improve their clinical outcome. Both pathological cardiac concentric and eccentric remodeling involve responding to a stress in an attempt to sustain adequate function. In pathological hypertrophy, in addition to strain caused by chronic hemodynamic load, additional factors including endothelin, cytokines, nitric oxide production, and oxidative stress also participate in the cardiac remodeling process [23].

One of the most common cardiovascular malignancies is hypertension which can manifest into concentric hypertrophy as a response to increased afterload [1]. Even individuals who experience morning hypertension or have normotensive blood pressure with a difference between morning and evening pressures greater than 15 mmHg have a significant increased risk of developing concentric LV hypertrophy [24]. In fact, concentric LV hypertrophy resulting from hypertension has an increased likelihood to be detrimental for cardiovascular prognoses with higher risks for cardiovascular death and mortality, even in uncomplicated essential hypertension, as it contributes to diastolic malfunction [25–27]. It was found that the degree of end-diastolic wall stress acts as a signal regulating hypertrophy level where hypertensive patients with low end-diastolic wall stress have more pronounced concentric LV geometry which is indicated by a significantly increased relative wall thickness [28]. Specifically, the cardiomyocytes present in concentrically remodeled myocardium are thicker than normal, indicating increased diameter, but are not significantly longer so the average length to width (L/W) ratio is significantly decreased [21, 22] as seen in Fig. 8.1. This is a result of the orientation in which contractile sarcomere units are added to cardiomyocytes. In PO hearts, sarcomeres are added in parallel which explains why the L/W ratio changes [21]. This contributes to observed thickening of the muscle walls without changes in ventricular chamber dimensions, although LV posterior wall dimensions were significantly increased during both systole and diastole [29–33]. When evaluating ventricular relaxation, it was found that that there were few significant changes in diastolic filling velocities until the heart failure stage [33]. One feature to look for when determining concentric LV hypertrophy is elevated pulse arterial pressure [34–36]. A consequence commonly associated with concentric hypertrophy is increased ventricular diastolic stiffness which impairs cardiac performance [37]. It is interesting to note that although the occurrence LV hypertrophy is not increased when comparing patients with or without depression, patients with depression that have hypertrophy exhibit a 2.1x risk of exhibiting concentric LV hypertrophy when compared to patients without depression [38]. This result was found to be independent of blood pressure and age and partially affected by the use of anti-depressants as patients medicated for depression had a decreased likelihood of presenting LV concentric geometry [38].

Cardiac pathologies associated with eccentric hypertrophy tend to be a result of increased preload, such as valve regurgitation or VO [39]. In infarcted rats, the heart has also been shown to undergo eccentric cardiac remodeling with 77 % of these animals presenting systolic dysfunction. When comparing the length and width measurements of individual cardiomyocytes, the L/W ratio remained the

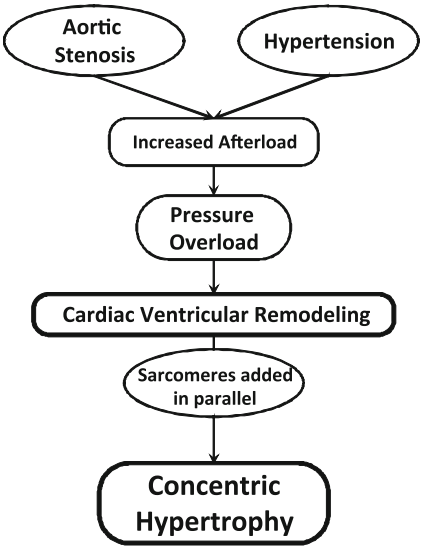

Fig. 8.1 Schematic representation of hypertrophic remodeling due to chronically increased afterload resulting in concentric hypertrophy

same as normal hearts, but this was due to both thickening and lengthening of the cells. The mechanism behind this overall increase in size is a result of sarcomeres being added both in series and in parallel in response to VO [21] as noted in Fig. 8.2. In addition, when evaluating specific LV parameters, it was found that the internal diameter was increased in VO rats with no changes in interventricular septal or LV posterior wall dimensions [33]. When evaluating ventricular relaxation time, it was observed that the ratio of peak early diastolic filling velocity and peak late diastolic filling velocity gradually decreased over time in VO rats [33]. The activation of MEK5–ERK5 signaling pathway can also lead to eccentric LV remodeling by lengthening cardiomyocytes, although when MEK5 or ERK5 was deleted in mice, the reciprocal relationship of increased width was not observed [41]. These aspects contribute to a phenotype which has increased ventricular volume with only a slightly thickened cardiac muscle wall known as chamber dilation [29, 30, 39, 41].

When investigating cardiac remodeling, it is important to consider the possible alterations in the collagen network and capillary bed. In addition to alterations in cardiomyocyte parameters, in hearts observed at autopsy, it was found that those showing concentric hypertrophy had an increased volume of connective tissue present. Furthermore, when compared to both control hearts and those with an eccentric hypertrophy pattern, there were more cardiomyocyte connections between individual cells in concentric hypertrophy. It was suggested that the increase in junctions is an attempt to counter connective tissue proliferation in order to compensate for hemodynamic overload [22]. In an experimental hypertension model evaluating collagen remodeling in the heart of non-human primates, it was found that early on in hypertrophy development, reactive fibrosis, and collagen remodeling occurred prior to the presence of cardiac damage caused by

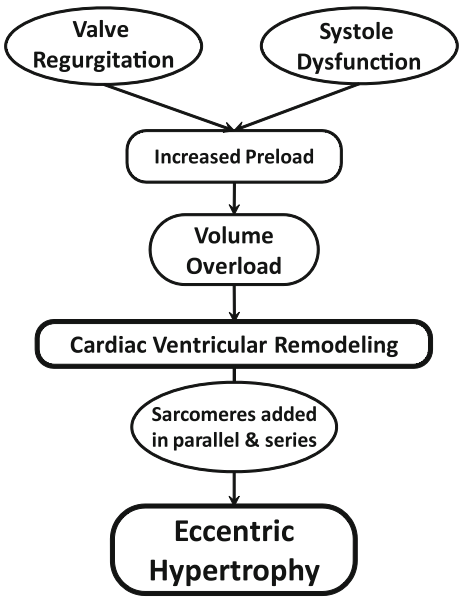

Fig. 8.2 Schematic representation of hypertrophic remodeling due to chronically increased preload resulting in eccentric hypertropohy

hypertension [42]. Collagen increased at 4 weeks with thick and dense collagen septae developing at 35 weeks. Necrosis was not evident until 88 weeks where structural remodeling became reminiscent of scar formation as the proportion of collagen types I and III reflected that found in scars [42]. In renovascular hypertension in rats, there was a marked increase in fibrosis in addition to a significant decrease in subendocardial density with concentric mass increase. This phenotype was seen at only 1 month and did not appear to alter over time [44]. The increase in collagen caused decreased cardiac distensibility [44]. In eccentrically hypertrophic hearts evaluated post-mortem, the prominence of fibrosis impaired cardiomyocyte connectivity and it was found that, when compared to control, there were significantly fewer lateral connections [22]. During the development of cardiac hypertrophy in the aorto-caval fistula rat model, eccentric remodeling occurred also with a significant decrease in subendocardial capillary density, which was negatively correlated with time although there was no difference in collagen content when compared to control. Interestingly, unlike hypertensive rats, there was a significantly positive correlation between the extent of cardiac hypertrophy due to aorto-caval fistula and LV cavity area [43]. The decrease in capillary density was attributed to an imbalance between the increasing thickness of the cardiac muscle and capillary formation. From these findings, it was concluded that the thickness of the LV wall and the geometry of the LV cavity affect capillary and fibrosis density in concentric and eccentric remodeling in VO and PO models [43].

The remodeling differences that occur between eccentric and concentric hypertrophy have been hypothesized to be the heart's specific response to normalize systolic wall stress, but not diastolic wall stress [1]. Some commonalities between

concentric and eccentric remodeling include that series branches of cardiomyocytes are increased per cell and both series and lateral branches are significantly longer and thicker compared to normal cardiomyocytes [21]. Additionally, both these forms of cardiac remodeling exhibit a linear decrease in overall interstitial space percentage area from the endocardium, where there is an initial increase in the collagen volume fraction, to the epicardium [11, 22]. Interestingly, this study found that all the hearts displaying the eccentric hypertrophy phenotype presented clinical features of congestive heart failure which was not seen in any of the hearts with concentric hypertrophy [22]. In an interesting study on a PO rat model and comparing animals that experienced heart failure to those that did not, different hypertrophic remodeling was observed to occur. Those without heart failure (NHF) exhibited concentric remodeling characterized by normal chamber size and increased relative wall thickness whereas rats that had heart failure (HF) showed eccentric remodeling marked by LV enlargement with normal relative wall thickness [45]. Blood pressures were found to be high in the NHF group with higher diastolic chamber stiffness; however, LV systolic function was lower in the HF group which had significantly increased levels of myocardial collagen content. This study suggests that when evaluating the transition to heart failure, it is important to analyze the combined effects of aging and hypertension [44].

8.3 Molecular Mechanisms of Pathological Concentric and Eccentric Hypertrophic Remodeling

In addition to structural changes, there are a number of molecular factors which have been found in both patients and animal models that influence the hypertrophic phenotype with variations between eccentric and concentric remodeling. The ERK1/2 pathway has been implied to be necessary in order for cardiomyocytes to undergo hypertrophic growth; however, in an in vivo model, this appears to be specific for concentric LV hypertrophy [39]. For example, patients with eccentric LV hypertrophy have low basal plasma renin activity in addition to downregulation of the endogenous inhibitor of MAP kinase [18, 34]. Patients suffering from aortic stenosis with concentric hypertrophy also show a downregulation of the MAP kinase inhibitor; however, there is upregulation of vitamin D3 upregulated protein (VDUP)-1 and myocyte-enriched calcineurin-interacting protein (MCIP)-1, as well as endogenous inhibitors of thioredoxin and calcineurin; these targets were unchanged in patients suffering from mitral regurgitation with eccentric LV remodeling [18]. In genetically modified mouse models, ERK1/2 signaling is associated with a unique form of concentric hypertrophy which appears to be caused by the expression of activated MAPK kinase (Mek)-1 and PO [46, 47]. It is interesting to note that loss of ERK1/2 activation did not prevent physiological hypertrophy induced by swimming, but instead caused an increase in the length of cardiomyocytes in ERK1-/- mice without an increase in width [40]. Interestingly, it was found that there was no significant difference in the plasma level of brain

natriuretic peptide or creatinine in either form of LV hypertrophy [20]. Franz M et al. [48] have suggested that splice variants of the large extracellular matrix glycoprotein tenascin-C could help to determine the difference between concentric and eccentric LV hypertrophy because the levels of the B-domain containing tenascin-C were higher in eccentric LV hypertrophy when compared to concentric LV hypertrophy. Although levels of matrix metalloproteinase, (MMP)-9, were found to be higher in LV hypertrophic patients overall, levels were significantly lower in eccentric hypertrophy compared to concentric hypertrophy [48]. Both hypertrophic remodeling groups had increased levels of tissue inhibitor of MMPs (TIMP)-1, TIMP-2, and TIMP-4 [49]. It should be noted that isoproterenol, a non-selective beta-adrenergic agonist, is widely used to cause concentric LV remodeling [49–51].

In an interesting experiment involving the activation of leukocyte tyrosine kinase (LTK), a tyrosine kinase receptor belonging to the insulin receptor superfamily caused severe concentric hypertrophy in mice [52]. There was significant cardiomyocyte degeneration and it was suggested that this specific receptor upon activation may evoke growth signals and produce concentric hypertrophy [53, 54]. Another unique study evaluated the response of different hypertrophic stimuli to three-dimensional engineered heart tissue (EHT) cultures and found that the tissue responded by forming either concentric or eccentric hypertrophy [55]. Specifically, if stimulated during the maturation phase in the presence of phenylephrine and angiotensin II, concentric hypertrophy occurred with significant increases in cell width, but not cell length with markedly increased atrial natriuretic peptide gene expression. In addition, these cells were found to have a low alpha/beta-myosin heavy chain ratio and high alpha-actin transcript levels and these findings provide evidence for a MEK1–ERK1/2 pathway having specific varying effects in concentric and eccentric cardiomyocyte hypertrophy [39, 55]. Concentric hypertrophy can also occur when stimulated cells with endothelin-1 (ET-1) whereas eccentric hypertrophy happens upon stimulation with leukemic inhibitory factor (LIF) exposure [56]. When stimulating cells with ET-1, there were found to be 8 different proteins expressed at a higher level including small heat shock protein (HSP) αB-crystallin, which has been implicated in cardioprotection [57], and atrial natriuretic factor (ANF), which is a marker for cardiac damage [56, 58]. An isoform of HSP-60 was found to be decreased in ET-1 treated cardiomyocytes and then increased in LIF-treatment myocytes which is vice versa from the presence of the light chain of clathrin. It is interesting to note that this study found that neither eccentric nor concentric hypertrophy was determined by a distinct change in a specific functional group of proteins [56]. When knocking out cardiac β-adrenergic receptors, the hypertrophy that ensues is reminiscent of concentric remodeling [59]. When looking at myocardial PCr/ATP ratios and creatine kinase in concentric LV hypertrophy in a porcine model, it was found that abnormal myocardial high-energy phosphate metabolism is related to decreased mitochondrial isozymes of creatine kinase which relates to the severity of hypertrophy [60]. This has been found to correlate with decreases of myocardial phosphocreatine/ATP ratios in patients with LV hypertrophy indicating an altered myocardial oxidative phosphorylation mechanism [61, 62].

Not only it has been observed that a variety of signaling transduction mechanisms are involved in remodeling, different metabolic interventions also appears to have some influence on LV ventricular remodeling. Changes in cardiac hypertrophy have been investigated in Wistar rats on and off a fructose-rich diet both with and without severe aortic regurgitation (AR). Both AR animals developed LV hypertrophy, although in those on the fructose diet had developed more hypertrophy with a slightly decreased LV ejection fraction [63]. In addition, when evaluating the effects of resveratrol, a phenolic phytoalexin present in grapes and berries, in a PO rat model, it was found that chronic cardiac hypertrophy and impaired cardiac function were reversed [64]. PO animals exhibited a greater heart-to-body weight ratio with resveratrol treatment but the wall thickness was attenuated at 14 and 28 days post-surgery. Interestingly, resveratrol treatment did not reduce elevated blood pressure but did prevent the increase in oxidative stress level and was able to reverse it when resveratrol treatment started 14 days post-surgery. When administering resveratrol to rats subjected to VO, the only significant change that was noted was a reduction in oxidative stress indicating that pathological eccentric LV remodeling may not be affected by oxidative stress [65]. In studies evaluating glucose uptake in PO rats, it was found that glucose uptake appeared to be linked in determining both the growth and phenotype of the heart [66]. In addition, when PO rats were fed sucrose, it was found that the decreases in SR Ca^{2+}-stimulated ATPase activity as well the proportion of myosin V1 were attenuated. This indicates that sucrose affects both the myosin and ATPase pump activity in hypertrophied cardiac muscle [67]. In order to further evaluate how oxidative stress affects or is affected by hypertrophy, endothelial nitric oxide synthase (eNOS) protein levels were evaluated after 4 weeks of aortic banding and were found to be decreased. This was prevented upon treatment with resveratrol [65] which increased NO levels by upregulating eNOS and may be involved in the regression of the development of PO-induced alterations [65]. When eNOS protein level/activity was evaluated in VO models, there was found to be no change, suggesting a reason why resveratrol had no effect on eccentric LV remodeling [68]. In a unique study evaluating nutrient deficiencies in animals, it was found that iron-deficient rats had elevated heart rates, yet lower norepinephrine levels and started to display eccentric LV remodeling whereas those on a copper-deficient diet exhibited concentric hypertrophy. In the copper-deficient animals, concentric remodeling happens regardless of whether or not the animal is anemic [69].

In a rat PO model, LV hypertrophy may initially exhibit a concentric phenotype which then progresses to eccentric hypertrophy resulting in cardiac failure [70]. In various animal models, numerous molecules have been found to be activated as a result of biochemical strain during hypertrophy including mitogen-activated protein (MAP) kinase, JAK/signal transducer and activator of the STAT (transcription) pathway, calcineurin, thioredoxin, as well as NFkappaB [71–74]. Transverse aortic constriction (TAC) also results in early concentric and then late eccentric hypertrophic phenotypes which is temporally associated with increases in fibrosis, and cardiomyocyte size and apoptosis [75]. Phosphorylated ERK/12

Table 8.1 Changes in the expression and/or presence of various proteins between concentric and eccentric hypertrophy

Protein/mRNA	Specific change in content or expression LV Hypertrophy	
	Concentric	Eccentric
VDUP-1	Increased	Unchanged
MCIP-1	Increased	Unchanged
Matrix metalloproteinase-9	Increased	Slightly increased
TIMP-1, TIMP-2, TIMP-4	Increased	Increased
Heat shock protein 60	Decreased	Increased
Endothelial nitric oxide synthase	Increased	Unchanged
GSK3β	Increased	Decreased
Transgelin	Decreased	Increased
pyruvate dehydrogenase lipoamide kinase-1	Increased	Decreased

Abbreviations VDUP-1—vitamin D3 upregulated protein-1; MCIP-1—myocyte-enriched calcineurin-interacting protein-1; TIMP—tissue inhibitor of matrix metalloproteinase

was evaluated over a 16 week time period and was found to be increased in the first 1–4 weeks post-TAC, and then decreased significantly in the final 12–16 weeks. In addition, GSK3β was increased from 1 to 8 weeks and then declined over the final weeks. The presence of collagen increased 12-fold by week 16 with a significantly altered deposition patter where weeks 4–12 showed diffuse myocardial fibrosis which then progressed to focal fibrosis at 16 weeks [75]. This indicates that there are numerous subtle molecular changes occurring in the transition from concentric to eccentric hypertrophy which should be monitored in order to establish the level of progression toward heart failure in patients [76]. A summary of changes in various biochemical parameters during the development of concentric and eccentric hypertrophy are noted in Table 8.1.

Changes in mRNA expression and genetics have also been discovered in patients with hypertrophy. This was observed when investigating different genotypes of the angiotensin-converting enzyme (ACE) genes which have been associated with changes in LV mass. The I (insertion)/D (deletion) polymorphism is associated with different levels of ACE activity [76–78] whereby the D/D genotype had a significantly higher prevalence in eccentric LV remodeling when compared to the I/D genotype [79]. When looking at gene expression between PO and VO rat models, it is clear that there are numerous genetic products that are differentially expressed. Upregulation of various extracellular matrix-related proteins such as osteopontin and tropoelastic were found including those that bind to actin, such as tropomyosin 4, thymosin beta-4, and transgelin in VO. This may help explain the differences between eccentric and concentric LV remodeling [80]. There is also mRNA that was found to be upregulated in VO hearts and downregulated in PO hearts including transgelin [81, 82] whereas the vice versa occurs with pyruvate dehydrogenase lipoamide kinase (PDK)-1 which is part of a family of proteins that phosphorylate the mitochondrial pyruvate dehydrogenase complex to inactivate pyruvate [80, 83]. Other genetic changes include collagen isoforms,

MMPs, expression of the beta-myosin heavy chain, transcriptional regulation of adrenomedulin, and the expression of growth-factors and beta-tubulin [84–91].

8.4 Hormonal Influences on Concentric and Eccentric Hypertrophic Remodeling

Among the numerous factors that differentiate hypertrophic remodeling when looking at PO versus VO, one of the most significant influences on cardiac physiology is the effect of hormones. It has been found that α- and β-adrenoceptor agonists, angiotensin II, endothelin-1, and thyroid hormone play a role in causing cardiac hypertrophy [92, 93]. These hormones increase the level of PLC activity, which has been found to be elevated in cardiac hypertrophy. Such alterations result in the formation of phosphatidic acid which is responsible for stimulating both transcription and translation processes contributing to accelerated protein synthesis in cardiomyocytes [92]. In both PO and VO animals, protein synthesis play a major role in increasing both protein content and LV volume which is partially a result of extracellular signaling associated with phospholipase C [94]. In PO animals, it is evident that both the SNS and renin-angiotensin system (RAS) are activated [95, 96]. When comparing cardiac function it was found at in PO animals, LV diastolic pressure and end-diastolic pressure were significantly increased, whereas, in VO animals, LV end diastolic pressure was found to be increased [97]. It has also been demonstrated that β-adrenoceptor-mediated signal transduction mechanisms are likely upregulated in VO and downregulated during PO. This view is based on various observations including increases in contractile activity and stimulation of adenylyl cyclase activity [97]. However, it is pointed out that PO has also been found to have increased β-adrenoceptor density at early stages of cardiac hypertrophy [95, 97, 98]. Alterations in β-adrenoceptors have also been reported to influence various downstream subcellular organelle function including decreasing LV Ca^{2+}-stimulated ATPase and myosin Ca^{2+}-ATPase activities in addition to changes in SR Ca^{2+}-uptake and release activities [99]. It was found that only the initial rate and not the capacity of SR Ca^{2+}-uptake was increased at 4 weeks post PO-inducement whereas at 8 weeks, both the rate and capacity of SR Ca^{2+}-uptake were decreased [100, 101]. Interestingly, in Dahl salt-sensitive rats with hypertension, it was observed that at the concentric remodeling stage, myocardial contractility and intracellular capability to regulate Ca^{2+} remained normal, and it was not until the chronic heart failure stage was the impaired SR Ca^{2+} handling observed [102]. Specifically, there were no significantly changes found in cardiac contractility, Ca^{2+} transients, the SR protein capability to regulate Ca^{2+}, and protein and mRNA levels of SERCA2, phospholamban, and calsequestrin [102].

Another significant change that appears at 8 weeks of inducing PO is related to the content of myosin light chain kinase (MLCK), which increases during this time, seemingly as a response for depressed MLC phosphorylation activity [103]. Changes in MLCK activity have been implied to contribute to impaired cardiac

function [104, 105]. There are also changes that have been observed regarding mRNA levels of α- and β-myosin heavy chains (MHC). At 8 weeks of PO, the level of α-MHC was decreased whereas that of β-MHC was increased. These changes were found to respond to the inhibition of angiotensin II formation by captopril and antagonism of angiotensin action by losartan [106, 107]. While evaluating the role of catecholamines and their receptors in PO, it was found that the increase in LV mass was associated with a decrease in norepinephrine (NE) stores in the heart and an increase in plasma levels of dopamine β-hydroxylase (DBH) activity in the early stages of hypertrophy. Thereafter, NE content in the heart increased and both plasma NE and DBH were elevated [96]. This indicated an increase in SNS activity during the initial stages of cardiac stress sustained by the heart emphasizing the role that catecholamines and adrenoceptors contribute to LV hypertrophic remodeling [96]. In addition, regression of LV hypertrophy in PO animals has been found to occur following propranolol and verapamil treatment where MHC gene expression returns to normal [107]. There has been a decrease in tissue catecholamines in both clinical and experimental cardiac hypertrophy, where the degree of depletion may correlate with impaired cardiac contractility [93, 108, 109]. Since the depletion of catecholamines present in tissue tends to associate with an increase in plasma levels, it has been suggested that high levels of circulating catecholamines in hypertrophy can become oxidized, subsequently causing cell damage and impairing cardiac contractile properties [93]. It is pointed out that the majority of research regarding the influence of hormones has been carried out in PO animals; however, there is still much to be done in this regard in VO models.

In addition to hormones released from the SNS, female sex hormones appear to have an influence on hearts subject to VO. When comparing male and female rats, males had increased LV end diastolic pressure with a significant decrease in cardiac output possibly as a result of changes in internal diameter [110, 111]. Female rats, unlike male rats, appeared to only have increases in wall thickness due to VO, although both had increased stroke volumes. It is interesting to note that the expression of ACE was increased in both female and male rats [110]. When looking at AR gene expression, it was found that β1-AR expression decreased in males whereas β2-AR expression increased in females [111]. Increases in ACE did not affect apoptosis which was not found until the chronic heart failure stage of VO hypertrophy [112]. Expression of G-coupled protein receptors (GRKs) was also found to be differentiated between female and male rats as GRK2 expression was increased in both genders but GRK3 was found to be increased in females only. Finally β-arrestin2 was increased in both genders, whereas β-arrestin1 was only increased in females [111]. From these observations, it can be appreciated that female sex hormones, such as estrogen, may be playing a protective role in the development of eccentric hypertrophy as it appears that female rats are able to better compensate for the stress and strain imposed by VO and maintain cardiac contractile function [110]. There is still much research needs to be carried out on the adaptive effects of female sex hormones in PO animals.

8.5 Physiological Concentric and Eccentric Hypertrophic Remodeling

Similar to pathological cardiac hypertrophy, physiological cardiac hypertrophy is a response of the heart to stress where concentric and eccentric remodeling occurs. VO leads predominantly to LV cavity dilatation and appears to be the result of endurance training [3, 7, 113]. Hearts of animals subjected to an endurance training program have been shown to add new sarcomeres in series to existing sarcomeres mirroring that of pathological cardiac hypertrophy [6]. On the other hand, concentric physiological remodeling is the result of PO which occurs in a resistance training program and is characterized by increased myocardial mass and wall thickness without changes in cavity size [4, 7, 113]. In strength training programs there are massive increases in systolic blood pressure and sarcomeres are formed in parallel to existing sarcomeres which cause an increase in wall thickness in a manner similar to pathological concentric hypertrophy [9]. Interestingly, triathlon training incorporates both PO and VO aspects as it combines the endurance sports, running and swimming, with the resistance sport, cycling, which results in a mixed hypertrophic phenotype [2]. Different types of physiological stimuli have thus been observed to result in different types of cardiac hypertrophy (seen in Fig. 8.3). It is important to keep in mind that it takes over 3 h of weekly exercise in order to change myocardial mass which means a consistently substantial amount of stress needs to be put on the heart in order for remodeling to occur [114]. Remodeling of the right ventricle (RV) also occurs with endurance training, unlike strength training, where RV dilatation happens with an increase in both systolic and diastolic function [7]. However, strength training has been shown to increase systemic blood pressure, aortic remodeling, and reduced arterial compliance [9, 115–117]. When comparing the effects of swimming and running in young athletic males, it was found that eccentric LV hypertrophic remodeling was more pronounced in the swimming athletes [5]. One of the unique attributes of physiological hypertrophy is that morphological alterations that occur during training were reduced upon detraining, although LV end-diastolic volume remains elevated [118, 119]. Unfortunately, patients with hypertrophic cardiomyopathy do not respond to detraining [119].

Physiological remodeling of the heart in athletes is not associated with interstitial fibrosis as an inflammatory response as seen in pathological cardiac hypertrophy [120]. Furthermore, myocardial inflammation occurring as a result of repeated injections of isoproterenol was prevented by exercise training [51]. In addition, exercise training prevented myocardial dysfunction induced by β-adrenergic hyperactivity [49]. It is interesting to note that such an inhibition of myocardial hypertrophy by exercise was achieved by attenuation of increases in TGF-β1 and NFκB mRNA expression [49]. Physiological remodeling as a response to increased exercise load is an indication that training is an important aspect in altering cardiac morphology [7]. When comparing specific values of cardiac dimensions between physiological and pathological remodeling, it has been found that these parameters in athletes were higher than those in non-athletes,

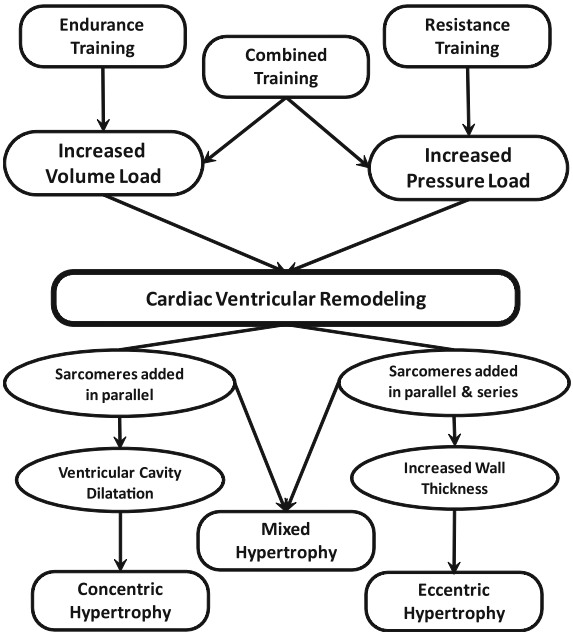

Fig. 8.3 Schematic representation of physiological hypertrophic remodeling due to either endurance and/or resistance training resulting in eccentric, concentric, and mixed hypertrophy

but were lower when compared to patients with essential hypertension and malignant hypertrophy [121–125]. Systolic function and diastolic relaxation and function have also been shown to be normal, despite remodeling changes, in athletes compared to control [8, 113, 121].

8.6 Conclusions

There are numerous differences with respect to structure of concentric and eccentric LV hypertrophy. There are also a number of factors such as hemodynamic overload as well as both molecular and metabolic events that have been shown to influence LV remodeling phenotypes. One of the most influential of factors contributing to LV hypertrophy pertains to changes in the status of SNS and subsequent increases in circulating catecholamine levels; however, the possibility of various other hormones which may contribute to differential remodeling between eccentric and concentric remodeling cannot be ruled out. Physiological remodeling shares many attributes of pathological remodeling at early stages of induction; however, the overall extent of dimensional changes is less and there is a lack of inflammation in the heart during the development of physiological hypertrophy. Although it appears that differences in the degree of ventricular wall stress superimposed upon the local release of some growth factors may determine the full extent of the biochemical and molecular

characteristic differences between eccentric and concentric remodeling, the exact mechanisms for these adaptive alterations in the heart remain to be discovered.

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Chapter 9
Cardiac Adaptation to Volume Overload

Vojtech Melenovsky

Abstract Cardiac adaptation to increased volume load is encountered in physiologic scenarios such as growth, pregnancy and aerobic exercise training and in pathological conditions like atrio-ventricular valve insufficiency or systemic arterio-venous shunt. Experimental models of pure volume overload due to chronic mitral regurgitation in dogs or aorto-caval fistula in rodents show that this type of remodelling differs in many ways from pressure overload. The early response is characterised by increased diastolic wall stress, activation of specific signalling pathways (Akt, Wnt), cardiomyocyte elongation, mast cell infiltration and degradation of extracellular matrix which allows increasing the chamber volume. Volume overload leads to eccentric cardiac hypertrophy with increased ventricular diameter but reduced relative wall thickness, enhanced diastolic function and relatively preserved systolic function. Myocardial collagen content is normal, in contrast to fibrosis due to pressure overload. Although initially well tolerated, pathological volume overload is an inevitably progressive condition. As a result of neurohumoral activation, cardiomyocyte dysfunction, abnormal Ca^{2+} handling and cell loss due to apoptosis, overt end-stage heart failure develops after long latent period. Understanding the pathophysiology of volume-overload cardiac remodelling is a mandatory step for development and proper timing of novel surgical and pharmaceutical therapies which may prevent or postpone the onset of heart failure.

Keywords Heart failure · Volume overload · Mitral regurgitation · Aorto-caval fistula · Diastolic function · Systolic function · Cardiac remodelling · Cardiac hypertrophy · Experimental models · Hemodynamics

V. Melenovsky (✉)
Department of Cardiology, Institute for Clinical and Experimental Medicine: IKEM, Videňská 1958/9, 140 21 Prague, Czech Republic
e-mail: vojtech.melenovsky@ikem.cz

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Abbreviations

LV	Left ventricle
ACF	Aorto-caval fistula
Ees	End-systolic elastance
ROS	Reactive oxygen species
ACE	Angiotensin-converting enzyme
MMP	Matrix metalloproteinase
TNF	Tumour necrosis factor

9.1 Introduction

The heart has just a few options of how to handle an increased volume load and how to provide a larger-than-normal cardiac output. First, it can use Frank-Starling autoregulation, by which an increase of end-diastolic sarcomere length instantly boosts contractile performance. This mechanism operates on a beat-to-beat basis and adjusts ventricular contractility to subtle variations in loading but has limited ability to contain larger fluctuations. Second, an increased preload with some delay activates the neurohumoral autonomic response that augments cardiac output by increasing heart rate, relaxation and contractility of the ventricle, mediated by sympathetic stimulation. Third, longer exposure to an increased load initiates changes in chamber ventricular geometry and increases the mass of the heart, both of which represent the main long-standing compensation [1]. Chamber remodelling and hypertrophy are initially adaptive allowing the organism to survive in an altered hemodynamic state, but over the long-term, these have adverse effects on survival. In contrast to cardiac remodelling due to myocardial infarction or pressure overload, cardiac adaptation to increased volume load is also encountered more frequently in physiologic scenarios such as growth, pregnancy and aerobic exercise training.

At the tissue level, cardiac hypertrophy is defined as an increase in ventricular or heart mass produced by the increased cell size of differentiated cardiomyocytes. Cardiac cells represent only a third of the total cardiac cell number but they occupy more than 70 % of cardiac volume. Therefore, changes in cardiomyocyte cell volume translate into changes of heart weight. In mammals, heart growth before birth is characterised by cardiomyocyte proliferation (hyperplasia) which ceases postnatally. Then the number of cardiomyocytes in the heart remain almost constant for the rest of life ($2–4 \times 10^9$) and all further postnatal heart growth occurs through cardiomyocyte size expansion (hypertrophy) [2]. The remarkable increase in cardiomyocyte cell volume during postnatal ontogenesis (by up to 40×) is often associated with multiplication of genetic material within the nucleus (polyploidy). Adult human cardiomyocytes are mostly (75 %) tetraploid, but the ploidy may

further increase with pathological heart growth [3]. Adult cardiomyocytes are terminally differentiated cells and posses only negligible (or no) mitotic capacity. Recent studies of carbon isotope composition of nuclear DNA of cardiomyocytes provided evidence for slow intrinsic postnatal cardiomyocyte renewal (replacement 0.45–1 % of cardiomyocytes/year) [4]. The presence of cardiac progenitor cells or the possibility of dividing more differentiated cardiomyocytes still remains a matter of intensive debate [3]. At the level of organism, the definition of hypertrophy is more complicated, because heart weight responds to changes of body weight, body composition and the degree of physical activity. Scaling to lean body mass is probably the most accurate way how to assess appropriateness of heart weight.

Cardiac hypertrophy can be functionally categorised as physiologic or pathologic. The cardinal features of both types are summarised in Table 9.1. Physiological hypertrophy responds to metabolic factors or peptide factors governing normal growth (Insulin-like growth factor-1, IGF-1) [5] and is driven by signalling pathways (protein kinase Akt) that are distinctive from those activated in pathological heart growth. Physiological hypertrophy is characterised by preserved chamber geometry, by absence of maladaptive features (fibrosis, foetal gene re-expression, apoptosis, metabolic remodelling), and by its full reversibility [6]. Pathological hypertrophy is a consequence of a persistent abnormal elevation of the hemodynamic load, combined with pro-hypertrophic effects of elevated circulating neurohormones (norepinephrine, angiotensin-II). In large prospective studies in a human population, pathological cardiac hypertrophy has been associated with increased risk of heart failure and arrhythmic death [7].

9.2 Physiological Volume Load-Induced Hypertrophy

9.2.1 Aerobic Exercise Training

An ample case of physiological hypertrophy is aerobic exercise training. While the maximal heart rate is intrinsically dependent on age and cannot be increased by training [8], increased stroke volume by training is the only mechanism for boosting maximal cardiac output and exercise performance. The mode of exercise and ensuing response of systemic circulation govern the pattern of cardiac adaptation. Strength training (isometric exercise) increases systemic vascular resistance, so the left ventricle (LV) must perform against it as a pressure pump, a situation which favours concentric LV remodelling. In contrast, endurance training (isometric exercise) increases cardiac output, while systemic vascular resistance is either normal of reduced [8]. In this situation, the heart operates as a flow pump and the whole organ adopts to handle increased venous return, developing hypertrophy with either preserved or mildly eccentric geometry. Highly trained endurance athletes (rowers, long-distance runners, cyclists, swimmers) have

Table 9.1 Differences between physiological and pathological hypertrophy

	Physiological hypertrophy	Pathological hypertrophy
Upstream signal	Peptide growth factors (IGF-1)	Catecholamines, ANG-II, Endothelin
	Metabolic stimuli, Nutrients?	Abnormal mechanical stress
Signal transduction	Phosphatidylinositol-3 kinase	Calcineurin/NFAT pathway
		Mitogen-activated protein kinase (MAPK)
		Ca^{2+}/calmodulin-dependent kinase (CaMKII)
	Akt-kinase	Protein kinase-C (PKC)
	mTOR	
Reversibility	Complete	Limited
Wall thickness/ diameter	Normal	Increased or decreased
Foetal gene activation	Absent	β-myosin heavy chain ↑
		α-skeletal actin ↑
Cardiomyocytes function	Normal	Abnormal excitation/contraction coupling SERCA, RYR2 downregulation
Extracellular matrix	Normal	Perivascular and interstitial fibrosis
Inflammatory cell infiltration	Absent	Mast cells, Monocytes
Microcirculation	Proportional to cardiomyocytes	Inadequate capillarisation
Apoptotic cell death	Absent	Increased
Metabolic remodelling	Absent	Decreased fatty acid oxidation
		Increased glucose oxidation
Electrical remodelling	Absent	Slowed conduction
		Delayed after depolarisations

Abbreviations: *mTOR* mammalian target of rapamycine, *ANG-II* angiotensin II, *SERCA* sarcoplasmic reticulum Ca^{2+} ATP-ase, *RYR*2 ryanodine receptor, *IGF*-1 insulin-like growth factor, *NFAT* Nuclear factor of activated T-cells

increased LV mass in the range of 10–20 % [9, 10], but sometimes up to 37 % [11]. The increase in LV mass is proportional to increases in LV volume and is associated with preservation or even enhancement of indexes of systolic and diastolic function (systolic and early diastolic longitudinal velocities or strains) [12]. Long-term studies in Olympic athletes performing uninterrupted high-intensity training over prolonged periods of time (~8 years) did not show any deterioration of LV function over time [13]. Other cardiac chambers adapt to exercise induced volume load as well-endurance training leads to a parallel increase of right ventricular volume [14] and left atrial size [15]. In contrast to the left ventricle, left atrial diameter regresses less with detraining—atrial dilatation may explain the higher incidence of atrial fibrillation in older endurance athletes. Exercise-induced changes of LV are also affected by various cofounders such as ethnicity, gender and genetic background. As a result, some trained subjects may end up in a shadow zone where physiological adaptation overlaps with pathologic

hypertrophy [16]. In a cross-sectional study of 947 elite athletes performing various types of aerobic activity, 38 % exceeded the normal LV chamber value for the population (<54 mm), but only 4 % had clearly abnormal end-diastolic dimension (>60 mm), while abnormal septum thickness (>13 mm) was even more rare (1.3 %) [17]. In subjects with abnormal hypertrophy, prolonged detraining led to a 28 % reduction of LV mass and to normalisation of LV thickness in all. However, the resolution of LV diameter was incomplete and substantial chamber dilatation persisted in more than 20 % of detrained athletes, particularly in those who gained weight or engaged in recreational sport activities [18].The prognostic impact of residual heart hypertrophy or chamber dilatation after cessation of intensive training remains unknown.

9.2.2 Pregnancy

Pregnancy leads to expansion of blood volume by 40–45 % and to an increase of cardiac output by 30–50 % that starts in the 6th week, reaches a plateau by the 2nd trimester and then stays until delivery [19]. This temporary increase in volume loading leads to mild dilatation of the left ventricle, a decrease in wall thickness/diameter ratio and to a 10–20 % increase in LV mass [20], that is fully reversible within 8th weeks post-delivery [21]. Molecular mechanisms responsible for heart hypertrophy during pregnancy remain understudied. In addition to mechanical stress, the rise of estrogens towards the end of pregnancy seems to contribute to pregnancy-related heart hypertrophy by increased activity of stretch-responsive c-Src kinase activity [22]. The heart response to pregnancy-induced overload is also modulated by the neuroregulin/ErbB system which has cardioprotective and cardioregenerative properties. Inhibition of this system with kinase inhibitor lapatinib in rodents led to pregnancy-associated maternal LV dysfunction and death [23].

9.2.3 Postprandial Hypertrophy

An extreme and intriguing form of physiological adaptation to increased volume and metabolic demands is the postprandial cardiac hypertrophy in large carnivorous snakes. The Burmese python's heart increases its mass by 40 % within 48 h after consumption of a large meal, driven by a large increase in cardiac output and a 7-fold increase in oxygen consumption related to digestion of the prey [24]. Postprandial heart enlargement is not due to cardiomyocyte division but purely due to hypertrophy which is fully reversible in the postprandial period. As recently demonstrated, heart growth occurs due to activation of physiological signal transduction pathways (Akt and AMPK kinases) and is paralleled by high upregulation of fatty acid transport and oxidation enzymes (CD36/fatty acid translocase, medium-chain acyl-coenzyme A dehydrogenase). This massive

Table 9.2 Causes of volume overload

Mechanism	Examples	Overloaded ventricle
Physiological		
	Isotonic exercise training	Both LV and RV
	Pregnancy	Both LV and RV
Pathological		
Pure volume overload	Mitral regurgitation	LV
	Tricuspid regurgitation	RV
	Systemic arterio-venous shunt	Both RV and LV
	Dialysis fistula	
	Post-traumatic or post-surgical	
	Vascular malformations	
	Rendu-Osler disease	
	Telangiectasia	
	Ventricular septal defect	Both RV and LV
	Atrial septal defect	RV
Mixed pressure and volume overload	Aortic regurgitation	LV
High-output state	Low systemic vascular resistance	Both RV and LV
	Sepsis	
	Hypercapnia	
	Paget's bone disease	
	Metabolic	
	Anaemia	
	Thiamine deficiency	
	Thyrotoxicosis	

LV left ventricle, *RV* right ventricle

growth response is triggered not only by mechanotransduction and metabolic hormones, but also by a specific combination of circulating fatty acids (nutrient signalling) that directly promote cardiomyocyte hypertrophy even in a mammalian heart [25].

9.3 Pathological Volume-Overload Heart Hypertrophy

Pathological volume overload-induced cardiac hypertrophy is often a result of structural defect that creates an inefficiency of circulation, such as mitral regurgitation or a shunt between the systemic vein and artery [1]. Similar cardiac remodelling can also develop in high cardiac output states due to low systemic arterial resistance. The causes of non-physiological cardiac overload are summarised in Table 9.2.

9.3.1 Valve Regurgitation

Frequent causes of mitral regurgitation are age-related or myxomatous degeneration, infective endocarditis, rheumatic disease or altered valve geometry by other cardiac heart pathology ("functional mitral regurgitation"). In mitral regurgitation, a part of the stroke volume escapes back into the atrium and then re-enters the ventricle in the following cardiac cycles. Because regurgitation occurs into the low pressure chamber, the contracting ventricle is exposed to pure volume overload which triggers an increase in LV diameter, mass and compliance. Mitral regurgitation is initially well tolerated, but if severe enough (regurgitant fraction >50 %) it leads to heart failure or premature death [26, 27]. Isolated volume overload can occur also in the right ventricle (due to atrial septal defect or tricuspid regurgitation) which tolerates it better than pressure overload [1]. Detailed mechanisms of early and late remodelling of the heart due to mitral regurgitation will be discussed below. Aortic insufficiency is noted only briefly here, because it represents a state of mixed volume and pressure overload—the left ventricle is exposed to higher systolic and diastolic pressures than in pure volume overload due to mitral regurgitation or systemic arterio-venous shunt [1].

9.3.2 Systemic Arterio-Venous Shunt

Another structural defect that leads to volume overload of both ventricles is an abnormal connection (shunt, fistula) between the large systemic artery and vein. Small arterio-venous fistula is often created surgically in patients with renal failure to allow easy vascular access for haemodialysis. Sometimes the feeding artery undergoes eccentric remodelling so that fistula flow considerably increases which may cause considerable volume overload for the heart. A shunt flow of at least 20–50 % of cardiac output is needed to develop heart failure [28]. Arterio-venous fistula can be traumatic due to a penetrating injury of proximal limb [29] or as a consequence catheterisation in the groin. Intraabdominal arterio-venous fistula can also develop after ligation of vascular stub during nephrectomy [30] or adnexectomy. Rarely, significant shunting develops as a consequence of large vascular malformations, either as isolated haemangioma or multiple arterio-venous malformations often affecting the liver (hereditary telangiectasia, Rendu-Weber-Osler disease) [31].

9.3.3 High Cardiac Output States

Volume overload can also occur as a consequence of a high-output circulatory state (cardiac index >3.9 l min m^{-2} in humans) [32], driven primarily by

excessive systemic vasodilatation. Cardiac output redistribution and relative hypotension due to low systemic vascular resistance trigger neurohumoral activation, fluid retention and development of clinical heart failure [32, 33]. In sepsis, low systemic vascular resistance is as a consequence of endotoxin-induced, NO-mediated systemic vasodilatation. In some patients with pulmonary disease or hypoventilation, low systemic vascular resistance is a consequence of vasodilatory properties of retained carbon dioxide [34]. Low vascular resistance can also be observed in some severely obese subjects [35] who may have simultaneously an alveolar hypoventilation resulting in increased cardiac output and congestion [36]. In anaemia, increased cardiac output compensates the diminished oxygen-carrying capacity of the blood. In severe chronic anaemia, diminished inactivation of endothelium-derived NO by erythrocytes may additionally contribute to excessive vasodilatation [37]. Other states of high cardiac output state include thyrotoxicosis, severe thiamine deficiency and advanced Paget's bone disease (Table 9.2).

9.4 Animal Models of Volume Overload

9.4.1 Models of Mitral Regurgitation

Carabello's group developed a model of chronic volume overload due to mitral regurgitation in dogs, created by partial destruction of mitral valve chordae using forceps under fluoroscopic control [38]. After 3 months of severe mitral regurgitation (regurgitant fraction >50 %), end-diastolic volume increased by 46 %, left ventricular mass increased by 36 %, mean end-diastolic LV pressure increased to 25 mmHg and cardiac output was reduced [38, 39]. This model was used in seminal studies that studied the relations between cardiac remodelling, wall stress and indices of ventricular function in chronic mitral regurgitation [40]. A similar model was later adopted also in pigs [41]. Due to high costs and ethical concerns of research use of dogs, a rodent model of chronic mitral regurgitation in rats has been recently developed. Mitral regurgitation is created by a puncture of mitral valve leaflets with a needle that is advanced from the cardiac apex and exposed from a small left thoracotomy. The amount of regurgitation created can be visually checked during the procedure with a human intracardiac echocardiographic (ICE) probe inserted into rat's oesophagus [42–44]. The procedure has acceptable mortality rate of 17–27 %. Twelve weeks after the procedure, LV end-diastolic dimension increased by 26 %, LV end-diastolic volume doubled, LV mass index increased by 35 % while LV end-diastolic pressure also increased in a part of the animals. The myocardium of rats with mitral regurgitation demonstrated a myosin heavy chain isotype switch (from α to β) and downregulation of sarcoplasmic reticulum Ca^{2+} ATP-ase 2 (SERCA2), confirming the presence of features of maladaptive remodelling. One-year survival of rats with mitral regurgitation was only 38 % [42].

9.4.2 Systemic Arterio-Venous Shunt Models

Systemic shunt due to artificial aorto-caval fistula (ACF) in rodents is now the most widely used volume overload model. Previously, a surgical anastomosis approach was used in rats [45] and pigs [46, 47]. More recently, Garcia and Diebold developed a simplified needle-puncture technique in which infrarenal aorta and adjacent vena cava inferior is punctured with a 18G phlebotomy needle (1.2 mm outer diameter) from midline laparotomy [48]. The puncture site is sealed with cyanoacrylate glue during brief abdominal aortic clamping and the abdominal cavity is then closed with a suture. The procedure is rapid (<20 min) and has low procedural mortality (<10 %). Shortly after ACF creation, pronounced cardiac hypertrophy with chamber dilatation develops: the heart weight/body weight increases by 52 % by the 2nd week and by 95 % by the 16th week, compared to sham-operated animals [49]. Volume overload is initially well tolerated, but after 12–15 weeks, congestion and heart failure signs such as tachypnoea, lethargy, piloerection and ascites, gradually develop [50]. The slow progress of symptoms mimics well the insidious onset of heart failure in humans [51]. After 21 weeks, 80 % animals are either in heart failure or dead [52]; 1 year mortality approaches to 72 % [50].

The course of morbidity after ACF induction depends on the shunt size, gender, genetic background [53] and strain differences [54]. The large fistula from a large needle leads quickly to heart failure, while small fistula from thinner needle induces only milder, less symptomatic hypertrophy [55, 56]. Females seem to tolerate volume overload better than males and develop less pronounced heart hypertrophy. This protective gender effect is removed by ovariectomy [57] and is restored back by oestrogen administration. The beneficial effects of oestrogens can be explained by their inhibitory effects on mast cell-mediated extracellular matrix degradation [58]. Oestrogens also increase the active form of Bcl-2 protein that confers the resistance to apoptosis [59].

In a small fraction of animals after ACF procedure (<10 %), the fistula closes itself over time, with resulting cardiac mass that falls within normal limits (<mean + 2*SD of control group); these animals should probably be post hoc excluded from experiments. The needle technique for ACF creation was later adopted also for mice [60, 61], which has higher surgical mortality and ACF failure rates.

9.4.3 Models of Aortic Insufficiency

Magid at all developed a rabbit model of aortic insufficiency created by retrograde transcarotid puncture of aortic leaflets under echocardiographic guidance [62, 63]. Arsenaut et al. more recently adopted a similar technique for rats with a favourable procedure mortality <20 % [64]. The procedure leads to more isolated hypertrophy of the LV (LV weight/body weight increase by 60 % 26 week) [65],

Table 9.3 Differences between pressure overload and volume overload-induced cardiac remodelling

	Pressure overload	Volume overload
Left ventricle		
Mass	↑↑↑	↑↑
Geometry	Concentric	Eccentric
	↑ relative wall thickness	↓ relative wall thickness
Systolic wall stress—acute	↑↑	↑
—chronic	→	→ or late ↑
Diastolic wall stress—acute	→	↑
—chronic	→ or ↓	↑
Coronary flow reserve	↓	→
Capillary density	↓↓	→ or ↓ (subendocardial)
Cardiomyocytes		
Pathway activation	CaMKII	Akt
	ERK	Wnt
	Calcineurin/NFAT	
Morphometry	↑ cross-sectional area	↑ length
Ca^{2+} abnormalities	↓ SR calcium fractional release	→
	↑ amplitude of Ca^{2+} transients	→
Apoptosis	↑↑↑ activated caspase 3	↑↑ activated caspase 3
	↑↑ TUNEL labelling	↑ TUNEL labelling
Protein turnover	↑ synthesis	↓ degradation
Extracellular matrix		
Fibrosis	↑ interstitial, ↑↑ perivascular	→ or ↑ (subendocardial)
Inflammatory cell infiltration	↑↑↑	↑
	monocytes, CD45 + cells	mast cells
Survival	↓↓↓	↓↓

CaMKII $Ca^{2+/}$ calmodulin-dependent kinase, *TUNEL* terminal deoxynucleotidyl transferase, dUTP nick-end labelling, *SR* sarcoplasmic reticulum, *ERK* extracellular signal-regulated kinase, *NFAT* Nuclear factor of activated T-cells

but it does not result in overt of heart failure. The model has been successfully used in worthy studies documenting beneficial effects of beta-blockade [66], moderate exercise [67] or angiotensin-converting enzyme (ACE) inhibition [68]. However, these findings can be condition-specific and cannot be generalised to other volume overload states.

9.5 Differences Between Volume and Pressure Overload

The principal differences between volume overload and pressure overload-induced cardiac adaptation and remodelling are summarised in Table 9.3.

9.5.1 Wall stress and Chamber Geometry

According to Laplace's law, the mechanical wall stress in the LV wall can be approximated as $\sigma =$ (LV pressure $\times$ LV radius)/(2 $\times$ LV wall thickness). In a landmark study, Grossmann invasively measured LV wall stress in 30 patients with various forms of overload due to valve lesions [69]. Patients with pressure overload, most often due to aortic stenosis, had higher systolic LV pressure, more concentric LV hypertrophy, but normal systolic and diastolic wall stress. In contrast, patients with volume overload, in this study most often due to aortic regurgitation, had an eccentric hypertrophy, normal systolic wall stress, normal LV thickness/diameter ratio, but increased diastolic wall stress. The authors proposed that increased systolic tension leads to cardiomyocyte thickening and feedback return of systolic wall stress to normal. On the other hand, increased diastolic tension leads to cardiomyocyte elongation that helps to maintain stroke volume, but it does not fully normalise diastolic wall stress.

By direct comparisons of invasive data from dogs with pressure or volume overload with matched levels of the LV stroke work, Carabello et al. showed that for the same work demand, LV hypertrophic response to volume is smaller than to pressure [40]. This implicates that in mitral regurgitation, each unit of myocardium performs more work and the dysfunction occurs at smaller degree of LV hypertrophy than in pressure overload. Patients with mitral regurgitation have reduced LV mass/volume ratio, supporting the experimental observations of limited hypertrophic response to volume overloading [70].

Based on the Grossmann study [69], concentric hypertrophy due to pressure overload was long viewed as an necessary adaptation, allowing to normalise elevated systolic wall stress [69] and to prevent early failure of the ventricle. This notion has been more recently challenged by experiments where hypertrophy in pressure-overloaded hearts was abolished by gene manipulations [71] or by anti-hypertrophic drugs [72]. In these models, heart failure did not develop despite persistent LV load elevation, suggesting that hypertrophy itself, independently of wall stress, is the factor responsible for progression towards heart failure. This idea has some support in clinical data from patients with aortic stenosis, where the degree of hypertrophic response for a given hemodynamic overload predicts the presence of systolic dysfunction [73]. Whether similar concept applies also to volume overload remains to be studied.

9.5.2 Mechanosensing

The driving force for cardiac hypertrophy are mechanical forces that act on cardiomyocytes and activate mechanosensing signalling pathways [69, 74]. Mechanical forces are sensed both inside and at surface of the cardiac cell. At the sarcolemma, stress is sensed by stretch-sensitive transient receptor potential

channels (TRP channels) and by membrane-spanning integrins that interconnect intracellular load-bearing cytoskeleton to the extracellular matrix and interact with sub-sarcolemmal focal adhesion kinases (FAK). Intracellularly, mechanical forces are sensed by protein complexes at Z-disc (muscle-LIM protein and others) and by titin, a gigantic protein that spans from Z-disc to M-band as a single molecule ($\sim$1 μm distance) and forms a myofibrillar backbone important for regulation of diastolic stretch [75].

Mechanical forces differ between pressure and volume overload, being responsible for two distinctive patterns of remodelling response. Not only the magnitude, but also the timing, of wall stress within cardiac cycle is important. In tissue culture experiments, Yamamoto showed that cardiomyocytes exposed to strain during systolic phase had a more prominent activation of mitogen-activated protein kinase (MAPK), increased B-type natriuretic peptide (BNP) expression and protein synthesis rate than cardiomyocytes exposed to strain during diastolic phase [76]. Imposing a deformation during systole (when cell stiffness is greater) could deliver more strain on cardiomyocyte and increase the stretch-responsive signalling. Alternatively, the coincidence of mechanical strain with electrical activation or intracellular calcium level of cell may be important in determining the difference between pressure overload and volume overload.

9.5.3 Signalling Pathway Activation

The molecular biology of these two different remodelling patterns has been examined in detail by Toisher et al. who studied mice with severe pressure overload (transaortic constriction) or volume overload (ACF) [60]. Mean LV wall stress/cycle early after surgery was equally elevated in both groups of animals (+69 and +67 %). Increased systolic or diastolic wall stress (measured immediately after surgery) led in both cases to similar cardiac hypertrophy (+22 % and +29 % by 1st week), but otherwise largely different molecular myocardial phenotypes. Pressure overload led to concentric hypertrophy with increased diffuse myocardial fibrosis, inflammatory cell infiltration (CD45+) and cardiomyocyte apoptosis measured by activated caspase-3 and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL). In contrast, volume overload led to eccentric hypertrophy without increased fibrosis, without inflammation and with less cardiomyocyte apoptosis. In the long-term, pressure-overload hypertrophy resulted in more severe LV dysfunction and higher mortality compared with shunt. The analysis demonstrated striking differences in signalling pathways that were activated by different overload conditions. Pressure overloaded hearts showed increased activation of extracellular-signal-regulated kinase (ERK) and even more persistent activation of calcium/calmodulin-dependent protein kinase II (CaMKII). On the other hand, volume overload showed early activation of Wnt signalling pathway and persistent activation of Akt-kinase (protein kinase B). Increased CaMKII activation in pressure overload resulted in altered Ca^{2+} cycling, increased

L-type calcium current, calcium transients, fractional sarcoplasmic reticulum calcium release and calcium spark frequency. None of these changes happened in the shunt model. This important experimental study indicates that differences in the loading pattern result in distinct load-dependent regulation of gene expression and therefore may require different overload-specific pharmacological interventions.

9.5.4 Protein Turnover

Both types of overload have also a diverse impact on protein synthetic rate. Myocardial contractile proteins have rapid turnover (5–10 days) so stable heart mass is preserved by maintaining a constant equilibrium between synthesis and degradation rate [1]. By measuring ^{3}H-labelled leucine incorporation into a heavy chain of myosin, Carabello et al. showed that acute pressure overload in dogs lead to a 30 % increase in synthesis of this sarcomeric protein and returns to normal when LV mass is increased. Interestingly, in volume overload, myosin synthesis is not increased either in the acute [77] or chronic phase [78]. Therefore, myocardial growth after induction of mitral regurgitation is accounted for by a decrease in the fractional rate of protein degradation [78]. Similar findings were also reported in rabbit model of aortic regurgitation. Total cardiac protein synthesis contributed only to the early phase of hypertrophy (3 days), whereas progressive chronic eccentric hypertrophy (at 1 month) was due to suppression of protein degradation [63]. Reduction of protein degradation rate implicates a longer life-span of myocardial contractile proteins and also longer exposure to oxidative damage that may contribute to functional impairment [1, 79].

9.5.5 Coronary Circulation

Both overload states may differ in the impact on coronary circulation. In pressure overload, coronary perfusion is typically diminished due to decreased coronary flow reserve [80], capillary rarefaction [81], decreased capillary density [82] and insufficient angiogenesis [83]. Substantially less information is available on volume overload states. In a dog model of severe chronic mitral regurgitation, coronary blood flow and flow reserve is preserved [84] and abnormalities in coronary blood flow do not explain the resting contractile dysfunction. However, a progressive reduction of subendocardial capillary density was also found in ACF model of volume overload [82, 85].

9.6 Function of Volume-Overloaded Ventricle

Assessment of ventricular performance in volume overloaded states is complicated by changed chamber geometry, mass and loading conditions. Most studies that used accurate load-independent measures of LV function were performed with the canine mitral regurgitation model [38–40, 78, 86]. More recently, similar data became available from the ACF model in rodents [60, 87].

9.6.1 Afterload

Immediately after creation of experimental mitral regurgitation, afterload (expressed as wall stress) is reduced due to partial emptying of left ventricle into the low pressure left atrium. Within hours, afterload returns to normal due to adaptive changes in chamber geometry [88]. In chronic compensated mitral regurgitation, mean systolic wall stress is similar to controls. With progression of the disease and with development of LV dysfunction, systolic wall stress actually increases, which may have further deleterious effects on chamber performance [70]. Volume overload due to advanced stage ACF is also characterised by increased systolic wall stress [89].

9.6.2 Diastolic Function

In compensated chronic volume overload, diastolic wall stress is increased [1, 40, 89], but the diastolic properties of the left ventricle are enhanced due to increased LV distensibility [90]. Corin et al. performed a detailed study of diastolic indexes in patients with mitral regurgitation using pressure-volume analysis [91]. In patients with compensated mitral regurgitation, the adaptations to chronic volume overload led to supernormal diastolic function due to the right-shift of end-diastolic pressure-volume relationship and enhanced diastolic compliance, enabling the left ventricle to operate at lower levels of filling pressures. However, in patients with mitral regurgitation and LV dysfunction, both myocardial and chamber stiffness were again increased, indicating that the late increase of LV stiffness may contribute to the clinical manifestations of congestive heart failure [91].

9.6.3 Acute Changes of Systolic Function

Berko et al. examined LV function in acute severe mitral regurgitations in dogs. After creation of valve insufficiency, LV stroke work and ejection fraction increased, but the slope of end-systolic pressure-volume relation (end-systolic elastance, Ees) was reduced or unchanged if Ees was normalised to end-diastolic volume [88]. The post-procedural increase in LV ejection fraction was not due to increased contractility or decreased afterload (that quickly returned to normal), but rather a consequence of increased preload. The observed disparity between LV ejection fraction and Ees supports the concept that LV function is overestimated by ejection indexes in the presence of mitral regurgitation and that ejection indexes are poor predictors of myocardial function in hearts with mitral regurgitation. Numerous clinical studies demonstrated a drop of ejection fraction in patients undergoing mitral valve replacement for severe mitral regurgitation, suggesting that after the surgery, irreversible myocardial dysfunction may become unmasked when the regurgitation is gone [88].

9.6.4 Chronic Changes of Systolic Function

Prolonged volume overload is associated with the insidious onset of intrinsic myocardial dysfunction. In a dog model of mitral regurgitation lasting for 3 months, Urabe et al. examined myocardial tissue composition, global LV function and contractility of isolated cardiomyocytes [92]. Reduced LV function (Ees) correlated with impaired sarcomere shortening velocity of isolated cardiomyocytes from the same individual, indicating that the contractile defect is present both at the ventricular and cellular level. Quantitative morphology showed no difference in LV interstitial volume fraction, but decreased myofibrillar volume fraction. Therefore, LV ventricular function occurs due to a combination of cardiomyocyte cell loss, intrinsic cardiomyocyte contractile dysfunction and perhaps inadequate compensatory hypertrophy.

The cause of gradual cardiomyocyte loss in a volume overloaded heart can be an increased rate of apoptosis, not matched by adequate cell replacement [93]. In patients with advanced mitral regurgitation, cardiomyocytes show an activation of pro-apoptotic signalling (Bax protein, protein p53, caspase 3, 8 and 9), although no evidence of DNA fragmentation [94]. Activation of apoptosis (increased Bax protein, caspase 3/9, TNF-α) has also been demonstrated in the advanced stage of volume overload due to ACF in rats [59, 60]. When directly compared, volume overload induces apoptosis less than a pressure overload of similar degree [60].

Intrinsic abnormalities of cardiomyocyte function due to impaired Ca^{2+} homeostasis and excitation/contraction coupling contributes to systolic dysfunction due to volume overload. Myocardial strips from patients undergoing mitral valve replacement demonstrated severe depression of tension generation and

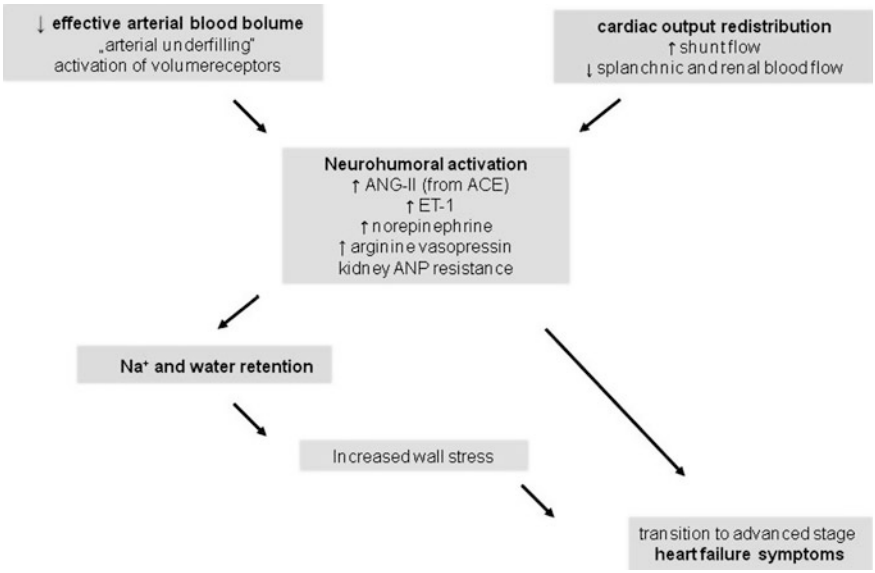

Fig. 9.1 The three stages of cardiac adaptation and remodelling caused by volume overload from ACF in rats. For abbreviations, see text. Modified according to Hutchinson [104] and Chen [103]

blunted force-frequency curve with a negative slope in exercise range of heart rate [95] and had downregulated sarcoplasmic Ca2+ -ATPase (SERCA) and Na+/Ca2+ exchanger (NCX) genes [96]. Similarly, in shunt-induced volume overload, rats with advanced ACF (10–28 weeks post-procedure) have reduced systolic function, reduced Ca^{2+} release from sarcoplasmic reticulum and decreased protein levels of ryanodine receptor 2 (RyR2) [97, 98] or SERCA [99]. Activated caspases [100] or calpain proteases [101] can negatively impact cardiomyocyte contractility due to partial proteolysis of contractile proteins. Increased oxidative stress from xanthine oxidase-derived ROS can also contribute to myofibrillar damage [102].

9.7 Time Course of Cardiac Adaptation and Remodelling in Response to Volume Overload

The temporal pattern of volume overload-induced cardiac remodelling and the transition of compensated cardiac hypertrophy into heart failure is best described in the rat ACF model [51, 52, 103, 104]. Severe mitral valve insufficiency induces similar cardiac changes [42, 105, 106], but heart failure syndrome is less pronounced than in shunt-induced overload. The progressive development from the early, adaptive phase to decompensated end-stage heart failure will be described in three phases for both models below (Fig. 9.1).

9.7.1 Early, Adaptive Phase of Volume Overload (0–2 Weeks of ACF Model)

Immediately after ACF creation, cardiac output is increased by 30–100 %, leading to enhanced venous return and an increase in cardiac filling pressures. A marked increase of diastolic wall stress activates stretch-sensitive responses which turn on pro-hypertrophic signalling pathways (Akt kinase and Wnt signalling pathway), increases ROS generation and leads to inflammatory cell infiltration [60]. The confirmed source of ROS is mitochondrial xanthine oxidase, which activity considerably increases within 24 h after ACF induction [107]. Gene expression analysis demonstrated increased transcription of genes regulating extracellular matrix, cell cycle, inflammation and apoptosis [103] which is followed by eccentric hypertrophy and cardiomyocyte elongation.

9.7.1.1 Extracellular Matrix Loss

The most rapid and profound changes in the early phase occurs in the extracellular matrix [104]. Partial degradation and remodelling of the extracellular matrix is responsible for enhanced diastolic ventricular compliance and biventricular enlargement, allowing to accommodate increased volume load and to maintain effective cardiac output. Ryan et al. [89] showed that immediately after ACF creation, eccentric LV remodelling is mediated by loss of interstitial collagen (by −50 % at 2nd day) and by loosening of extracellular matrix scaffold by activated matrix metalloproteinases. After several days and after the enlargement of the ventricle, the collagen volume fraction returns to normal. Bradykinin inhibitors prevented extracellular matrix loss, but ACE inhibitors potentiate it via increased bradykinin production. The lack of effect of ACE inhibitors on early volume overload-induced remodelling [89, 108] is in striking contrast to pressure overload-related or infarct-related remodelling.

9.7.1.2 Inflammatory and Mast Cells Infiltration

An infiltration of the myocardium with inflammatory cells, particularly mast cells, seems to play a central role in triggering the early response to volume overload. Brower et al. documented a close association between myocardial mast cell density and matrix metalloproteinase (MMP) activity after creation of ACF [109]. Mast cell accumulation after ACF induction occurs due to in situ maturation and differentiation of resident mast cells [110] and due to mast cell recruitment driven by tumour necrosis factor α (TNF-α) produced by cardiomyocytes in response to stretch [111]. Inhibition of mast cell degranulation with cromolin or nedocromyl prevented the increase in mast cell number, MMP activation and volume-induced

structural changes of the heart [109, 112]. Mast cells-deficient rats displayed reduced MMP-2 activity, higher collagen content and less LV dilatation after ACF [113]. Mast cells store and release many biologically active mediators, including TNF-α, and proteolytic enzymes (tryptase, chymase) capable of activating MMPs. TNF-α neutralisation with infliximab (TNF-α antibody) attenuated myocardial inflammatory response, mast cell recruitment and prevented MMP activation and collagen depletion although it did not completely prevented cardiac remodelling [111].

This sequence of events in the early phase is not unique to the rat ACF model. Early mast cell infiltration, increased chymase and MMP-2 activity and ensuing extracellular collagen depletion has also been described in a dog model of mitral regurgitation [114]. Gene expression profiling showed marked upregulation of MMP-1, MMP-9 and extensive downregulation of pro-fibrotic growth factors (TGF-β, CTGF) and non-collagen components of extracellular matrix such as decorin, fibulin and fibrillin [105]. Patients undergoing valve replacement for severe chronic mitral regurgitation have increased myocardial and plasma levels of TNF-α and its receptors. The study also found a relationship between myocardial TNF-α expression and LV remodelling [115]. In summary, early response to volume overload is quite specific and distinctive than other causes of abnormal loading. However, it remains a question whether this phase represents a good target for therapy—lack of adaptive increase in LV end-diastolic volume after acute volume overload might be associated with poorer short-term survival [109].

9.7.2 Intermediate Stage of Compensated Ventricular Hypertrophy (3–10 Week in ACF Model)

After several weeks, early phase of remodelling resolves, leaving the heart in the stage of compensated eccentric hypertrophy with still relatively preserved systolic function and enhanced diastolic function. Inflammatory response in the myocardium is less intense, MMP-2 activity is lower and the extracellular matrix has become more stabilised. At this stage, animals show no clinical signs of heart failure [50], but neurohumoral activation gradually develops. By the 9th week, ACF rats have a significant increase in plasma renin concentration and activity, 50 % increase in norepinephrine, 4-times higher vasopressin concentration [116], increased aldosterone and natriuretic peptides [117]. Persistent elevation of circulating norepinephrine contributes to catecholamine-driven β-adrenergic receptor pathway desensitisation [46].

The sequence of events responsible for neurohumoral activation and transition from compensated hypertrophy into overt heart failure is summarised in Fig. 9.2. The reason for neurohumoral activation is the “underfilling” of the arterial part of vascular tree due to low systemic vascular resistance which triggers volume baroreceptor activation [33]. In addition, ACF rats have an abnormal distribution

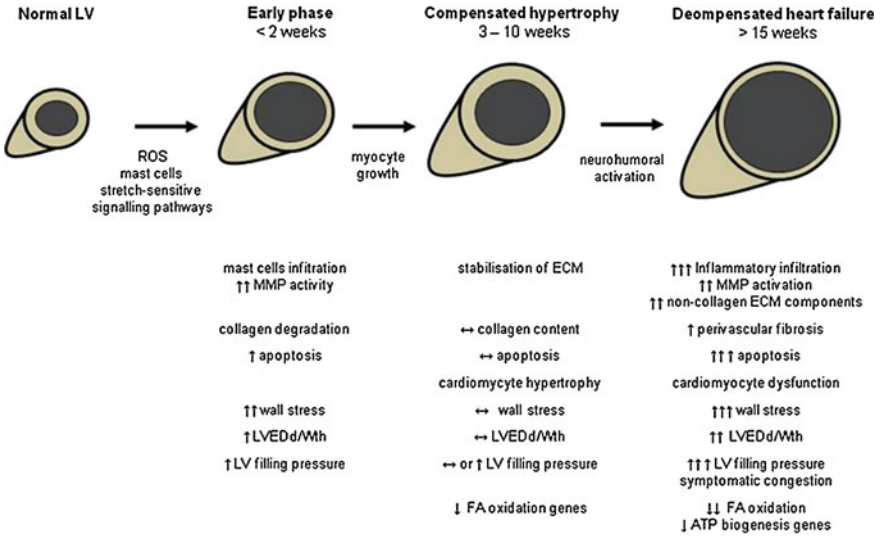

Fig. 9.2 The sequence of events responsible for neurohumoral activation and transition from compensated hypertrophy into overt heart failure in rats with aorto-caval fistula. Modified according to Schrier [33] and Flaim [118]

of cardiac output. Using radioactive microspheres, Flaim et al. showed that ACF rats have increased cardiac output due to high shunt flow (37 % of total output), but simultaneously have splanchnic and renal hypoperfusion [118]. Diminished renal perfusion and baroreceptor activation triggers neurohumoral response, causing sodium and water retention. Renal resistance to endogenous natriuretic peptides contributes to fluid retention—kidneys of rats with ACF have attenuated natriuretic response to acute volume loading [119] and to exogenous atrial natriuretic peptide administration [120]. The ACF model is therefore suitable for studies of cardio-renal interactions in chronic heart failure [117].

9.7.3 Late Stage of Decompensated Heart Failure (>15 Week of ACF Model)

Over time, the indexed heart mass (heart/body weight ratio) increases more than twice in comparison to sham-operated animals [121], the ventricle become markedly dilated but relatively thin, with decreased LV diameter/wall thickness ratio [52]. Chamber dilatation in the advanced stage is a consequence of the reappearance of local inflammatory response [103]. Total collagen content in myocardium is not increased, but MMP-2 activity is again increased, similarly as in early phase [103]. Ventricular filling pressures, lung and liver wet weights are

increased due to congestion in pulmonary and systemic circulation [52]. The increase of LV wall stress and simultaneous intense neurohumoral activation promotes the vicious cycle of adverse myocardial remodelling. As a result of apoptosis-mediated cardiomyocyte cell loss [122] and of intrinsic cardiomyocyte dysfunction, LV contractile dysfunction now becomes obvious [49, 103].

In ACF model, the right ventricle became dilated and dysfunctional as well, because the right heart has to pump similar surplus of volume. Moreover, rats with advanced ACF develop moderate pulmonary hypertension (RV systolic pressure ~40–50 mmHg) [123] due to a combination of post-capillary venous hypertension and increased pulmonary arterial resistance. From that reason, right ventricular myocardium is exposed not only to excessive volume but also to pressure, leading to more right ventricular collagen accumulation [108] and higher angiotensin-II and endothelin levels than in the left ventricular myocardium [47]. Ventricular dilatation leads to functional mitral and tricuspid regurgitations that further exacerbate volume overload. Molecular features show typical heart failure fingerprint—a switch of myosin heavy chain expression from α- to β-isoform [49] and decreased calcium handling proteins (SERCA, RyR2) [99, 124]. Quantitative histology showed myocardial perivascular fibrosis, leukocyte infiltration [103], apoptosis (by TUNEL assay) and decreased subendocardial capillary density [82]. Gene expression analysis from the late stage of ACF shows the reappearance inflammatory response genes and an increased expression of genes controlling non-collagen components of the extracellular matrix [103]. One of the most upregulated proteins, both at the mRNA and protein level, was matricellular protein periostin which modulates interactions between the cardiomyocyte and extracellular matrix [103, 125]. Unbiased proteomic analysis demonstrated increased expression ROS generating enzyme monoamine oxidase A (MAO-A) and attenuated expression of two NADPH-generating enzymes (nicotinamide nucleotide transhydrogenase-NNT and isocitrate dehydrogenase-IDH2) that may contribute to an observed reduction of redox reserve, reflected by the ratio of reduced and oxidised glutathione (GSH/GSSG ratio) [124].

9.7.3.1 Metabolic Remodelling

Characteristic features of advanced stage heart failure are metabolic and bioenergetic abnormalities of the myocardium [103, 125]. Progressive downregulation of enzymes controlling myocardial fatty acid transport and β-oxidation have been observed in most experimental models and in patients with moderate-to-advanced heart failure [126]. Attenuated myocardial palmitate oxidation has recently been demonstrated in a volume overload ACF model [125, 127, 128]. Changes in myocardial substrate utilisation may represent a return to foetal metabolic programme when glucose is the predominant energetic source [129], or can be a

consequence of secondary humoral influences due to elevated neurohormones and cytokines (angiotensin II, TNF-α) [130]. Whether downregulation of fatty acid oxidation in the failing heart has adaptive, maladaptive or neutral consequences for the course of heart failure is still intensively debated [131]. Interestingly, the increase in myocardial fatty acid utilisation induced by chronic administration of metformin in rats with ACF-induced heart failure was not associated with any change of cardiac function or prognosis [121]. A time-series examination of the gene transcription profile in the ACF model found that intense upregulation of NFκB-driven inflammatory genes in advanced ACF stage (15th week) coincided with downregulation of transcription factor PPAR-α which controls fatty acid metabolism [103]. Despite gene expression analysis which suggested downregulation of several components of respiratory chain and of ATP biogenesis, functional studies of cardiac mitochondria from compensated, intermediary stage (12 weeks) [132] or from decompensated heart failure stage (22nd week) [127] showed a preserved resting mitochondrial respiratory function, but increased vulnerability to anoxia-reoxygenation injury [132].

The advanced stage of ACF also served to explore changes in systemic metabolism induced by the presence of heart failure. Rats with ACF and established heart failure (22nd week) had elevated circulating levels of free fatty acids and a depletion of intraabdominal fat as consequence of increased fatty acid mobilisation. However, glucose tolerance and insulin sensitivity was preserved and postprandial insulin levels were actually attenuated in ACF animals, perhaps due to splanchnic and pancreatic hypoperfusion. Despite elevated free fatty acid levels in serum, ACF animals had markedly reduced myocardial triglyceride content (by 50 % compared to sham), indicating that myocardial triglyceride levels are not dependent on fatty acid delivery and that myocardial lipid overload does not occur in this heart failure model [125].

9.7.3.2 Electric Remodelling

Volume-overload induced heart hypertrophy is associated with an increased risk of arrhythmias. In long-term survival studies in the ACF model, a third of rats with ACF died suddenly [50, 52], presumably due to arrhythmic death. Given the known lack of fibrosis, myocardial substrate for increased electrical instability is unknown. Recent immunohistochemic analysis of junctions in rats with ACF demonstrated normal localisation and distribution of myocardial connexins, but a 60 % reduction of phosphorylated connexin-43 isoform which may be responsible for slower conduction velocity and an increased risk of re-entrant arrhythmias [121]. The connexin-43 phosphorylation state was normal in earlier ACF stage (11th week), suggesting that heart failure, but not cardiac hypertrophy itself, contributed to connexin hypophosphorylation.

9.7.3.3 Advanced stage of Mitral Regurgitation

Only a few studies have examined the molecular characteristics of late stages of mitral regurgitation. Pu et al. showed that chronic mitral regurgitation in rats led to a myosin heavy chain shift, SERCA2 downregulation and impaired LV contractility. One year survival in the rats with mitral regurgitation was only 38 %. From echocardiographic variables, increased end-diastolic LV diameter at 6th week, but not LV mass or fractional shortening, was the first parameter that significantly predicted mortality [42]. Kim et al. recently reported a more detailed description of the course of heart changes of the same model at 4-month duration. Immediately after creation of mitral regurgitation, an increase of LV end-diastolic dimension and ejection fraction was observed, followed by LV thinning, progressive LV dilatation and finally by a modest decrease in ejection fraction by the 12th week. By the end of the study, end-systolic elastance was reduced and lung weights were increased, suggesting the onset of heart failure. Structural analysis of the myocardium showed perivascular fibrosis, increased apoptosis and upregulation of inflammatory genes (interleukin-6 and interleukin-16). Despite the fact that the authors did not provide any evidence for phosphodiesterase-5 upregulation in volume overloaded heart, chronic sildenafil therapy in this experiment reduced LV dilatation and LV mass, improved LV contractility, increased exercise tolerance, attenuated apoptosis (by 50 %) and inflammatory response [44]. This study suggests that enhancement of cyclic guanosine monophosphate (cGMP)-dependent signalling deactivates pro-hypertrophic pathways similarly as in pressure overload [72].

9.8 Possible Therapeutic Targets

Because volume overload is primarily a defect of hemodynamics, the most effective therapy is surgical correction of increased hemodynamic stress. In an experimental model of severe chronic mitral regurgitation in dogs, valve replacement restored previously depressed load-independent indexes of contractile function [133]. Similarly, Hutchinson et al. recently reported a reversal of LV ventricular structure and function at 4 weeks of volume overload due to aortocaval fistula using pressure-volume analysis. After closure of the shunt with aortic stentgraft, LV dimensions returned early to normal within 4 weeks, but LV systolic and diastolic function needed 11 weeks to normalise [87]. In clinical scenarios, a radical repair of hemodynamic defect is sometimes not possible and attempts to alleviate unfavourable course of the disease with drugs are then mandated. Potential pharmaceutical targets for therapy are briefly summarised in Table 9.4. It should be noted that the beneficial effects of drugs may be specific for a particular disease phase. For example, ACE inhibitors have no direct effect on the early phase of volume remodelling, but they attenuate renin-angiotensin system activation and decrease mortality in the late stage of ACF [134].

Table 9.4 Potential therapeutic targets in volume overload

Target	Model	Intervention	Early VO	Late VO	Reference
Circulatory defect	MiR, dog	Valve replacement	++	+	Spinale [135]
					Nakano [133]
	ACF, rats	ACF closure	++	+	Hutchinson [87]
					Gerdes [136]
					Abbasi [137]
Renin-angiotensin system	ACF, rats	Lisinopril	0	+	Oka [134]
		Enalapril	0	+	Ruzicka [108, 138]
	ACF, rats	Losartan	0	+	Ruzicka [108, 138]
	MiR, dogs	Irbesartan	0	?	Perry [139]
Mineralocorticoid receptor	ACF, rats	Spironolactone	+	?	Karram [140]
Endothelin system	ACF, rats	ET-A receptor antagonist	+	?	Francis [141]
Bradykinin system	ACF, rats	BK_2R antagonist Hoe 140	+	?	Ryan [89]
	ACF, rats	BK_2R antagonist Hoe 140	+	?	Wei [142]
Matrix metalloproteinases	ACF, rats	MMP-inhibitor PD166793	+	?	Ulasova [143]
β-adrenergic receptor	MiR, dogs	Metoprolol	+	?	Sabri [144]
	MiR, dogs	Atenolol	0	+	Tsutsui [86]
	ACF, rats	Carvedilol	+	?	Shyu [145]
	AoI, rats	Carvedilol	?	+	Zendaoui [66]
Mast cell degranulation	ACF, rats	Nedocromil	+	?	Brower [112]
	MiR, dogs	Cromolyn	+	?	Brower [109]
		Ketotifen	-	?	Pat [146]
Chymase	MiR, dogs	Chymase inhibitor	?	+	Pat [147]
Substance P/Neurokinin A	ACF, rats	Neurokinin receptor antagonist	+	?	Melendes [148]
TNF- α	ACF, rats	Infliximab	+	?	Chen [111]
	ACF, rats	Etanercept	0	+	Jobe [149]
Phosphodiesterase 5	MiR, rats	Sildenafil	?	+	Kim [44]

(continued)

Table 9.4 (continued)

Target	Model	Intervention	Early VO	Late VO	Reference
ROS-xanthine oxidase	ACF, rats	Allopuriol	+	?	Gladden [107]
ROS-monoamine oxidase B	ACF, rats	TVP 1022, rasagiline isomer	+	?	Abassi [150]
Ca^{2+} handling	MiR, pigs	SERCA2a gene transfer	?	+	Kawase [151]
Natriuretic peptides	ACF rats	Ecadotril (NEP inhibitor)	+	+	Wegner [152]
	ACF rats	Omapatrilat (ACE and NEP inhibitor)	+	0	Abbasi [153]
	ACF rats	Atrial natriuretic peptide	+	?	Abassi [154]
Vasopressin system	ACF, rats	V1a V2 receptor antagonists	+	+	Bishara [155]
Oestrogen receptor	ACF, rats	17β-oestradiol	+	+	Gardner [156]
Fatty acid metabolism	ACF, rats	Rosiglitazone	+	+	Goltsman [157]
	MiR, dogs	Rosiglitazone	?	+	Nemoto [158]
Fatty acid transport	ACF, rats	Propionyl-L-carnitine	?	+	Alaoui-Talibi [159]
Exercise training	AoI, rats	Moderate exercise	?	+	Lachance [67]

ACF aorto-caval fistula, *MiR* mitral regurgitation, *AoI* aortic insufficiency. *BKR* bradykinin receptor, *TNF* tumour necrosis factor, *ET-A* endothelin receptor type A, Early VO: volume overload <1 month, Late VO: volume overload > 1 month from the model induction; (+) positive effect, (0) no effect,(-) negative effect, (?) not tested. Due to space limitations, the list is not complete

9.9 Conclusions

Cardiac adaptation to a pathological increase in volume load differs in many ways from pressure overload or post-myocardial remodelling. The early response to volume overload is characterised by increased diastolic wall stress, activation of specific signalling pathways, cardiomyocyte elongation, inflammatory infiltration and degradation of extracellular matrix which allows for the increase of the chamber size. Volume overload leads to eccentric cardiac hypertrophy with increased ventricular volume, reduced relative wall thickness, enhanced diastolic function and relatively preserved systolic function. Although initially well tolerated, pathological volume overload is inevitably a progressive condition. As a result of neurohumoral activation, cardiomyocyte dysfunction and cell loss due to apoptosis, overt end-stage heart failure develops in the end. Understanding the pathophysiology of volume-overload cardiac remodelling is a mandatory step for the new development and proper timing of novel surgical and pharmaceutical therapies which may prevent or postpone the onset of heart failure.

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Chapter 10
Functional Adaptation During the Development of Cardiac Hypertrophy and Heart Failure in Females

Melissa R. Dent, Vijayan Elimban, Amrit Malik, Paramjit S. Tappia and Naranjan S. Dhalla

Abstract Cardiac hypertrophy is an adaptive response of the heart to hemodynamic overload, during which terminally differentiated cardiomyocytes increase in size without undergoing cell division. Although the hypertrophic response may serve to maintain cardiac function for a certain period, prolonged hypertrophy becomes detrimental, resulting in cardiac dysfunction and heart failure. It is well known that both cardiac hypertrophy and heart failure are associated with cardiac remodeling as a consequence of the activation of β-adrenoceptor systems and apoptotic pathways; however, gender disparities in the type and extent of cardiac hypertrophy and occurence of cardiac dysfunction exist. The risk of death from heart failure in women lags about 10 years behind men, but the gap in the incidence rates is narrowed with advancing age. The mechanisms for the gender differences in the development of cardiac hypertrophy and heart failure as well as the mechanisms involved in cardioprotection in adult females are not completely understood. This article highlights some of the gender-related differences in the β-adrenergic system and cardiomyocyte apoptosis with respect to cardiac remodeling in cardiac hypertrophy and heart failure. Furthermore, the role of ovaries and

A. Malik
Internal Medicine, Faculty of Medicine, St. Boniface Hospital Research, University of Manitoba, Winnipeg, Canada

P. S. Tappia
Asper Clinical Research Institute, Faculty of Medicine, St. Boniface Hospital Research, University of Manitoba, Winnipeg, Canada

V. Elimban (✉)
Institute of Cardiovascular Sciences, St. Boniface Hospital Research, 351 Tache Avenue, Winnipeg, Manitoba R2H 2A6, Canada
e-mail: velimban@sbrc.ca

M. R. Dent · N. S. Dhalla
Institute of Cardiovascular Sciences, Departments of Physiology, Faculty of Medicine, St. Boniface Hospital Research, University of Manitoba, Winnipeg, Canada

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estrogen replacement therapy in attenuating changes in β-adrenergic system, cardiomyocyte apoptosis, and cardiac remodeling during hypertrophy and failing stages of the female heart are also discussed.

Keywords Cardiac hypertrophy · Heart failure · Cardiac remodeling · Sex differences · β-Adrenergic system · Cardiomyocyte apoptosis · Estrogen

10.1 Introduction

Cardiac hypertrophy is defined as the change in phenotype, which includes the process of enlargement of ventricular cells [1]. When an excessive workload or hemodynamic overload on the heart is sustained, ventricular myocytes grow in response to a complex series of events; this cardiac hypertrophy is an adaptive mechanism by which the heart compensates for the increase in hemodynamic overload. Cardiac hypertrophy is associated with a variety of cardiovascular diseases (CVD) including myocardial infarction, cardiomyopathy and endocrine disorders. It is well known that different stimuli including mechanical stress, ischemia, and neurohormones activate multiple intracellular signaling pathways leading to the development of cardiac hypertrophy and subsequent heart failure. Hypertrophy occurs as an adaptation prior to chronic heart failure caused by excessive workload (pressure or volume overload), heart dysfunction and/or genetic mutation [2]. The remodeling of the myocardium during hypertrophy leads to an increased risk of ultimately developing cardiac failure [3]. Although cardiac hypertrophy may initially be a beneficial response that normalizes wall stress and maintains normal cardiac function, prolonged hypertrophy is considered to be a leading cause of heart failure and sudden death [4]. The transition of cardiac hypertrophy to heart failure is triggered by marked ventricular dilatation occurring once the myocardial hypertrophic response is exhausted [4]. It is noteworthy that heart failure is a medical condition in which the heart cannot pump enough oxygenated blood to meet the needs of other organs in the body. The loss in the pumping action of the heart is a result of an underlying cardiac defect [5, 6] and is the final clinical presentation of a variety of CVD. Patients presenting with Class III and IV heart failure have approximately 40–50 % probability of dying within 5 years after the symptom onset despite optimal therapy; this prognosis is worse than many cancers [7]. The progressive deterioration of left ventricular (LV) function is a characteristic feature of the heart failure state; however, the mechanisms responsible for myocardial deterioration are unknown and are attributed to a so-called vicious circle of compensatory mechanisms intended to maintain homeostasis. Ventricular remodeling (hypertrophy) and cardiac cell death (apoptosis) are considered to be the major factors that accelerate the process of LV dysfunction. Since females are more resistant to the processes associated with cardiac hypertrophy, cardiac apoptosis and heart failure in comparison to males [8–11], this article is focused on

the gender-dependent disparities in cardiac adaptation during the development of cardiac apoptosis, cardiac hypertrophy, and heart failure.

10.2 Apoptosis in Cardiac Hypertrophy and Heart Failure

Apoptosis, known as programmed cell death, is a physiologic and pathologic mechanism to allow for elimination of normal but no-longer useful or damaged cells [12]. It is an active process which is highly regulated and requires energy; however, it is silent and progressive [13]. Cardiomyocyte apoptosis is an important mechanism that may contribute to the development of heart failure due to myocardial infarction (MI) as well as heart failure of non-ischemic origin [14–20]. In addition, it has also been detected in atherosclerotic lesions of the vasculature [21]. The exact role of apoptosis in the heart is unknown; however, given the limited ability for self renewal of the myocardium, cardiomyocyte loss through apoptosis may profoundly influence cardiac structure and function. It should be noted that apoptosis is controlled by two main signaling pathways that process apoptotic stimuli; the intrinsic mitochondrial and extrinsic death receptor mediated pathways [22]. In the intrinsic mitochondrial pathway, the mitochondria generate the initial apoptotic cascade by releasing various proteins including cytochrome c [23–27], that in turn activate caspase 3 and 9 [28], and ultimately lead to cardiomyocyte apoptosis. Activation of caspase 9 is controlled both by the proapoptotic and antiapoptotic members of the *Bcl*-2 family [22]. Propagation and execution of apoptotic signals in cells is largely dependent upon active caspases [29]. It is generally believed that cytochrome c release by mitochondria is a crucial mediator of the mitochondrial cell death pathway by forming the apoptosome which allows for processing and activation of pro-caspase 9 leading to caspase 3 activation and eventual DNA fragmentation [29].

Bcl-2 proteins belong to a large family of homologous proteins that can either promote or suppress apoptosis [29]; these proteins have variable BH1–BH4 domains which functionally account for the given *Bcl*-2 family member to promote or prevent cell death. Proapoptotic members are subdivided into the *Bax* subfamily containing the BH1, BH2, and BH3 domains. *Bax* is a member of the "BH3-domain-only" subgroup of the *Bcl*-2 family of proteins; this group is responsible for the proximal death signal to the core apoptotic pathway [23, 30]. Recent studies suggest that *Bax* enter the outer mitochondrial membrane and subsequently forms a large conductance channel, which allows the release of cytochrome c [23, 30]. Furthermore, *Bax* also can induce transport of cytochrome c in liposomes [31]. On the other hand, *Bcl*-2 and other members of the antiapoptotic family are required for the binding of glucokinase to the mitochondrial complexes to protect the release of cytochrome c [27]. Mitochondria from cells that overexpress *Bcl*-2/*Bcl*-*XL* are resistant to loss of mitochondrial membrane potential and increased mitochondrial outer membrane permeability [32]. It is currently unknown how these *Bcl*-2 family members mediate these effects but it is

known that physical association with the mitochondrial membrane is required. Expression of *Bcl*-2 appears to be inversely related to the extent of apoptosis [33]. In contrast, the pro-apoptotic factor *Bax* binds to *Bcl*-2 and inhibits its protective effect [34].

It is known that cardiomyocyte apoptosis occurs during heart failure due to MI [14–16], pressure overload [17–19], as well as in other non-ischemic models of heart failure [20]. Previously, we have shown that there occurs a down regulation in the Akt dependent survival signal involving *Bcl*-2 whereas the proapoptotic protein *Bax* is upregulated; these alterations may play a role in the occurrence of cardiomyocyte apoptosis seen in heart failure due to volume overload [35]. Recently, Moorjani et al. [36] have found that activation of the cardiomyocyte apoptotic cascade occurs during the development of volume overload induced heart failure; specifically, *Bax*, p53 and caspases 3 and 9 increased progressively without completion of DNA fragmentation. There are many triggers to induce apoptosis such as angiotensin II and activation of its receptor (AT_1) that lead to apoptosis in adult rat cardiac fibroblasts; this was dependent on Ca^{2+} influx [37]. Angiotensin II induced a significant increase in *Bax* protein and decrease in *Bcl*-2 protein; losartan alone or in combination with simvastatin blocked the increase in *Bax* and caused an increase in *Bcl*-2 [38]. It has also been shown that treatment with a β_1-adrenoceptor (AR) antagonist in combination with a β_2-AR agonist further improved cardiac function and prevented cardiac remodeling as opposed to a β_1-AR antagonist alone in heart failure; this improvement is thought to be partially due to a downregulation in *Bax* and upregulation in the *Bcl*-2/*Bax* expression, indicating a reduction in cardiomyocyte apoptosis [39]. It is thus evident that angiotensin II through the activation of AT_1 receptors [37, 38] and catecholamines via the activation of β_1-AR [39, 40] participate in the genesis of apoptosis and heart failure.

10.3 Gender Differences in Cardiovascular Disease

Although heart failure is a significant health problem, its incidence and prevalence are expected to continue to rise with the ageing population [41]. Furthermore, gender differences exist in the prevalence of CVD and have been identified both clinically and experimentally. In this regard, pre-menopausal women show a reduced risk of CVD, but the incidence and severity of CVD increase after menopause [42]. Since women tend to develop heart disease some 10–15 years later than men, it can be argued that there are some special mechanisms in females which reduce the risk of developing heart disease. It has been suggested that female hormones such as estrogen serve as cardioprotective factors in women [43]. Female rats had 10-fold less mortality as compared to their male counterparts, and females, unlike males, showed no signs of heart failure [44]. Females have also been shown to be less susceptible and have improved survival following

myocardial ischemia–reperfusion injury; some studies have reported that this may be due to increased phosphorylated Akt and PKC ε levels [45, 46]. In fact, the resistance to ischemia–reperfusion injury has been seen in female dogs, rats, mice, and rabbits. Blood pressure levels are lower in female than in male spontaneously hypertensive rats and this is considered to be due to differences in vascular reactivity as well as the receptor gene expression for angiotensin II. It was reported that estrogen modulates AT_1 and AT_2 receptor gene expression and this may be responsible for the lower blood pressure seen in female spontaneously hypertensive rats. Female rats showed a different pattern of LV remodeling than males with less of an increase in thickness of the noninfarcted portions of the LV than males, but comparable LV cavity enlargement and systolic dysfunction. Despite similar infarct size, females developed less pronounced abnormalities of LV diastolic filling. In addition, when male and female healthy rats were given an intravenous bolus of epinephrine it was shown that males had a higher frequency and occurrence of epinephrine-induced premature ventricular contractions, missed beats, and blocks than female rat hearts; ovariectomy abolished the female advantage [47].

The incidence of heart failure is higher for men in all age groups when men and women are compared [48]; however, lifetime risk is similar in men and women because women tend to live longer [49]. Hypertension and diabetes represent the major risk factors for the development of heart failure in women, unlike men, and the clinical course of heart failure is generally more benign and more frequently characterized by preserved systolic function [48]. Women tend to be older and more hypertensive, but are less likely to demonstrate any clinical signs of coronary heart disease [49]. In fact, older women more often have diastolic dysfunction than men [48], which can present challenges in both its detection and treatment. The combination of obesity and hormonal disturbances in postmenopausal women is of serious concern [50]; indeed it has been shown that diabetic women have a 3–6-fold risk of MI whereas diabetic men are at an increased risk of 2–4-fold [50]. Following the induction of a mechanical load, women have a lower tendency to develop myocardial hypertrophy than men [51] and seem to be better protected against apoptotic death signals and showed a later onset of cardiac decompensation than men with heart failure [52]. There is gender bias in the diagnosis of heart failure because only 20 % of women are given an objective test for diagnosis [53]. Women are more frequently prescribed diuretics and digitalis and are much less often prescribed a combination therapy of the two [53, 54]. In fact, digoxin was associated with a significantly higher risk of death in women as compared to placebo [55] and diuretics may cause more adverse effects in women [56]. Older adult women with heart failure had significantly better survival than men irrespective of race [57]. In women, an increased LV mass index or degree of hypertrophy was a stronger predictor of mortality than traditional measures of LV size and function [58]. This situation underscores the need to perform either gender specific clinical trials or to include sufficient numbers of women in trials [50].

10.4 Mechanisms of Resistance to Cardiovascular Disease in Females

The mechanisms responsible for the cardioprotection observed in pre-menopausal women are far from clear; however, one of the main hypotheses is that estrogen may be responsible for this cardioprotection. Estrogen receptors (ERs) are believed to modulate hypertrophy and the progression of heart failure [50, 59, 60]. To support this, hormone replacement therapy was shown to reduce myocardial hypertrophy in women [61] and some of the proposed signaling pathways involve phosphorylation of intracellular kinases and production of nitric oxide. There are two main ERs, ER-α and ER-β; ER-α was found to be the predominant receptor in the adult rat heart whereas ER-β expression was more prominent in neonatal animals and then decreased later in life [62]. It has recently been shown that there is a significant upregulation in ER-α mRNA and protein and ER-β mRNA in human heart hypertrophy [50, 63]. 17 β-estradiol signaling modulates expression of natriuretic peptides in the atria and the LV of intact hearts and signals via mitogen activated protein kinases (MAPKs) in cardiomyocytes; it also increases expressions of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and connexin 43 [59, 64]. Specifically, estrogen may reduce the activity of a major hypertrophic pathway, the p38 MAPK pathway, by stimulation of its inhibitor MAPK phosphatase1 [65]. It has also been reported that chronic β-adrenergic stimulation caused severe cardiac injury in ovariectomized rats through calpain activation and impairment of Akt and eNOS signaling pathways [66]. Witt et al. [67] found that female specific genes were related to mitochondria and metabolism whereas those in males were related to matrix and biosynthesis. There was less downregulation of metabolic genes in female hearts and increased protein synthesis capacity and deregulation of matrix remodeling in male hearts characterize the gender differences in the early response to pressure overload [67]. In several experimental models, more severe cardiovascular phenotype in male transgenic animals or ovariectomized females can be rescued by the administration of estrogen; suggesting that estrogen prevents or at least slows down the development of cardiac hypertrophy and heart failure. Estrogen was responsible for attenuating the pressure overload-induced cardiac hypertrophy [68], and antagonized cardiomyocyte hypertrophy in vitro by ER-dependent mechanisms [50, 69]. Other investigators have found that there occurs a disproportionate amount of females with a more pronounced degree of remodeling in different forms of hypertrophic cardiomyopathy, but it should be noted that gender did not appear to influence the quantity of fibrosis [70].

Female cardiomyocytes were observed to exhibit a lower density of β-AR than male cells, and were less responsive to isoproterenol leading to reduced calcium influx after β-AR stimulation [71]. It has also been shown that there is a decreased β-AR density and β-AR inotropic response in the aging female heart [50]. Increased angiotensinogen levels, increased angiotensin converting enzyme activity and AT_1 expression and increased collagen content may play a role in

changing the myocardial structure and function in the aging female heart [50]. Another study has shown that estrogen was able to suppress cardiomyocyte apoptosis possibly through the PI3K/Akt pathway; it seems that ER-β mediates myocardial protection by upregulation of the PI3K/Akt and antiapoptotic signaling in the myocardium [46]. The most persuasive evidence in favor of a protective effect for estrogen comes from the large number of cohort studies comparing coronary heart disease risk in postmenopausal women currently using estrogen to never-users. These studies have shown consistently that coronary heart disease risk is 35–50 % lower in estrogen users after adjusting for other risk factors [72, 73]. Hormone therapy may only be beneficial when initiated early during a narrow window of opportunity around the time of menopause and before women develop an excessive buildup of atherosclerotic plaque. It should be mentioned that several studies have discounted any benefit of hormone replacement therapy and thus a great deal of caution should be exercised while initiating the hormone therapy in women of different age groups. Nonetheless, it is evident that the incidence of CVD is substantially lower in young women than in men, but increases disproportionally in women after menopause [74, 75].

In order to gain some detailed information regarding the mechanisms of gender difference with respect to cardiac hypertrophy and heart failure due to volume overload, we employed an experimental model of arteriovenous fistula (AV-shunt) in rats [8–11]. It was found that gender difference in cardiac function was due to differences in the type of cardiac remodeling due to volume overload whereas estrogen appeared to play an important role in preventing cardiac dysfunction and cardiac remodeling in female rats [8]. Furthermore, remodeling of myocardium in cardiac hypertrophy was not associated with cardiomyocyte apoptosis [9]. While the pro-apoptotic proteins, *Bax* as well as caspase 3 and 9 were increased, anti-apoptotic protein, *phospho-Bcl*-2 was decreased in heart failure due to volume overload in male rats. In contrast, females did not show heart failure or cardiomyocyte apoptosis upon inducing AV-shunt for a prolonged period; such cardioprotection in females was conferred by estrogen [9]. On the other hand, increases in β_1-AR, β_2-AR, G-protein coupled receptor kinase (GRK) and β-arrestin 1 mRNA levels as well as epinephrine-stimulated adenylyl cyclase activities were observed in female hearts. In contrast, a decrease in β_1-AR and increase in the inhibitory G-protein were detected in the hearts of male rats only. Both GRK2 and β-arrestin 2 gene expression were increased in male and female hearts [10]. In heart failure, both β_1-AR and β_2-AR protein and mRNA levels were decreased in the male, while increases were seen in the female hearts. Adenylyl cyclase protein content as well as the epinephrine-stimulated adenylyl cyclase activities were greater in the females. While adenylyl cyclase and β-arrestin 2 mRNA levels were decreased in males, mRNA level for GRK2 was increased in females. Treatment of ovariectomized rats with estrogen attenuated the AV-shunt induced changes in β-AR, adenylyl cyclase protein content and cardiac dysfunction. Thus, estrogen appears to play an important role in the upregulation of β-AR mechanisms and maintenance of cardiac function [11]. It should be mentioned that the significance of the AV-shunt-induced changes in some of the components of the β-adrenergic

system, particularly with respect to the increase in β_2-AR, is that upregulation of β_2-AR has been linked to cardioprotection and decreased cardiomyocyte apoptosis [76–78]. In addition, estrogen is reported to regulate cardiomyocyte β-AR gene expression [79]. Therefore, modulation of β-AR levels due to estrogen may represent a mechanism of cardioprotection against cardiac remodeling and cardiomyocyte apoptosis.

10.5 Conclusions

Sex differences in the type and extent of cardiac hypertrophy following volume overload due to AV shunt are considered to maintain cardiac function and delay transition to heart failure in females. Significant gender differences also exist in cell death and cell survival pathways following induction of volume overload. Females appear to have a lower level of cardiomyocyte apoptosis as compared to males, which is attributed to disparities in pro- and anti- apoptotic factors. From the aforementioned discussion, it is apparent that females, unlike males, are able to endure the over activation of the β-adrenergic system and thus prevent the occurence of down regulation of key components of the β-adrenergic system including β_1-AR and adenylyl cyclase as well as cardiac dysfunction. Estrogen appears to play an important cardioprotective role likely through attenuation of cardiomyocyte apoptosis and down regulation of the β_1-adrenergic system. It is evident that gender-dependent disparities in cardiac apoptosis, cardiac hypertrophy and heart failure exist; however, despite extensive work, the mechanisms for such differences still remain incompletely understood. Accordingly, it is suggested that a comprehensive study on the mechanisms of gender difference in cardiac hypertrophy and heart failure should be undertaken if we are to understand the mechanisms of cardiovascular adaptation in females during development of heart failure.

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Chapter 11
Impact of Gender and Exercise on Cardiac Adaptation to Pathological Situations: Sex Hormones, Exercise and Cardiac Adaptation

Feriel Azibani, Claude Delcayre and Jane-lise Samuel

Abstract The cardiovascular diseases are the leading cause of death in men and women in industrialized countries. The profound impact of biological sex on cardiovascular physiology or pathology has long been known, but the biological mechanisms responsible for sex-related differences have emerged more recently. Hormone therapy replacement is classically used to prevent cardiovascular pathologies in postmenopausal women but more recently, the beneficial effect of exercise begins to be taken into account. This review aimed to focus on the respective impact of sexual hormones, and/or exercise on the adaptive response of the heart to pathological situations such as severe pressure overload.

Keywords Estrogen · Heart · Exercise · Cardiac adaptation

11.1 Introduction

Cardiovascular diseases (CVD) are one of the leading cause deaths and hospital discharge diagnoses in the modern world. Major risk factors include the age, the presence of diabetes, hypertension, left ventricular hypertrophy (LVH), and/or

F. Azibani · J. L. Samuel (✉)
UMR-S942 Inserm and Univ-Paris Diderot, Lariboisière Hospital,
75010 Paris, France
e-mail: jane-lise.samuel@inserm.fr

J. L. Samuel
Lariboisière Hospital AP-HP, 75010 Paris, France

C. Delcayre
UMR-S942 Inserm and CNRS, Lariboisière Hospital, 75010 Paris, France

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myocardial infarction but the frequency distribution of risk factors differs according to the biological sex. In the Euro Heart Failure (HF) survey, systolic HF was found predominantly in men whereas women presented HF with preserved ejection fraction (EF) or diastolic HF [1–3]. Pre-menopausal women have a lower risk of CVD than age-matched men, but this advantage no longer applies after women reach menopause with about 2–4-fold more risk of ischemic heart disease than premenopausal women [4]. In fact, estrogen (E2) deficiency considerably increases the cardiovascular risk of hypertension, diabetes, and myocardial infarction [4, 5] supporting that ovarian hormones have a cardioprotective and antioxidant effect [6].

In human, the hormone replacement therapy (HRT) not only alleviates the metabolic consequences of menopause [7–9] but improves and/or maintains the cardiac performance [10]. However, the safety of HRT using synthetic estrogen and progesterone are subject of debates following the report of increased risk of heart disease, stroke, and venous thromboembolism with HRT in postmenopausal women [11]. Recently, the treatment of postmenopausal women starts to take into account the lifestyle patterns since adequate exercise and nutrition programs were shown to benefit in the prevention and the treatment of obesity, diabetes, and CVD in postmenopausal women [12].

In healthy subjects, exercise training is known to positively affect maximal VO2, hemodynamic function, peripheral vascular and muscle function. Regular physical exercise has also a beneficial impact on patients with chronic heart failure [13] and reduces significantly the incidence of myocardial infarction (MI) [14]. Thus, exercise training is recognized as a valuable assistant in the therapeutic approach to the HF patient [15].

In this review we aimed to focus on the respective impact of sexual hormones, and/or exercise on the adaptive response of the heart to pathological situations.

11.2 Sex-Based Differences in Cardiac Structure and Function in Adult

Before puberty there is no difference between men and women in terms of heart size, number and size of cardiomyocytes. However, after puberty the size of cardiomyocytes is higher in male than in female whereas the myocyte number is similar (review in [16]). With age, the number of cardiomyocytes significantly decreases in men (−64 millions/year) [17] through different processes including apoptosis and necrosis (review in [18]). Of note, women demonstrate a marked increase in the incidence of LV hypertrophy after menopause, when the prevalence of arterial hypertension increases [19]. This cardiac hypertrophy can be significantly prevented by HRT [16].

It is well assessed that beside E2 production by ovary, both men and women might synthesize E2 locally particularly in adipose tissue, through the conversion

of testosterone by aromatase [20]. As a matter of fact, E2 levels rise in older and/or obese men. Such elevated E2 levels have been proposed to decrease the risk for the development or progression of cardiovascular disease [21].

Recent studies demonstrated that E2 actions differ between the sexes with direct sex- and cardiomyocyte-specific effects in the heart [21]. The candidates to mediate sex-specific effects in the cardiovascular system are receptors for estrogen, progesterone, and androgens (ERs, PRs, and ARs, respectively) system. The two known ERs—ERα and ERβ—have been described in the human and rodent heart, review in [2]. ER, AR, and PR act by a number of genomic and non-genomic pathways. They are transcription factors able to initiate the transcription of hormone-sensitive genes or to modulate the activity of other transcription factors. ER, AR, and PR upon binding of hormones might activate or interfere with multiple signaling pathways including phosphatidylinositol 3-kinase (PI3K). In addition many cardioprotective genes such as Hsp (Heat Shock Protein) 72 or Hsp70 are upregulated either directly or indirectly by estrogen, review in [16].

One of the major cellular targets for the sex-based differences in the cardiovascular system is the endothelial cells, mainly through the modulation of the endogenous vasodilator nitric oxide (NO). At baseline, the endothelial NO synthase (NOS3) is present in both coronary vascular endothelium and cardiac endothelium. Regarding the control of NOS3 activity in endothelial cells, the estrogen plays a key role. Chambliss et al. [22] have shown in endothelial cells the non-genomic control of NOS3 via the ER-α within the caveolae. Estrogen absence significantly affects the NOS3 activity [23, 24]. As reviewed by Fleming and Busse [24] chronic changes in estrogen status can differentially affect NOS3 and caveolin-1 protein levels in endothelial cells. In estrogen-depleted rat heart, a significant reduction in NOS3 activity without change in the NOS3 expression but with enhanced NOS3-caveolin-1 interactions has been described [25]. Thus the estrogen-dependent NOS3-caveolin interactions play an important role in the control of NOS3 activity and in turn in the endothelium-dependent vasodilation.

In addition to the action on NO, the regulating effects of estrogen on artery myogenic tone appear to involve regulation of calcium-activated potassium (BKCa) channels [26, 27]. In line with these results, sex–based differences in coronary artery function have been observed in response to moderate increase in cardiac aldosterone production without alteration of cardiac function [28]. Indeed, a coronary dysfunction in aldosterone synthase-transgenic mice is observed only in male [29] and is demonstrated to be related to altered (1) BKCa channels expression in vascular smooth muscle, and (2) coronary BKCa-dependent relaxation [30]. Results from our laboratory indicate that estrogen might counteract the effect of hyperaldosteronism on the BKCa-mediated coronary relaxation (C Delcayre et al., unpublished data).

At the level of cardiomyocytes, there are sex-differences in excitation–contraction (E–C) coupling in cardiac cells from adult rats; Ca^{2+} transients are smaller and the gain of E–C coupling is lower in female cardiomyocytes than in male cells and in addition, aging-induced alterations of cardiac E–C coupling are more prominent in cardiomyocytes from males than in cells from females [31]. Other sex-based differences in intracellular calcium handling have been reported such as

phosphorylation state of phospholamban and L-Type Ca^{2+} channel density. Revisiting gender-related differences in K(+) currents in mouse ventricle, it has been recently proposed that downregulation of Kv4.3 and Kv1.5 transcripts by estrogen are one mechanism defining gender-related differences in ventricular repolarization [32].

Besides, sex-based differences are found also in the uptake of Ca^{2+} by cardiac mitochondria; mitochondria from female rat heart having lower Ca^{2+} uptake rates, for a review see [33]. In addition, female rats exhibit lower cardiac mitochondria content; they are more efficient and generate less H_2O_2 than the males. Finally female cardiomyocytes have a lower density of β-adrenergic receptors and thus also a decreased inotropic response to β-adrenergic stimulation [33]. Of note, across the life span various biochemical characteristics (including telomerase activity and several components of the insulin-like growth factor system) vary differently in male and female cardiomyocytes [34].

It has been clearly established that a decrease in ovarian hormones, in particular estrogen, promotes fat deposition and accumulation [35] and participate in the regulation of the pancreatic secretion of insulin, insulin sensitivity, and carbohydrate metabolism.

Data from observational and clinical trials indicate that estrogen has favorable metabolic effects by decreasing body weight gain and fat accumulation in both animals and humans [36, 37]. Thus, E2 deficiency increased heart and muscle lipid content with an increase in the atherogenic index [38, 39] and is associated with metabolic disorders such as insulin resistance and altered glucose metabolism [40–43]. Estrogen supplementation in ovariectomized rats lowered food intake [44, 45], decreased LPL activity of adipose tissue [46], and increased lipolysis [47].

In addition to the sex-based differences listed above, significant differences in the way male and female hearts respond to various challenges bring important insight into the mechanisms by which female gender may influence favorably the remodeling and the adaptive response to myocardial insult.

11.3 Sex-Based Differences in Cardiac Benefit Following Exercise Training

Randomized controlled trials have documented the benefits of exercise training on body weight, muscle strength and endurance, oxygen consumption, systolic blood pressure (SBP), and metabolic control in postmenopause women. Table 11.1 summarizes the most important clinical studies on exercise training in postmenopausal women.

It is important to point out that exercise reveals clearly sex-related differences in both healthy subjects and patients with asymptomatic aortic stenosis despite similar hemodynamic properties of the heart at rest [48–50]. During exercise, the

Table 11.1 Clinical studies on exercise training action in postmenopausal women

Clinical status	Training protocol	Findings	References
Healthy	Long-term lifestyle dietary and physical activity	Decreased waist circumference and weight gain	[136, 137, 138]
Healthy	Two daily walking exercise training	Decreased diastolic blood pressure and blood glucose	[139]
Healthy ± HRT	High intensity endurance training	Decrease of total and regional body fat values than HRT alone.	[140]
Healthy ± HRT	Resistance training program (2 days/week)	Preventing of menopause-related osteoporosis and sarcopenia by training better than with HRT alone. There was no additional benefit in combining HRT with exercise training.	[141]
Overweight	Moderate-intensity sports/ recreational activity or biking and walking for transportation	Lower body fat and less central adiposity	[142, 143]
Overweight or obese	Moderate-intensity exercise (walking or 45 min moderate-intensity aerobic activity 5 days/week)	Improvement of insulin resistance	[144]
Obese with elevated blood pressure	Three different doses of aerobic exercise training	Improvement of blood pressure and endothelial function	[145]

HRT hormone therapy replacement

ejection fraction trends to increase more in men than in women whereas the cardiac output increases similarly. Women tend to increase their cardiac output primarily by increasing end-diastolic volume index without significantly increasing ejection fraction whereas men primarily decrease end-systolic volume index and raise ejection fraction (reviews in [16, 50]). Possible interactive effects exist between exercise training and female sex hormones on cardiac performance [51–54]. For example, Bupha-Intr et al. [55] demonstrated that regular exercise maintains the molecular activation of cardiac SR Ca^{2+} uptake under normal physiological conditions and is able to induce a protective impact on cardiac SR Ca^{2+} uptake in E2-deprived status through regulation of SERCA expression and PLB phosphorylation. Of note, exercise training had no effect on female intact mice. Such results support the idea that exercise training exerts much more benefit on cardiac function after menopause. However, the clinical studies revealed more conflicting results. According to Swank et al. [56], who analyzed the influence of gender and age on the effects of resistance training and aerobic training, both men and women significantly increased their strength together with decreased levels of

SBP and heart rate. Endurance training in healthy postmenopausal women, who remain in energy balance, results in many of the classic cardiopulmonary training effects without a reduction in body weight [57]. Larger study indicates that adding aerobic exercise training to caloric restriction will result in the greatest metabolic benefits for overweight and impaired glucose-tolerant obese postmenopausal women [58]. In addition, exercising reduces inflammation and cell adhesion molecules in postmenopausal women [59].

Experimental studies clearly demonstrated the gender influence on exercise effects on cardiac structure and function. In line with the gender dependent expression of Hsp72 described above, Paroo et al. [60] proposed that males have greater exercise-induced cardiac Hsp70 than do females probably because of the attenuated signaling of Hsp70 induction by estrogen. In E2-deprivated rats, swimming exercise was able to prevent the shift in cardiac myosin heavy chain (MyHC) isoenzymes without effect on female animals [61].

In addition, exercise training in estrogen-deficient rats improved resting hemodynamic status and arterial baroreflex sensitivity, probably associated with oxidative stress reduction [62]. However, the exercise type may influence the responses: aerobic training increased high frequency oscillations, whereas, resistance training produced no effect [63].

Experimental studies also demonstrated that exercise training can restore cardiac reserve function and all antioxidant levels in estrogen-deficient animals [64, 65]. Chronic exercise heightened the expression of natriuretic peptide (NP) systems, iNOS, and eNOS, which may play a role in the cardioprotection role of exercise training [66, 67]. Interestingly, chronic exercise also enhanced iNOS expression in normal and estrogen-deficient rats, whereas the effect on eNOS was observed only in control animals [68]. Such training in female rats induced cardiac hypertrophy and a decrease in myofilament Ca^{2+} sensitivity without changes in myofibrillar ATPase activity and MyHC pattern of expression. Conversely, under estrogen deficiency, the running program prevented the β-MyHC expression and induced an increase of maximum myofibrillar ATPase activity [52].

Several of the metabolic disorders provoked by the E2-deficiency (as described in 1) are prevented by exercise training [69, 70]. In fact, resistance training decreased lipid content in the liver and skeletal muscle, decreased mesenteric fat, and changed the lipid profile, independently of estrogen status [71]. There is a growing evidence that these effects are dependent on AMP-activated protein kinase (AMPK) pathway [72, 73]. Estrogen-deprivated rats submitted to short-term resistance exercise exhibited an improvement in insulin sensitivity [74, 75]. One of the best evidence of the effects of exercise training on estrogen deficiency-induced insulin resistance comes from Saengsirisuwan's study [76] who showed that estrogen-deprivated rats developed a metabolic syndrome. They also provided evidence that insulin resistance is corrected by endurance exercise training alone and estrogen replacement alone. As a matter of fact, no additive effect was observed in response to both treatments. These results are in line with the observations that transcripts encoding estrogen receptor in cardiac muscle and liver are upregulated by regular exercise [77]. Pighon et al. [78] found that

exercise training acts as estrogen supplementation for properly decreasing in liver several genes of lipogenesis (SREBP-1c, ChREBP, SCD-1, ACC) as well as decreasing several biomarkers of inflammation (IL-6, NFkB, TNFα) in estrogen-deprivated rats.

11.4 Cardiac Adaptability to Pressure Overload, Impact of Sex-Based Differences

Hypertrophy *per se* is an independent risk factor for heart failure (HF) and sudden death, review in [18]. Mechanical pressure overload being secondary to either hypertension or to aortic stenosis, we will refer to both etiologies in this chapter. Albeit, we know for a long time the sex-specific ability of the myocardium to adapt to mechanical overload [79, 80], sex differences in cardiovascular disease have been receiving increasing interest in the recent past [2, 16, 25, 33, 81–83]. It is well assessed that premenopausal women have a better prognosis than do men in response to hypertension and to aortic stenosis [49]. Based on clinical trial, lines of evidence indicated that heart failure with normal ejection fraction (HFNEF) is much more common in women than in men [3, 84, 85]. It emerged from Devereux study that patients with HFNEF were older and overweighted, in majority women, had renal dysfunction, impaired early diastolic LV relaxation, and concentric LV geometry. Conversely, patients with congestive heart failure and severe LV dysfunction were more often men exhibiting a restrictive pattern of LV filling, and eccentric LV hypertrophy. Other studies provide more nuanced results, suggesting equal or greater prevalence of diastolic dysfunction in men, review in [3]. Thus, the greater rate of HFNEF in women might be related to sex-based differences in ventricular diastolic distensibility, in vascular stiffness and ventricular/vascular coupling, in skeletal muscle adaptation to HF, and in the perception of symptoms [3]. When focusing on patients with aortic stenosis (AS), review in [81], women and particularly elderly ones develop a more concentric form of hypertrophy than men, with smaller ventricular diameters and less ventricular dilatation. In some, but not all studies, women had higher transvalvular gradients, greater relative wall thickness, and better systolic function. Furthermore, women with congestive heart failure have also been shown to survive better than men in some studies [81]. Interestingly enough, when analyzing hypertrophy regression after aortic valve replacement LV hypertrophy reversed more frequently in women than in men [82].

Experimental data confirm and extend these sex-based differences in the development of cardiac hypertrophy to pressure overload. At the very early phase of a mechanical pressure overload female rats develop more cardiac hypertrophy than male [79, 83] and that only male exhibit signs of acute heart failure [83]. In mice, the sex-based differences were observed only at later stages of cardiac hypertrophy (2 weeks after surgery) [86]. Thus, the differences in the adaptation of

female and male hearts to pressure overload draw attention on the underlying mechanistic pathways.

The sex-based differences in remodeling of the whole heart are mirrored by differences in signaling pathways and/or patterns of gene expression. One of the pioneer work on sex-based differences in cardiac response to thoracic aortic stenosis (TAC) demonstrated that β MyHC expression is greater in male than in female hypertrophied hearts, whereas the SERCA mRNA levels are depressed in male only, these transcriptional changes being associated with a preserved contractile reserve in female hypertrophied hearts [87]. In contrast, mRNA microarray analysis using the TAC model in mice do not evidence differences in a selection of hypertrophy markers, such as α-actin, ANP, and BNP [86, 88]. However, several genes controlling mitochondrial function including PGC-1 had lower expression in males [86]. A whole female/male gene network analysis reveals that female-specific genes are mainly related to mitochondria and metabolism and male-specific ones to extracellular matrix and biosynthesis [86]. Of note, the number of differentially regulated genes in response to acute pressure overload is greater (>2-fold) in males than in females, whereas the response to chronic pressure overload is similar between males and females [88].

Ten years ago it was elegantly demonstrated that estrogen might prevent the hypertrophic response to Ca^{2+} dysregulation such as that induced in FKBP12 deficient mice [89]. Estrogen can prevent development of cardiac hypertrophy indirectly by counteracting hypertension, directly by triggering the release of ANP [90–92], and by blocking the p38—mitogen-activated protein kinase (MAPK) phosphorylation [91]. Conversely E2-deficiency enhanced adverse cardiac remodeling (capillary rarefaction, cardiomyocyte hypertrophy and loss) in pressure overloaded rats [25, 93].

Marked sex-based differences in the development of fibrosis associated with cardiovascular disease, and particularly with pressure overload, have been observed both in human [94] and in experimental models [86, 95]. In these microarray analysis [86, 95], genes associated with extracellular matrix remodeling exhibited relative lower expression in female heart (collagen 3, MMP 2, TIMP2, and TGFbeta2) after TAC in mice. Although the molecular mechanisms underlying gender dimorphism are complex and are still not well understood, it emerged that sex steroid hormones and their respective receptors play a key role, review in [96]. It is established that estrogen reduces the turnover of the extracellular matrix, especially the collagen network [97, 98]. Insights into the sex-specific regulation of fibrosis-related genes were brought using genetic models and in vitro approaches. ß-estradiol significantly increases collagen-I and -III gene expressions in male fibroblasts and had opposite effects in female cells [82]. Using the genetic model ER-β(-/-), it is demonstrated that sex-based difference in cardiac fibrosis after TAC was abolished in ER-β (-/-) mice [95]. The sex-based differences observed in the regulation of genes encoding ECM proteins and metalloproteinase might represent one of the major mechanisms slowing the progression to heart failure in female.

Other lines of evidence of estrogen-induced cardioprotection were provided by studies devoted to NO bioavailability and/or endothelial function. It has been demonstrated that NO had direct systolic as well as diastolic myocardial effects, review in [99]. The reduction in the bio-availability of NO is a key feature of endothelial dysfunction, classicaly described during heart failure. Besides, role of the protein–protein interactions, or post-translational modification controlling NOS3 activity such as caveolin-1 binding, NOS3 phosphorylation have been proposed [100, 101] to modulate NOS3 activity. At least, the NO reduction could be due to the increased peroxynitrites or to NOS3 decoupling, review in [99]. In response to a severe TAC, gender differences in changes in NOS3 activity are observed [83]. In female rat, the NOS3 activity in the hypertrophied heart remains constant before appearance of HF signs [25]. After TAC, the absence of estrogen prevents the increase in NOS3 expression and, worsens the cardiac dysfunction without affecting the development of cardiac hypertrophy. These data highlight the role of NOS3 through the estrogen in the cardiac adaptation to new load conditions [25].

Besides the putative role of NOS3-derived NO, the implication of NOS1-derived NO have been demonstrated during the development of cardiac hypertrophy and failure [102–104]. Thus, male mice lacking both NOS isoforms NOS1/3(-/-) have 2-fold increased mortality compared to females. Notably, the development of cardiomyocyte hypertrophy and interstitial fibrosis with age in NOS1/3(-/-) mice is independent of the gender [105].

Conversely to failing heart, in rats as well as in human, the sub-cellular relocalization of NOS1, is not observed in hypertrophying-hearts following TAC [102, 103]. Sex-based differences have been observed: NOS1/caveolin-3 association is significantly higher in female versus male in response to cardiac injury in mice [106] or following pressure overload in rats [83]. In these models, estrogen *per se* modulates neither NOS1 expression nor activity whatever the hypertrophy status [25]. Thus, according to Murphy et al. [107] the increase in NOS1 near caveolin-3 in females under stress conditions (I/R) associated with increased calcium (which activates NOS) results in increased S-nitrosylation of the L-type calcium channel, less calcium entry and therefore less calcium loading, constituting a cardioprotective mechanism as previously discussed [108].

In addition it was demonstrated that NOS1 expression in cardiac muscle following TAC was independent of estrogen level [25]. It is proposed that the mechanotransduction pathway is mainly involved in the induction of NOS1, whereas the ER pathway regulates NOS3 activity and in turn cardiac function.

The association of natriuretic peptides (NPs) such as BNP, ANP with gender was examined in several studies, despite disparity, some studies report a higher BNP concentration in females compared to males. Indeed, in normal patients, NT-proBNP, like BNP, tends to be higher in female patients and older individuals, through mechanisms involving either the clearance receptor for BNP or increased expression [109, 110]. On the other hand, a population-based study indicated that in women, LV mass and NP concentrations increase to a lesser extent and only with severe LV dysfunction when compared to men [111]. Regarding

postmenopausal women, HRT has been associated with higher BNP levels, suggesting that BNP expression is sensitive to estrogen [110]. In line with these findings, it has been shown that in vitro estrogen exerts antihypertrophic effects on cardiomyocytes, by transactivation of the ANP gene [112, 113]. Estrogen-induced ANP accumulation in the ventricular cardiomyocytes most likely results in ANP receptor activation in an autocrine/paracrine manner [112] leading to an increase in cGMP that prevents cardiomyocyte hypertrophy [113].

Taken together it emerged that the tight regulation of NP expression is of importance for the sex-based differences in the development of cardiac hypertrophy.

11.5 Sex-Based Differences in Cardiac Benefit Following Exercise Training During Cardiac Pathological Situation

Conversely to data regarding normotensive postmenopausal women [58], a prospective, randomized, controlled exercise trial indicated that exercise training had no major impact on the cardiometabolic risk profile of overweight or obese, postmenopausal women with moderately elevated SBP, despite considerable improvements in maximal oxygen consumption [114]. This contrast with data in aging men indicating that progressive resistance training can be used as antihypertensive therapy as well as for control of metabolic diseases such as obesity or Type 2 diabetes [115].

The 4-fold higher prevalence of hypertension is observed in postmenopausal women than in premenopausal women [116] not only underlined the role of estrogen deficiency to induce endothelial and vascular dysfunction, but also highlighted that exercise training might bring beneficial effects in this context. In rats, 8–13 weeks of exercise training dramatically reduced systolic blood pressure of both normo- and hypertensive E2-deficient rats [62, 93]. Postmenopausal women who engage in intermittent, moderate-intensity physical training experience demonstrate a significant reduction in SBP [117]. However, the best program of exercise training to achieve SBP lowering is still debated [118], requiring additional study better taking into account the previous physical activity and the hormonal status of women.

Regarding ischemic disorder, in rats, the endurance exercise protects myocardium against ischemia–reperfusion (I–R) injury by improving the recovery of left ventricular pressures, by preserving coronary blood flow, and by reducing oxidative stress in cardiomyocytes [119–121]. A majority of AS mice that overexpress aldosterone in the heart [29] dies after 3 weeks of intensive swimming (stress-induced exercise). All (100 %) AS males died whereas less than 20 % of WT male did. Interestingly, estrogen appears to slightly protect AS mice, since 20 % of survival of female AS was noticed after swimming period (Delcayre et al.

Table 11.2 Exercise training effect on cardiac structure and function in physiological and pathological conditions

	Basal conditions		Hypertension	
	+E2	−E2	+E2	−E2
SBP	↓	↓	=	↓
Heart rate	↓	↓	ND	ND
β/α − MyHC	=	↓	ND	ND
ANP, BNP	=	↑	ND	ND
eNOS	↑	=	ND	ND
Myofilament Ca^{2+} response	=	↓	ND	ND
Cardiac hypertrophy	↑	↑	=	↓
Hsp	=	↑	–	↑
Fibrosis	ND	ND	↓	↓
Inflammation	ND	↓	ND	ND
Insulin resistance and glucose intolerance	=	↓	ND	ND
Vascularization	ND	ND	↑	↑
SERCA expression	=	↑	ND	ND
Baroreflex sensitivity	↓	↓	ND	ND
Heart lipid content	↓	=	ND	ND

ANP atrial natriuretic peptide, *BNP* brain natriuretic peptide, *E2* estrogen hormone, *eNOS* endothelial nitric oxide synthase, *Hsp* heat sock protein, *ND* not determined, *SBP* systolic blood pressure, *SERCA* sarco/endoplasmic reticulum Ca^{2+} -ATPase

Unpublished data) confirming the role of estrogen in the control of coronary function. A short-term exercise reduced myocardial infarct size following ischemia–reperfusion injury only in male rats [51]. Also in SHR rats, exercise training reduced blood pressure only in males [53]. Among the molecular and biochemical mechanisms responsible for this protection, it has been postulated that these exercise-induced changes in the myocardium may result from local increases in antioxidant defenses and/or levels of Hsp [119, 120, 122]. Exercise training increases also antioxidant defenses through the expression of catalase and glutathione peroxidase [123], and nitric oxide (NO) bioactivity through an enhanced NOS3 expression [124, 125].

Beside the decrease in oxidative stress, exercise training by increasing transiently synthesis and release of atrial natriuretic peptide (ANP) as shown in humans and animals [126–130] might trigger the diuretic, natriuretic, and vasorelaxant effects of the hormone together with its anti-inflammatory, antihypertrophic, and antifibrotic properties [131, 132]. Exercise training also reduced levels of the inflammatory cytokines (TNF—alpha and IL-6) [133, 134]. The increase in both ANP and NO bioactivity likely result in increased cGMP, which exerts beneficial effects on the heart, is associated with a reduced risk of cardiovascular diseases.

Recently, a new hypothesis was proposed by Gutkowska et al. [68]. According to their study, the cardioprotective effects of exercise training may be due, at least in part, to the stimulation of the cardiac oxytocin system, which in turn will induce locally the subsequent upregulation of the ANP and NO systems and thus trigger some beneficial effect.

Interestingly, Marques et al. [93] demonstrated that exercise training prevent the adverse remodeling observed in estrogen-deficient SHR rat. A major effect of exercise training was the prevention of estrogen deprivation-enhanced myocyte loss in SHR. It is hypothesized that as under normal condition, the regular exercise induces a protective impact on cardiac SR Ca^{2+} uptake in E2-deficient [54]. Additional benefit arised through their BP-lowering effects by increasing the capillary density in heart and muscle and physiological cardiac hypertrophy activation (AKT pathway). Thus exercise training results in maintenance and/or upregulation of the heart's oxygen supply through adapted angiogenesis and hypertrophy [135]. Thus, exercise training has beneficial effects diminishing the adverse remodeling induced by pressure overload, mainly by reducing the interstitial myocardial fibrosis, improving the myocardial vascularization, and sustaining the number of cardiomyocytes.

11.6 Conclusion

In summary, all these studies demonstrate the important role played by exercise in the prevention and treatment of CVD and comorbities (obesity, diabetes) particularly in postmenopausal women by maintaining homeostasis of various subcellular and molecular functions of cardiomyocytes (Table 11.2).

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Chapter 12
Cardiopulmonary Adaptation to High Altitude

Jean-Paul Richalet

Abstract The reduction in inspired oxygen pressure with increasing altitude triggers a full array of mechanisms allowing the human body to adapt to the lower oxygen availability. The adrenergic system is activated via the stimulation of peripheral chemoreceptors, leading to an increase in heart rate and cardiac output. With acclimatization, the cardiac response to adrenergic activation is blunted leading to an autoregulation of cardiac function that protects the heart against an imbalance between O_2 availability and O_2 consumption. Altogether, the cardiac inotropic function in normal subjects is not altered by altitude exposure, while diastolic filling might be slightly impaired. Hypoxia induces a pulmonary vasoconstriction that may be harmful when pulmonary hypertension develops, favoring the occurrence of High Altitude Pulmonary Edema. No major change is observed in the peripheral circulation except a transient increase in cerebral blood flow. High altitude natives may develop with aging an excessive polycythemia frequently associated with pulmonary hypertension that might lead to cardiac failure. In sea-level cardiac patients, oxygen lack might be detrimental although there is very little evidence of aggravated cardiovascular diseases at least at low and moderate altitudes.

Keywords High altitude · Heart · Pulmonary circulation · Hypoxia · Adrenergic system · Downregulation of β-adrenoreceptors · Cardiac patients · Pulmonary hypertension · Heart failure

J.-P. Richalet (✉)
UFR SMBH, Université Paris 13, 74 rue Marcel Cachin,
93017 Bobigny Cedex, France
e-mail: richalet@smbh.univ-paris13.fr

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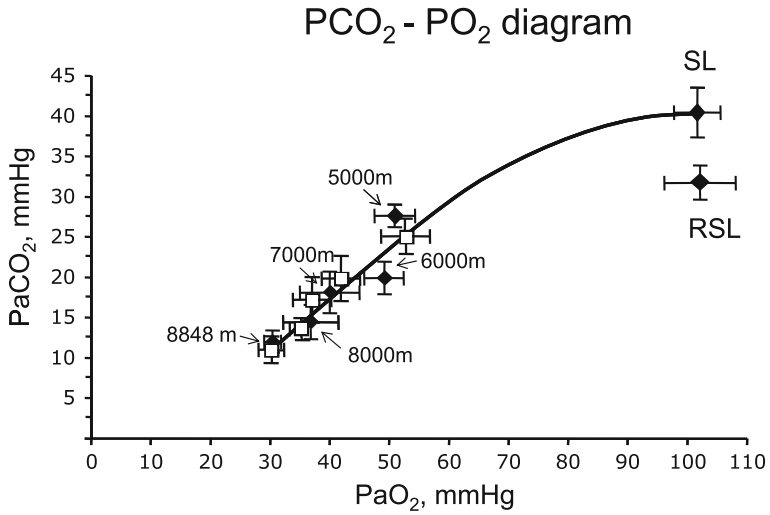

Fig. 12.1 Resting PaO_2 and $PaCO_2$ at various altitudes during of Operation Everest III (*filled diamonds*, arterialized blood gases) and Operation Everest II (*open squares*, arterial blood gases). SL: sea level; RSL: 2 days after return to normoxia (Adapted from [7]).

12.1 Introduction

The reduction in barometric pressure with altitude induces a parallel reduction in inspired O_2 pressure that triggers hyperventilation, which will result in hypocapnia and alkalosis. Therefore, the environment of high altitude can be characterized, from a physiological point of view, as hypoxic and hypocapnic (Fig. 12.1).

Since 1978 when Reinhold Messner and Peter Habeler reached, for the first time, the summit without oxygen, only 142 climbers (until 2010) realized the same achievement with success. It is important to note that the mortality among this category of high altitude climbers is around 8 %, suggesting that exercising with an arterial PO_2 of around 30 mm Hg or less for hours causes severe damages and is a threat to life. Does it cause a specific damage to the heart? We will try to discuss this point in the present review.

Living for a few days above 8,000 m and even exercising at higher altitudes is possible only if time is given to the organism for acclimatization to hypoxia. Bringing a non-acclimatized human being by helicopter to the top of Everest without supplementary oxygen leads to inevitable death within minutes. One-month acclimatization allows surviving and even exercising at the same altitude. All the processes that make possible this different response to the same constraint (adaptation process) is called “acclimatization”, the term “adaptation” being generally limited to the permanent, especially genetic, changes. These processes are accompanied by a decrease in the overall energy cost of the responses to the hypoxic stress [1].

A complex machinery of responses is triggered when an aerobic organism is exposed to hypoxia, involving all cells through the activation of genes presenting a Hypoxia Responsive Element (HRE). Many factors respond to hypoxia, such as HIF-1α (hypoxia inducible factor), VEGF (vascular endothelial growth factor), EPO, etc. These factors are responsible for initiating physiological processes that, for some of them, will reduce the level of tissue hypoxia [2]. One of the principal response systems to hypoxia is triggered by the stimulation of the peripheral chemoreceptors and involves, from one side the activation of ventilatory control centers, and from the other side the activation of the cardiovascular control centers. The stimulation of the adrenergic nervous system is responsible for the acute cardiovascular response to hypoxia, i.e., tachycardia and increase in cardiac output that will try to compensate the acute decrease in blood oxygen content. As one of the most powerful responses to the hypoxic stress, the sympathetic system and its counterpart, the parasympathetic system, will play a crucial role in the cardiovascular adaptation to acute and chronic hypoxia, especially during exercise.

On the vascular side, hypoxia has contrasting effects on systemic and pulmonary circulation: vasodilation in most organs, vasoconstriction in pulmonary circulation. Hypoxic pulmonary vasoconstriction and vascular remodeling may have deleterious effects and may lead to pulmonary hypertension and cardiac failure.

Finally, we will rapidly review the possible limitations induced by hypoxia for cardiac patients who aim to visit regions of high altitude.

12.2 Adaptation of the Heart to Altitude Hypoxia

The cardiac response to hypoxia is conditioned by the activation of the adrenergic system via the stimulation of the peripheral chemoreceptors and the afferent fibers to the medullary cardiac centers.

The activation of the adrenergic system in acute hypoxia has been evidenced by direct and indirect observations. Plasma and urine norepinephrine concentrations have been found elevated in most studies performed in acute and chronic hypoxia, at rest or at a given absolute level of exercise [3, 4]. The direct measurement of the activity of adrenergic fibers has also evidenced an increase in sympathetic activity in hypoxia [5, 6].

Operation Everest III is interesting to consider for illustrating the effect of prolonged and severe hypoxia on cardiac function in normal healthy subjects [7, 8]. Eight male volunteers, aged 23–37, were selected to participate in a simulated ascent to 8,848 m in a hypobaric chamber. The chamber was progressively decompressed down to 253 mm Hg barometric pressure (equivalent to the altitude of Mount Everest), with a recovery period of 3 days at 5,000 m from day 20–22. They spent a total of 31 days in the chamber. Cardiac function was evaluated by echocardiography at rest up to 8,000 m. Aortic and left atrial diameters, left ventricular (LV) diameter, right ventricular end-systolic diameter fell progressively

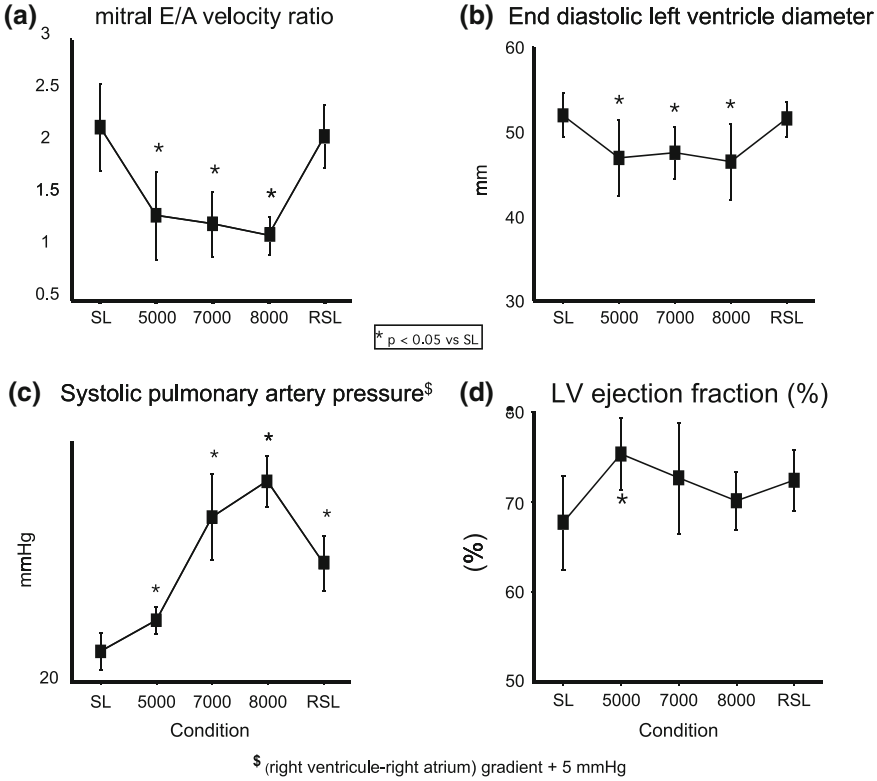

Fig. 12.2 Cardiac function during Operation Everest III. **a** mitral E/A velocity ratio (index of early/late ventricular filling); **b** end-diastolic left ventricle diameter (index of LV filling); **c** systolic pulmonary artery pressure; **d** left ventricle ejection fraction (index of contractility). Note that contractility is normal, pulmonary pressure increases and filling of left ventricle is slightly impaired (Adapted from [8]).

with increasing altitude. Mitral peak E velocity (early LV filling) decreased, peak A velocity (filling due to auricular contraction) increased, and E/A ratio (an index of early/late LV filling) decreased (Fig. 12.2a). Systolic pulmonary arterial pressure increased: the right ventricular—right atrial gradient increased from 19 ± 2.4 mm Hg at sea level to 40.1 ± 3.3 at 8,000 m, and remained elevated 2 days after return to normoxia (Fig. 12.2c). All indices of LV contractility were normal (Fig. 12.2d). However, there was a modification of the LV filling pattern, with a decreased early filling and a greater contribution of the atrial contraction, without elevation of LV end-diastolic pressure (Fig. 12.2b). These modifications of LV filling, assessed for the first time in hypoxia, could be an adaptive response to tachycardia and reduced preload, or could be the consequence of an impaired LV relaxation due to ventricular interdependence or hypoxia per se [8]. Tissue Doppler imaging was also used to evaluate left and right ventricular diastolic function at high altitude and confirmed altered diastolic filling pattern although it is not clear if

the observed changes are adaptive to the increase in RV afterload or express an alteration in diastolic function [9, 10]. Healthy volunteers ($n = 14$) were studied immediately before, and within 4 d of return from a 17-d trek to Mt. Everest Base Camp (5,300 m) [11]. Left ventricular mass, evaluated by magnetic resonance imaging, adjusted for changes in body surface area, had decreased by 11 %. Alterations in diastolic function were also observed, with a reduction in peak left ventricular filling rates and mitral inflow E/A. Compared to pretrek, cardiac phosphocreatine (PCr)/ATP ratio, measured using ^{31}P magnetic resonance spectroscopy, had decreased by 18 %. All variables had returned to pretrek levels after 6 months. To confirm these observations, the same authors exposed 12 healthy male volunteers to 20 h of normobaric hypoxia. Hypoxia caused a 15 % reduction in cardiac PCr/ATP and a parallel decrease in diastolic function [12]. It is therefore suggested that the reduction in LV mass was due to a reduction in protein synthesis and an adaptation of the heart muscle to decrease oxygen diffusion distance. The reduction in PCr/ATP observed in acute [11] or chronic [12] hypoxia might be linked to a decreased mitochondrial oxidative capacity in response to low oxygen supply.

Acclimatization to hypoxia induces polycythemia secondary to erythropoietin (Epo) release by the kidney to increase arterial oxygen content [13]. However, despite a marked increase in O_2 carrying capacity, VO_{2max} changes little after acclimatization [14, 15]. This lack of effect of acclimatization on VO_{2max} partly attributed to a decrease in maximal cardiac output (Q_{max}) observed in both rats and humans which offsets the increase in arterial blood O_2 content [16, 17]. Supporting Q_{max} as a factor limiting VO_{max} is the observation that increasing maximal heart rate (HR_{max}) and Q_{max} by cardiac pacing increases VO_{2max} of rats acclimitized to prolonged hypoxia [18].

The reduction in Q_{max} in prolonged hypoxia has been attributed to several factors [19]: (1) reduction in blood volume and cardiac filling, (2) increased blood viscosity and vascular resistance, (3) alterations in control by the autonomous nervous system, and (4) passive reduction due to a tight coupling between muscle O_2 consumption (which is reduced for metabolic causes) and cardiac output. The reduction in HR_{max} at high altitude has been observed by many authors (review in [3] and [20]). Above 4,000 m, the reduction in maximal cardiac output and heart rate becomes an important limiting factor. However, the importance of this reduction has been debated since the advantage of rising cardiac output to increase O_2 transport to the periphery can be offset by the disadvantage of increasing diffusion impairment in the lungs [19]. The mechanisms of reduction of HR_{max} are to be looked for in the control of the cardiovascular system by the autonomous nervous system.

In humans as well as in other mammals, prolonged hypoxia tends to reduce exercise heart rate while circulating catecholamines remain elevated [21]. These results can suggest either a decrease in the responsiveness of the adrenergic system to stimulation or an increase in parasympathetic activity [22–24]. Both hypotheses can be validated by some experimental evidence that have been recently reviewed [25].

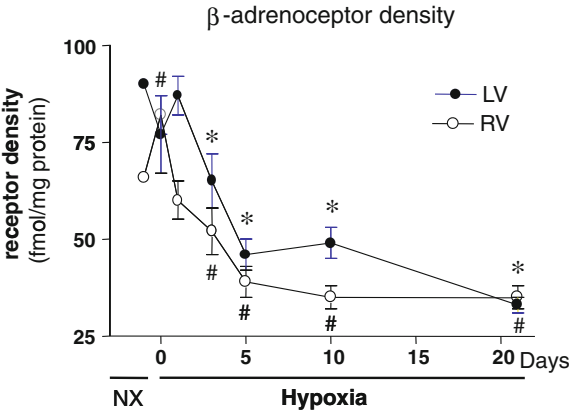

Fig. 12.3 Variations with time of β-adreceptor density in the left (*LV*) and the right (*RV*) ventricles of rats exposed for 21 days to a hypobaric hypoxia at 280 mm Hg (Adapted from [16]).

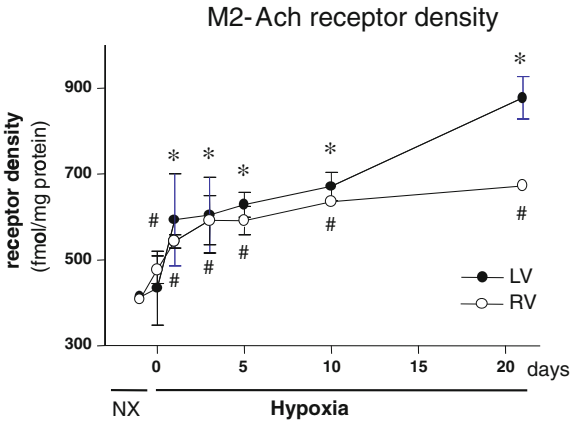

Fig. 12.4 Variations with time of M2-muscarinic receptors density in the left (*LV*) and the right (*RV*) ventricles of rats exposed for 21 days to a hypobaric hypoxia at 280 mm Hg. Adapted from [16]

The responsiveness of the adrenergic system to endogenous (exercise) or exogenous (isoproterenol infusion) stimulation is decreased in prolonged hypoxia [26–29]. For example, for a given increase in norepinephrine plasma concentration from rest to exercise, the corresponding increase in heart rate is lower in prolonged hypoxia than in normoxia [27]. The infusion of increasing doses of isoproterenol leads to a lower increase in heart rate in prolonged hypoxia vs normoxia in humans [28, 29]. This blunted response to adrenergic activation is partly and rapidly reversible with re-oxygenation [29].

This hypoxia-induced blunted responsiveness of the adrenergic system has been explored through the study of signal transduction in the main receptor systems controlling cardiac chronotropic function: β-adrenergic (β-AR), muscarinic (M-Ach-R) and adenosinergic receptors. Acute hypoxia (1–5 days at 4,559 m) has been found to reduce HR_{max} and this reduction was fully reversible with O_2 inhalation, which suggests an uncoupling of β-AR, as a result of phosphorylation

of G protein or second messenger, rather than a downregulation of the receptor [20]. Chronic hypoxia leads to a downregulation of β-AR in the rat myocardium [30, 31] and in human lymphocytes [21]. This decrease is associated with a decrease in adenylate cyclase activity in rats [31–34] and in guinea pigs [35]. The effects of hypoxia on the adrenergic pathway could be mediated by the desensitizing effect of permanently increased catecholamine levels or to a direct effect of hypoxia on one or several elements of the transduction pathway. To test this hypothesis, chronically hypoxic rats were compared with rats exposed to prolonged norepinephrine infusion [36]. There were some clear differences between the two models, especially at the level of Gi inhibitory protein that was more activated in hypoxic rats than in rats exposed to norepinephrine. Several studies have also observed that chronic exposure to hypoxia led to an increase in the density of myocardial M-Ach-R [16, 37, 38]. These results are consistent with the hypothesis that myocardial β-adrenergic receptors as well as muscarinic receptors are involved in the reduction of HR_{max} after acclimatization to hypoxia. [16, 25]. Favret et al. [16] provided clues of the possible role of myocardial β-AR and M-Ach-R on Q_{max} by studying the time course of acclimatization (Fig. 12.3 and 12.4). They showed a strong correlation between ventricular β-AR as well as M-Ach-R density and HR_{max}. These results suggest that β-AR downregulation and M-Ach-R upregulation could be considered as a possible mechanism leading to the reduction of HR_{max} and Q_{max}. Boushel et al. [23] have shown, by parasympathetic blockade using glycopyrrolate, that the parasympathetic nervous system was involved in the decrease in HR_{max} in human acclimatized to 9 weeks at 5,260 meters. In subjects acclimatized for 2 weeks at a much lower altitude (3,800 m), glycopyrrolate also increased HR_{max} but failed to significantly increase maximal cardiac output [39]. However, in rats acclimatized for 3 weeks at 5,500 m, Clancy et al. [40] did not observe that the M-Ach-R was responsible for the low HR_{max}. Prolonged exposure to severe hypoxia (3 weeks at 6,542 m) leads to a permanent increase in adrenergic activity (although plasma norepinephrine decreases from the first to the third week of exposure), a decrease in the density of β-AR in circulating lymphocytes, and a decrease in the heart rate response to isoproterenol infusion [21]. The desensitization of the β-adrenergic pathway is not only linked to the decrease in the β-AR density but also to an alteration of the Gs protein coupling to adenylate cyclase [41]. The impaired function of Gs could be due to a reduction in the biologically active form Gsα-small and/or an increase in the biologically inactive form Gsα-large of the Gs protein [34]. Moreover, the bioactivity of the membrane-bound Gsα would be reduced [33]. The stimulation of adenosinergic receptors, by activating the inhibitory Gi protein can also reduce the adenylate cyclase activity, although these receptors are also downregulated in hypoxia [41]. The Gi protein has been found increased in the heart of rats exposed to 5 days of hypoxia [32]. An impairment of norepinephrine intravesicular uptake in hypoxia could also contribute to increase the concentration of norepinephrine in the synaptic space and contribute to the desensitization of the adrenergic pathway [32, 42]. Opioid receptors can also be involved since a cross-talk exists between

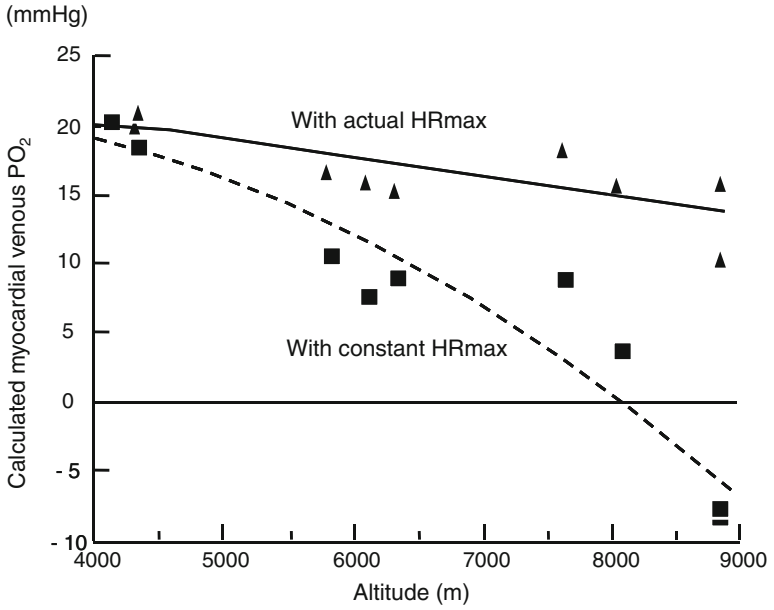

Fig. 12.5 Variations of calculated venous myocardial PO_2 as a function of altitude, at maximal exercise. Values were calculated using data obtained at maximal exercise from various studies in the literature. When HR_{max} is entered in the equation for the computation of PO_2 as actual HR_{max} (which decreases with altitude), myocardial venous PO_2 is almost kept constant whatever the altitude. When an invariable constant value (sea level value) of HR_{max} is used, predicted PO_2 is negative above 8,000 m. For details and references, see [3]

these receptors and β-AR: the pertussis toxin-sensitive G protein of the opioid pathway inhibits the Gs protein of the adrenergic pathway [43]. The release of dynorphins from the heart in hypoxia can activate the kappa opioid receptors and blunt the adrenergic pathway, therefore protecting the heart from too high energy consumption [44].

In a model of oxygen transport in the myocardium, it was clearly shown that the maintenance of a normal myocardial tissue PO_2 at maximal exercise in hypoxia was possible at the only condition that the myocardial energy expenditure decreases that is to say that maximal heart rate decreases [3, 45]. Therefore, the currently observed decrease in HR_{max} in chronic hypoxia would be a homeostatic mechanism contributing to the maintenance of myocardium tissue oxygenation, despite a decrease in oxygen supply (Fig. 12.5). By reducing maximal O_2 transport, the decrease in HR_{max} can be considered as a limiting factor of performance at high altitude. However, this mechanism is protective for the heart, a vital organ that is also demanding for a high O_2 supply at exercise. The coronary flow reserve has been shown to be limited to 33 % above what is prevailing during maximal exercise at sea level [46]. Therefore, the compensation of decreased arterial O_2

content by increasing coronary blood flow is not possible above a certain altitude and the only option to preserve cardiac integrity is to decrease myocardial O_2 demand and therefore maximal heart rate. The downregulation of the β-adrenoceptors and upregulation of the muscarinic receptors within the myocardium are sufficient to explain the decrease in HR_{max} and are purely local homeostatic mechanisms that protect the myocardium against a too high energy expenditure. It is important to note that all these changes in cardiac chronotropic function are not associated with alterations in the inotropic function. Stroke volume and myocardial contractility, explored by echocardiography up to the simulated altitude of 8,848 m, are not diminished, at least at rest [8].

12.3 Adaptation of Cerebral Circulation to Altitude Hypoxia

Cerebral blood flow increases acutely when ascending to high altitude, then returns to normal sea-level values. These variations are explained by the balanced influence of PaO_2 and $PaCO_2$ on cerebral vasculature [47]. Initial hypoxia induces a cerebral vasodilation; then, the hyperventilation-induced hypocapnia leads to a substantial vasocontriction, while the hypoxic stimulus decreases with progressive ventilatory acclimatization. After two weeks of exposure to 5,050 m, the cerebrovascular reactivity to hypercapnia was reduced while the reactivity to hypocania was enhanced [47]).

As cerebral hemodynamics may play an important role in circulatory adaptation to high altitude and in the pathophysiology of high altitude cerebral edema (HACE), transcranial Doppler measurements were performed during the Operation Everest III experiment, up to 8,000 m in basal conditions and after 3–5 s compression of the left common carotid artery to evaluate the transient hyperemic response of the middle cerebral artery (MCA). MCA blood flow velocity increased only at 8,000 m and the corresponding pulsatility and resistivity indices decreased over 5,000 m. The transient hyperemic response of MCA to compression was depressed at 8000 m, suggesting an impaired autoregulation of cerebral blood flow at extreme altitude. Through this mechanism, the brain could be more sensitive to rapid changes in arterial pressure and exposed to vascular damage [48].

12.4 Adaptation of the Pulmonary Circulation to Altitude Hypoxia

12.4.1 Sea-Level Natives

Hypoxia induces a rise in pulmonary artery pressure by two main mechanisms. First, hypoxia provokes a vasocontriction of pulmonary arterioles by inhibiting the O_2 sensitive K+ channels, second a progressive remodeling of the arterioles by

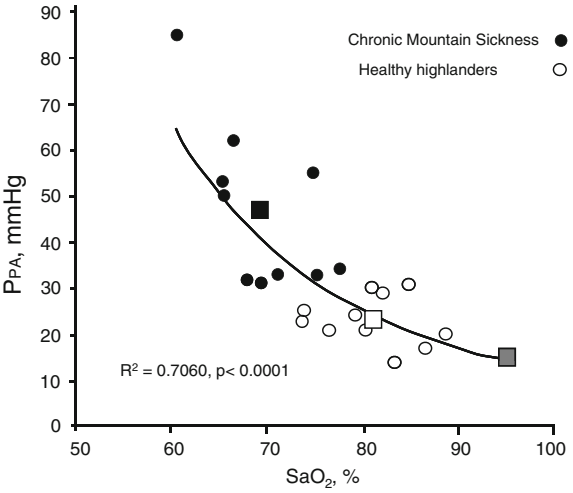

Fig. 12.6 Relationship between pulmonary artery pressure and arterial oxygen saturation in patients with chronic mountain sickness (*filled circles*) and healthy highlanders (*open circles*). *Squares* represent corresponding mean values and *gray square* corresponds to the mean value of sea level residents (Adapted from [58]).

proliferation of smooth muscle cells and a thickening of the arterial wall maintains or even increases pulmonary vascular resistance [49]. Pulmonary vasoconstriction can also be favored by the release of vasoactives substances such as histamine, serotonin, leucotrienes, endothelin, thromboxane, and reactive oxygen species, whereas nitrogen monoxide (NO) modulates this vasoconstriction [50]. The hypoxia-induced rise in pulmonary artery pressure is exaggerated by exercise [51] and blunted by the phosphodiesterase 5 inhibitors [52]. Echocardiographic measurements of pulmonary hemodynamics and a cardiopulmonary exercise test were performed in 22 healthy subjects after acclimatization to an altitude of 5,050 m [51]. The measurements were obtained after the intake of placebo or the endothelin A receptor blocker sitaxsentan. Hypoxia increased pulmonary vascular resistance and decreased VO_{2max}. Sitaxsentan decreased PVR and restored VO_{2max} by 10 % in hypoxia suggesting that high pulmonary artery pressure limits exercise capacity at high altitude.

In some cases, a high altitude pulmonary edema (HAPE) may develop but its cause is not only related to pulmonary hypertension. Various pathophysiological pathways have been evoked to explain the development of HAPE in some highly susceptible subjects. Apart from heterogeneous pulmonary vasoconstriction, HAPE is also associated with a hypoxia-induced increase in endothelial permeability and a dysfunction of pneumocyte II cells leading to an enhance leak of fluid from the capillary space to the interstitium, then from the interstitium to the alveolar space [53, 54]. Clinical and therapeutic aspects of this disease have been developed elsewhere [53, 55].

12.4.2 High Altitude Natives

Monge's disease or chronic mountain sickness (CMS) is characterized by an excessive polycythemia (haemoglobin >21 g/dL in men and >19 g/dL in women) in high altitude dwellers, with a prevalence of 5–18 % above 3,200 m. Clinical signs include headache, fatigue, sleep disturbances, dyspnea, dizziness, tinnitus, paresthesia and digestive complains [56, 57]. Pulmonary hypertension is frequently associated, which may lead to congestive heart failure.

Mean pulmonary artery pressure (PPA), measured by catheterization, was found elevated in various studies performed in Peru, Bolivia, or Tibet [58–60]. Mean values of PPA often rise above 47 mm Hg. Pulmonary wedge pressure is normal, pulmonary vascular resistance is increased, cardiac output is normal or reduced. There is an inverse relationship between PPA and arterial oxygen saturation (Fig. 12.6). An extensive study of pulmonary circulation and cardiac function has been recently performed, using echocardiography, in a group of CMS patients compared to normal high altitude natives (HA) and normal sea level natives (SL) [61]. None of the CMS patients had overt cardiac failure symptoms. They exhibited elevated pulmonary pressures as assessed by high tricuspid pressure gradients (34 ± 10 mm Hg vs. 25 ± 4 mm Hg for HA and 19 ± 3 mm Hg for SL). They also showed right ventricular dilation (end-diastolic right ventricular area: 17 ± 2 cm^2 vs. 13 ± 2 cm^2 for HA and 12 ± 2 cm^2 for SL) but did not display impaired systolic ventricular function. However, right ventricular Tei index was increased in altitude subjects (0.56 ± 0.15 for CMS, 0.52 ± 0.12 for HA vs. 0.21 ± 0.12 for SL). These results raise the question on the actual prevalence of cardiac failure in CMS [61]. Recent studies have suggested that genetic factors could contribute to the development of CMS [62]. Some alleles seem more prevalent (G allele of eNOS polymorphism Glu298Asp in Sherpas and ACE I allele in Kyrgyz with pulmonary hypertension) or less prevalent (ACE D allele in Andeans) in various groups of high altitude populations. Pyruvate dehydrogenase kinase I and HIF prolyl hydroxylase 3 mRNA expressions were lower in 10 children of CMS fathers (4,300 m) compared to children of non-CMS fathers [63]. However, no clear association is actually in favor of a genetic cause of CMS.

12.4.3 Cardiac patients and High Altitude

The facility of travel and the growing interest for high altitude regions expose more and more people to the acute effects of altitude hypoxia, including aging people and patients with cardiovascular diseases. The scientific literature is unable to give us clinical evidence regarding the risk of all types of cardiovascular diseases at moderate or high altitude [64–66]. However, the basic knowledge of the physiology of hypoxia and of the pathophysiology of cardiac or vascular diseases

allows us to propose four simple rules that may help the practitioner to take a decision and give an appropriate advice to his cardiac patient [67].

1. Any disease that may be aggravated by an overactivation of the adrenergic system (arrhythmias) might be at risk at high altitude
2. Any disease associated with a pulmonary hypertension will be at high risk even at moderate altitude.
3. Any disease presenting, even at sea level, a certain degree of arterial hypoxemia (right-to-left shunt) will be at risk at high altitude
4. For a given absolute level of power output at exercise, heart rate (and therefore the energetic demand of the myocardium) increases with altitude, lowering the ischemic threshold in coronary patients.

Arrhythmias. Although rapid ascent to high altitude may increase the frequency of supraventricular and ventricular arrhythmias in patients with underlying heart disease [68], no demonstrable clinical impact has been found [69]. However, it is reasonable to limit the access to altitude above 2,500 m for patients with severe arrhythmias associated with underlying heart disease.

Pulmonary hypertension. Preexisting pulmonary hypertension at sea level may deteriorate at even moderate altitude, whatever the origin of the hypertension. Patients with congenital or acquired anomalies of the pulmonary circulation are also at high risk [70–72]. A transient perinatal insult to the pulmonary circulation leaves a persistent and potentially fatal imprint, which when activated in adult life predisposes to a pathological response, especially when exposed to high altitude [73].

Right-to-left shunt. Right-to-left atrium shunting through a persistent foramen ovale (PFO) might be aggravated in hypoxic conditions due to increased pressure in the pulmonary artery and the right heart. The presence of PFO was found in 56 % of patients susceptible to HAPE while was only 11 % in non-susceptible subjects [74]. Cyanotic congenital heart diseases are theoretically at high risk at even moderate altitude, although the only study that explored this condition (in air travel environment) failed to evidence any significant cardiovascular damage [75].

Coronary artery disease. It is reasonable to think that in patients with a reduced coronary reserve, the decrease in O_2 availability due to altitude exposure will increase the risk of myocardial ischemia. However, the clinical observations related in the literature show no evidence of increased incidence of acute myocardial ischemic events at low and moderate altitude [76, 77]. In nine men with coronary artery disease and exercise-induced angina and/or ST segment depression, a treadmill testing was performed at 1,600 and 3,100 m. Clinical or EKG signs of ischemia occurred at lower work loads at 3,100 m than at 1,600 m, but for the same level of heart rate or heart rate systolic pressure product [76]. The authors conclude that a target heart rate range of 70–85 % of the ischemic end-point rate at lower altitude predicts an appropriate level of tolerable exercise at high altitude, so that patients should limit their activity at high altitude rather by controlling their heart rate that their workload. A rapid ascent and submaximal exercise can be considered safe at an altitude of 3,454 m for low risk patients 6 months after

revascularization for an acute coronary event and a normal exercise stress test at low altitude [77]. A study performed in Switzerland showed that mortality from coronary heart disease, from 1990 to 2000, in men and women living at altitudes of 259–1,960 decreased by 22 % every 1,000 m. The protective effect of living at higher altitude on coronary heart disease and stroke mortality was consistent and became stronger after adjustment for potential confounders [78].

Congestive heart failure. Very few studies are available about heart failure and high altitude [65, 69, 79]. However, a chamber study showed in a group of 38 patients with a mean LV ejection fraction of 35 % that the acute exposure to 300 m did not induce any signs of ischemia, arrhythmias, or acute heart failure [79]. Altogether, it seems that up to 3,000 m, there is no substantial increase in cardiovascular risk for patients with stable, compensated heart failure [69, 79].

Systemic hypertension. Peripheral circulation is submitted at high altitude to two opposite effects: local hypoxia-induced vasodilation and general sympathetic-induced vasoconstriction. The result is variable among subjects and therefore among published studies [69, 77, 80, 81]. Finally, in well-controlled hypertensive patients, the initial slight increase in blood pressure is modest and no complication of systemic hypertension at high altitude has been published.

12.5 Conclusions

The exposure to altitude hypoxia triggers a full array of mechanisms allowing the human body to adapt to the lower oxygen availability. The activation of the adrenergic system is on the first line of defense mechanisms against hypoxia and triggers an increase in heart rate and cardiac output. However, with acclimatization, the cardiac response to adrenergic activation is blunted leading to an autoregulation of cardiac function that protects the heart against an imbalance between O_2 availability and O_2 consumption. Altogether, cardiac function in normal subjects is not altered by altitude exposure. In cardiac patients, oxygen lack might be detrimental although there is very few evidence of aggravated cardiovascular diseases at least at low and moderate altitude.

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Chapter 13
Cardiac Hypertrophy in Hypertension

Jaroslav Kuneš, Ivana Vaněčková, Michaela Kadlecová, Michal Behuliak, Zdenka Dobešová and Josef Zicha

Abstract Hypertension often results in left ventricular hypertrophy (LVH), defined as a major risk factor for cardiovascular morbidity and mortality. In its initial stages, the hypertrophied ventricle compensates an overload, but later on, the diastolic and eventually also systolic properties of left ventricle (LV) become impaired causing the decompensation and finally the heart failure (HF). Although in animals the LVH can be prevented by pharmacological therapy, in man LVH can be reduced only to a limited extent. This could be related to the fact that most of the human studies are too short and the duration of particular stimuli is not exactly known. Moreover, if the therapy is not applied in a precise developmental window, the results may be subtle or none. There is no doubt that angiotensin converting enzyme (ACE) inhibitors and AT_1 blockers are currently the drugs of the first choice in the treatment of essential hypertension. Besides the effect of these drugs on blood pressure (BP) *per se* they also have a potential to normalize cardiovascular structural changes. HF is continuously increasing in the Western world. The population-based Framingham study suggested that the prognosis in women is significantly better than in men after the onset of HF. What is the reason for such gender differences is not fully understood.

Keywords Heart · Hypertrophy · Hypertension · Renin-angiotensin system · Gender · Rat

J. Kuneš (✉) · I. Vaněčková · M. Kadlecová · M. Behuliak · Z. Dobešová · J. Zicha
Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague 4, Czech Republic
e-mail: kunes@biomed.cas.cz

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13.1 Introduction

Essential hypertension affects 20–30 % of the population in industrialized countries and contributes to cardiovascular mortality and morbidity. Heritability of blood pressure (BP) is around 40 % and some environmental factors also play a significant role. A recent study concluded that 54 % of stroke, 47 % of ischemic heart disease, 75 % of hypertensive disease, and 25 % of other cardiovascular disease around the world are attributable to high BP [1]. What does normal or high BP mean?

The target BP at all stages of hypertension recommended by many national societies for hypertension is $\leq$140/90 mm Hg. In elderly patients, no evidence exists on the benefit of the reduction in systolic BP below 140 mm Hg. Moreover, earlier recommendations to reduce BP below 130/80 in diabetics and those with high cardiovascular risk are not clearly supported by clinical studies [2]. Based on the current knowledge, it seems reasonable to recommend a reduction of pressure in hypertension to the values of 130–139/80–85 mm Hg, respectively [2].

As it was demonstrated by Lewington et al. [3], among people with no previous vascular disease recorded, the usual BP is positively related to the risks of death from vascular disease not only among hypertensive individuals, but also among those who are usually considered normotensive (at least down to usual BP levels of 115/75 mm Hg). For example, the long-term systolic BP decrease by 10 mm Hg or diastolic BP decrease by 5 mm Hg is associated with about 40 % lower risk of stroke death and about 30 % lower risk of death from ischemic heart disease or other vascular causes throughout middle age [4, 5].

The solution of all problems associated with hypertension and cardiac hypertrophy requires the use of appropriate animal model(s). Such model must meet certain criteria as it was suggested by Doggrell and Brown [6]. An ideal model of cardiovascular diseases should have five characteristics: (1) mimic human disease, (2) allow studies in chronic, stable disease, (3) produce symptoms which are predictable and controllable, (4) satisfy economical, technical, and animal welfare considerations, and finally (5) allow measurement of relevant parameters. The use of laboratory rats as animal model in cardiovascular research is rational from the economic viewpoint and because many techniques have been developed to measure relevant functional parameters. However, some ethical problems and legislation within communities could be the limitations for the use of animals in research. One should keep in mind that not always the results can be simply transferred from animals to human beings. The results of genetic studies may be a typical example of such a disagreement [7]. According to our knowledge there is no example of direct transfer of findings in animals to human pathology. Recently, a number of reviews were written about animal (mainly rat) models of cardiovascular disease [7–9]. Although these papers discuss exclusively experiments in living animals, it should be pointed out that the alternative methods exist for the study of cardiovascular system. Cell dispersion from adult hearts, tissue cultures of cardiomyocytes, and cardiac fibroblasts could replace animal studies and give us

many answers for particular questions. Spontaneously hypertensive rats (SHR) are still the mostly used model to study cardiovascular disorders as evidenced by thousands of Medline references. The great advantage of this model is that there are many similarities with human essential hypertension. Although there are currently numerous colonies of these animals, they have two common features—high BP and cardiac hypertrophy. It has been shown that, in the early stages of hypertension, they have increased cardiac output with normal total peripheral resistance (for more details see [10]). As hypertension progresses into the established state, the cardiac output returns to the normal and the constriction of hypertrophied blood vessels causes an increase of total peripheral resistance. If this early hyper-circulation is a signal for cardiac hypertrophy in SHR is still a question as can be seen from the studies in newborns.

SHR is generally considered as a good model in which hypertension and cardiac hypertrophy goes in parallel. Nevertheless, it was demonstrated that approximately 30 % increase of heart weight is found in all studies on untreated SHR and that many rats progress to heart failure (HF) between the ages of 18 and 24 months [11]. Since not all SHR rats exhibit signs of HF it is clear that there exists the inconsistency of this model. However, numerous models have also been developed to study only particular syndromes of HF without the close relation to hypertension [12]. There are two general types of models: those occurring naturally (e.g., cardiomyopathic hamster, etc.) and those experimentally induced (e.g., pressure and/or volume overload, myocardial ischemia and infarction, toxic cardiomyopathy, etc.) (for more details see [12]).

In this review, we shall focus on the role of BP as a primary event in cardiac hypertrophy, on the role of renin-angiotensin system in this process as well as on the gender differences in cardiac hypertrophy.

13.2 Cardiac Hypertrophy: Common Mechanisms

Cardiac hypertrophy involves a sequence of events beginning with initiating signals through gene expression to physiological consequences (Fig. 13.1). One of the strongest initiating signal for cardiac hypertrophy can be pressure or volume overload. However, cardiac hypertrophy associated with hypertension does not seem to be a simple functional response of the myocardium to the mechanical stress after an overload because dissociation between the degree of cardiac hypertrophy and BP level in humans and experimental animals has been shown [13–16]. These finding have led to the speculation that factors other than BP *per se* may regulate cardiac hypertrophy [13, 17]. The sympathetic nervous system and renin-angiotensin-aldosterone system may be the potential ones. Angiotensin II (Ang II) plays a pivotal role in cardiac remodeling and induces cardiac hypertrophy and fibrosis (Fig. 13.2). Mechanisms of Ang II-dependent hypertrophy involves receptor activation and subsequent modification of several pathways (e.g., TGF-β pathway) leading to transcriptional activation in the nucleus and to

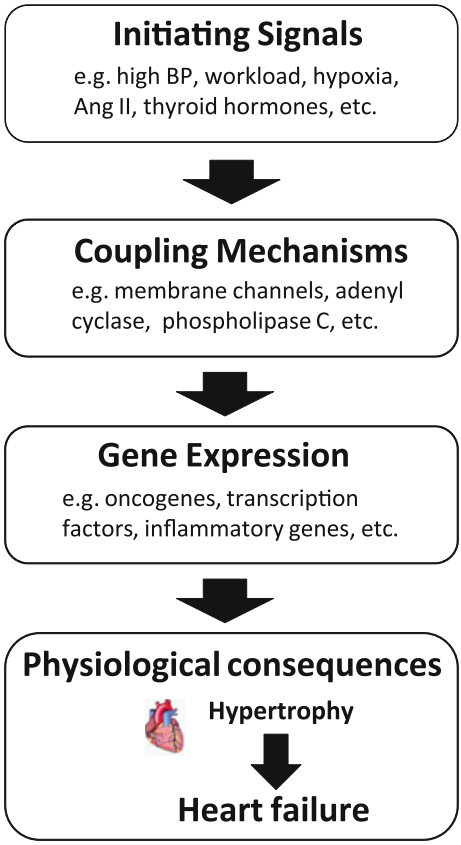

Fig. 13.1 Possible mechanisms associated with the process of cardiac hypertrophy and heart failure

hypertrophic myocyte growth [18]. It was demonstrated that AT_1 receptor blockers attenuated cardiac hypertrophy in humans [19, 20] and in experimental animals [21]. A recent study suggests that mechanical stress can induce cardiac hypertrophy by activating AT_1 receptors through calcineurin pathway independent of Ang II [22]. In addition to AT_1 receptors, AT_2 receptors are also involved in Ang II-dependent cardiac hypertrophy and fibrosis as it was demonstrated in mice lacking AT_2 gene [23]. These animals did not develop cardiac hypertrophy after the infusion of Ang II. A sympathetic nervous system contributes to the pathophysiology of cardiac hypertrophy as it was demonstrated by the administration of β-adrenoreceptor blockers [24]. In rats with dilated cardiomyopathy, carvedilol, an α1- and β-adrenergic receptor blocker, attenuates inflammation, oxidative response, myocardial fibrosis and apoptosis, and preserves the left ventricular function [25]. Recently, it was shown that carvedilol reduced left ventricular mass index (LVMI) and improved coronary flow reserve in patients with hypertensive LVH [26].

At the molecular level, cardiac hypertrophy is mediated by many extrinsic and intrinsic molecular growth signals [18]. This induces the expression of genes that

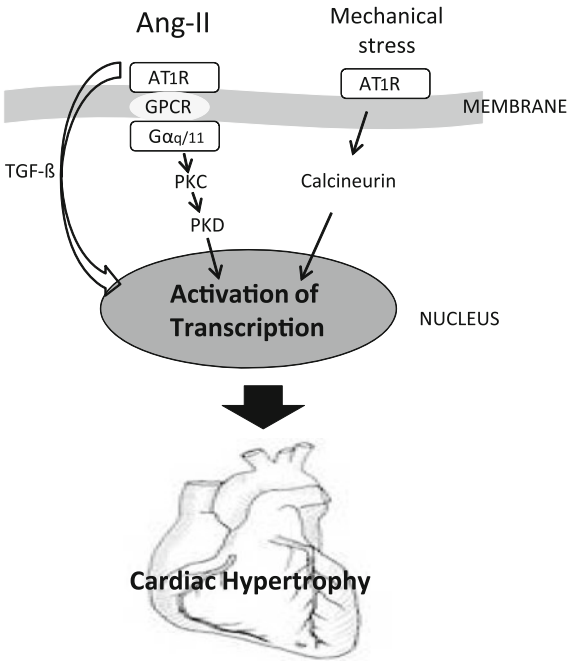

Fig. 13.2 Scheme of Ang II-dependent and Ang II-independent AT_1 receptor (AT_1R) activation involved in the induction of cardiac hypertrophy. Ang II via AT_1R activates PKC and/or TGF-β pathways. AT_1Rs can be activated independently of Ang II in response mechanical stress and induce cardiac hypertrophy through calcineurin pathway (for more details see [18])

include the upregulation of fetal isoforms of genes whose products regulate cardiac contractility and calcium handling, which is concomitantly associated with downregulation of their adult isoforms [27]. Transgenic and knockout (KO) technology in combination with other experimental models has demonstrated very powerful signaling cascades that play a role in the regulation of physiological and pathophysiological heart growth [18, 28]. For example, the activation of proto-oncogenes (e.g., c-fos, c-myc, c-jun, etc.) that encode for peptide transcription factors is believed to induce the transcription of other hypertrophy-related genes [29]. This so-called "early response" by the proto-oncogenes induces a "fetal gene program" characterized by the re-expression of several genes normally expressed predominantly in the embryonic heart [30]. Moreover, it was demonstrated that chr 2 near D2Mgh15 influences heart weight independent of BP in F_2 hybrids derived from SHR and Donryu rats [31]. The same was found on X chromosome in Lyon rats [32]. Suggestive evidence was shown for linkage of heart weight on chr 14 near D14Mgh3 [33] and on chr 17 near the dopamine 1A receptor [34].

13.3 Cardiac Enlargement and High Blood Pressure

High BP triggers the pathophysiological cascade which proceeds to left ventricular hypertrophy (LVH) defined as a major risk factor for cardiovascular morbidity and mortality. In its initial stages, the hypertrophied ventricle compensates an

overload, but later on, the diastolic and eventually also systolic properties of left ventricle (LV) become impaired causing decompensation and finally the HF. HF is a pathophysiological state in which the heart is unable to supply the body with sufficient amount of blood. The most common clinical form of chronic HF is idiopathic dilated cardiomyopathy, which is hereditary in 20–30 % of individuals [35]. According to epidemiological data, ∼30 % mortality occurs at 1 year after HF diagnosis [36, 37].

It is well known that cardiac mass increases if afterload lasts for considerable period. The common statement that there is a relation between BP and LVMI [38] has recently been denied [39]. Harrap and colleagues [40] established a colony of inbred rats with LV hypertrophy and normal BP (hypertrophied heart rat—HHR). The isolated cardiomyocytes from these animals were significantly longer and wider than those isolated from rats without LV hypertrophy. On the contrary, a positive correlation between BP and LV mass was found in adult SHR, whereas no such relation was observed in WKY or Wistar rats [41]. Another interesting finding of this study was that WKY rats presented LV hypertrophy and fibrosis when compared with Wistar rats while the systolic BP of both strains was the same (Wistar—118.5 mm Hg, WKY—119.8 mm Hg, respectively). An enhanced expression of the local renin-angiotensin system at LV tissue level might be the explanation for this increased cardiac mass. In connection with this, an increased activity of the angiotensin-converting enzyme in the left but not in the right ventricle was reported in rats with cardiac hypertrophy [42]. Previous experiments from the same laboratory showed regression of LV hypertrophy in WKY rats after chronic treatment with the Ang II type 1 receptor antagonist losartan [43]. Moreover, Magga and co-authors [44] also observed that the ratio of heart to body weight was reduced in WKY rats chronically treated with losartan or the angiotensin-converting enzyme inhibitor enalapril. Obviously, the conclusion of whether or not a pharmacological intervention induces the regression of myocyte size and/or fibrosis will depend on which control hearts are selected. An inappropriate selection of the "controls" would probably lead to a misinterpretation of the results because a given intervention may decrease these parameters of hypertensive rats below those of the considered "normal" control hearts.

Moreover, if clinical BP in humans is used, only 9–16 % of the LVMI is accounted for BP level and this increases to 16–36 % using ambulatory BP [45]. Some of the residual variance is accounted for age, obesity, physical activity, alcohol consumption, etc. Indeed in hypertensive, much of the variance in cardiac mass remains unexplained. This unexplained variance in the response to pressure overload should be accounted to gene-environmental interaction and mainly to the role of so-called "cardiac mass-modifying" genes [46]. Moreover, recent studies have shown that circulating inflammatory markers are associated with BP variability in hypertensive patients and these factors could be responsible even for the variability in cardiac mass [47, 48].

There is a rat model of hypertension, with large short-term BP variability and cardiac remodeling [49] which is based upon bilateral sinoaortic denervation (SAD) in SHR and Wistar Kyoto rats. SAD disrupts the afferent pathway of the

arterial baroreflex system. This procedure includes ablation of the aortic depressor nerve and superior laryngeal nerve, resection of the superior cervical ganglia and cervical sympathetic trunks as well as denervation of the aortic and carotid sinus baroreceptors [50, 51]. It was demonstrated that SAD aggravates left ventricular/myocyte hypertrophy and myocardial fibrosis to a greater extent and impairs left ventricular systolic function without changing average BP in SHR [48].

Spontaneously hypertensive heart failure (SHHF) rats were bred from Koletsky rat and SHR progenitor [52]. These rats are hypertensive and develop congestive HF at 16–20 months of age featuring the typical hallmarks of the human disease [53]. Using this inbred strain, the gene encoding soluble epoxide hydrolase (*Ephx*2) as a HF susceptibility gene was demonstrated [53].

13.4 The Role of Renin-Angiotensin System in Cardiac Hypertrophy

Cardiac hypertrophy or LVH is the consequence of either pressure overload (due to, e.g., hypertension) or volume overload (due to, e.g., renal failure). Hypertension-induced LVH is characterized by an increase in relative wall thickness (given by the rise in the volume of existing cells), and is known as concentric hypertrophy [54]. Following volume overload, left ventricular mass increases without the increase in relative wall thickness; in this case cardiomyocytes elongate inducing eccentric hypertrophy. Both types of hypertrophy have been shown to effectively respond to RAS inhibition in large clinical trials [55–57], with similar effects of angiotensin receptor blockers (ARB) and angiotensin converting enzyme inhibitors (ACEi), and with lower effects of either β blockers or calcium antagonists. Moreover, there are some studies supporting synergistic effects of the combined ARB and ACEi therapies due to a better inhibition of renin-angiotensin system. In addition, some clinical trials indicated that RAS inhibition has favorable effects that go beyond its BP lowering effects and these effects are marked as BP independent.

However, although the term cardiac hypertrophy is widely used especially in clinical content, it lacks specificity, precision, and resolution [58]. The problem is that cardiac hypertrophy is complex and it is necessary to define it on functional, histological, and molecular basis. Therefore, it was suggested to restrict the use of "hypertrophy" better to cardiac myocyte status and to use the term remodeling for the whole heart. Remodeling means the reorganization of different cardiac tissue components (i.e., cardiac myocytes, fibroblasts of the stroma, and blood vessel cells). Cardiac remodeling comprises three major processes—the growth of cardiomyocytes, accumulation of extracellular matrix, and fibrosis. All these processes are somehow connected with renin-angiotensin system. Previously, all the effects were ascribed to Ang II, the main effector of RAS. Recently, more attention is being focused on type 1 of angiotensin receptor (AT_1R), angiotensin 1–7 and angiotensin converting enzyme 2 (ACE2).

In the RAS cascade, there are two opposing parts—one producing vasoconstrictor Ang II, and the second one—the vasodilator part which through the cleavage of Ang II by ACE2 produces Ang 1–7. The effects of Ang II, regarded as the most important substance of the RAS due to its potent vasoconstrictor activity, is mediated through two types of receptors with opposite functions. Most of the deleterious effects of Ang II (growth, fibrosis, inflammation) are mediated through AT_1 (AT_1R) receptors, while AT_2 (AT_2R) receptors have cardioprotective effects. Activation of AT_1R triggers intracellular events leading to phospholipase activation, resulting in the activation of protein kinase C and phosphorylation of various proteins [59]. Ang II binding to AT_1R results in their internalization. Unlike AT_2R, AT_1R move between endosomal vehicles and plasma membrane. Internalized Ang II is then either degraded or exerts its effect [60]. AT_2 receptors are ubiquitously expressed in fetal tissue, but their expression declines rapidly after birth, being preserved in low densities in ventricular myocytes and vascular endothelium [61].

Recent studies demonstrated the importance of ACE2 for maintaining the balance between the vasoconstrictor and vasodilator loops in both health and disease. Thus, Tikellis et al. [62] demonstrated that the deficiency of ACE2 resulted in increased tissue and circulating levels of Ang II and reduced levels of Ang 1–7. They postulated ACE2 as a key modulator of RAS in cardiovascular and renal disease [63] suggesting that the most important fact is the balance between ACE and ACE2 and their ability to counterbalance the effects of Ang II and Ang 1–7. Moreover, Oudit et al. [64] demonstrated that ACE2 deficiency resulted in early cardiac hypertrophy. On the other hand, several studies indicated that the heptapeptide Ang 1–7 attenuated cardiac remodeling through the reduction of MAP kinases ERK1 and ERK2 [65] and the reduction of proliferation and collagen production by cardiac fibroblasts [66].

Previous studies have shown that Ang II stimulation induced marked cardiac hypertrophy and interstitial fibrosis [67]. However, the AT_1R were suggested as mediators of these effects. Thus, AT_1R KO mice had severe cardiac developmental defects [68], while mice overexpressing AT_1R had marked cardiac hypertrophy and fibrosis [69]. Moreover, it has been demonstrated that the stimulation of AT_2R results in the suppression of cardiac hypertrophy, fibroblast proliferation, and hyperplasia of ventricular myocytes [60], which is in line with the hypothesis that AT_2R is generally regarded as the opponents to AT_1R. However, the experiments with KO mice provided conflicting results. Yan et al. [70] have shown that mouse model overexpressing AT_2R in the ventricle produced cardiac hypertrophy, while AT_2R expressed both in atria and ventricles did not induce any abnormality in the myocardial development [71]. When targeted disruption of AT_2R was performed, Ichiki et al. [72] found BP increase and LVW/BW decrease in KO mice using C57BL/6 strain, while BP and cardiac morphology did not change in KO mice using FVB/n strain [73]. The different results were ascribed to different mouse strains used in these studies.

An interesting insight about the roles of Ang II and AT_1R in cardiac remodeling came from the studies of Crowley et al. [74] who used transplantation approach producing four groups of mice, in which inherent kidneys were removed and

replaced by kidneys either with normal or with inactivated AT_1R. Thus, they had *wild -type* group (with kidneys transplanted from wild-type donors, i.e., AT_1R expressed both in the kidneys and in the systemic tissues), *systemic KO* group (AT_1R only in the kidneys), *kidney KO* (AT_1R in the systemic tissues), and *total KO* group (lacking AT_1R in all tissues). They infused high doses of Ang II for four weeks and monitored BP and evaluated final cardiac remodeling. They found that two groups of mice with inactivated AT_1R in the kidney were unable to respond to Ang II infusion with increasing BP. These groups also did not develop cardiac remodeling despite large quantities of circulating Ang II and the presence of AT_1R in the heart. They concluded that kidney, especially AT_1 receptors, plays an important role in hypertension which causes cardiac remodeling.

Finally, Yasuda et al. [75] in an elegant study demonstrated that cardiac-specific upregulation of human AT_1R ($hATR_1$) expression lead to spontaneous cardiac remodeling and fibrosis in mice in which Ang II production was genetically deficient by KO of angiotensinogen gene. Cardiac remodeling in these transgenic mice was prevented by AT_1R antagonist candesartan. They concluded that the constitutive activity of AT_1R contributes to cardiac remodeling even in the absence of Ang II.

13.5 Ontogenetic Aspects of Cardiac Hypertrophy Development

Critical developmental periods (developmental windows) exist during which experimental animals are more vulnerable to the effects of environmental stimuli. This is true for different models of genetic and experimental hypertension [10, 76]. It has been shown that in humans the intrauterine growth and maternal "environment" could be responsible for BP level and cardiovascular problems in adulthood [77, 78]. Furthermore, studies in humans have shown that men and women whose birth weights were at the lower end of the normal range or small in relation to placental size have increased rates of coronary heart disease [79]. Recently, the inverse association between the birth weight and cardiovascular mortality in a large cohort of adult mothers and fathers was demonstrated [80]. Surprisingly, the association between the birth weight and other than cardiovascular diseases was also observed. In men, higher birth weight was strongly associated with an increased risk of cancer deaths [81]. All these findings can be interpreted according to the hypothesis that hypertension and/or other cardiovascular diseases are the late consequences of abnormal ontogeny of particular systems [82] suggesting that, e.g., fetal undernutrition may increase the susceptibility to diseases that occur later in life [83]. The development of hypertension and other cardiovascular diseases should therefore be studied with respect to the maturation of the organism [10, 82].

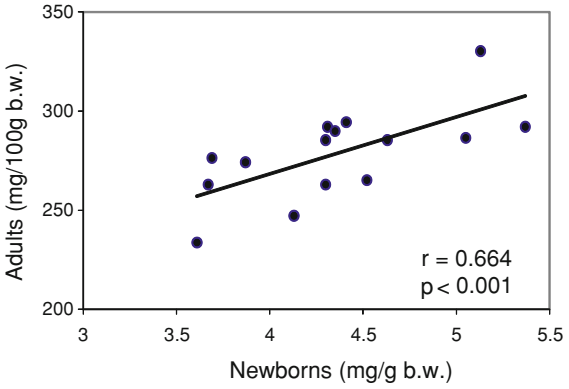

Fig. 13.3 Correlation between newborn and adult relative heart weight in recombinant inbred strains

Embryonic and fetal periods are important for the growth of the vertebrate heart. Strong hyperplasia is typical for fetal life and persists shortly after birth. In this period the heart grows more rapidly than the rest of the body, so that the heart/body weight ratio maximizes at 4–5 days of age and then it declines [84]. The LV grows faster than the right one. One of the reasons for this may be to prepare the LV for a greater burden during postnatal life. We have demonstrated that cardiac hypertrophy is already present in newborn rats with genetic hypertension but not in those ones with renal hypertension or in the offspring of mothers with DOCA-salt hypertension [85]. On the other hand, it was observed in hypertrophic heart rat (HHR) model (without hypertension) that at postnatal day 2, HHR hearts were even considerably smaller than those of controls [16]. This cardiac growth restriction in HHR neonates was associated with reduced myocyte size and an increased proportion of binucleated cardiomyocytes. This is in a good agreement with our results obtained much earlier in recombinant inbred (RI) strains developed by inbreeding of F2 cross derived from SHR and Brown Norway (BN) [86]. To study the relation between cardiac hypertrophy and blood pressure in more detail, we have used progenitor strains and 31 RI strains. It was shown that relative heart weight of newborns was related positively to blood pressure of their mothers only in the strains derived from SHR females but not in those ones derived from BN mothers. Surprisingly, there was no relation of newborn cardiac weight and adult BP.

We have demonstrated that RI strains are a very useful tool to search for genetic determinants of target organ damage in hypertension [17, 87]. The analysis of the results in adult RI strains has confirmed that relative organ weight in hypertensive individuals reflects both intrinsic organ growth capacity and the growth induced by deleterious effects of increased BP [17]. Figure 13.3 demonstrate a strong correlation between newborn and adult relative heart weight. This is in a good agreement with results concerning relative kidney weight [87].

13.6 The Role of Gender in Cardiac Hypertrophy

It is well known that blood pressure is higher in men than in premenopausal women, but the incidence of hypertension in postmenopausal women is similar or higher than in men [88]. Recently, it has been demonstrated that the prevalence of hypertension is relatively stable in men but it is increasing in women [89]. Moreover, the level of blood pressure is much better controlled in men than in women [90], suggesting that mechanisms responsible for hypertension could be gender specific. This seems to be true also for myocardial hypertrophy and HF. The population-based Framingham study suggested that the prognosis is significantly better in women than in men after the onset of HF. What is the reason for such gender differences is not fully understood. Gender-specific differences have been demonstrated even in experimental studies.

It was demonstrated that male SHR have higher blood pressure than do females of the same age [91, 92]. This is also true for other models of experimental hypertension [93, 94]. It is of interest that only few gender-specific results exist in normotensive animals. In 12-week-old Wistar Kyoto rats, Calhoun et al. [95] have demonstrated lower BP in males than in females by approximately 9 mm Hg and this difference was not confirmed in 14-week-old rats. As has been mentioned above, the proper mechanisms for gender differences in blood pressure control are not clear. However, there is a significant evidence that androgens (namely testosterone) could play an important role in gender-associated differences in BP regulation. Castration of young SHR males at the age of 3–5 weeks attenuates the development of hypertension [96]. The same is true for other models of experimental hypertension: Dahl salt-sensitive rats [97], Ren-2 transgenic rats [98], in males subjected to 2-kidney 1-clip procedure [99], or reduction of renal mass [100]. Moreover, it was demonstrated that chronic blockade of the androgen receptor with its antagonist flutamide attenuated BP of male SHR to the level of females [101]. These results from experimental animals are in a good agreement with the results in human beings. Studies in children have shown that after the onset of puberty, the boys had higher BP than the age-matched girls [102, 103]. At the age of 18 years this difference was almost 15 mm Hg [103]. Surprisingly, blood pressure of boys at this age does not dip as low at night as in girls and this reduction in nocturnal dipping was recognized as very important for the dysfunction in BP regulation [102, 103].

In general, female hearts are smaller and stiffer than male hearts and this may contribute to the different function under normal conditions or in pathology. Although the number of cardiomyocytes at birth is similar in men and women, in the aging female heart, hypertrophy, apoptosis, and fibrosis are less pronounced than in age-matched male hearts [102]. In animal models, it was described that male mice subjected to pressure overload had higher relative heart weight than females [104]. Two weeks after transverse aortic constriction, classical marker genes of hypertrophy were upregulated in both sexes, but the genes controlling mitochondrial function had lower expression in males than in females. Moreover,

an important role was suggested for estrogen receptor-β in attenuating the hypertrophic response to pressure overload in females [104]. Nine weeks after the onset of overload, male hearts showed more eccentric hypertrophy than female ones and the same was also true for the development of fibrosis [105].

13.7 Conclusions

Recent data from experimental animals and human beings support the idea that cardiac hypertrophy together with hypertension represent a major problem in cardiovascular morbidity and mortality. Cardiac hypertrophy involves a sequence of events beginning with initiating signals through gene expression to physiological consequences. One of the strongest initiating signal for cardiac hypertrophy can be pressure or volume overload. However, cardiac hypertrophy associated with hypertension does not seem to be a simple functional response of the myocardium to the mechanical stress after an overload because dissociation between the degree of cardiac hypertrophy and BP level in humans and experimental animals has been shown. These findings have led to the speculation that factors other than blood pressure *per se* may regulate cardiac hypertrophy. We have previously demonstrated that cardiac hypertrophy is already present in newborn rats with genetic hypertension but not in those ones with renal hypertension or in the offsprings of mothers with DOCA-salt hypertension. Moreover, it was demonstrated that on postnatal day 2 hearts in a model of hypertrophic heart (without hypertension) were even considerably smaller than those of controls.

The sympathetic nervous system and renin-angiotensin-aldosterone system may be the potential systems playing the important role in cardiac hypertrophy. However, it was shown that Ang II *per se* has not an exclusive role in cardiac remodeling, and that most importantly AT_1 receptors are the crucial players in these processes.

It is well known that blood pressure is higher in men than in premenopausal women, but the incidence of hypertension in postmenopausal women is similar or higher than in men and is increasing nowadays. This seems to be true also for myocardial hypertrophy and HF. The population-based Framingham study suggested that the prognosis is significantly better in women than in men after the onset of HF. What is the reason for such gender differences is not fully understood. In the era of personalized medicine, there is a need for better understanding of gender differences not only in HF mechanisms but also in hypertension as well as other socially important diseases.

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Chapter 14
Exercise Training and Adverse Cardiac Remodeling and Dysfunction in Mice

Dirk J. Duncker, Elza D. van Deel, Monique C. de Waard and Jolanda van der Velden

Abstract Cardiac remodeling in response to a myocardial infarction or chronic pressure-overload is an independent risk factor for the development of angina pectoris and heart failure. In contrast, cardiac remodeling produced by regular physical exercise is associated with a decreased risk for coronary artery disease and heart failure. There is evidence that exercise training has a beneficial effect on disease progression and survival in patients with cardiac remodeling and dysfunction, but concern has also been expressed that exercise training may aggravate pathological remodeling and dysfunction. Here, we present studies from our laboratory on the effects of exercise training on pathological cardiac remodeling and dysfunction in mice. The results indicate that even in the presence of a large infarct, exercise training exerts beneficial effects on the heart. These effects were mimicked in part by eNOS overexpression and abrogated by eNOS deficiency, demonstrating the importance of nitric oxide signaling in mediating the cardiac effects of exercise. Exercise prior to a myocardial infarction was also cardioprotective. In contrast, exercise tended to aggravate pathological cardiac remodeling and dysfunction in the setting of pressure-overload produced by an aortic stenosis. These observations emphasize the critical importance of the underlying pathological stimulus for cardiac hypertrophy and remodeling, in determining the effects of exercise training. Future studies are needed to define the influence of exercise

D. J. Duncker (✉) · E. D. van Deel · M. C. de Waard
Division of Experimental Cardiology, Deptartment of Cardiology, Thoraxcenter, Erasmus MC, University Medical Center Rotterdam, 3000 DR, P.O. Box 1738 Rotterdam, The Netherlands
e-mail: d.duncker@erasmusmc.nl

J. van der Velden
Laboratory for Physiology, VU University Medical Center, Amsterdam, The Netherlands

M. C. de Waard
Department of Intensive Care Medicine, VU University Medical Center, Amsterdam, The Netherlands

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type, intensity, and duration in different models and severities of pathological cardiac remodeling. Together such studies will aid in optimizing the therapy of exercise training in the setting of cardiovascular disease.

Keywords Cardiac remodeling · Cardiac hypertrophy · eNOS overexpression · eNOS deficiency · Exercise training · Myocardial infarction · Pressure-overload

14.1 Introduction

Myocardial hypertrophy is a compensatory mechanism by which the left ventricle (LV) adapts to an increased systolic load, which serves to restore LV wall stress to normal levels and maintain cardiac pump function [1, 2]. Clinically, chronic systolic overload of the LV most commonly results from regional loss of myocardial tissue (myocardial infarction, MI) or elevated impedance to LV outflow (hypertension, aortic stenosis) [3, 4]. Despite the apparent appropriateness of hypertrophic remodeling in response to an increased systolic workload, LV hypertrophy has been shown to be an independent risk factor for the development of angina pectoris, congestive heart failure, and sudden death [1, 5, 6]. The mechanism underlying the progression from LV remodeling to overt heart failure remains incompletely understood, but may include perfusion abnormalities [7, 8], loss of cardiomyocytes through apoptosis [9], reduction in contractile function of the surviving myocardium [10, 11] and/or alterations in extracellular matrix leading to LV dilation [12].

In contrast to pathological LV remodeling, physiological LV remodeling produced by regular dynamic exercise is associated with a decreased risk for coronary-artery disease and heart failure [13], an increased myocardial perfusion capacity [14], and with normal or even increased contractile function in the normal heart [15]. In addition, there is clinical evidence that exercise training has a beneficial effect on disease progression and survival in patients with LV dysfunction [16, 17]. For example, physical conditioning in patients with LV dysfunction results in an increased exercise capacity. The improved exercise capacity was initially ascribed to skeletal muscle adaptations [18], but evidence has since accumulated that exercise training may also ameliorate cardiac dysfunction [19–22]. Nevertheless, concern has also been expressed regarding potentially detrimental effects of exercise training on cardiac remodeling and function, particularly when started too early after a large MI or in case of strenuous exercise [23–27].

In this chapter, we present the results of a series of studies in mice, in which we explored the effects of exercise training on pathological LV remodeling and dysfunction produced by a large MI (with a special emphasis on the mechanisms by which exercise exerts its effects) or produced by pressure-overload.

14.2 Experimental Models of Pathological LV Remodeling

14.2.1 Myocardial Infarction

In our studies, we produce MI by permanent ligation of the proximal left coronary-artery (LCA), which results in infarction of ~40 % of LV mass [28, 29]. Eight weeks after induction of MI, LV remodeling has occurred, characterized by LV dilation and myocardial hypertrophy, as well as increased interstitial collagen deposition and apoptosis, and decreased capillary density in remote surviving myocardium [28–31]. MI also produces marked LV dysfunction, characterized by a pronounced decrease in LV pump function (fractional shortening) and decreases in indices of systolic (dP/dt_{P30}) and diastolic (dP/dt_{min} and τ) function, resulting in pulmonary congestion reflected in pulmonary edema and RV hypertrophy (Fig. 14.1) [28–31].

14.2.2 Aortic Stenosis

For our studies on pressure-overload we apply transverse aortic constriction (TAC) with a 6-0 silk suture, between the truncus brachiocephalicus and the arteria carotis communis sinistra [32]. A 25G needle is used to induce mild TAC (mTAC) and a 27G needle is used to produce severe TAC (sTAC). Eight weeks of exposure to sTAC results in LV remodeling, characterized by ~85 % LV hypertrophy, capillary rarefaction, and interstitial fibrosis [33]. In addition, LV systolic dysfunction is present, characterized by a decrease in fractional shortening and LV dP/dt_{P40} as well as LV diastolic dysfunction, characterized by reduced LV dP/dt_{min} and increased time constant of relaxation (τ) and LV end-diastolic pressure. The latter is associated with pulmonary congestion reflected in pulmonary edema and secondary RV hypertrophy. In contrast, mTAC resulted in only ~40 % LV hypertrophy that is not associated with LV systolic or diastolic dysfunction, reflecting a state of compensated hypertrophy [33].

14.3 Exercise Training and LV Remodeling and Dysfunction After MI

In our exercise studies, we expose mice to an 8 week period of voluntary wheel running. Voluntary exercise is our training method of choice in order to minimize stress factors, which are present during forced running and particularly during swimming [34]. Furthermore, the C57Bl6/J strain, which is the typical background for our transgenic mouse models, is known to perform excellent in voluntary wheel running, while being poor performers during forced treadmill running [34].

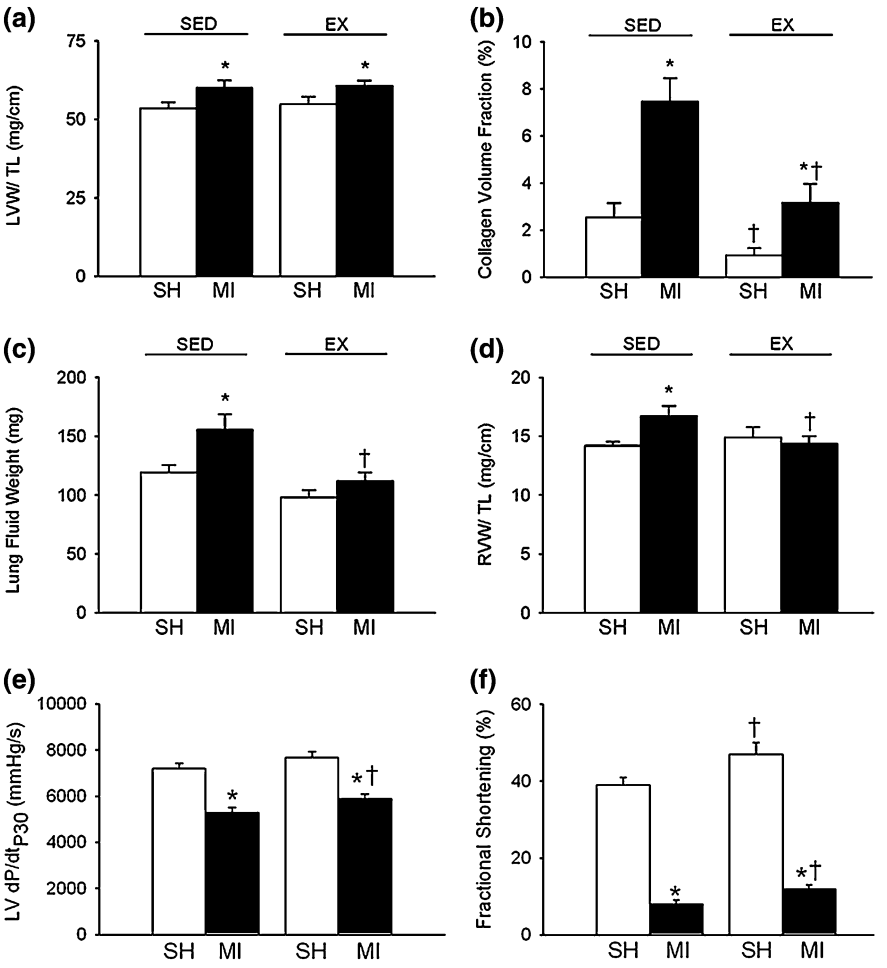

Fig. 14.1 Effects of myocardial infarction (MI) and exercise (EX) on relative LVmass **a**. collagen content **b**. lung fluid weight **c**. relative RVmass **d**. global LV contractility (dP/dt_{P30}) **e**. and global LV pump function (fractional shortening) **f**. $*P < 0.05$ vs. corresponding sham (SH); $\dagger P < 0.05$ vs. corresponding sedentary (SED) mice. Modified from de Waard et al. [29] with permission

Voluntary wheel running, up to 6 km/24 h, had negligible effects on LV geometry and function in sham mice. Voluntary wheel running, starting gradually early after a large MI, did not aggravate post-MI mortality, LV remodeling, capillary rarefaction, and cardiomyocyte hypertrophy of remote myocardium [29, 31], and even reduced myocardial interstitial fibrosis and apoptosis, while blunting LV dysfunction and pulmonary congestion (Fig. 14.1). The mechanisms underlying the improvement of LV function likely include the reductions in apoptosis and collagen deposition as well as improved cardiomyocyte function. Thus, shortening of isolated cardiomyocytes was increased by exercise [29, 31]. Ca^{2+}-transient

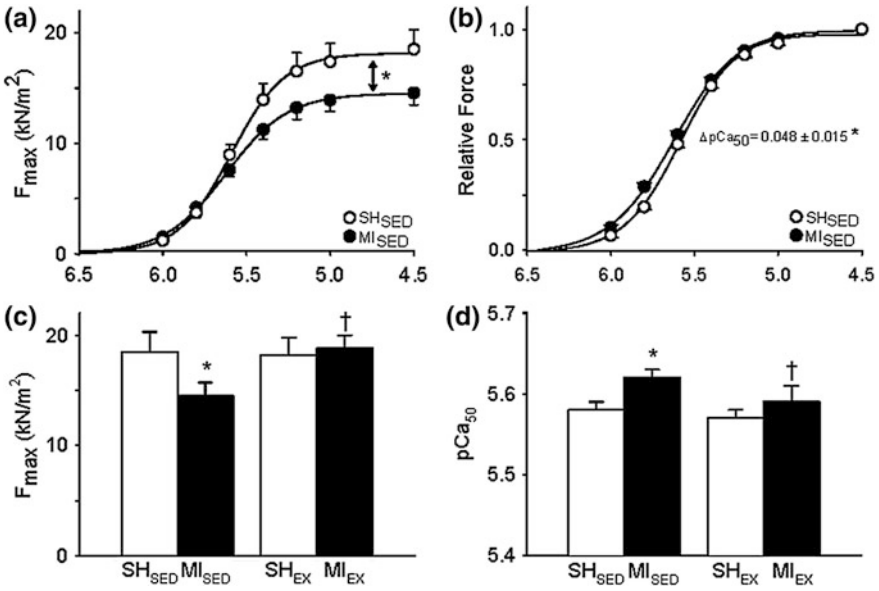

Fig. 14.2 Absolute (**a** and **c**) and normalized (**b** and **d**) force-pCa curves and bar charts. Panels (**a** and **b**) show the effects of myocardial infarction (MI) in sedentary (SED) mice. The panels (**c** and **d**) show the effects of EX in sham (SH) and MI. $*P < 0.05$ vs corresponding SH; † $P < 0.05$ vs corresponding SED. Modified from de Waard et al. [29] with permission

amplitude remained unaltered [29, 35], despite a small increase in sarcoplasmatic reticulum calcium ATPase (SERCA) 2a expression in exercise trained mice [31]. However, basal (diastolic) calcium concentrations were reduced by exercise after MI [29, 35], which was likely the result of the slight increase in SERCA2a [31] in conjunction with the increased expression of Na^+/Ca^{2+}-exchange [29]. Both in our pig and mouse model of MI, we typically observe a reduction in the maximal force generating capacity (F_{max}) and an increase in calcium sensitivity (pCa_{50}) of myofilaments in post-MI remodeled myocardium [10, 29]. Indeed, in trained MI mice, an increase in F_{max} and normalization of pCa_{50} was principally responsible for the improved isolated myocyte shortening in vitro and global LV fractional shortening in vivo (Fig. 14.2).

14.4 Role of NO Signaling in the Beneficial Effects of Exercise Training after MI

In view of the critical role of the endothelium in modulating the progression of ventricular and vascular remodeling in heart failure, the beneficial effects of exercise on cardiac function could be due, at least in part, to an exercise-induced increase in

expression and activity of endothelial nitric oxide synthase (eNOS) [36], which is the major isoform of NOS that is expressed in coronary vascular endothelium and cardiac myocytes [37]. For example, an increase in nitric oxide (NO) bioavailability has been shown to improve many of the perturbed processes in LV remodeling, including angiogenesis [38], cardiac fibrosis [39], and hypertrophy [40].

For our studies into the role of eNOS in the beneficial effects of exercise in mice with MI, we took a two-pronged approach. First, we investigated whether eNOS overexpression could mimic the effects of exercise training [30, 41], and secondly we investigated whether eNOS knockout would abrogate the protective effects of exercise [31].

14.4.1 eNOS Overexpression

To test the hypothesis that eNOS overexpression mimics the beneficial effects of exercise training, we investigated the effects of eNOS overexpression on LV remodeling and dysfunction in mice with a large MI [30, 41]. We found that 10–12 fold overexpression of eNOS improved survival, global LV pump function, attenuated LV dilation, myocardial interstitial fibrosis, and pulmonary edema after MI [30, 41]. Conversely, eNOS overexpression did not attenuate cardiac hypertrophy or improve global cardiac contractility and relaxation parameters. Interestingly, all the improvements by elevated eNOS expression occurred without significant differences in baseline LV geometry, morphology, or function in Sham mice.

The mechanism underlying the improved cardiac function in eNOS overexpressing mice after MI was likely twofold. First, the lower systemic vascular resistance and hence lower aortic and LV systolic pressure [42] likely facilitated fractional shortening of the post-MI LV. In addition, cardiomyocyte-specific overexpression of eNOS has been shown to exert a beneficial effect on LV structure and function after MI [43], suggesting that eNOS overexpression improved LV function by systemic vascular as well as local myocardial effects. To further investigate the effects of eNOS overexpression on cardiomyocyte function, we investigated myofilament function and myofilament protein phosphorylation status. Interestingly, we noted that similar to exercise training, elevated eNOS expression increased the maximal force generating capacity of isolated permeabilized cardiomyocytes. However, in contrast to exercise, elevated eNOS expression did not correct the high myofilament Ca^{2+}-sensitivity in MI and had no effect on MLC-2 protein phosphorylation and β_1-adrenergic receptor and SERCA2a protein levels [30]. These findings suggest that in addition to the afterload reduction, an improvement in myofilament F_{max} could have contributed to eNOS overexpression-induced improvement in LV pump function after MI. The mechanism by which eNOS overexpression increased F_{max} remains to be determined, but it is of interest to note that S-nitrosylation of myofilament proteins, including the myofilament protein alpha-myosin heavy chain, myosin light chain kinase 1 and myomesin, may be involved in the cardioprotection by ischemic preconditioning against ischemia–reperfusion induced sarcomeric

damage in mice [44, 45]. It remains to be investigated whether S-nitrosylation of myofilaments is altered and contributes to the enhanced myofilament contractility in our model.

In order to ascribe the beneficial effects of exercise on cardiac function to an exercise-induced upregulation of eNOS expression and activity, the effects of exercise and eNOS overexpression should be similar. However, while the effects of either exercise or eNOS overexpression in MI mice on LV geometry, collagen content, and function were indeed similar, survival in MI mice was improved by eNOS overexpression but not by exercise. Interestingly, mortality in C57Bl6/J mice after permanent coronary-artery-ligation is principally caused by cardiac rupture of the infarct-area within the first 2 weeks after MI [46]. It is thus possible that the lower systemic vascular resistance and mean aortic pressure in eNOS overexpressing compared to Wt mice [42] may have prevented cardiac rupture thereby enhancing post-MI survival in eNOS overexpressing mice.

14.4.2 eNOS Deficiency

In order to unequivocally demonstrate a critical role for eNOS in the beneficial effects of exercise after MI, it is mandatory to show that the effects of exercise training are absent in eNOS-deficient mice. For this purpose, we repeated our studies in wild-type ($eNOS^{+/+}$) and eNOS-deficient mice lacking either one ($eNOS^{+/-}$) or both ($eNOS^{-/-}$) functional eNOS alleles [31]. We also included $eNOS^{+/-}$ mice, in view of observations by Kojda et al. [47] that basal levels of eNOS expression and hemodynamics are not different from $eNOS^{+/+}$ mice, but the exercise-induced increase in eNOS expression is selectively abolished [47, 48]. Indeed, we observed not only that basal LV protein levels of eNOS were maintained, but also that aortic blood pressure, as well as systolic and diastolic function, was normal in $eNOS^{+/-}$ mice. In addition, levels of apoptosis and cardiomyocyte cross-sectional area were also similar to $eNOS^{+/+}$ mice. However, we did observe that collagen content was higher in $eNOS^{+/-}$ mice and that, similar to the $eNOS^{-/-}$ mice, capillary densities were lower compared to wild-type mice.

Scherrer-Crosbie et al. [49] reported that 4 weeks after a small MI (resulting in a decrease in fractional shortening from 58 ± 2 to 44 ± 2 % in $eNOS^{+/+}$ mice), mortality as well as LV remodeling and dysfunction were more pronounced in $eNOS^{-/-}$ compared to $eNOS^{+/+}$ mice. In contrast, we found that mortality, LV remodeling, and dysfunction, as well as cardiomyocyte hypertrophy, interstitial fibrosis, and increased apoptosis that occurred after a large MI (decreasing fractional shortening from $\sim$40 to 10 %) were also similar in $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ mice (Fig. 14.3) [31]. Our observations are in agreement with the study of Liu et al. [50] in which mortality, LV remodeling, and the development of heart failure over a 6-month follow-up period after a large MI (decreasing fractional shortening from $\sim$65 to 20 %) were similar in $eNOS^{-/-}$ and $eNOS^{+/+}$ mice. Taken together these findings are consistent with the concept that lack of eNOS

does not aggravate myocardial remodeling and LV dysfunction after a large MI and suggest that in more chronic post-MI remodeling an adaptation through compensatory mechanisms has occurred. Such compensatory factors might include prostacyclin [51], nNOS [52–54] and iNOS [55, 56]. However, we failed to observe compensatory increases in expression of nNOS or iNOS [31]. An explanation for the lack of a compensatory increase is not readily found, but could be related to the genetic background of the $eNOS^{-/-}$ mice. Thus, Sharp et al. [55] previously observed that iNOS upregulation in $eNOS^{-/-}$ depended critically on the strain of mice employed. Future studies are necessary to investigate the exact mechanisms that act to compensate after MI in eNOS deficient mice.

To investigate the role of eNOS in mediating the beneficial effects of exercise training, $eNOS^{+/+}$, $eNOS^{+/-}$, and $eNOS^{-/-}$ mice were exposed to either sedentary housing or voluntary exercise training [31]. Although, exercise after MI in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice did not influence LV myocardial hypertrophy, the beneficial effects of exercise on MI-induced LV dilation and LV systolic dysfunction, as observed in $eNOS^{+/+}$ mice, were lost in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice (Fig. 14.3). Furthermore, the exercise-induced amelioration of LV backward failure that was observed in $eNOS^{+/+}$ mice was lost in $eNOS^{-/-}$ mice and even reversed to a worsening of pulmonary congestion in $eNOS^{+/-}$ mice. At the myocardial level, the beneficial effects of exercise in $eNOS^{+/+}$ mice on interstitial fibrosis and apoptosis within non-infarcted remote myocardium were lost in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice [31]. These findings indicate that eNOS is critical for the exercise-induced improvement of LV geometry (both at the macroscopic and microscopic level) and LV function after MI.

Interestingly, $eNOS^{+/-}$ mice which lack only one eNOS allele appeared to respond even slightly worse to exercise after MI than $eNOS^{-/-}$ mice, as reflected in the exacerbated pulmonary congestion and LV interstitial fibrosis (Fig. 14.3). Kojda et al. [47] proposed that when two eNOS alleles are present, as in $eNOS^{+/+}$ mice, under basal conditions both are being transcribed at a submaximal rate. Exercise thus increases transcriptional activity of both genes. The absence of one eNOS allele results in the opposite allele functioning at near peak transcription rate, so that it cannot be further activated by exercise [48]. It could be speculated that in the complete absence of eNOS expression, other factors, including prostacyclin [51], may be upregulated or increased in $eNOS^{-/-}$ mice and act to compensate for the absence of eNOS, thereby preventing further worsening of cardiac remodeling and function. If one eNOS allele is still functional and results in normal basal eNOS expression, such compensatory mechanisms may not occur, leaving the heart more vulnerable to the effects of exercise. Future studies are needed to study the influence of potential compensating factors.

From this study we could not determine the exact location of eNOS involved in the exercise-induced amelioration of LV dysfunction after MI. First, an exercise-induced increase in vascular eNOS expression in the systemic vascular bed may have contributed to the beneficial effects of exercise on LV function after MI. Indeed, we have previously shown that eNOS overexpression, that was particularly pronounced in endothelial cells of various regional vascular beds [57], partly

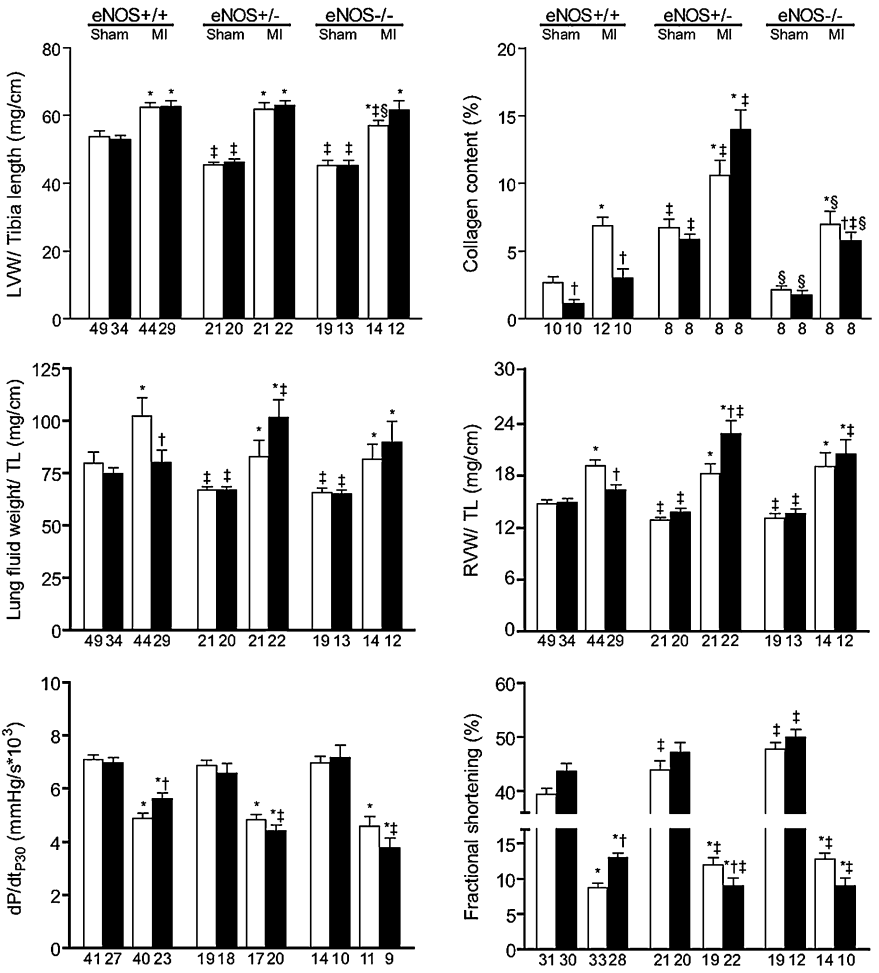

Fig. 14.3 Effects of myocardial infarction (MI) and exercise in eNOS$^{+/+}$, eNOS$^{+/-}$, and eNOS$^{-/-}$ mice on relative LV mass (**a**), collagen content (**b**), and relative lung fluid weight (**c**), relative RV mass (**d**), global LV contractility (dP/dt_{P30}) (**e**) and global LV pump function (fractional shortening) (**f**), $*P < 0.05$ versus corresponding sham, $\dagger P < 0.05$ versus corresponding sedentary, $\ddagger P < 0.05$ versus corresponding eNOS $^{+/+}$, and $\S P < 0.05$ versus corresponding eNOS$^{+/-}$ □ Sedentary and ■ exercise. Numbers of animals are indicated below the figures. Reproduced from de Waard et al. [31] with permission

mimicked the beneficial effects of exercise on cardiac function after MI [30]. However, eNOS overexpression could not explain all the effects of exercise, suggesting that in addition to systemic vascular eNOS, cardiac eNOS (in the coronary endothelium and/or cardiomyocytes) also contributed to the beneficial effects of exercise. Indeed, a recent study reported that eNOS in bone marrow-derived coronary endothelial cells is critical for ameliorating pathological cardiac remodeling [58, 59]

The mechanism by which eNOS mediated the beneficial effects of exercise in the heart likely includes reduced fibrosis and apoptosis, possibly as a result of activation of survival pathways and reduced senescence [58, 60]. In addition, the observations in a previous study that the MI-induced myofilament dysfunction was prevented by exercise [29], in conjunction with the observation that exercise blunted MI-induced oxidative stress [31], could be interpreted to suggest that eNOS-derived NO prevented oxidative modifications of myofilaments and thereby contributed to the beneficial effects of exercise on LV function. The exact location where eNOS exerts its beneficial effects, as well as the mechanism(s) involved in the eNOS-mediated exercise-induced amelioration of LV dysfunction after MI, remains the subject of future studies.

14.5 Exercise Training Prior to a Myocardial Infarction

Physical inactivity has been proposed to be an independent risk factor for cardiovascular disease [61, 62]. Indeed, prospective epidemiological data indicate that moderate (e.g., walking) and vigorous exercise in healthy subjects are associated with substantial reductions in the incidence of cardiovascular events [63, 64]. The beneficial effects of exercise extend to patients with established coronary heart disease in which regular physical activity also reduces the incidence of cardiac events and all-cause mortality [65]. Furthermore, there is evidence that exercise initiated after a cardiac event, such as MI, ameliorates LV remodeling and dysfunction [21, 29, 66–71], and improves clinical outcome [72, 73].

In contrast, there is substantially less information available as to whether prior exercise affords any protection in situations when, despite regular exercise, a major cardiovascular event such as MI occurs. Animal studies suggest that prior exercise can precondition the myocardium, thereby protecting the heart against irreversible damage produced by ischemia-reperfusion in rats [74–77] and dogs [78]. In addition, prior exercise may modulate post-infarct remodeling independent of any myocardial preconditioning effect. Thus, two studies in rats using a permanent coronary-artery-ligation (in which preconditioning cannot limit acute myocardial necrosis) [79, 80] reported that 5–7 weeks of prior swimming had no effect on post-MI mortality, but reduced infarct-size and attenuated LV remodeling, as determined at 2 days [81] or 4 weeks [82] after induction of MI. Swimming-induced increases in capillary [81] and arteriolar [82] densities were observed in the remote region, which led the authors to speculate (collateral blood flow was not actually measured) that prior exercise may have resulted in increased collateral blood flow to the area at risk, thereby limiting infarct size. Although myocardial vascular densities are known to be enhanced in young male rats by swim training [83], there is no evidence to suggest that collateral vessel growth is stimulated by exercise in healthy hearts. Thus, studies pertaining to exercise of healthy dogs, swines, or rats have consistently shown that exercise does not increase innate collateral blood flow capacity in healthy hearts [83]. An alternative explanation

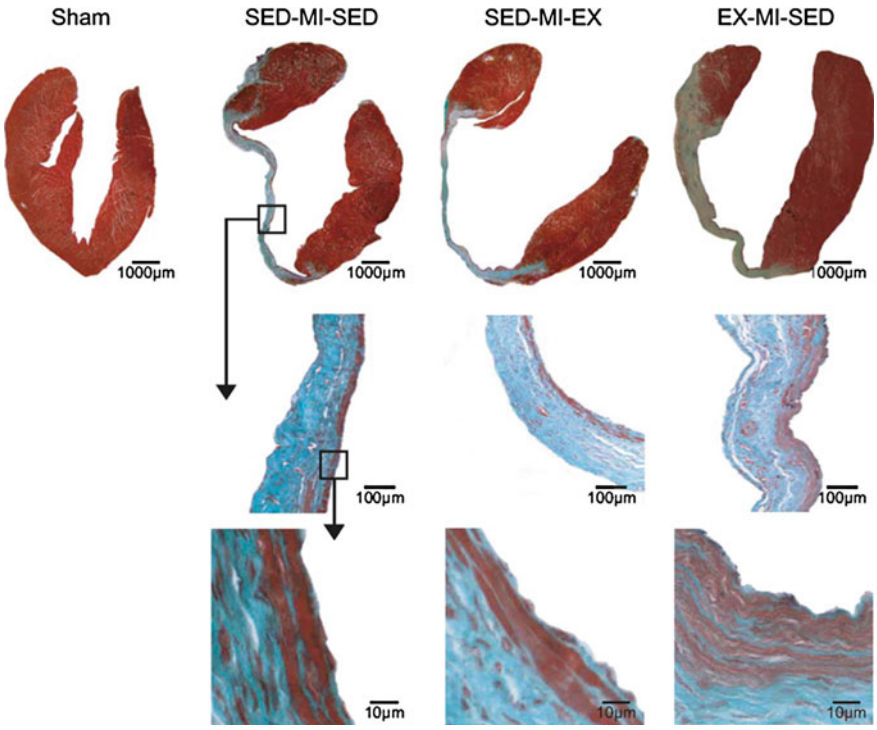

Fig. 14.4 Macroscopic and microscopic representative examples of the LV-infarcted myocardium. Masson's trichrome staining of longitudinal sections of the LV is shown. Red staining represents viable cardiomyocytes, whereas blue-green staining represents collagen. Reproduced from de Waard et al. [82] with permission

may be that prior exercise affected healing and remodeling of the infarct area (contracture), thereby leading to a reduction of infarct length [81, 82]. Consequently, we tested the hypothesis that a daily exercise regimen started 2 weeks prior to an acute MI is associated with improved survival and blunted LV remodeling and dysfunction after MI [84].

Prior exercise reduced post-MI mortality from ~40 to 20 % and attenuated LV dysfunction, but did not enhance capillary density in remote myocardium or limit infarct-size as measured 8 weeks later [84]. Mortality in C57Bl6/J mice after permanent coronary-artery ligation appears to be principally caused by cardiac rupture of the infarct area within the first 2 weeks after MI [46]. Interestingly, we observed that infarct thickness was increased (Fig. 14.4), consistent with exercise-induced modulation of infarct healing and remodeling. Consequently, it could be speculated that the increased infarct thickness acted to reduce systolic wall stress and thereby prevented cardiac rupture, thus enhancing post-MI survival in mice that had been subjected to prior exercise.

The precise mechanism for the blunted LV dysfunction in MI mice exposed to prior exercise was not determined. However, there is evidence that the hearts of

exercised animals respond more effectively to stress. For example, rats subjected to 8 weeks of forced treadmill exercise were able to maintain or increase myocardial contractility more effectively in the face of a sustained pressure overload than sedentary animals [85]. More importantly, these results suggest that even when regular exercise fails to prevent an acute MI, it can still act to improve cardiac function and survival after MI. These observations warrant an even greater emphasis on lifestyle changes in patients with an increased risk of or already established coronary heart disease, and which are at increased risk of encountering an acute MI.

14.6 Effects of Exercise Training in Pressure-Overload LV Hypertrophy

There is ample evidence from both clinical and experimental studies that aerobic exercise training has a beneficial effect on cardiac function and remodeling secondary to ischemic cardiomyopathy [22, 29, 86]. Similarly, the majority of experimental studies in genetic models of systemic hypertension [87–89] have shown a beneficial effect of regular exercise on cardiac remodeling and function, which is supported by recent clinical studies [90, 91]. In contrast, little is known about the effects of exercise on LV pressure-overload hypertrophy as a result of mechanical obstruction to outflow. This is important because there is an increasing number of patients with (congenital) aortic stenosis that are chronically exposed to LV pressure-overload and will ultimately require surgery during their adult life [92]. Furthermore, as life expectancy in general is increasing in Western society and since the prevalence of aortic stenosis increases with age, the number of patients suffering from pressure-overload-induced cardiac hypertrophy due to aortic stenosis is inevitably growing [93]. It is therefore imperative to investigate new therapies for the prevention and treatment of cardiac dysfunction in these patients. So far the potentially beneficial effects of exercise on cardiac function and hypertrophy have been insufficiently examined in this group of patients. Several studies recommended patients with pressure-overload hypertrophy to participate only in mild physical training [94, 95] to minimize the increase in workload to the heart during exercise, but solid clinical evidence for such guidelines is lacking. The effects of regular physical exercise in such patients may well be different from that in patients with cardiac hypertrophy due to systemic hypertension. Thus the presence of an aortic stenosis results in exaggerated LV pressure responses to exercise thereby producing aggravated increases in afterload during each exercise bout [8]. In contrast, relatively normal LV hemodynamic responses to exercise occur in patients with systemic hypertension [96]. It is therefore possible that exercise training in case of a chronic aortic stenosis does not recapitulate the beneficial effects that are observed in ischemic heart disease or systemic hypertension.

In light of these considerations, we investigated the effects of dynamic exercise training on aortic stenosis-induced LV hypertrophy and dysfunction. Since the

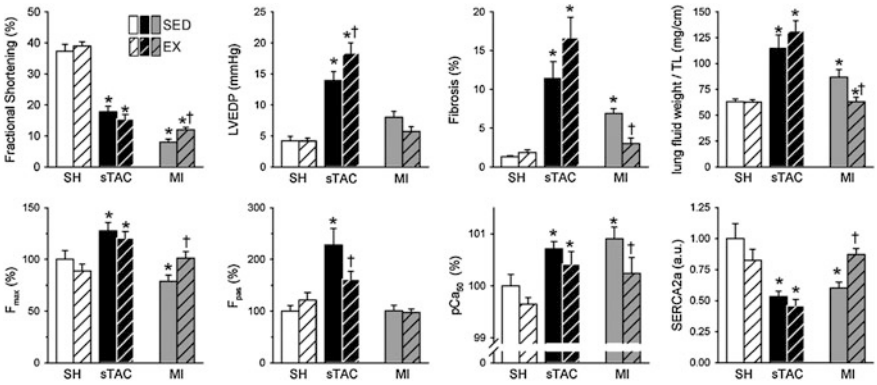

Fig. 14.5 Comparative data on the effect of exercise (EX) on cardiac function and geometry after severe transverse aortic constriction (*sTAC*) and myocardial infarction (*MI*). *LVEDP* = LV end-diastolic pressure; *TL* = tibia length; Data on myofilament function are normalized to corresponding sedentary sham mice (SH_{SED}). *Fmax* = maximum force; *Fpas* = passive force; *pCa*50 = calcium sensitivity; S*ERCA*2*a* = Sarco-Endoplasmatic Reticulum Calcium ATP-ase. $*P < 0.05$ vs corresponding SH; $\dagger P < 0.05$ vs corresponding SED. Reproduced from van Deel et al. [33] with permission

effects of exercise training might depend on the severity of the aortic stenosis, we assessed the effects of exercise in mice subjected to either mild (mTAC) or severe aortic stenosis (sTAC) [33].

Eight weeks of voluntary wheel running had no significant effect on LV hypertrophy and remodeling in either mTAC or sTAC mice. In contrast, while LV function was well maintained in exercise-trained $mTAC_{EX}$ mice (data not shown), a trend toward aggravated LV dysfunction and backward failure was apparent in $sTAC_{EX}$ mice (Fig. 14.5). These effects of exercise in sTAC mice could not be explained by alterations in myofilament function, as F_{max} and pCa_{50} were not altered. Interestingly, a decrease in passive stiffness of myofilaments (F_{pas}) was noted, which was however not associated with a reduction in LV end-diastolic pressure. The latter may, at least in part, be related to an increase in collagen content in the $sTAC_{EX}$ mice, which may have offset the exercise-induced lowering of passive cardiomyocyte stiffness. Survival rate was not significantly altered by exercise training but tended to improve with exercise in mTAC and sTAC mice. This trend toward an improved survival with exercise without improvements in LV function is in agreement with another rodent study of pressure-overload hypertrophy [87] and indicates that exercise could potentially improve survival in models of pressure-overload hypertrophy without beneficial effects on LV function. The results of this study contrast with the beneficial effects of exercise on LV hypertrophy and dysfunction reported for ischemic [22], systemic hypertensive [90, 91], and hypertrophic [97] cardiomyopathy. Experimental studies in Dahl salt-sensitive or spontaneously hypertensive rats, have generally shown beneficial effects of regular treadmill exercise [87, 88] or swimming [89, 98] on cardiac function, fibrosis, the expression of hypertrophy marker genes and survival

although not on LV hypertrophy per se. Only Schulz et al. [27] reported that excessive long-term (up to 16 months) wheel running aggravated cardiac hypertrophy and dysfunction, fibrosis, and expression of hypertrophy marker genes in spontaneously hypertensive female rats.

An explanation for the lack of a beneficial effect is not readily found. However, it should be noted that the effects of exercise training on the development of LV dysfunction and hypertrophy depend at least in part on the mode, frequency, and intensity of exercise [22, 27]. For example, dynamic exercise, which exposes the heart to a volume-overload, reverses LV remodeling and improves cardiac function in patients with heart failure [22, 99]. In contrast, when static and dynamic exercise are combined (resulting in more pronounced increases in systemic pressure) the beneficial effects of physical training are no longer observed [22]. Since dynamic exercise in the presence of an aortic stenosis will result in exaggerated increases in LV systolic pressures [8] this will likely add a pronounced static component to the exercise response. It is thus possible that the excessive increase in LV systolic workload that occurs in response to acute exercise may have offset the positive effects of the 8 weeks voluntary wheel running protocol, which we previously observed in mice with a MI [29]. In view of our observations that eNOS overexpression in part mimics the beneficial effects of exercise training in mice with MI [30, 41] it will be of interest to evaluate the effects of eNOS overexpression (i.e., the beneficial molecular effects of exercise, but without the hemodynamic overload) on LV hypertrophy and dysfunction in sTAC mice in future studies.

14.7 Conclusions

Our studies investigating the effects of exercise training on pathological cardiac remodeling and dysfunction have shown that in the presence of a large MI, exercise training (started early after MI) exerts a beneficial effect, an observation that is now increasingly recognized in clinical research [100]. The beneficial effects of exercise training on the heart were (in part) mimicked by eNOS overexpression and abrogated by eNOS deficiency. The results clearly point toward the importance of nitric oxide signaling in the beneficial effects of exercise. Interestingly, exercise prior to MI was also cardioprotective, likely by improving infarct healing. In contrast to the beneficial effects of exercise training in the setting of an acute MI, exercise tended to aggravate pathological cardiac remodeling and dysfunction in the setting of aortic stenosis, which emphasizes the critical importance of the underlying pathological stimulus for cardiac hypertrophy and remodeling. Future studies are needed to better define the influence of exercise intensity and duration on different models and severities of pathological cardiac remodeling. Such studies will aid in optimizing the therapy of exercise training in the setting of cardiovascular disease.

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Chapter 15
The Athlete's Heart

Aaron L. Baggish

Abstract There are numerous cardiovascular adaptations to exercise. The notion that repetitive exercise affects the heart dates back to the initial descriptions of cardiac enlargement in trained athletes in the 1890s. It is now well-established that prolonged exposure to vigorous physical exercise leads to numerous changes in cardiac structure and function. The majority of these changes represent adaptations that facilitate preserved or enhanced cardiovascular function during the hemodynamic stress of exercise. This chapter will provide an overview of the adaptive cardiac physiology associated with exercise training.

Keywords Athlete's heart · Ventricular hypertrophy · Cardiac remodeling

15.1 Introduction

The association between sport participation and cardiac "abnormalities" has been appreciated for more than a century. The initial descriptions of cardiac enlargement in healthy trained athletes, later characterized as cardiac chamber dilation and myocardial hypertrophy, lead to the concept of the athlete's heart. Scientific understanding of how repetitive exercise affects heart structure and function has advanced considerably since the initial descriptions in the 1890s. It is now well-established that prolonged exposure to vigorous physical exercise leads to numerous changes in cardiac structure and function. The majority of these changes

A. L. Baggish (✉)
Cardiovascular Performance Program, Division of Cardiology,
Massachusetts General Hospital, 55 Fruit Street, Yawkey 5, Boston, MA 02114, USA
e-mail: abaggish@partners.org

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represent adaptations that facilitate preserved or enhanced cardiovascular function during the hemodynamic stress of exercise. This chapter provides an overview of the adaptive cardiac physiology that is associated with exercise training.

15.2 Historical Perspective

The first report of cardiac adaptation in trained athletes is credited to the Swedish clinician Henschen, who utilized the physical examination skills of auscultation and percussion to demonstrate increased cardiac dimensions in elite Nordic skiers [1]. Initial reports describing cardiac enlargement in athletes date back to the late 1890s. In the United States, Eugene Darling of Harvard University made similar observations in university rowers [2]. In the early 1900s, Paul Dudley White studied the radial pulse contour among Boston Marathon competitors, [3] and was the first to report marked resting sinus bradycardia in long distance runners [4]. Further advances in our understanding of the relationship between athletic activity and cardiac morphology have paralleled developments in diagnostic technology. Early use of chest radiography confirmed the physical examination finding of global cardiac enlargement in trained athletes [5–7]. The development of electrocardiography enabled widespread study of the electrical activity in the heart of the trained athlete [8–14]. In addition to the morphologic patterns of cardiac hypertrophy, early ECG work documented brady- [15] and tachyarrhythmias [16], in exercise-trained individuals. The development and rapid dissemination of two-dimensional echocardiography lead to important further advances in our understanding of the athlete's heart. Descriptions of biventricular chamber enlargement, myocardial hypertrophy, and atrial dilation lead to a more comprehensive understanding of the typical cardiac findings in the trained athlete. Most recently, advanced echocardiography and magnetic resonance imaging have begun to clarify important functional adaptations that accompany previously reported changes in structure.

15.3 Exercise Physiology and the Athlete's Heart: Overview

Successful performance of physical exercise relies on coordinated activity of the lungs and pulmonary vasculature (oxygen uptake), the heart and systemic vasculature (oxygen transport), and the skeletal muscle (oxygen utilization and force generation). Numerous superb reviews of clinically relevant exercise physiology are available [17–19]. Only key aspects relevant to exercise-induced cardiac remodeling (EICR) will be reviewed here.

There is a direct relationship between exercise intensity (external work) and the body's demand for oxygen. This oxygen demand is met by increasing pulmonary oxygen uptake (VO_2). The cardiovascular system is responsible for transporting oxygen rich blood from the lungs to the skeletal muscles, a process quantified as cardiac output (L/min.). The Fick equation (Cardiac Output = VO_2 × Arterial-

Venous O_2 Δ) can be used to quantify the relationship between cardiac output and VO_2. In the healthy human, there is a direct and inviolate relationship between VO_2 and cardiac output.

Cardiac output, the product of stroke volume and heart rate, may increase 5 to 6-fold during a maximal exercise effort. Coordinated autonomic nervous system function, characterized by rapid and sustained parasympathetic withdrawal coupled with sympathetic activation, is required for this to occur. Heart rate in the athlete may range from less than 40 beats per minute at rest to greater than 200 beats per minute in a young maximally exercising patient. Maximal heart rate varies innately among individuals and decreases with age [20]. Heart rate increase is responsible for the majority of cardiac output augmentation during exercise and peak heart rate is a fundamentally limiting factor of peak exercise capacity in healthy humans. However, maximal heart rate does not increase with exercise training, and thus this is not considered and adaptable property [21].

In contrast, stroke volume both at rest and during exercise characteristically increases significantly with prolonged exercise training. Cardiac chamber enlargement and the accompanying ability to generate a large stroke volume is a direct result of EICR and is one of the cardiovascular hallmarks of the endurance-trained athlete. Stroke volume rises during exercise due to increases in ventricular end-diastolic volume and to a lesser degree, due to sympathetically mediated reduction in end systolic volume (particularly during upright exercise) [18]. Left ventricular (LV) end-diastolic volume is determined by diastolic filling, a complex process that is affected by a variety of variables including heart rate, intrinsic myocardial relaxation, ventricular compliance, ventricular filling pressures, atrial contraction, and extra-cardiac mechanical factors including pericardial and pulmonary constraints. At the present time, to what degree each of these factors contributes to stroke volume augmentation during exercise remains uncertain.

Hemodynamic conditions, specifically changes in cardiac output and peripheral vascular resistance, vary widely across sporting disciplines. Although some overlap exists, exercise activity can be segregated into two forms with defining hemodynamic differences. Isotonic exercise, also referred to as endurance exercise, involves sustained elevations in cardiac output with normal or reduced peripheral vascular resistance. Such activity represents primarily a volume challenge for the heart, which affects all four chambers. This form of exercise underlies activities including long distance running, Nordic skiing, cycling, rowing, and swimming. In contrast, isometric exercise, also referred to as strength training, involves activity characterized by increased peripheral vascular resistance and normal or only slightly elevated cardiac output. This increase in peripheral vascular resistance causes transient but potentially marked systolic hypertension and LV afterload. Strength training is the dominant form of exercise in activities such as weightlifting, track and field throwing events, and American-style football. Many sports, including popular team-based activities such as soccer, lacrosse, basketball, hockey, and field hockey, involve significant elements of both endurance and strength exercise. As will be discussed, sport-specific hemodynamic conditions may play an important role in EICR.

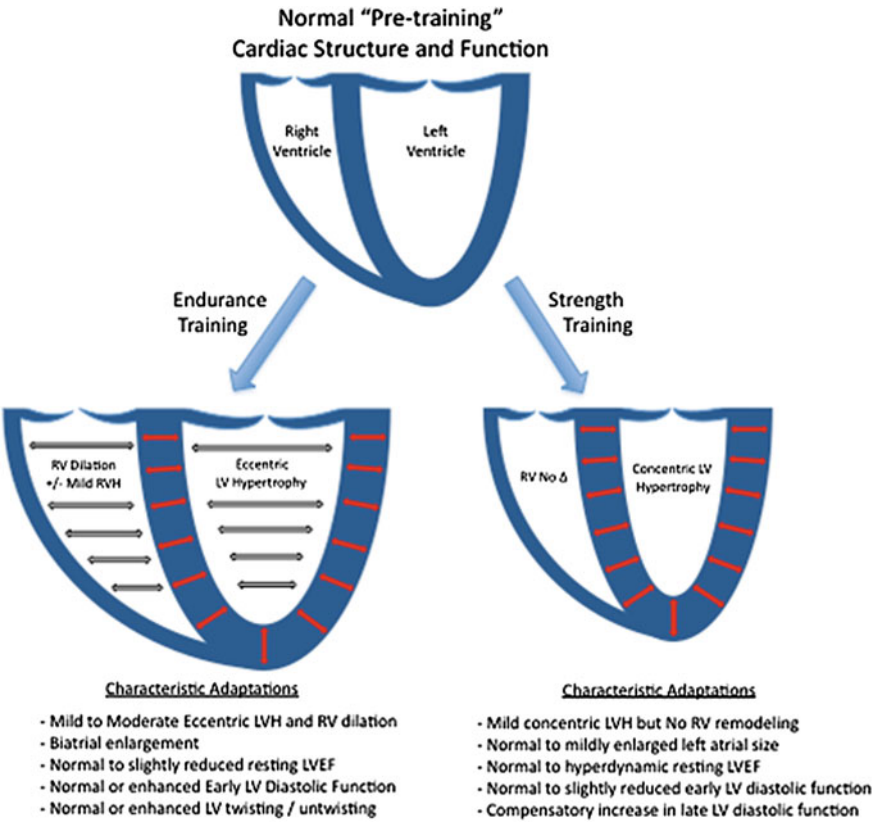

Fig. 15.1 Summary of ventricular remodeling during sustained exercise training highlighting the sport-specific nature of EICR. Adopted with permissions from Weiner and Baggish, progress in cardiovascular disease 2012

15.4 Exercise-Induced Cardiac Adaptations

An overview of exercise-induced cardiac adaptations is presented in Fig. 15.1. The following sections address specific cardiac chambers in detail.

15.4.1 The Left Ventricle

The impact of exercise training on LV structure has been the topic of extensive study. Early studies utilizing electrocardiography in trained athletes demonstrated a high prevalence of increased cardiac voltage suggestive of LV enlargement [9, 22]. Subsequent work with 2-D echocardiography confirmed underlying LV hypertrophy and dilation [23]. Most of these early studies utilized a cross-sectional

design comparing small groups of endurance-trained athletes to sedentary controls. More recently, newer noninvasive cardiovascular imaging techniques have been used to examine LV structure and function in a wide variety of athletes including cyclists [24], triathletes [25], runners [26], canoeists [27], weight lifters [28, 29], orienteers [30], tennis players [31], tumblers [32], American-style football players [33], soccer players [34], baseball player [35], hockey players [36], skiers [37], and swimmers [38].

Italian physician scientists have contributed a great deal to our understanding of LV structure in athletes using data derived from their long-standing pre-participation screening program. Pelliccia et al. reported echocardiographically derived LV end-diastolic cavity dimensions in a large group (n = 1,309) of Italian elite athletes [39]. This cohort was comprised predominantly of male athletes (73 %) and included individuals from 38 different sports. LV end-diastolic diameters varied widely from 38 to 66 mm in women (mean = 48 mm) and from 43 to 70 mm in men (mean = 55 mm). Importantly, LV end-diastolic diameters were $\geq$54 mm in 45 % and >60 mm in 14 % of the cohort. Markedly, dilated LV chambers (>60 mm) were most common in athletes with higher body mass and those participating in endurance sports (cycling, cross-country skiing, and canoeing).

Pelliccia et al. similarly reported echocardiographic measurements of LV wall thicknesses among 947 elite athletes. Within this cohort, a small but significant percentage of athletes (1.7 %) had LV wall thickness $\geq$13 mm and all of these individuals had concomitant LV cavity dilation [40]. Sharma et al. also reported a low incidence (0.4 %) of LV wall thickness >12 mm among 720 elite junior athletes and confirmed that increased LV wall thickness is typically associated with increased chamber size consistent with adaptive remodeling even in these young athletes [41]. Although studies such as these demonstrate a low incidence of increased wall thickness measurements among athletes, a small but significant number of trained individuals do have wall thickness values in the 13–15 mm range. This finding may be particularly common among elite athletes and those that engage in exercise training with both significant isometric and isotonic physiologic stress such as rowers [42]. It is has been consistently shown the most marked LV hypertrophy occurs in athletes with relatively large body size.

Copious data show that sporting discipline impacts the LV response exercise. The notion that endurance-based and strength-based exercise lead to distinctly different changes in LV structure was first proposed by Morganroth et al. in 1975 [43]. These authors compared echocardiographically derived LV measurements in wrestlers (isometric training), swimmers (isotonic training), and sedentary controls and found significant differences with different forms of exercise training. Specifically, athletes exposed to isometric hemodynamic stress demonstrated concentric LV hypertrophy, whereas individuals exposed to isotonic hemodynamic stress demonstrated eccentric LV enlargement. This study led to the concept of sport-specific cardiac remodeling, often referred to as the "Morganroth Hypothesis". Although some data have been presented which refute the concept of sport-specific LV remodeling [44–46], the majority of cross-sectional data and more

recently, carefully designed longitudinal work, support the Morganroth Hypothesis [47]. The interested reader is referred to a comprehensive review of this topic by Naylor and colleagues [48].

Exercise-induced adaptations in LV function have also been studied. Numerous investigators have examined resting LV systolic function in athletes using cross-sectional, sedentary control study designs [25, 49–51]. These studies and a large meta-analysis show that LV ejection fraction is generally normal among athletes [52], although at least one study of 147 cyclists participating in the Tour de France found that 17 (11 %) had a calculated LV ejection fraction $\leq$52 % [53]. Such results suggest that endurance athletes may occasionally demonstrate mildly diminished LV function at rest. Recent advances in functional myocardial imaging, including tissue Doppler echocardiography and strain echocardiography, have also suggested that exercise training may lead to changes in LV systolic function that are not detected by assessment of a global index like LV ejection fraction [54–56]. Preservation or mild enhancements of LV strain [57] and twist (torsion) [58] have been documented among endurance athletes. The importance of these findings with respect to our understanding of exercise physiology and performance is an area of active investigation.

LV diastolic function has also been extensively evaluated in trained athletes. Most studies of diastolic function in athletes have utilized conventional 2-D (transmitral) and tissue Doppler echocardiography. It is now well-recognized that, endurance exercise training leads to enhanced early diastolic LV filling as assessed by E-wave velocity and mitral annular/LV tissue velocities [34, 42, 59–61]. It is likely that improved LV diastolic function, particularly the ability of the LV to relax briskly at high heart rates, is an essential mechanism for stroke volume preservation during exercise. There are sparse data examining diastolic function in strength trained athletes, but one longitudinal study suggested that the concentric LV hypertrophy associated with strength training is accompanied by impaired relaxation [47].

15.4.2 The Right Ventricle

Exercise training induced cardiac remodeling is not confined to the LV. Endurance exercise requires both the LV and right ventricle (RV) to accept and eject relatively large quantities of blood. In the absence of significant shunting, both chambers must augment function to accomplish this task. Recent advances in noninvasive imaging have begun to clarify how the RV responds to the repeated challenges of exercise.

Henriksen et al. examined RV and LV cavities and wall measurements using M-mode and 2-D echocardiography in 127 male elite endurance athletes [62]. Compared to historical controls, endurance-trained athletes demonstrated significantly larger RV cavities and a trend toward thicker RV free walls. In an elegant MRI-based study, Scharhag et al. confirmed that RV enlargement is common

among endurance athletes [63]. Data from this study and others [64–67] suggest that RV enlargement parallels LV enlargement supporting the concept that endurance-based EICR is a balanced, biventricular phenomenon.

The impact of strength training on the RV remains unclear as the limited available data are inconsistent. Perseghin et al. compared RV and LV structure in endurance athletes (marathon runners), strength athletes (sprinters), and sedentary controls and found the largest RV volumes among the strength athletes. However, there were no significant difference between the RV dimensions in strength and endurance athletes after adjustment for body surface area [68]. One study compared RV structure in collegiate endurance-trained (rowers) and strength-trained (American-style football players) athletes before and after 90 days of team-based exercise training [47]. There was statistically significant RV dilation in the endurance athletes, but no changes in RV architecture in the strength athletes. Further elucidation of how the RV responds to different forms of exercise and its contribution to exercise capacity is an important area for future work.

15.4.3 The Aorta

The aorta experiences a significant hemodynamic load during exercise. The nature of this load is dependent on sport type with endurance activity causing high-volume aortic flow with modest systemic hypertension and strength activity resulting in normal volume aortic flow with potentially profound systemic hypertension. It is logical to assume that such conditions may result in variable aortic remodeling along athletic individuals. This premise has been the topic of numerous studies, but no definitive conclusions are provided by the few existing studies. For example, Babaee and colleagues compared aortic dimensions in 100 elite strength-trained athletes to those in 128 age-matched controls [69]. They reported significantly larger aortic dimensions at the valve annulus, sinuses of Valsalva, sinotubular junction, and proximal root in the strength-trained athletes and the largest dimensions were observed in those with the longest duration of exercise training. Similarly, D'Andrea et al. used transthoracic echocardiography to measure aortic dimensions in 615 elite athletes (370 endurance-trained athletes and 245 strength-trained athletes; 410 men; mean age 28.4 $\pm$ 10.2 years, range of 18–40). These authors found that aortic root diameter was significantly higher among strength-trained athletes [70]. In contrast, Pelliccia et al. reported aortic root dimensions in a heterogeneous group of 2,317 Italian athletes and found the largest aortic root measurements in endurance-trained athletes, specifically swimmers and cyclists [71]. Such contradictory data make definitive conclusions about the impact of exercise training on aortic dimensions impossible. Of note, these and other studies [72] have found that the ascending aortic root rarely exceeds the clinically accepted upper limits of normal (40 mm). As such, it seems reasonable to conclude that athletic training is not a common cause of marked aortic dilation.

15.4.4 The Left Atrium

Numerous authors have examined left atrial structure in trained athletes. Hauser et al. presented an early echocardiographic study demonstrating larger left atria in endurance athletes (n = 12) than in sedentary controls (n = 12) [73]. A similar early study documented relative left atrial enlargement in older individuals with a history of exercise training [74]. Hoogsteen et al. subsequently, compared atrial dimensions in young competitive cyclists (17 ± 0.2 years, n = 66) to those in older, presumably more experienced cyclists (29 ± 2.6 years, n = 35) and found larger dimensions in the older athletes [75]. Pelliccia et al. presented the largest data set of atrial measurements in athletes (n = 1,777) and demonstrated that left atrial enlargement (>40 mm in an anterior/posterior transthoracic echocardiographic view) was present in 20 % of the athletes [71]. D'Andrea et al. recently confirmed a high prevalence of left atrial enlargement in trained athletes and demonstrated an association with endurance sports training [70].

15.5 Determinants of Cardiac Adaptation Magnitude

The extent of cardiac adaptation varies considerably across individuals. Obvious explanatory factors include sport type, prior exercise exposure, and training-intensity/duration. However, these contributors do not explain all of the variability [47]. Additional factors including gender, ethnicity, and genetics are contributory. Available data suggest that female athletes exhibit quantitatively less physiologic remodeling than their male counterparts [44, 48–53]. This appears to be true even after cardiac dimensions are corrected for the typically smaller female body size. Definitive explanation for the gender-specific magnitude of EICR remains elusive and on-going work, specifically designed to examine the relationship between sex hormones and cardiac structure, is necessary.

Race may also be an important determinant of adaptation. Several recent studies suggest that black athletes tend to have thicker LV walls than Caucasian athletes. Basavarajaiah and colleagues studied a Caucasian and black athletes utilizing echocardiographic imaging and found that nearly 20 % of the black athletes were found to have LV wall thickness of at least 12 mm as compared to 4 % of white athletes [54]. Importantly, 3 % of black athletes in this cohort were found to have wall thickness of >15 mm. Similarly, Rawlins and colleagues studied ethnic/race-related differences in a group of 440 black and white female athletes using echocardiography [55]. Black female athletes demonstrated significantly higher LV wall thickness and mass compared to the white women (LV wall thickness = 9.2 ± 1.2 mm, LV mass = 187.2 ± 42 g in black athletes versus LV wall thickness = 8.6 ± 1.2 mm, LV mass = 172.3 ± 42 g in the white athletes).

The genetics of cardiac adaptation are an area of active investigation. Polymorphisms within genes coding for proteins of the renin-angiotensin-aldosterone

axis have been examined in the context. Among military recruits, the angiotensin converting enzyme-DD polymorphism was associated with more LV hypertrophy than the II polymorphism during 10 weeks of exercise training [56]. Specific polymorphisms of the angiotensinogen gene have been similarly associated with LV remodeling [57]. Further work is underway to examine alternative gene candidates. We recently demonstrated that familial hypertension, a complex genetic trait, is associated with both the magnitude and geometry of LV adaptation in young normotensive offspring [58]. Further work will be required to determine the genetic and hemodynamic mechanisms underlying this observation.

15.6 Cellular Mechanisms of Cardiac Adaptation

The cellular pathways responsible for cardiac adaptation remain poorly understood. There are currently no mechanistic studies in humans that explain why the myocardial cells remodel in the face of repeated exercise bouts. The lack of in vivo data relates to the challenges inherent in acquiring cardiac tissue from healthy subjects. However, animal data examining pathologic forms of ventricular hypertrophy during experimental exercise intervention are instructive and hypothesis generating.

Cellular signals for cardiac hypertrophy can be broadly segregated into biomechanical ("stretch sensitive") and neurohumoral mechanisms [59]. Biomechanical stress, imparted by volume or pressure overload, has been shown to trigger LV hypertrophy in animal models by activation of integrin cell adhesion complexes [60], myocardial sarcomeric Z-disks [61], and transmembrane receptors [62]. Several neurohumoral agents that are up-regulated during exercise in humans including catecholamines, natriuretic peptides, and fibroblast growth factor have been implicated as mediators of ventricular hypertrophy in animal models [59]. At the present time, the relative contributions of these factors for determining the magnitude of cardiac adaptation remain completely speculative. This constitutes an important area of future work.

15.7 Exercise-Induced Cardiac Adaptation: Physiology vs. Pathology

The significance of cardiac enlargement in athletes has been debated since the time of its initial description. Although EICR is most often regarded as a beneficial adaptation to exercise, this view has not been universally accepted. It was postulated as early as 1902 that cardiac enlargement in athletes is a form of overuse pathology and that prolonged participation in sport could lead to premature cardiovascular system collapse [63]. This concept has resurfaced numerous times

over the last century of scientific inquiry despite the fact that there is no definitive evidence to substantiate its validity.

This debate has most recently focused on the RV. We previously reported physiologic RV dilation with focal deterioration of interventricular septal function after a period of intense exercise training [29]. Specifically, we found significant reductions in systolic function within the interventricular septum, specifically reductions in circumferential vector systolic shortening, among collegiate rowers after 90 days of team-based training. In this setting, the magnitude of circumferential strain impairment was tightly correlated with the degree of concomitant RV dilation. While this observation requires replication and its clinical significance remains uncertain, we hypothesized that RV dilation, by the nature of the way its fibers insert into the LV, may compromise septal function. Recently, La Gerche et al. used cardiac magnetic resonance imaging to document septal fibrosis in the locations of RV fiber insertion among a small group of experienced endurance athletes. Not surprisingly based on our prior work, this septal fibrosis was encountered among individuals with the most marked RV dilation [64]. Thus, it appears possible that select individuals may experience some adverse forms of cardiac remodeling (i.e. fibrosis) following long-term training.

Although this debate is ongoing [65] and more prospective, long-term studies of athletes are necessary, the modern view of the athlete's heart must considered one that implicates adaptive and beneficial physiology, not pre-clinical disease. One recent observational longitudinal study of Olympic-caliber Italian athletes ($n = 114$) demonstrated no deterioration in LV function or occurrence of cardiovascular events over an extended period (8.6 ± 3 years) of intense training [66]. The most reassuring data come from the numerous cross-sectional studies which document normal or increased life expectancy among former elite athletes [67, 68] and recreationally active individuals [72, 76]. Although these studies do not directly define the relationship between cardiac adaptation and clinical outcomes, they document favorable prognosis in the populations most likely to harbor significant these changes.

15.8 Conclusions

Participation in vigorous recreational exercise and competitive athletics leads to numerous changes in cardiac structure and function. These changes can largely be considered adaptive as they facilitate preserved or enhanced cardiac function during the hemodynamic stressors of intense exercise. While the last several decades of work have lead to comprehensive understanding of the basic structural and functional characteristics of exercise-induced cardiac adaptation, little is understood about the underlying cellular mechanics, genetics, and individual variability. These three principal areas of uncertainty represent important areas of future work.

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Part III
Molecular and Cellular Aspects of Cardiac Adaptations

Chapter 16
Role of β-Adrenoceptor/Adenylyl Cyclase System in Cardiac Hypertrophy

Adriana Adameova, Paramjit S. Tappia and Naranjan S. Dhalla

Abstract Activation of the sympathetic nervous system (SNS) releases norepinephrine, stimulates β-adrenoceptors, and plays a crucial role in the development of cardiac hypertrophy upon activation of various cellular signaling pathways in the heart. At early stages, norepinephrine-induced cardiac hypertrophy serves as an adaptive mechanism for maintaining heart function whereas at late stages, it is associated with contractile dysfunction, alterations in electrical activity, and programmed cell death. Activation of G_s-protein coupled β_1- or β_2-adrenoceptors produces an increase in cardiac contractility and some deleterious effects whereas that of G_i-protein coupled β_2- or β_3-adrenoceptors is known to result in beneficial adaptive actions in the heart. While the increase in G_s-protein involves the downstream activation of adenylyl cyclase (AC), the activation of G_i-proteins is associated with either a depression in AC or augmentation of guanylate cyclase activity. In this article, we discuss the physiological aspects of β-adrenergic signaling pathways and their modification in the hypertrophied heart as well as their participation in the transition of cardiac hypertrophy to heart failure. Furthermore, we highlight the actions of some components of the β-adrenoreceptor signaling cascade that may participate in the genesis of cardiac hypertrophy and thus serve as pharmacological targets for the prevention of cardiac hypertrophy or treatment of the hypertrophied failing heart.

A. Adameova (✉)
Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University, Odbojarov 10, 832 32 Bratislava, Slovak Republic
e-mail: adameova@fpharm.uniba.sk

P. S. Tappia
Asper Clinical Research Institute, St. Boniface Hospital Research Centre, Winnipeg, Canada

N. S. Dhalla
Institute of Cardiovascular Sciences and Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Canada

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16.1 Introduction

Cardiac hypertrophy develops as a consequence of physiological or pathological stimulus, which induces activation of different signal transduction pathways to increase muscle mass. Since adult myocardial cells are terminally differentiated, cardiac growth during the hypertrophic process is due to an increase in the size of individual myocardial cells with little or no change in cell number [1]. In highly trained athletes, the growth of the cardiac ventricle refers to physiological hypertrophy, which is not associated with any predisposition to heart failure. In contrast, pathological hypertrophy of mechanically overloaded heart is linked with specific changes that make the heart more sensitive to injury. It is noteworthy that pathological hypertrophy at initial stages is a physiological response of the heart to an increased workload. However, at late stages the heart is unable to maintain cardiac function and exhibits certain distinct features of pathological hypertrophy. Although in addition to hemodynamic overload, different circulating hormones including those released upon the activation of sympathetic nervous system (SNS) are known to play important roles in the genesis of cardiac hypertrophy, this review will be focussed to discussion on the involvement of circulating catecholamines in the development of both physiological and pathological forms of hypertrophy.

16.2 Role of the SNS and Catecholamines in Cardiac Hypertrophy

Development of heart hypertrophy is a multifactorial process, which is characterized by an increase in contractile protein content, myofilament organization, and expression of embryonic markers for the genes encoding these proteins [2–4]. Furthermore, in addition to mechanical factors, different hormones play a crucial role in the genesis of cardiac hypertrophy. In this regard, catecholamines are considered as “hypertrophy hormones” and the activation of SNS and subsequent elevations in circulating catecholamines contribute to changes in cardiac function [5, 6]. Although these alterations in the SNS can be initially considered as compensation for increased hemodynamic load and exhibits functional benefits associated with increased muscle mass, the hypertrophied heart ultimately dilates and fails in a process referred to as decompensation [7, 8]. Indeed, a direct correlation between plasma levels of catecholamines including norepinephrine and mortality in patients with chronic congestive heart failure (CHF) has been documented. In addition to catecholamines *per se*, their oxidation products, such as amino chromes

and adrenolutin, have been suggested as an independent predictor of survival in CHF patients [9]. It should be noted that despite a considerable effort, molecular mechanisms involved in the transition from cardiac hypertrophy to heart failure are incompletely understood. It is therefore, that both pathological states (cardiac hypertrophy and heart failure) with respect to the involvement of the SNS in their pathogenesis are discussed in this article. Furthermore, although both α-adrenoceptors and β-adrenoceptors (β-ARs) are known to mediate cardiomyocyte growth, we will discuss only those mechanisms associated with the β-adrenoceptor complex. In particular, we will focus on the role of adenylyl cyclase (AC) and protein kinase A (PKA) in signaling pathways leading to cardiac hypertrophy.

16.3 AC as a Mediator of the β-Adrenoceptor Signaling Pathway in the Heart

Stimulation of β-ARs leads to the activation of AC. There are three subtypes of β-ARs, designated as β_1-AR, β_2-AR and β_3-AR, identified in the heart, which differ in function as well as molecular and pharmacological characteristics. The β_1-AR is the most abundant subtype expressed within the mammalian heart (about 75–80 % of the total β-ARs), while β_2-ARs account for about 20–25 % of total β-ARs. Expression of β_3-ARs in cardiac tissue is variable across species; low expression levels are found in human myocardium, while these are absent in rats [10, 11]. In addition, localization of the β-AR subtypes is different within the cardiomyocytes with higher concentration of β_2-ARs within caveolae, while most β_1-ARs are distributed throughout the cell membrane [12].

The β_1 and β_2 ARs are normally coupled to *Gs* proteins to stimulate AC activity and generate cyclic AMP (cAMP), a second messenger, which in turn activates PKA. The activated PKA mediates phosphorylation of target proteins involved in metabolic regulation, growth control, muscle contraction, and cell survival or death. The signal transduction mechanisms associated with G_s-protein coupled β_1- and β_2-ARs are depicted in Fig. 16.1. Stimulation of the β-ARs is the most potent mechanism to augment cardiac function in response to "fight -or- flight". As a consequence, there is an increase in cardiac contractility (positive inotropic effect), acceleration of cardiac relaxation (positive lusinotropic effect), and increase in heart rate (a positive chronotropic effect). These effects in cardiac cells upon stimulation of the β-ARs are mediated by phosporylation of Ca^{2+}-handling proteins, including sarcolemmal L-type Ca^{2+} channels, sarcoplasmic reticulum (SR) proteins (phospholamban and ryanodine receptors) as well as myofilament components (troponin I and C proteins).

Several studies have shown that β_2-ARs are coupled to the inhibitory G_i protein under certain conditions, in addition to interacting with *Gs* protein. This leads to attenuation of interaction with AC and activation of an extracellular signal-regulated kinase (ERK) cascade involving Src tyrosine kinases and Ras [13, 14]. The β_2-ARs-G_i signaling has also been demonstrated to induce the recruitment and

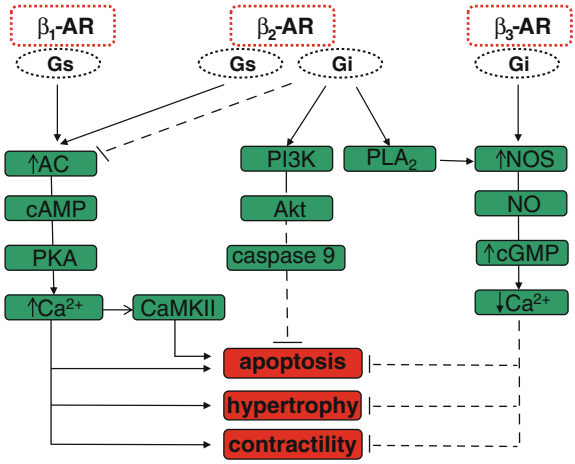

Fig. 16.1 Different signaling pathways activated upon stimulation of different types of *β*-adrenoceptors and their physiological responses. *β*-ARs, *β*-adrenoceptors; G_s, stimulative regulative *G*-protein; G_i, inhibitory regulative *G*-protein; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; Ca^{2+}, calcium; CaMKII, calcium/calmodulin-dependent protein kinase II; PI3 K, phosphatidylinositol 3-kinase; Akt, protein kinase B; PLA_2, phospholipase A_2; NOS, nitric oxide synthase; NO, nitric oxide; cGMP, cyclic guanosine monophosphate

activation of phosphatidylinositol 3-kinase (PI3 K) and the serine-threonine kinase (Akt) which regulates cardiomyocyte growth and apoptosis. Furthermore, stimulation of the β_2-ARs-G_i signaling axis results in the activation of phospholipase A_2 (PLA_2), inhibition of nitric oxide synthase (NOS) activity, alterations in calcium handling, and decreased cardiac contractility [15]. The signal transduction pathway associated with β_2-ARs-G_i-protein complex is shown in Fig. 16.1. In addition, there is evidence that β_2-ARs mediate responses through a G-protein-independent manner. In fact, Na^+/H^+ exchange regulatory factor (NHERF), an inhibitor of Na^+/H^+ exchanger type 3 (NHE3) has been found to bind to the cytoplasmic domain of the β_2-ARs resulting in a release of the inhibitory effects of NHERF on NHE3 [16]. These findings provide a putative mechanism by which the the β_2-ARs exert opposing effects on NHE3 activity and thus induce alkalization. However, there is no clear evidence for a catecholamine-dependent inotropic response due to β_2-ARs-dependent alkalization. As shown in Fig. 16.1, β_3-ARs have also been implicated as inhibitors of contractile function. In fact, coupling of β_3-AR to G_i-proteins has been shown to lead to activation of endothelial NOS expressed in ventricular myocytes and production of NO, resulting in the synthesis of cGMP and consequent reduction in cardiac contractility [17]. Interestingly, overexpression of β_3-ARs in transgenic mice has been found to increase cardiac contractility in heart via coupling to G_s-proteins [18]. Thus, a great deal of information needs to be obtained concerning the β_3-AR associated signal transduction mechanisms for making a meaningful conclusion.

16.4 Pathological Aspects of Sustained Stimulation of the β-Adrenergic Receptors

16.4.1 Cellular Growth and Apoptosis Regulated by Stimulation of β-ARs

It is well known that receptors for different hypertrophic agents including angiotensin II, endothelin-1, and α-AR agonists are coupled to G_q-proteins. However, D'Angelo et al. [19] have also suggested a link between β-ARs and G_q-proteins. Indeed, β-AR insensitivity is now considered to be a hallmark of the G_q-phenotype. Impaired G_s-coupling attributed to PKC-mediated receptor phosphorylation, increased G_i-protein content, and decreased AC content are observed in the G_q-heart. These findings have raised an important question of whether restoration of β-AR responsiveness can rescue cardiac contractility in $G\alpha_q$-mice. Genetically modified G_q-mice overexpressing β-ARs experienced 30-fold enhanced basal left ventricular function, and diminished fetal cardiac gene expression. In case of 140-fold increase of β-ARs, ventricular function, morphometric hypertrophy and fetal gene expression were exacerbated in G_q-mice. Consequently, most of these mice died at 5 weeks of a rapidly progressive dilated cardiomyopathy. Based on these findings, it is apparent that G_q-mediated hypertrophy exhibits multiple molecular defects in β-AR signaling and that normalization of cardiac contractility through restoration of β-AR responsiveness increases myocardial fibrosis, and lead to the development of heart failure [20–22].

Although β_1-ARs are the predominant β-AR subtype in mammalian ventricular cardiomyocytes, and that β_2-AR-dependent signal may only represent a relatively minor component of catecholamine response under normal physiological conditions, the β_2-AR linked mechanisms become more relevant with respect to contractile function in the hypertrophied and failing heart [23, 24]. Of note, we have shown that the volume overload-induced changes in the different components of β-AR complex in cardiac hypertrophy due to arteriovenous shunt are gender dependent [25]. In addition, the expression of β_2-ARs has been found to be up-regulated in denervated, transplanted human hearts [26]. Clinical studies have shown that non- selective β-blockers reduce sudden cardiac death in post-myocardial infarction patients, while selective β_1-AR blockers did not exert such a beneficial action [27]. Subsequently, studies employing transgenic mice have shown that overexpression of β_1-ARs results in a dilated cardiomyopathy phenotype, even in young mice, whereas mice overexpressing β_2-ARs (up to 150-fold over endogenous receptor expression) did not exhibit any significant cardiac pathology [28, 29]. Furthermore, cardiac function in mice with mild overexpression of β_2-ARs has been shown to be enhanced, perhaps as a result of the dual coupling of these receptor subtypes [30]. On the other hand, higher overexpression of β_2-ARs (>200-fold) resulted in a similar negative effect on heart function as the overexpression of β_1-ARs [31]. The hypothesis that the β_1-ARs are the predominant mediators of catecholamine-induced chronotropy and inotropy is

also supported by evidence that β_1-AR knockout in mice is generally embryonic lethal, while β_2-AR knockout mice are healthy and the physiological consequences are only observed under conditions of stress and exercise [32, 33].

Although α-ARs were believed to be main mediators of growth-promoting signaling, there is now evidence that β-ARs induce expression of proto-ocogenes (c-fos, c-jun), expression of the hypertrophic marker, atrial natriuretic factor (ANF), and stimulate protein synthesis in cardiomyocytes [34, 35]. A wide variety of calcium-dependent kinases and phosphatases have been implicated in signal transduction leading to transcriptional activation and/or hypertrophic growth responses [36, 37]. It has been suggested that the β_1-AR hypertrophic effects are not associated with either the activation of cAMP/PKA, or ERK activation [38, 39], but requires Akt-glycogen synthase kinase 3b (GSK-3b)-GATA4-signaling and activation of tyrosine kinase. In contrast, β_2-AR stimulation has been shown to inhibit β_1-AR mediated cardiomyocyte hypertrophy [38].

The β_1- and β_2-ARs differ in their role in apoptotic cell death, a process thought to be important in the transition from cardiac hypertrophy to heart failure. Stimulation of the β_1-ARs leads to apoptosis, while stimulation of the β_2-ARs may produce anti-apoptotic signals. Several studies have reported that the cAMP/PKA signaling pathway plays a critical role in β_1-AR-mediated apoptotic cell death. This was confirmed by the finding that a PKA inhibitor, H-89, was capable of abolishing programmed cell death [40, 41]. On the other hand, although the β_2-AR stimulation enhances cAMP formation, it elicits cardioprotective effects [40, 42]. It is noteworthy that some studies have reported that apoptosis due to sustained β_1-AR stimulation is PKA independent. In fact, recent studies have demonstrated that activation of calcium/calmodulin-dependent protein kinase II (CaMKII) due to β_1-AR stimulation constitutes a novel PKA-independent pro-apoptotic stimulus [43]. In addition, the expression of ANF was found to be correlated to the activation of CaMKII [44]. However, the exact molecular mechanisms underlying PKA-independent activation of CaMKII leading to apoptosis during sustained β_1-AR stimulation remains to be explored.

The main anti-apoptotic mechanism of the β_2-AR has been suggested to be linked to PI3 K-Akt signal transduction. Overexpression of PI3 Kα has been demonstrated to increase cardiac hypertrophy, while deletion of the PI3 Kα regulatory subunits attenuates myocardial hypertrophy in response to exercise training [45]. Concurrently, chronic activation of Akt leads to a decrease in PI3 K activity at the cell membrane due to a negative feedback mechanism involving insulin receptor substrate (IRS)-1 and 2. This molecule binds to the regulatory subunit of PI3 Kα and recruits it to the activated growth hormone receptor. Of note, Akt-mediated degradation and decreased synthesis of IRS-1 and -2 prevents recruitment of PI3 Kα to the cell membranes, which consequently attenuates the PI3 Kα-mediated beneficial effects; however, introduction of constitutively active PI3 K to failing hearts restores cardiac function and decreases ischemia/reperfusion injury [46]. In addition to this mechanism, the β-AR-mediated survival signal was found not only to counteract the concurrent G_s-induced apoptosis, but also to protect cardiomyocytes against a wide range of apoptotic insults, such as due to reactive oxygen species [42].

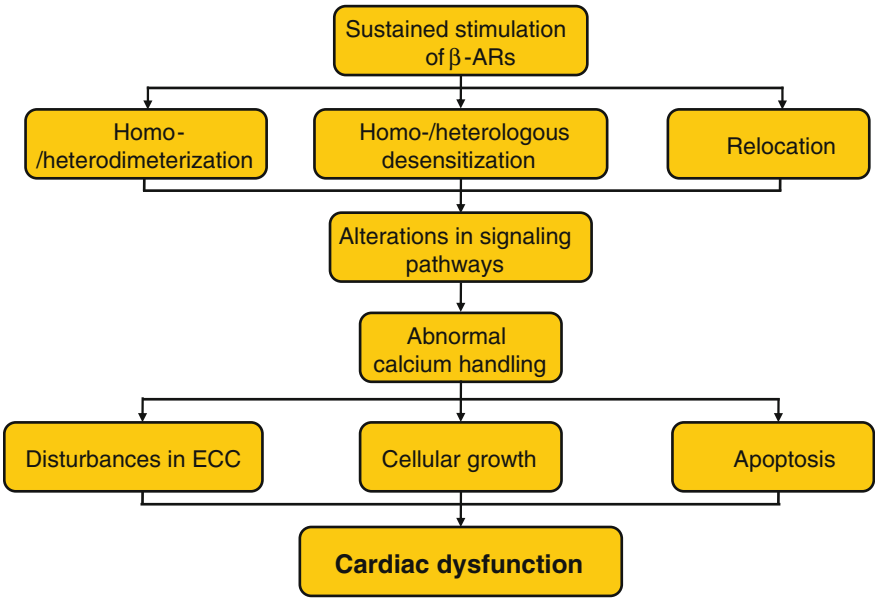

Fig. 16.2 Consequences of sustained stimulation of beta-adrenergic receptors leading to alterations in signaling pathways and modification of cardiac function. Excitation–contraction coupling (*ECC*)

16.4.2 Dimerization, Desensitization, and Relocalization of β-ARs

While the SNS activation provides essential inotropic support, sustained AR stimulation by catecholamines leads to β-AR dimerization and desensitization culminating in alterations in receptor responsiveness (Fig. 16.2). β-AR homo- and heterodimerization leads to altered cardiomyocyte responsiveness due to switching of *G*-protein coupling. In fact, β_1/β_2-ARs dimers are linked to G_s-proteins and produce synergic effects with respect to production of cAMP and increased cardiac contractility compared with either receptor alone [47]. While both β_2- and β_3-ARs can couple with G_i-proteins alone, heterodimers are unable to produce signal through G_i-proteins [48]. This β-AR association may help to understand the pathophysiology of cardiac hypertrophy as well as to explain the effectiveness of selective/non-selective β-AR agonists/antagonists.

Another consequence of sustained β-adrenergic stimulation is desensitization, which can be often seen in various G-protein coupled receptors (GPCRs). If it occurs in the context of diminished responsiveness to a diverse array of other agonists for GPCRs, it is referred to as heterologous desensitization and results from a negative feedback regulation by PKA-mediated phosphorylation. In addition to phosphorylation of the β-AR itself, phosphorylation of downstream protein targets such as *G*-proteins, and AC, also occurs. Furthermore, switching the

expression of *G*-proteins in favor of G_i-proteins over G_s-proteins also decreases β-AR-mediated response resulting in an attenuation of AC activation [49]. Likewise, induction of cAMP-hydrolyzing phosphodiesterases (PDEs) also blunts the effects of β-AR-stimulated cAMP signalling in cardiomyocytes producing a decrease in the SNS control of cardiac output [50]. Homologous or agonist-specific desensitization occurs during chronic exposure to an agonist resulting in a diminished response to that specific agonist. This type of desensitization is generally mediated by GPCR kinase family (GRK1-6), particularly GRK2, which is also known as β-AR kinase (β-ARK1) [51]. GRKs phosphorylate the activated ARs to alter their conformation and prevent further Signallng via G_s-proteins [52].

Early findings from experimental studies using the isoproterenol-induced cardiac hypertrophy model have suggested that a reduction in β-AR-mediated contractile response at the initial stage of cardiac hypertrophy is attributed to post-receptor modifications of the β-AR-cAMP signaling pathway rather than down-regulation of the myocardial β-ARs *per se*. In fact, short-term isoproterenol exposure produced a 53 % reduction in β-AR-mediated stimulation of AC, but the content of these receptors was unchanged [53]. Such a reduction in AC activity was shown under basal conditions and upon stimulation by β-AR agonists as well as non-adrenergic stimuli such as forskolin, sodium fluoride, and GTP [54–57]. Furthermore, since responses to these stimuli were reduced by various substances, it would appear that heterologous rather than homologous desensitization of AC was induced. In accordance to this, molecular studies have revealed that mRNA levels of AC are decreased in hearts chronically exposed to isoproterenol [57]. Desensitization of AC upon stimulation of the β-ARs is attributed to the increased activity of inhibitory G-proteins, as increased G_i-protein α-subunit mRNA level was found [58, 59]. On the other hand, the alterations in G_i- subunit mRNA levels were prevented by β-AR blockade [60]. Based on these results, it has been suggested that the increased functional activity of inhibitory G_i-proteins contributes to reduced β-AR-mediated inotropic responses upon chronic β-AR-stimulation. Of note, β_3-ARs are unlikely to undergo desensitization; in fact they are desensitization-resistant [61]. In contrast, the data regarding alterations in G_s-protein in hearts exposed to β-AR agonists are not clear as reduced level of G_s-proteins has been reported, while others have observed that reduced β-AR-mediated AC stimulation originates from decreased expression of G_s-proteins [62–64].

Sustained SNS stimulation, results in downregulation of the β-ARs due to redistribution of the receptors from the cell membrane to endosomal compartments (relocalization) and thereby it leads to an overall reduction in the number of receptors available for activation at the cell surface [65]. Despite numerous investigations exploring the mechanisms responsible for β-ARs desensitisation, the question of whether chronic β-AR desensitization in heart failure is considered as a deleterious or beneficial adaptive mechanism remains unanswered. From studies employing human tissue and a wide variety of models of experimental cardiac hypertrophy or heart failure, there is a general consensus that both pathologies are associated with alterations in the β-ARs. With respect to cardiac hypertrophy, it has been suggested that alterations in β_1-AR signal transduction

can be dependent on the type and stage of heart hypertrophy. In view of the activation of the SNS and renin-angiotensin-aldosterone system (RAAS), it appears that compensated and decompensated forms of cardiac hypertrophy may depend on the duration of exposure of the heart to different humoral and other growth factors circulating in the body. It has been shown that β-AR-mediated signal transduction mechanisms are up-regulated or unchanged in the compensated stages of cardiac hypertrophy, while these are down-regulated in the decompensated stages of cardiac hypertrophy [66]. Such findings are in line with the hypothesis that upregulation of β-AR mechanisms in compensated cardiac hypertrophy may play an adaptive role in maintaining heart function, while down-regulation of these mechanisms in decompensated cardiac hypertrophy may reflect the loss of its support to the failing heart. In a series of studies using various models of heart failure, it has been documented that β_1-ARs are downregulated, while β_2-AR expression is preserved [67, 68]. Furthermore, the coupling of both β-AR subtypes has been found to be impaired in human failing heart, presumably as a result of the up-regulation of GRK, namely GRK2 and/or GRK5 [69].

Although activation of the SNS plays a critical role in the pathogenesis of cardiac hypertrophy, excessive amount of circulating catecholamines may serve as a trigger for the transition of myocardial hypertrophy to heart failure. In this regard, treatment of rats for 24 h with a high dose (40 mg/kg) of a synthetic catecholamine, isoproterenol has been shown to result in increased LVEDP, depressed rates of pressure development and pressure decay as well as intracellular Ca^{2+}-overload [70]. Excessive amounts of catecholamines released from sympathetic nerve endings as well as from the adrenal medulla under stressful conditions are considered to also produce intracellular Ca^{2+}-overload and cardiac dysfunction through the β_1-AR signal transduction pathway [71]. Interestingly, β-AR kinase (GRK2) has been suggested to be a key molecule in the transition of myocardial hypertrophy to heart failure [72]. In patients with dilated cardiomyopathy the β-adrenergic responsiveness of the myocardium is diminished. It was shown that in these patients the expression of the β_1-AR is reduced at the mRNA and protein level whereas the expression of the inhibitory G_i- protein is increased. Furthermore, the expression of the GRK is elevated and induces uncoupling of the β_1-ARs. These alterations of the β_1-AR signal cascade may be induced by an elevated catecholamine release in patients with dilated cardiomyopathy [73]. Thus, excesses in catecholamines may be viewed as a mechanism for the transition of myocardial hypertrophy to heart failure. Indeed, it is likely that a threshold catecholamine concentration triggers this maladaptive response.

Recent studies have found that polymorphism of β_2-ARs may be a disease modifier. In fact, changes in the coding sequence of the β_2-AR gene (where isoleucine is substituted for threonine at 164 amino acid in the fourth transmembrane-spanning domain) have been suggested not to predispose heart failure, but individuals with heart failure harbouring this receptor are at significant risk for rapid decompensation [74]. This polymorphism leads to a decrease in the basal and agonist-stimulated AC activities as a result of defective coupling of the receptor to G_s-proteins [75]. It is plausible that other polymorphism processes may underlie

Table 16.1 Alterations in β-adrenoceptor complex in cardiac hypertrophy progressing into heart failure

Density of β-ARs	Healthy heart $\beta_1 \gg \beta_2$	Diseased heart $\beta_1 \cong \beta_2$
Localization of β-ARs	Membrane surface $\gg$ caveolae	Membrane surface $\ll$ caveolae
Coupling of β_2-ARs	$G_s \gg G_i$	$G_i \gg G_s$
Adenylyl cyslase isoforms	AC type V $\cong$ AC type VI	AC type V $\gg$ AC type VI

β-ARs β-adrenoceptors
AC adenylyl cyclase
G_s stimulative regulative *G*-protein
G_i inhibitory regulative *G*-protein

inter-individual variations in catecholamine responsiveness. From a clinical perspective, these data suggest that genetic polymorphism in β-ARs may account for differences in the clinical outcome of patients with chronic heart failure. In addition to these changes, some studies have shown abnormalities in *G*-protein expression. No changes in G_s-subunit were detected, while the G_i-subunit expression was found to be increased [76]. Furthermore, mRNAs for AC type V and VI tend to be reduced in the failing heart [77]. Thus, in the context of diseased heart, all these changes act to impair the signal transduction mechanisms via β_1 and β_2-ARs (Table 16.1).

16.5 Role of AC in Generation of cAMP and Regulation of PKA Activity

AC is a transmembrane protein that converts ATP to cAMP in response to stimulation of different GPCR, including β-ARs by various agonists. The mammalian AC gene family consists of 9 members, all of which are activated by $G\alpha_s$-proteins, but exhibit different patterns of regulation by other cofactors such as $G\alpha_i$- and $G\beta\gamma$-subunits, calcium, protein kinases (protein kinase C, calmodulin kinase), and phosphatases [78, 79]. Each isoform has a distinct tissue distribution, and biological as well as pharmacological properties. Types V and VI are designated as the cardiac subclasses of AC; both AC type V and VI mRNA are equally present at birth however; the AC V mRNA becomes predominant in the adult heart [80]. During heart failure, the levels of AC VI decrease while the content of AC V remains constant [77]. They have a comparable structural homology and patterns of regulation by cofactors [80, 81]. Both these AC isoforms are activated by the non-selective β-AR agonist, isoproterenol, but may have specialized functions in cardiomyocytes. The AC activity has been reported to be inhibited by submicromolar concentrations of calcium (less than 1 μM) [82]. The interaction between calcium and AC seems critical; indeed, co-localization of AC, and subunits of the L-type calcium channels along T tubules supports this hypothesis [83]. In addition, both AC isoforms can be phosphorylated by PKA resulting in a

desensitization and inhibition of AC activity [84]. Likewise, PKCα and PKCε isoforms can directly phosphorylate AC V, although PKCα is a less potent activator. PKCε activates AC V in a calcium-independent manner, whereas the PKCα requires calcium, indicating that AC V can be activated by distinct PKC isozymes under certain calcium-regulated conditions (physiological versus pathological levels of intracellular calcium). In addition, it has been suggested that cAMP production through activation of AC V is regulated by hormones and growth factors activating phosphatidyl-inositol-3,4,5 triphosphate which in turn activates PKCε [85].

During normal oncogenic development, there is a reciprocal change in the steady-state levels of mRNAs encoding type V and VI AC isoforms, while the expression of AC V increases and AC VI decreases with age [80, 86]. In the failing heart, the transcriptional level of AC V was found to be unchanged whereas the AC VI was decreased in various animal models of heart failure [77, 87, 88]. Since it is known that the proportion of β_1- and β_2-ARs is changed in the aging and diseased heart, it is possible that the abundance of AC isoforms may limit β-AR signaling in these states. In accordance, it has been suggested that these two AC isoforms may exert cardio protection through different mechanisms. In fact, overexpression of AC VI and depression of AC V are considered to be cardioprotective [89]. Cardiac-specific overexpression of AC VI has been found not to influence the basal cardiac activity, but does increase the response to β-AR stimulation [90]. In addition, enhanced survival after myocardial infarction was seen in mice with cardiac-specific overexpression of AC VI [91]. The predominant mechanism underlying this protection has been suggested to be linked with improved calcium handling as a consequence of increased phospholamban phosphorylation, reduced NCX1 and increased SR calcium content [92]. In contrast, AC VI deletion leads to impaired cardiac cAMP generation and calcium handling resulting in depressed left ventricle (LV) function (Fig. 16.3). The phenotype of AC VI knockout mice is somewhat difficult to interpret as the cardiac protein content of AC V was also found to be reduced, probably due to a post-translational mechanism. In these mice, basal contractile function and cAMP-levels were normal and β_1-AR-induced contractile function was only mildly reduced. In addition, phosphorylation of phospholamban was also reduced and the steady states SR Ca^{2+}-uptake and Ca^{2+}-storage were impaired [93]. It is pointed out that results regarding cardioprotective effects of AC VI overexpression are still not clear. Chronic aortic banding in mice with AC VI overexpression leads to impaired LV function, which is not consistent with the findings of Takahashi et al. [91] suggesting that distinct mechanisms are involved in the development of cardiac stress (pressure overload versus ischemia).

The AC V overexpression and deletion have been shown to produce opposite effects. In fact, the AC V overexpression leads to an increase in basal heart rate, an elevated LV ejection fraction and the development of cardiomyopathy with age. However, under conditions of chronic pressure overload or catecholamine stimulation, decreased LV function, increased LV hypertrophy, apoptosis and fibrosis as well as the development of pulmonary congestion and heart failure were

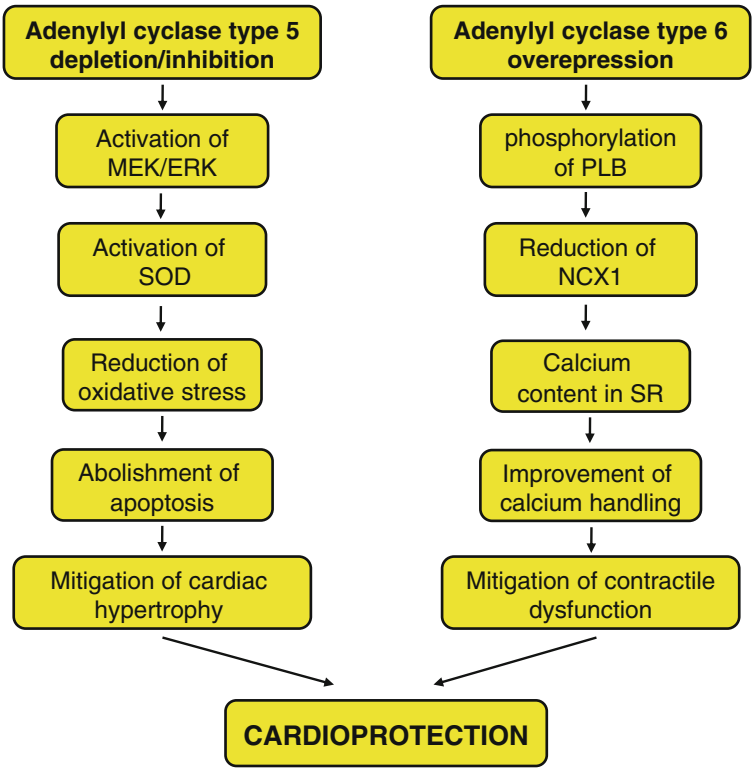

Fig. 16.3 Cardiac isoforms of adenylyl cyclase and their role in the protection of the heart against the development of cardiac hypertrophy and progression of heart failure. MEK/Erk, mitogen-activated protein kinase/extracellular-signal-regulated kinase; *SOD* superoxide dismutase 2; *PLB* phospholamban; *NCX*1 sodium/calcium exchanger 1; *SR* sarcoplasmic reticulum

documented [94, 95]. On the other hand, AC V knockout mice appear to be resistant against chronic pressure overload; these were found to have a more effective physiological response, desensitization to chronic isoproterenol infusion and a lower number of apoptotic cardiomyocytes after chronic stimulation [96]. Since abolishment of desensitization to chronic catecholamine stress is a major defence mechanism against transition of cardiac hypertrophy to heart failure, it seems that AC V is a crucial regulator of cardiac damage and induction of heart failure. In contrast, it seems that AC V does not take part in force-regulation and cAMP-production under physiological conditions [97].

It has been shown that in AC V knockout mice, despite the total protein levels of all AC isoforms in the heart and the maximal cAMP being reduced by about 50 %, both basal cardiac contractile function and heart rate were unchanged or even increased [96, 98, 99]. These interesting findings have suggested that AC V is the major G_i-inhibitable AC isoform in the heart and its absence is associated with the reduction of parasympathetic inhibition [100]. Of note, these genetically engineered

AC V knockout mice exhibit increased lifespan; they live a third longer than wild type [101]. Furthermore, these animals are resistant against aging-induced cardiomyopathy, and develop reduced cardiac hypertrophy, fibrosis, and apoptosis. In addition, AC V knockout mice have increased exercise capacity. A potential link associated with longevity of AC V knockout mice seems to be caloric restriction. Indeed, these mice and calorie restricted animals exhibit similarity in metabolism. Both these groups weigh less than their control littermates; the calorie restricted mice weigh less because of restricted food intake, however the AC V knockout mice weigh less despite increased food intake. In addition, a decrease in glycogen and blood glucose levels was observed in both groups [102, 103].

Another mechanism responsible for longevity of AC V knockout mice may be linked to attenuation of oxidative stress. In these mice, an up-regulation of manganese-superoxide dismutase (MnSOD) was detected [104]. This has been suggested to occur due to activation of the MEK/ERK signaling pathway. In addition to MEK/ERK-mediated activation of SOD resulting in decreased oxidative stress, this pathway also results in an increase in cell survival and therefore longevity (Fig. 16.3). Interestingly, bigenic mice with AC V knock out and β_2-AR overexpression do not develop the typical cardiomyopathy as is commonly observed in β_2 AR over-expressing mice [103]. In line with studies employing knockout AC V mice, a pharmacological inhibitor PMC-6, which was developed as a selective antagonist, was found to abolish β-AR-induced apoptosis. It is noteworthy that PMC-6 inhibited isoproterenol-induced cAMP accumulation only at concentrations higher than 100 nM, which is a concentration higher than that required for an maximal inotropic effect [105]. This is in agreement with an earlier report that showed the maximal force-generating effects of catecholamines in the heart occur at concentrations that are much lower than those required for maximal cAMP-increasing effects, indicating the existence of a large reserve of catecholamine-inducible cAMP [106].

16.6 Possible Benefits of Inhibiting Components of the β-AR Complex

Several groups of drugs have been designed to increase intracellular cAMP and consequently enhance the impaired myocardial contractility of the hypertrophied/failing heart. However, many of these strategies, such as cardiac directed expression of β-ARs, the stimulatory GTP-binding protein and PKA, which induce positive inotropic effects, have failed. In fact, they have resulted in LV chamber dilatation, cardiac fibrosis and heart failure [28, 107, 108]. Hence, other mechanisms related to β-AR complex have been investigated as potential targets for effective and safer pharmacological interventions. From the aforementioned discussion, it can been postulated that benefits in the treatment of cardiac hypertrophy/heart failure could be achieved by selective blockade of the β_1-ARs, inhibition of CaMKIIδC, and modulation of AC. Selective blockade of β_1-ARs maintains a β_2-mediated anti-apoptotic effect, while inhibition of CaMKIIδC directly prevents

activation of hypertrophic phenotype and apoptosis. With respect to AC, it has been suggested that selective AC VI agonists and selective AC V inhibitors could emerge as modulators of cardiac hypertrophy and heart failure. Although specific AC V inhibitors have been developed, their translation to the clinic is currently restricted due to high IC50, and low selectivity for isoform V [109, 110]. Moreover, although these inhibitors could exert similar effects as β-AR blockers the occurrence of side effects such as broncho- and vasoconstriction might limit their use since AC V and AC VI are also expressed within the bronchi and vessels [87].

16.7 Conclusions

Although a remarkable effort has been undertaken to describe pathomechanisms of cardiac hypertrophy, as well as identification of the signal transduction pathways involved, there are still many issues which need to be investigated. The involvement of β-ARs in the development of cardiac hypertrophy has been underestimated for many years, however it has become evident that they play a crucial role in the regulation of cardiac growth and apoptosis and thus account for, at least partially, decompensation of this pathological state resulting in heart failure. Molecular studies have revealed that alterations in the β-AR expression, desensitization, relocalization as well as alterations in coupling to G-proteins can underlie some phenotypes of cardiac dysfunction induced by the hypertrophic process. In addition, a different role of cardiac AC isoforms in the regulation of heart function has been recently highlighted to demonstrate that sustained stimulation of β-ARs under pathological conditions must be evaluated as a complex and not only as individual components of the signaling pathway. These findings will continue to encourage and stimulate the development of novel pharmacological agents designed to target specific proteins/isoforms.

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Chapter 17
Role of Phospholipase C in the α_1-Adrenoceptor Mediated Cardiac Hypertrophy

Paramjit S. Tappia, Adriana Adameova and Naranjan S. Dhalla

Abstract Phospholipase C (PLC) is considered to mediate the cardiomyocyte hypertrophic response to norepinephrine (NE) through activation of α_1-adrenoceptor (α_1-AR). In this review, the role of PLC isozymes in cardiac hypertrophy is highlighted and some of the mechanisms that are involved in the regulation of PLC isozyme gene expression, protein abundance, and activities are identified. The discussion is focussed to highlight the role of PLC in different experimental models of cardiac hypertrophy, transgenic mice, as well as isolated adult and neonatal cardiomyocytes with particular emphasis on α_1-AR-PLC-mediated hypertrophic signals. On the basis of the information available in the literature, it is suggested that molecular modulation of specific PLC isozymes is involved in the α_1-AR mediated response for the initiation and progression of cardiac hypertrophy. Furthermore, different molecular sites in the NE-induced signal transduction pathway are identified to serve as viable targets for the modification of this adaptive mechanism for maintaining cardiac function.

Keywords Phospholipase C · α_1-adrenoceptor · Signal transduction · Cardiac hypertrophy · Pressure overload · Volume overload · Transgenic mice · Neonatal cardiomyocytes Adult cardiomyocytes Phospholipase D

P. S. Tappia (✉)
Asper Clinical Research Institute, St. Boniface Hospital Research,
CR3129-369 Tache Avenue, Winnipeg, Manitoba R2H 2A6, Canada
e-mail: ptappia@sbrc.ca

A. Adameova
Department of Pharmacology and Toxicology, Comenius University, Bratislava, Slovakia

N. S. Dhalla
Institute of Cardiovascular Sciences and Department of Physiology, University of Manitoba, Winnipeg, Canada

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17.1 Introduction

Although the heart is known to adapt to increased work and hemodynamic load by increasing muscle mass as well as changing the size and shape of the heart, such a remodeling of the myocardium is compensatory at initial stages, but results in cardiac failure at late stages of the development [1, 2]. A moderate increase in the level of hypertrophic hormones including norepinephrine (NE) produces beneficial effects during early stages of cardiac hypertrophy, but prolonged exposure of the hearts to an excessive amount of NE produces deleterious actions at late stages of cardiac hypertrophy [1, 2]. A large body of evidence has revealed that various subcellular organelles including sarcolemma (SL), undergo varying degrees of changes in their biochemical composition and molecular structure in the development of cardiac hypertrophy as well as transition of cardiac hypertrophy to heart failure. This subcellular remodeling occurs due to alterations in cardiac gene expression as well as activation of different signaling proteins including phospholipases. The activation of phospholipase C (PLC) has a number of immediate consequences for signal transduction events in cardiomyocytes, and thus has an integral role to play in subcellular and cardiac remodeling (Fig. 17.1). Under physiological conditions, adrenergic responses are mediated predominantly by the β_1-AR to increase cardiac contractile activity and to influence hypertrophic growth in the long term [3]; however, under pathological conditions signal transduction mechanisms via the α-AR become more apparent and influential in the initiation and progression of cardiac hypertrophy [4]. Diminishing or reversing subcellular remodeling is now emerging as an important therapeutic goal in the treatment or prevention of cardiac hypertrophy and subsequent transition to heart failure in high-risk patients. Accordingly, it is our contention that pharmacological or molecular modulation of the different components of the α_1-AR-PLC signaling axis may represent a viable target.

17.2 The Myocardial α_1-Adrenoceptor Subtypes

The α-ARs are classified into two subtypes; $\alpha_{1A,B,D}$ and $\alpha_{2A/D,B,C}$ [5–7]. They belong to the superfamily of G-protein-coupled receptors (GPCRs), which contain a conserved structure of seven transmembrane α-helices linked by three alternating intracellular and extracellular loops. According to the classic paradigm of GPCR signaling, binding of the ligand to the receptor induces a sequence of conformational changes that result in its coupling to a heterotrimeric G protein. Activated G proteins then dissociate into G_α and $G_{\beta\gamma}$ subunits, each capable of modulating the activity of a variety of intracellular effector molecules. The protein expression levels of α_1-ARs in mammalian species including humans are considerably lower than for β-ARs [4, 8]. Interestingly, the α_{1A} is predominant α_1-AR in the human heart at the mRNA level, but not at the protein level [9]. Recent evidence suggests

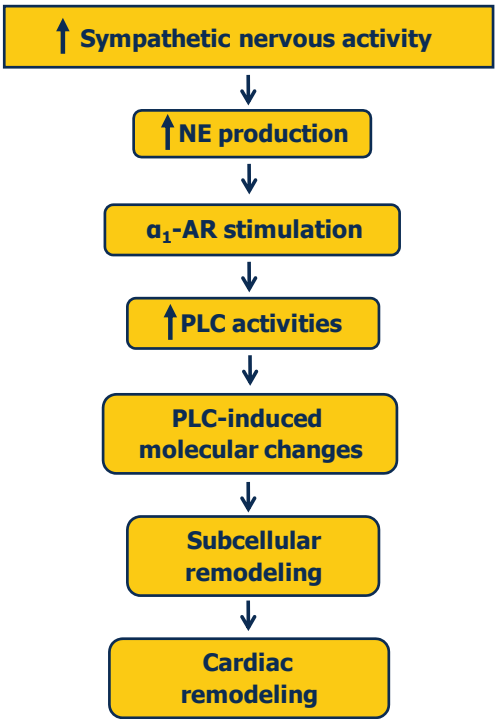

Fig. 17.1 Role of phospholipase C (*PLC*) activation in cardiac remodeling upon stimulation of sympathetic nervous system. *NE* norepinephrine, α_1-*AR* α_1-adrenoceptor

that expression of the α_{1B}-AR may also predominate in the left and right ventricles of the human heart [10].

Both the α_{1A} and α_{1B} subtypes couple to the Gq family of G proteins and are associated with the activation of the cardiac SL membrane-associated phospholipase C β (PLC β) that play a key role in initiation of intracellular signal transduction pathways and regulate a variety of cell functions [11–14]. It is interesting to note that, the proteins involved in targeting PLC β1b to SL membrane have been investigated in neonatal cardiomyocytes. It was found that PLC β1b co-immunoprecipitated with a high-MW scaffolding protein SH3 and ankyrin repeat protein 3 (Shank3) as well as the Shank3-interacting protein α-fodrin, indicating that PLC β1b associates with a Shank3 complex at the SL level [15, 16]. The protein caveolin-3 forms caveolae-flask-shaped invaginations localized on the cytoplasmic surface of the SL membrane [17, 18]. Caveolae have a key role in signal transduction and are gaining more interest as cellular organelles that may contribute to the pathogenesis of cardiac hypertrophy [17, 18]. Interestingly, the α_1-AR, Gq, PLC β_1, and PLC β_3 have been found to be confined exclusively to the same caveolin microdomain in the caveolar fraction isolated from rat heart [19].

The overexpression of α_1-ARs has demonstrated that an increase in α_{1B}-AR, but not α_1-AR activity predisposes the heart to hypertrophy [19]. There is some evidence that the α_{1A}-AR couples to Gq-PLC β more efficiently than the α_{1B}-AR subtype [20]. In this regard, cardiac-specific overexpression of the α_{1A}-AR exerts a

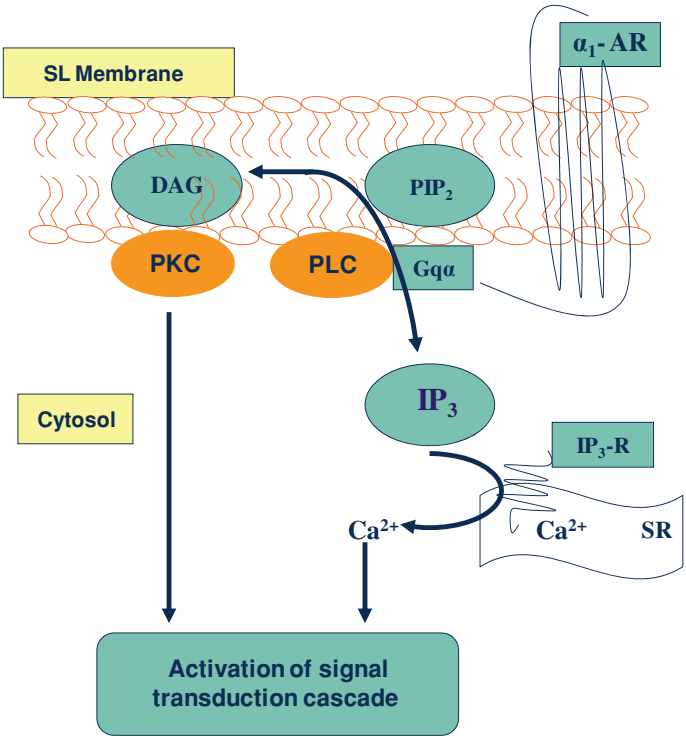

Fig. 17.2 Involvement of different signaling molecules due to the activation of phospholipase C (*PLC*) by α_1-adrenoceptor (α_1-*AR*) for the development of cardiac hypertrophy. *PIP*$_2$ Phosphatidylinositol-4,5-bisphosphate, *DAG* 1,2-diacylglycerol, *IP*$_3$ inositol-1,4,5-trisphosphate, *R* receptor, *Gqα* G-protein qα, *PKC* protein kinase C, *SR* sarcoplasmic reticulum, *Ca*$^{2+}$ calcium ion

higher activation of PLC as compared to α_{1B}-AR overexpression [19, 21]. While PLC β isozymes, β_1 and β_3 have been extensively characterized in cardiac tissue, recently higher PLC β_4 mRNA expression levels than PLC $\beta_{1\text{-}3}$ have been reported in human LV tissue [22]. Furthermore, it was demonstrated that PLC β_4 mRNA levels are increased in response to hypertrophic stimuli in mouse HL-1 cardiomyocytes, suggesting that this isoform may also have a role in the development of cardiac hypertrophy.

17.3 Phospholipase C-Mediated Signal Transduction

The α_1-AR mediated activation of PLC results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce 1,2 diacylglycerol (DAG), and inositol-1,4,5-trisphosphate (IP_3) (Fig. 17.2). The role of IP_3 in the cardiomyocyte has been a matter for contention as IP_3 generation in cardiomyocytes is low

compared to nonexcitable cells [23, 24]. The IP_3 receptors (IP_3R) are ubiquitous intracellular Ca^{2+} release channels [25]. However, relative to ryanodine receptor (RyR), which is the main source of Ca^{2+} in excitation–contraction coupling (ECC), low levels of IP_3R (approximately 1/50 of RyR) are present in the cardiomyocyte [26, 27]. The type 2 IP_3R, which is the predominant subtype in cardiomyocytes, is located mainly in the nuclear envelope in ventricular cardiomyocytes, but its role in the heart is poorly understood.

It has been suggested that the local Ca^{2+} release results in the activation of transcription, and thus providing a mechanism of how PLC-derived IP_3 may be involved in altered gene expression in cardiac hypertrophy; so-called excitation–transcription coupling [25]. Interestingly, overexpression of IP_3 5-phosphatase has been shown to result in reduced IP_3 responses to α_1-AR agonists acutely, but with prolonged stimulation, an overall increase in PLC activity was observed; this was associated with a selective increase in expression of PLC β_1 that served to normalize IP_3 content in neonatal rat cardiomyocytes [24]. It was suggested that the level of IP_3 selectively regulates the expression of PLC β_1. Furthermore, it was also demonstrated that hearts from type 2 IP_3R knockout mice showed heightened PLCβ_1 expression. Accordingly, it was concluded that IP_3 and type 2 IP_3R regulate PLC β_1 and thereby maintain levels of IP_3 [24], providing further functional significance for IP_3 in the heart. On the other hand, DAG acts in conjunction with phosphatidylserine and in some cases Ca^{2+} to activate different PKC isoforms containing a cysteine-rich C-1 domain that is known to be involved in cardiomyocyte growth [28–31].

17.4 Role of PLC in Different Animal Models of Cardiac Hypertrophy

The role of PLC in the development of different types of cardiac hypertrophy, in vivo, is well documented. For example, the development of cardiac hypertrophy in stroke prone spontaneously hypertensive rats has been reported to involve PLC signaling pathway [32, 33]. In addition, the development of cardiac hypertrophy in cardiomyopathic hamster (BIO 14.6) was found to be associated with an increase in PLC activity [34]. We have previously reported an increase in PLC isozyme gene and protein expression as well as activities in the hypertrophied rat heart; due to volume overload induced by an arteriovenous shunt [35, 36]. Of note, it was demonstrated that increases in PLC β_1 and PLC γ_1 were associated with the hypertrophic stage in this model [36]. Although PLC γ is activated through receptor tyrosine kinase [14, 37], we believe that a reciprocal cross-talk between tyrosine kinase and Gqα may exist in cardiomyocytes [37], linking α_1-AR with tyrosine kinase-associated receptors.

The status of PLC β_1, status in cardiac hypertrophy due to pressure overload induced by ligation of the descending thoracic aorta in the guinea pig, has also been

examined [38]. In this study, quantitative immunoblotting revealed that PLC β_1 and Gαq protein levels were unchanged during hypertrophy. However,translocation of PKC isozymes from cytosol to membranous fractions was elevated. These investigators suggested that PKC translocation occurred without changes in Gαq and PLC β protein abundance and that it might be due to increases in Gαq and PLC β_1 activity rather than upregulation of expression [38]; however, PLC β_1 activity was not determined in this study. Several studies have shown that antagonism of the α_1-AR results in mitigation of cardiac hypertrophy and its progression to heart failure [39–43], thus further implicating PLC β isozymes in the signal transduction mechanisms for cardiac hypertrophy. It should be noted that caveolin-3 expression has been shown to be significantly less in spontaneously hypertensive rats (SHR) as compared to Wistar-Kyoto (WKY) control rats [44]. These investigators suggested that the decrease in caveolin-3 expression may play a role in the development of cardiac hypertrophy in SHR through de-regulating the inhibition of growth signals in the hearts of SHR in the hypertrophic stage. Since α_1-AR and PLC β are located in caveolin-3 [44], it is likely that an increase in α_1-AR-PLC β signal transduction contributes to the cardiac hypertrophy in this model.

17.5 Role of PLC in Cardiac Hypertrophy in Genetic Models

Stimulation of signaling pathways via Gαq and rac1 provokes cardiac hypertrophy in cultured cardiomyocytes and transgenic mouse models [45–48]. The first transgenic murine cardiac hypertrophy model to support a Gαq mechanism of hypertrophy was overexpression of the wild-type Gαq in the heart using the α-MHC promoter [45]. Indeed, a 4-fold overexpression of Gαq resulted in increased heart weight and cardiomyocyte size along with marked increases in atrial natriuretic factor (ANF), α-skeletal actin, and β-myosin heavy chain expression. In view of the fact that an essential downstream effector for Gαq is PLC β [14], these observations would appear to implicate the activation of PLC β isozymes in cardiac hypertrophy. Indeed, Gαq expression in vivo constitutively elevates cardiac PLC β activity [49, 50]. The transgenic mouse line (αq*52) in which cardiac–specific expression of hemagglutinin (HA) epitope-tagged constitutively active mutant of the Gαq subunit (HAαq*) leads to activation of PLCβ, the immediate downstream target of HAαq*, with subsequent development of cardiac hypertrophy and dilation. However, in a second, independent line in the same genetic background (αq*44 h) with lower expression of HAαq* protein that ultimately results in the same phenotype of dilated cardiomyopathy, no correlation with PLC activity was seen [51]. In a different mouse model, loss of PLC ε signaling in PLC ε knockout mice has been suggested to sensitize the heart to the development of hypertrophy in response to chronic isoproterenol treatment [52].

G proteins are subject to direct regulation by RGS (regulators of G protein signaling) proteins, which shorten the duration of the cellular response to external signals and generally cause a reduction in hormone sensitivity [53]. Although the

primary mode of action of RGS proteins is to accelerate termination of the signal by decreasing the lifetime of active, GTP-bound Gα subunits, some RGS proteins can also inhibit signal generation by antagonizing Gα-mediated effector activation [54]. In this regard, recently it has been reported that endogenous ventricular RGS2 expression is selectively reduced in two different models of cardiac hypertrophy (transgenic Gαq expression and pressure overload), which was linked to elevated PLC β activity [55]. These investigators suggested that endogenous RGS2 exerts a functionally important inhibitory restraint on Gq/11-mediated PLC β activation and hypertrophy and concluded that loss of cardiac fine tuning of PLC β signaling by RGS2 down regulation could potentially play a pathophysiological role in the development of Gq/11-mediated cardiac hypertrophy.

The cardiac-targeted overexpression of α_{1A}-AR results in a small increase in the NE-stimulated, but not basal, PLC activity. However, no morphological, histological or echocardiographic evidence of LV hypertrophy was observed [19]. In addition, apart from an increase in ANF mRNA, expression of other hypertrophy-associated genes was unchanged. On the other hand, cardiac-specific expression of α_{1B}-AR in mice results in the activation of PLC as evidenced by an increase in myocardial DAG content [54]. Furthermore, a phenotype consistent with cardiac hypertrophy developed in the adult transgenic mice with increase heart/body weight ratios, cardiomyocyte cross-sectional areas and ventricular ANF mRNA levels [56]. Interestingly, cardiac expression of constitutively active mutant α_{1B}-AR, but not increased expression of α_{1A}-AR has been shown to be involved in the myocardial hypertrophic response to pressure overload in transgenic mice [57, 58]. Thus, it would appear that the α_{1B}-AR is primarily implicated in hypertrophy.

17.6 PLC-Mediated Hypertrophic Responses in Adult Cardiomyocytes

We have earlier reported that the NE-induced increases in ANF (a marker for cardiac hypertrophy), gene expression as well as protein synthesis that can be, in turn, attenuated by U73122, an inhibitor of PLC activities, as well as by an α_1-AR blocker, prazosin in isolated adult rat left ventricular (LV) cardiomyocytes [59]. We have also examined the signal transduction mechanisms involved in the regulation of PLC isozyme gene expression in adult cardiomyocytes in response to NE [60]. In this study, it was revealed that the NE-induced increases in PLC β_1, β_3, γ_1, and δ_1 isozyme mRNA and protein levels were attenuated in cardiomyocytes pretreated with either prazosin, or U73122, an inhibitor of PLC activities. The effects of prazosin and U73122 were associated with inhibition of PLC activity. The inhibition of NE-stimulated PLC protein and gene expression by bisindolylmaleimide-1, a PKC inhibitor, and PD98059, an ERK1/2 inhibitor, indicated that PKC-MAPK may be involved in this signal transduction pathway. Furthermore, significant increases in mRNA levels and protein contents for all PLC isozymes

were found in cardiomyocytes treated with phorbol 12-myristate 13-acetate, a PKC activator. Taken together, it was suggested that PLC isozymes may regulate their own gene expression through a PKC and ERK 1/2-dependent pathway.

An increased expression of the protooncogene, *c-fos* is associated with the initiation of some types of cardiac hypertrophy. In this regard, elevated levels of *c-fos* have been observed in rat heart following administration of NE [61, 62]. Similarly, it has been reported that the stretching of isolated neonatal cardiomyocytes or exposure to NE also elevates *c-fos* mRNA levels and produces cellular hypertrophy [63–65]. Although the pathway that mediates the NE induction of *c-fos* in other cell types has been shown to involve PKC, the identity of the specific PLC isozymes that may be part of this signaling pathway is not known. In addition, since ERK 1/2 is considered to play a major role in the upregulation of the mRNA and protein levels of the immediate early gene *c-jun* [65], it is possible that, this transcription factor may play a role in the regulation of PLC isozyme mRNA levels in response to α_1-AR stimulation in adult cardiomyocytes.

Although it is well-known that both *c-fos* and *c-jun* regulate the expression of a number of genes in the heart [66–69], our studies [70] using *c-fos* and *c-jun* siRNA have indicated that these transcription factors might also regulate the expression of specific PLC isozymes. It should be noted that under our experimental conditions, NE treatment of adult rat cardiomyocytes for 2 h did not induce any change in transcription factors such as NFAT3, NFκB, MEF2C, and MEF2D mRNA levels, suggesting that they may not regulate the early increase in PLC isozyme gene expression in response to NE [71]. Furthermore, our studies revealed that specific PLC isozymes may be involved in the regulation of *c-fos* and *c-jun* gene expression in response to NE [71]. This raises the intriguing possibility of a reciprocal regulation of PLC isozyme and *c-fos*/*c-jun* gene expression in adult cardiomyocytes. In fact, PLC may play an important role in a cycle of events that may be involved in the progression of the cardiomyocyte hypertrophic response (Fig. 17.3).

It should be noted that cardiac hypertrophy independent of PLC activation has also been reported [53, 72]. Nonetheless, from the aforementioned discussion it is possible that specific PLC isozymes might play a contributory role in the signal transduction pathways activated in cardiac hypertrophy. It is worth pointing out that we as well as others have reported that phosphatidic acid (PA), a product of phospholipase D activity, can stimulate PLC isozyme activities [73–75]. We also believe that PA can induce an increase in PLC isozyme gene expression [76]. Interestingly, we have previously reported that PA may be a potential signal transducer for cardiac hypertrophy [73]. In fact, we have also previously reported that PA is a potent stimulator of PLC isozyme activities. Accordingly, it can be suggested that the generation of PA in cardiac hypertrophy may be involved in the perpetuation and amplification of the cardiomyocyte hypertrophic response that might involve increases in PLC isozyme gene and protein expression as well as their activities (Fig. 17.4).

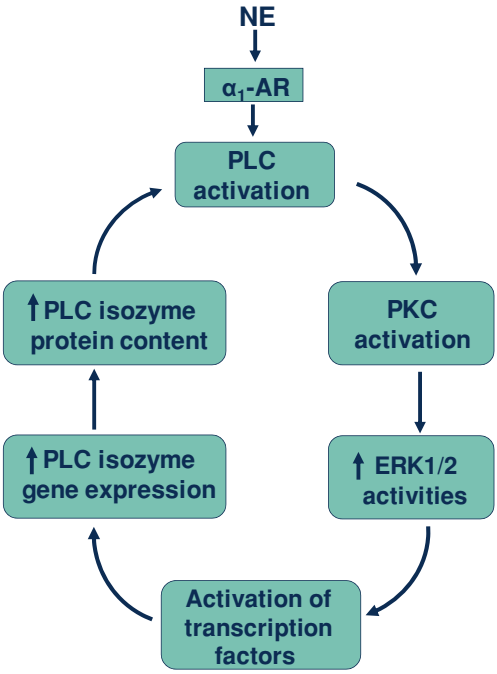

Fig. 17.3 Stimulation of phospholipase C (*PLC*) by norepinephrine (*NE*) mediated cycle of signal transduction events. α_1-*AR* α_1-adrenoceptor, *PKC* protein kinase C, *ERK*1/2 extracellular signal-related kinases 1 and 2

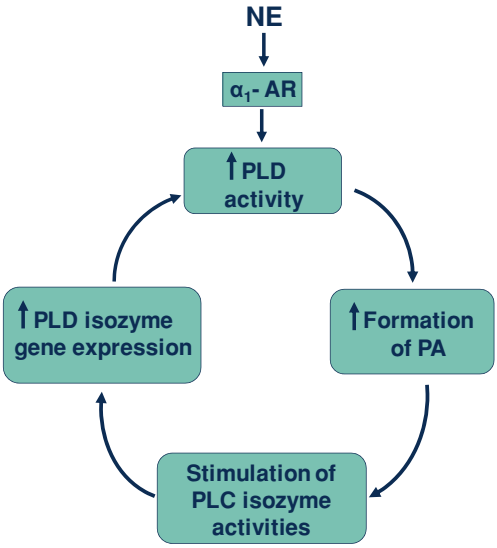

Fig. 17.4 Involvement of phospholipase D (*PLD*) in the activation of phospholipase C (*PLC*) through the formation of phosphatidic acid (*PA*) due to norepinephrine (*NE*). α_1-*AR* α_1-adrenoceptor

17.7 PLC-Mediated Hypertrophic Response in Neonatal Cardiomyocytes

The expression pattern and activation of PLC β isozymes in the development of hypertrophy in neonatal rat cardiomyocytes after stimulation with different hypertrophic substances has been investigated [77, 78]. Under control conditions and after stimulation with NE, cardiomyocytes expressed similar amounts of PLC β_3 mRNA. However, in the presence of fetal calf serum, additional expression of PLC β_1 was induced [77]. The induction of the immediate early genes c-myc, c-fos, and c-jun by IGF-I was also shown to be abolished by preincubation with antisense oligos against PLC β_3. These investigators concluded that the expression of PLC β isozymes in cardiomyocytes is differentially regulated by different hypertrophic stimuli [77]. It is pointed out that the NE-induced IP_3 generation in neonatal rat cardiomyocytes has been reported to be primarily due to α_1-AR mediated activation of PLC β_1 [78]. PLC β_1 exists as two splice variants, PLC β_{1a} and PLC β_{1b}, which differ only in their C-terminal sequences of 64 and 31 amino acids, respectively. While PLC β_{1a} is localized in the cytoplasm, PLC β_{1b} targets to the SL and is enriched in caveolae, where α_1-AR signaling is also localized [79]. Furthermore, in cardiomyocytes, responses initiated by α_1-AR activation involve only PLC β_{1b}, thus the selective action of this splice variant to the SL membrane provides a potential target to reduce hypertrophy [79]. Indeed, recently it has been shown that the overexpression of one splice variant of PLC β_1, specifically PLC β_{1b}, in neonatal rat cardiomyocytes causes increased cell size, elevated protein/DNA ratio, and heightened expression of the hypertrophy-related marker gene, atrial natriuretic peptide [80]. On the other hand, the other splice variant, PLC β_{1a}, had no such effect. Expression of a 32-amino acid C-terminal PLC β_{1b} peptide, which competes with PLC β_{1b} for sarcolemmal association, prevented PLC activation and eliminated hypertrophic responses initiated by Gq or Gq-coupled α_1-adrenergic receptors. In contrast, a PLC β_{1a} C-terminal peptide altered neither PLC activity nor cellular hypertrophy. It was concluded that hypertrophic responses initiated by Gq are mediated specifically by PLC β_{1b}. This study provided further evidence that preventing PLC β_{1b} association with the SL may provide a useful therapeutic target to limit hypertrophy.

PLC ε depletion, using siRNA has been demonstrated to dramatically reduce the hypertrophic growth and gene expression in neonatal rat cardiomyocytes induced by NE, ET-1, IGF-1, and isoproterenol [81]. Furthermore, it was observed that PLC ε catalytic activity was required for hypertrophy development, yet PLC ε depletion did not reduce global agonist-stimulated IP production, suggesting a requirement for localized PLC activity. In fact, these investigators went on to determine that PLC ε is scaffolded to a muscle-specific A kinase anchoring protein (mAKAPβ) that is localized to the nuclear envelope in neonatal rat cardiomyocytes. Accordingly, it was concluded that PLC ε may be involved in the integration of multiple upstream signaling pathways to generate local signals at the nucleus that regulate hypertrophy [81].

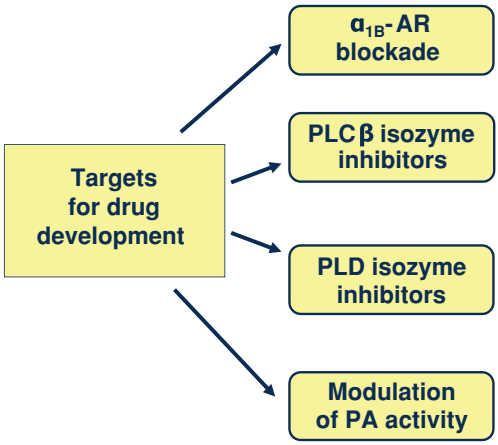

Fig. 17.5 Potential targets in the α_{1B}-adrenoceptor (α_{1B}-*AR*) mediated phospholipase C (*PLC*) signal transduction pathways for the modification of cardiac hypertrophy. *PLD* phospholipase D, *PA* phosphatidic acid

Mechanical stress induced by cell stretching in neonatal cardiomyocytes has also been reported to increase PLC activity [82]. However, in this study no attempt was made to identify the PLC isozymes responsible for such responses. Since mechanical stretch is an initial factor for cardiac hypertrophy in response to hemodynamic overload (high blood pressure) and that increases in Gqα and PLC β_1 activities [38] as well as enhanced NE release from sympathetic nerves [83] are involved in pressure-overload hypertrophy, it is likely that α_1-AR activates PLC β isozymes under conditions of mechanical stress. Indeed, it is important to note that while some studies have reported changes in the expression levels of PLC β isozymes in the hypertrophic response in neonatal cardiomyocytes the signaling function, i.e., PLC activities are determined by the interaction with Gαq, and thus increases in the myocardial PLC isozyme mRNA levels alone, does not necessarily signify a role of PLC isozymes in cardiac hypertrophy.

17.8 Conclusions

The involvement of PLC-mediated signal transduction in cardiac hypertrophy has been demonstrated at the cellular and organ level. While a number of different signal transduction pathways are activated in the myocardial hypertrophic response to different stimuli, it is evident that PLC may constitute additional targets for drug development for the prevention or regression of cardiac hypertrophy in high-risk patients. Although some studies have shown that blockade of the α_1-AR in mitigating the progression of cardiac hypertrophy to heart failure, a direct inhibition of PLC and regression of cardiac hypertrophy is yet to be demonstrated in vivo. Possible targets for drug development for minimizing or reversing cardiac hypertrophy are depicted in Fig. 17.5. The increased formation of PA due to α-AR activation not only stimulate PLC and produce cardiac

hypertrophy, but has also been demonstrated to increase protein synthesis [84]. Interestingly, trimetazidine, an anti-anginal drug has been reported to modulate phospholipid biosynthesis and to reduce IP_3 availability in a PLC-independent manner that results in a prevention of the hypertrophic response to chronic α_1 adrenergic stimulation with phenylephrine in cultured rat cardiomyocytes [85]. The majority of the published work is on describing the involvement of PLCβ isozymes in cardiac hypertrophy; however; since a number of PLC isozymes belonging to different subfamilies (β, γ, δ, and ε) are also expressed in the heart [52, 81, 86, 87] the distinct role of each isozymes, particularly with respect to cardiac hypertrophy, and the extent of their overlap has yet to be completely defined. Indeed, specific PLC isozymes could emerge as important contributors of signal transduction mechanisms for cardiac hypertrophy.

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Chapter 18
Cardiac Remodeling in the Hypertrophic Heart: Signal-Dependent Regulation of the Fibrotic Gene Program by CLP-1

Michael Wagner and M. A. Q. Siddiqui

Abstract The response of the heart to hypertrophic stress stimuli is designed to normalize heart function under conditions of increased demand. To achieve this, the heart must mount a genomic stress response that is itself responsive to the signal transduction pathways used by heart cells to transmit stress signals. The ability to adaptively link stress signal to genomic stress response is critical to how the heart responds to stress. Our laboratory has been studying the molecular events involved in this adaptive linkage. Our studies have shown that CLP-1 (Cardiac Lineage Protein-1), the mouse homolog of the human HEXIM1, acts as the molecular "go-between" linking stress signal with genomic stress response. Critical to this linkage is HEXIM1/CLP-1's control of cyclin-dependent kinase 9 (cdk9), the kinase responsible for activating RNA polymerase (pol) II to complete synthesis of nascent stress gene transcripts. Through its control of cdk9, HEXIM1/CLP-1 controls the transcriptional output of stress response genes by regulating the ability of RNA polymerase (pol) II, and as more recent data has shown, the activity of specific transcription factors such as those of the small mother against decapentaplegic(smad) family, to transcribe stress response genes. Together, these observations provide strong support for the idea that HEXIM1/CLP-1 plays a critical role in controlling the response of cardiac cells to hypertrophic stress stimuli.

M. Wagner · M. A. Q. Siddiqui (✉)
The Center for Cardiovascular and Muscle Research and Department of Cell Biology, State University of New York Downstate Medical Center, 450 Clarkson Avenue, New York, 11203, USA
e-mail: maq.siddiqui@downstate.edu

M. Wagner
e-mail: michael.wagner@downstate.edu

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Abbreviations

CLP-1	Cardiac lineage protein-1
HEXIM1	Hexamethylene bis-acetamide-inducible protein 1
Cdk9	Cyclin-dependent kinase 9
P-TEFb	Positive transcription elongation factor b
Smad	Small mother against decapentaplegic
Ang II	Angiotensin II
SHR	Spontaneously hypertensive rat
MAP kinase	Mitogen-activated protein kinase

18.1 Introduction

The heart responds to stress and increased demand by mounting an adaptive or compensatory hypertrophic response to normalize cardiac function. In this response, myocytes enlarge to increase their contractile ability and cardiac fibroblasts differentiate to produce and secrete collagen into the extracellular matrix to preserve the force generating capacity, or active (systolic) stiffness, of the hypertrophied myocardium [1, 2]. The collagen deposited into the extracellular matrix is predominantly perivascular and does not infiltrate the syncytial-like cardiomyocyte network. As a result, coordinated transmission of contractile activity between ventricular myocytes is preserved for continued cardiac output. This form of collagen deposition is called reactive fibrosis. With prolonged stress, however, this adaptive response turns maladaptive with cardiomyocytes becoming metabolically depleted and dying and cardiac fibroblasts undergoing a proliferative expansion to replace these lost cardiomyocytes [2]. The cardiac fibroblasts subsequently differentiate into collagen-producing myofibroblasts that produce and secrete excess collagen into the extracellular matrix for supporting the contractile function of increasingly fewer hypertrophic cardiomyocytes. Continued collagen production leads to a scarring fibrosis called replacement or reparative fibrosis that infiltrates the cardiomyocyte network to a point where cardiomyocytes become mechanically uncoupled and no longer able to coordinately contract. In addition, the increased presence of non-elastic collagen fibers makes the ventricular myocardium less compliant resulting in decreased systolic and diastolic function that eventually leads to heart failure, a progression and outcome impervious to most therapeutic options. Of the multiple processes underlying the progression to maladaptive or pathological hypertrophy, metabolic failure and scarring fibrosis appear to be the most consequential. Preventing their occurrence could be the most effective way to impede progression to pathological hypertrophy.

18.2 Hypertension and Activation of the AT1 Angiotensin II Receptor

One of the most prevalent causes of cardiac hypertrophy is hypertension. Hypertension results from increased production of the circulating hormonal peptide Angiotensin II (Ang II) in response to decreased blood volume and arterial blood pressure. Ang II acts to increase arterial blood pressure to normal levels in two ways. The first is by activating Ang II receptors (AT1 receptors) on arterial smooth muscle cells to increase vasoconstriction of the arterial system. The second is to increase plasma and overall blood volume by increasing salt and water retention in the kidney. However, persistent retention of salt and water in the kidney can lead to chronically high arterial blood pressure or hypertension. To maintain cardiac output against this chronically elevated peripheral resistance, the heart must work harder to pump blood. It achieves this by increasing the contractility of its cardiomyocytes by increasing the extent to which they can mechanically stretch. Under these conditions, local AT1 receptors on cardiomyocytes can act as stretch-sensitive receptors [3]. Stretch-induced activation of these receptors initiates production of angiotensinogen, the primary substrate of the rennin–angiotensinogen system or RAS that produces Ang II. Persistent autocrine activation in this way of this RAS system, called local RAS, leads to continued overproduction of Ang II, hypertension, and the onset of cardiac hypertrophy and its progression to decompensatory hypertrophy and heart failure.

In addition to acting as a hypertrophic stimulus [3–8], Ang II is a known inducer of cardiac fibroblast growth and collagen synthesis [9–12]. Ang II can activate cardiac fibroblasts to proliferate and secrete collagen into the extracellular matrix directly by activating AT1 receptors on cardiac fibroblasts [13, 14] or indirectly through increased expression and activity of the growth factor, TGF-beta, that acts through TGF-beta type I and II receptors [15–17]. In both cases, receptor activation leads to activation of receptor-linked signal transduction pathways that up-regulate expression of target genes. For Ang II, studies by Kojima and colleagues showed that blockade of AT1 receptors in hypertensive SHR rats leads to a decrease in blood pressure, myocyte hypertrophy, and interstitial fibrosis [3]. In vitro studies showed that activation of AT1 receptors increased hypertrophy-related protein synthesis, MAP kinase activity and expression of c-fos, an immediate early stress response gene in cardiomyocytes [10, 18]. In cardiac fibroblasts, findings from early studies paralleled these findings in cardiomyocytes with respect to MAP kinase activation but ruled out the involvement of either Ca^{2+} or PKC-dependent pathways in the hyperplastic growth of cardiac fibroblasts [19]. More recent studies of cardiac fibroblasts showed that activation of the AT1 receptor by mechanical stretch leads to activation of p38alpha kinase via regulation by RhoA, a RhoGTPase signal transducer that can signal independently of Ca^{2+} or PKC activation [20, 21]. Transduction of the Ang II cytokine signal in cardiac fibroblasts could be via a similar pathway or other pathways linked to the AT1 receptor by the Janus 2 kinase [22].

18.3 TGF-Beta, Activation of Smad Transcription Factors, and Fibrosis

As mentioned, the Ang II signal can also be transmitted via TGF-beta. TGF-beta signals via TGF-beta type I or II receptors to activate the small mother against decapentaplegic(smad) transcription factors. In cardiac fibroblasts, Ang II induces expression of both TGF-beta and a TGF-beta Type I/II co-receptor, referred to as a type III TGF-beta receptor known as endoglin [23]. Treatment of cardiac fibroblasts with Ang II increased expression of endoglin which was then shown to be required for modulating fibrotic processes such as matrix metalloproteinase 1 and type I collagen expression [24]. In other studies, TGF-beta expression and activity were shown to be increased in cardiac fibroblasts treated with Ang II and in an animal model of hypertension [25–27]. Together, these studies show that Ang II-induced fibrosis involves a type of autocrine activation of cardiac fibroblasts through cross-talk between the Ang II and TGF-beta signaling pathways.

From these studies it appears that in response to Ang II, the actual transcriptional mechanism responsible for turning on fibrotic genes in cardiac fibroblasts is the TGF-beta-smad signaling pathway. TGF-beta receptors are serine–threonine kinases that upon binding TGF-beta recruit and phosphorylate smad factors, in this case, smads 2 and 3, which then bind to smad 4 and enter the nucleus where they bind to specific binding sites in the promoter of smad-responsive genes. Several independent studies have confirmed the involvement of smad transcription factors in the expression of the fibrotic program. In Ang II-treated cardiac fibroblasts, phospho-smad2 was seen to rapidly translocate to the nucleus and this translocation was blocked by losartan, an AT1 receptor antagonist [28]. In TGF-beta-treated cardiac fibroblasts, lysyl oxidase, an enzyme that modifies collagens I and III to allow formation of fibrillar collagen, is up-regulated and this increase can be abolished by inhibition of smad 3 [29]. Other indications for smad involvement in fibrosis come from studies of Smad 7, an inhibitory smad factor that binds to activated TGF-beta receptors and prevents binding by other receptor-activated smads such as smads 2 and 3 [30]. In TGF-beta-treated cardiac fibroblasts, cytoplasmic smad 7 was increased (suggesting increased binding to plasma membrane TGF-beta receptors) and over-expression of smad 7 suppressed collagen type I and III expression suggesting that smad activity is required for fibrotic gene expression [31].

18.4 Regulation of Smad Transcription Factors by cdk9

Like most transcription factors, smads are comprised of distinct structural domains, each instrumental in regulating transcriptional activity [32]. One of these domains, the linker region joining the DNA binding domain with the protein interaction domain, is noteworthy in this regard in that it contains the transcriptional activation motif of smads and is a target of various kinases [32, 33]. Depending upon kinase

type, linker phosphorylation can either activate or attenuate smad transcriptional activity and some kinases such as cdk8/9 can both fully activate smads while also "priming" them for degradation [34–36]. Alarcon and colleagues have studied smad phosphorylation and its influence on transcriptional activation and have formulated a model of phosphorylation-dependent cycling of smads between full activity and turnover by proteasome-mediated degradation. According to their model, TGF-beta receptors phosphorylate smads on their C-terminal, promoting their import into the nucleus and binding to sites in promoters of smad-responsive genes. To fully activate these smads, cdk8 or 9 phosphorylates the linker region to create a binding site for co-activator proteins that initiate full transcriptional activity. After transcription is complete, linker phosphorylation of smads plays a second role by providing a binding site for ubiquitin ligases which target these smads for degradation and turnover by a proteasome-mediated degradation pathway. Alarcon and colleagues postulate that this cyclic recruitment and turnover of smads affords cells the opportunity to respond to changing developmental and homeostatic signals (see Fig. 7f of Alarcon et al.).

In the nucleus, cdk9 exists as a complex with cyclin T, usually cyclin T1, that together form a complex known as P-TEFb, or Transcriptional Elongation Factor b [37–40]. As a regulator of transcriptional elongation, the major substrate of P-TEFb's ckd9 kinase activity is RNA polymerase (pol) II and its associated negative elongation control factors, NELF (negative elongation factor) and DSIF (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole sensitivity-inducing factor). These factors can exist in either a native or phosphorylated state. In its native state, RNA pol II localizes to the transcriptional initiation start site of RNA pol II-dependent genes where it synthesizes a nascent RNA transcript of only several nucleotides in length and then stops. This process is referred to as "promoter proximal pausing" and the RNA pol II is said to be in an initiation state. Imposing this paused status on RNA pol II are the two negative elongation factors NELF and DSIF. To complete synthesis of full-length transcripts, RNA pol II must be released from this paused, initiation state. This requires the activity of cdk9 which phosphorylates RNA pol II on select serine residues in its carboxy terminal end as well as phosphorylating the DSIF and NELF elongation factors. Phosphorylation of NELF causes its dissociation from the transcriptional complex while phosphorylation of DSIF converts it into positive elongation factor. DSIF, together with phospho-RNA pol II shifts pol II into an elongation state that allows pol II to complete synthesis of full-length RNA transcripts [38].

18.5 HEXIM1/CLP-1 Controls cdk9 Kinase Activity in Response to Cellular Stressors

From the above, it can be seen that switching RNA pol II from its initiation state to its elongation state determines whether a gene will be expressed or not. Such gene control forms the basis of virtually all cellular processes requiring differential gene

expression such as growth and differentiation, cell homeostasis, the response to environmental factors and stresses, and eventually the decision to undergo programmed cell death. Because of its critical role in controlling RNA pol II activity and gene expression, P-TEFb kinase activity itself is under tight control. This control is exerted by a negative regulatory protein called HEXIM1 or Hexamethylene bis-acetamide inducible protein 1 for the human homolog or CLP-1 or Cardiac Lineage Protein-1 for the murine homolog [41, 42]. HEXIM1 can reversibly bind to P-TEFb. As a negative regulator, when HEXIM1 is bound to P-TEFb, its ckd9 kinase activity is repressed; when HEXIM1 dissociates from P-TEFb, cdk9 activity is de-repressed and free to phosphorylate RNA pol II, NELF, and DSIF. As a regulator of genes involved in cell homeostasis, HEXIM1 and its control of P-TEFb is likely to be responsive to the status of the cell through interaction with signaling pathways transmitting extracellular and intracellular signals as well as gene-specific transcription factors activated in response to such signals to transcribe their target genes.

The promoter proximal pausing mechanism lends itself readily to those genes requiring immediate expression from a basal state of transcriptional quiescence. Emerging evidence suggests that many of these genes are likely to be developmental, stress response, communication, adhesion, and differentiation genes in which "paused" transcripts can be rapidly re-initiated for immediate expression in response to changing cellular conditions [43, 44]. One of the most prominent examples of stress response is cardiac hypertrophy. Our laboratory initially cloned the CLP-1 cDNA and gene and has been examining the role of CLP-1 in mediating the genomic response to hypertrophic stress signals [45–48]. Underlying the hypertrophic response are distinct genetic programs regulating expression of immediate stress response genes, fetal genes and fetal isoforms of genes, glycolytic enzymes and metabolic genes, and genes directing myofibroblast formation, and collagen expression [49–54]. Each program is controlled by hypertrophic signals, cytokines and stress hormones directing cardiomyocytes and cardiac fibroblasts at each stage of hypertrophy [55–57]. Translating hypertrophic signal into genomic stress response requires a signal-regulated factor capable of modulating the expression of stress genes in response to a wide range of hypertrophic and cellular stress signals. As the molecular "go-between" linking extracellular signals to genomic output, CLP-1 is ideally suited for controlling the genomic response to hypertrophic and stress-generated signals.

To mount a rapid yet measured response to cardiac stressors such as pressure or volume overload requires a rapid and measured genomic stress response. One way to achieve this is to coordinate the activity of signal-activated transcription factors with that of the basal transcriptional apparatus responsible for transcribing all genes. Rapid and coordinated assembly of these components onto individual stress response genes will ensure rapid expression of these genes in response to cellular stress. To assure this rapid and coordinated assembly, we postulate that hypertrophic signals simultaneously activate both nuclear transcription factors and RNA polymerase II via P-TEFb. Since P-TEFb is under negative regulatory control by CLP-1, activation of P-TEFb by signal transducers is likely to involve their

promoting CLP-1 dissociation from P-TEFb or preventing its binding to P-TEFb in the first place. This synchronized, dual activation scheme would allow the rapid assembly of transcriptional complexes required for the immediate synthesis of stress gene transcripts. Signal-dependent dissociation of CLP-1 from P-TEFb may not only provide a rapid means of activating transcription but also a way of modulating stress gene transcriptional activity to match stress signal strength. Implicit in this notion is the idea that the extent of CLP-1 dissociation from P-TEFb may determine the level of P-TEFb activity and RNA polymerase II phosphorylation with less dissociation giving less activity, more dissociation giving more activity. In this way, the genomic response could be "scalable" with less stress provoking an attenuated response and greater stress provoking a robust response.

18.6 Decreased HEXIM1/CLP-1 Intensifies the Cardiac Hypertrophic Stress Response and Ang II-Induced Fibrosis

Our laboratory has examined these aspects of CLP-1 function within the context of the hypertrophic stress response through the use of defined genetic models. We sought to determine if different levels of CLP-1 activity would lead to different levels of hypertrophic response. Since it is presently unclear how CLP-1 activity is controlled, we surmised that the levels of CLP-1 protein relative to P-TEFb could act as a proxy for levels of CLP-1 activity with lower levels of CLP-1 phenocopying decreased CLP-1 activity, and higher levels phenocopying increased activity. To achieve this, we used CLP-1+/− heterozygous mice which express CLP-1 levels at approximately half that of wild-type mice. To further test our hypothesis, we produced an even greater disparity between CLP-1 levels and its target, the cyclin T1 component of the active P-TEFb complex, by raising cyclin T1 levels using transgenic mice over-expressing cyclin T1 in the heart (αMHC-cyclinT1 mice) [58]. By crossing these mice with CLP-1+/− mice, we effectively could produce mice with a greater decrease in CLP-1 levels relative to cyclin T1 than that in CLP-1+/− heterozygous mice alone. Examination of the hearts from wild-type, CLP-1+/−, αMHC-cyclinT1, and αMHC-cyclinT1 × CLP-1+/− mice showed wild-type and CLP-1+/− hearts to be normal, αMHC-cyclinT1 hearts to be slightly hypertrophic, and αMHC-cyclinT1 × CLP-1+/− hearts to be significantly enlarged relative to αMHC-cyclinT1 hearts (Fig. 18.1a, b). These data suggest that lowering CLP-1 levels relative to cyclin T1 levels increased the hypertrophic response. We further examined if lowering levels of the CLP-1 inhibitor led to derepressed activation of cdk9 and if this activation was greatest in the αMHC-cyclinT1 × CLP-1+/− hearts. Figure 18.1d shows that cdk 9 phosphorylation of serine 2 residues in the carboxy terminal end of RNA pol II directly correlated with the degree of hypertrophy exhibited by experimental hearts. This suggests that underlying the more robust hypertrophic response was a more robust

transcriptional activation of hypertrophic response genes presumably due to increased P-TEFb and RNA pol II activity.

As mentioned, underlying the hypertrophic response are distinct genetic programs directing various responses to stress, two of which, the switch in metabolic energy substrates and fibrosis, are key to determining if hypertrophic hearts progress to decompensatory or pathological hypertrophy and heart failure. To determine if increased P-TEFb activity can enhance expression of these stress response programs, we extended our analysis to another mouse model of hypertrophy, the αMHC-ANG mouse in which Ang II is overexpressed in the heart [59]. These mice chronically express Ang II establishing a state of hypertension leading to an attendant state of compensatory hypertrophy. To increase P-TEFb activity within the context of this hypertrophic mouse model, we crossed these mice with CLP-1+/− heterozygotes to produce αMHC-ANG × CLP-1+/− mice. Using the Masson-Trichrome method in which collagen deposits stain blue (or purple with eosin co-staining), we showed that αMHC-ANG × CLP-1+/− hearts had increased collagen deposits (11.9 % of total cell surface) over that seen in wild-type, αMHC-ANG, or CLP-1+/− hearts (7.2, 6.0, 5.0 %) (Fig. 18.2a). These data show that lowering CLP-1 levels leads to increased expression of collagen which is secreted into the ventricular extracellular matrix. This result suggests that decreased CLP-1 levels lead to de-repression of P-TEFb, increased cdk9 kinase and RNA pol II activity, and transcription of fibrotic genes such as collagen type I and III.

18.7 Decreased HEXIM1/CLP-1 Levels Increase Cardiomyocyte Metabolism and Decrease Reparative Fibrosis in Hypertrophic Heart

At this level of resolution, it is unclear as to what type of fibrosis, reactive or reparative, is being expressed in these hearts. Since reparative or scarring fibrosis often leads to a greater stiffening of the ventricle and a decreased compliance over what is seen in reactive fibrosis, we used echocardiographic analysis of ventricular function to assess the degree of fibrosis. Using fractional shortening as a measure of left ventricular function, αMHC-ANG × CLP-1+/− hearts showed virtually no difference in percent fractional shortening compared to all other mouse strains suggesting that these mice are more likely to have the reactive type of fibrosis associated with adaptive or compensatory hypertrophy (Fig. 18.2b). Another possible explanation for this result would be if decreased CLP-1 levels were in some way maintaining cardiomyocyte viability via sustained metabolic output such that reparative fibrosis would be prevented or at least lessened. To address this issue, we analyzed αMHC-ANG × CLP-1+/− hearts for expression of a key metabolic gene, p-AMPK, an activator of PGC-1α, the master regulator of mitochondrial biogenesis and energy production, fatty acid metabolism and glucose

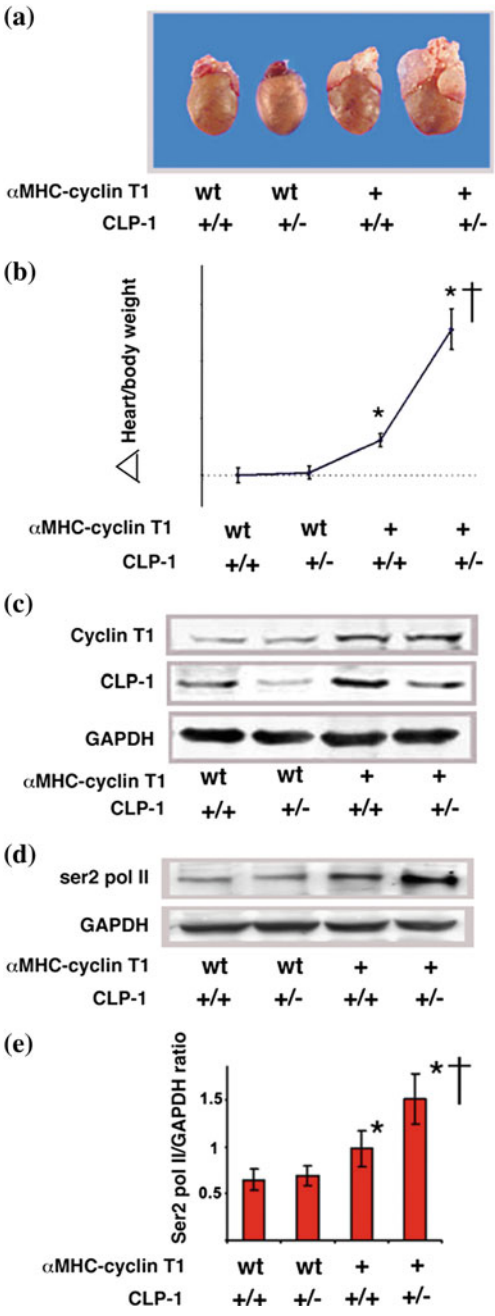
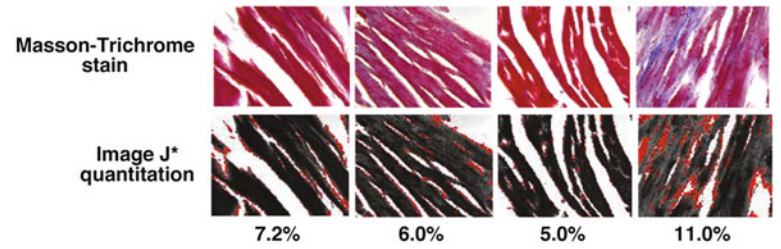


Fig. 18.1 Lowering CLP-1 levels in the αMHC-cyclin T1 mouse model of hypertrophy increases hypertrophy and phosphorylation of ser 2 residue in carboxy-terminal domain of RNA pol II. **a** αMHC-cyclin T1 mice with one allele of CLP-1 exhibit larger hypertrophic hearts than the other three mouse strains. **b** αMHC-cyclin T1 × CLP-1+/− mice have a significantly increased heart to body weight ratio. **c** Relative amounts of cyclin T1 and CLP-1 in αMHC-cyclin T1, CLP-1+/−, wild type, and αMHC-cyclin T1 × CLP-1+/− hearts. **d** Western blot showing increased phosphorylation of ser2 residue in the carboxy-terminal domain of RNA pol II. **e** Quantitation of Western blot in panel **d**

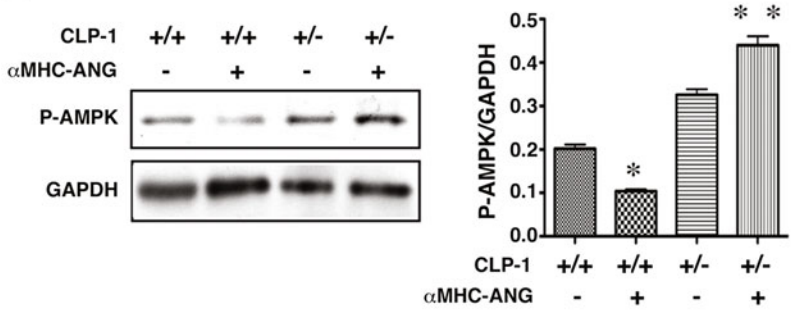
(a)



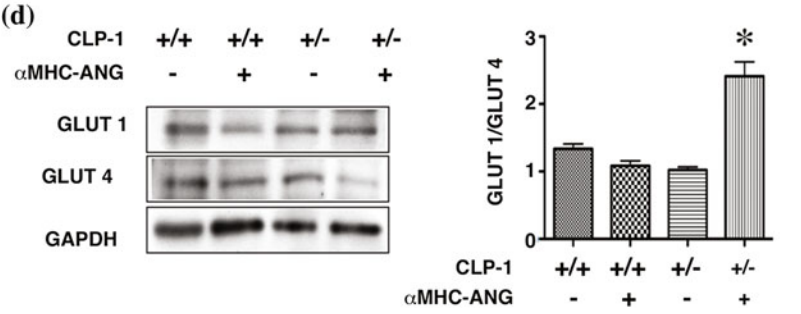
(b)

αMHC-ANG	WT	TG	WT	TG
CLP-1	+/+	+/+	+/-	+/-
	n = 8	n = 10	n = 10	n = 12
Heart rate (beats/min)	610 ± 13	590 ± 33	650 ± 17	630 ± 11
LVESD (mm)	1.08 ± 0.006	1.29 ± 0.004	0.93 ± 0.005	1.06 ± 0.003
LVEDD (mm)	2.18 ± 0.010	2.37 ± 0.011 *	2.03 ± 0.024	2.35 ± 0.009
ED-SWT (mm)	0.97 ± 0.020	1.26 ± 0.010*	0.88 ± 0.060	1.25 ± 0.040 **
ES-SWT (mm)	1.20 ± 0.030	1.60 ± 0.070	1.40 ± 0.030	1.98 ± 0.050**
LV mass (mg)	60 ± 3	80 ± 4 *	62 ± 2	93 ± 3 **
% FS	50 ± 2	45 ± 4	54 ± 1	54 ± 3

(c)



(d)



◀ **Fig. 18.2** Reduction of CLP-1 levels is associated with increased collagen deposition, septal wall thickness, and P-AMPK and GLUT1 expression, with little alteration in function. **a** Masson-Trichrome staining of sections taken from wild-type, CLP-1+/−, αMHC-ANG, and αMHC-ANG × CLP-1+/− hearts showing collagen deposition (*purple staining*) as opposed to no collagen deposition (*red staining*). NIH Image J quantitation of non-red regions of cells is shown in panels below. Collagen is present in αMHC-ANG heart (6.0 %) and to a greater degree in the αMHC-ANG × CLP-1+/− heart (11.0 %). * Connective tissue area per area of connective tissue plus cardiomyocyte. **b** Echocardiographic analysis of hearts showing little difference in percent fractional shortening among all mouse strains. Abbreviations: LVESD and LVEDD: left ventricular end-systolic and -diastolic dimension; ED-SWT and ES-SWT: end-diastolic and end-systolic septa wall thickness; %FS: % fractional shortening. **c** Immunoblot analysis of hearts of hypertrophic mouse models showing p-AMPK expression to be greatest in the αMHC-ANG × CLP-1+/− heart. P-AMPK expression is significantly increased in αMHC-ANG × CLP-1+/− versus αMHC-ANG × CLP-1+/+ hearts indicating increased P-AMPK expression with decreased CLP-1 gene dosage. * $P < 0.05$ versus control. **d** Analysis of GLUT1 and 4 levels in hearts of hypertrophic mouse models. GLUT4 shows decreased expression in αMHC-ANG × CLP-1+/− hearts whereas GLUT1 does not. GLUT1 is maintained at levels similar to wild-type hearts. Ratio of GLUT1 to GLUT4 is seen to be highest in αMHC-ANG × CLP-1+/− hearts. * $P < 0.05$ versus control. (Data from Espinoza et al.[48])

uptake [60, 61]. P-AMPK levels were seen to be greatest in αMHC-ANG × CLP-1+/− hearts suggesting that decreased CLP-1 levels could promote or at least maintain mitochondrial energy output (Fig. 18.2c). We also examined the expression of GLUT1 and 4 glucose transporters, two transporters that ordinarily decrease by 80 % in hearts enroute to metabolic insufficiency [62]. In mice with reduced CLP-1 levels, we found that GLUT4 levels decreased, while GLUT1 levels stayed roughly at or near wild-type levels (Fig. 18.2d). Together, the increase in p-AMPK activity and the maintenance of normal GLUT1 levels in αMHC-ANG × CLP-1+/− hearts suggests that reduced CLP-1 levels may promote metabolic sufficiency in cardiomyocytes that prevents their loss to necrosis and mitigates the attendant formation of reparative or scarring fibrosis [63].

18.8 Activation of cdk9 Kinase may Rapidly Promote Fibrotic Gene Expression by Coordinately Activating and Assembling RNA pol II and Smad Factors

The increase in collagen deposition seen in αMHC-ANG × CLP-1+/− hearts compared with all other experimental hearts could be due to increased formation of collagen-producing cardiac myofibroblasts. This would suggest that reduced CLP-1 levels promote expression of genes directing the differentiation of cardiac fibroblasts into myofibroblasts as well as the expression of fibrotic genes such as those encoding collagen types I and III. Since Ang II is a known inducer of fibrosis in hypertensive hearts, the increase in collagen deposition in αMHC-ANG × CLP-1+/− versus αMHC-ANG hearts may reflect the effect reduced CLP-1 levels have

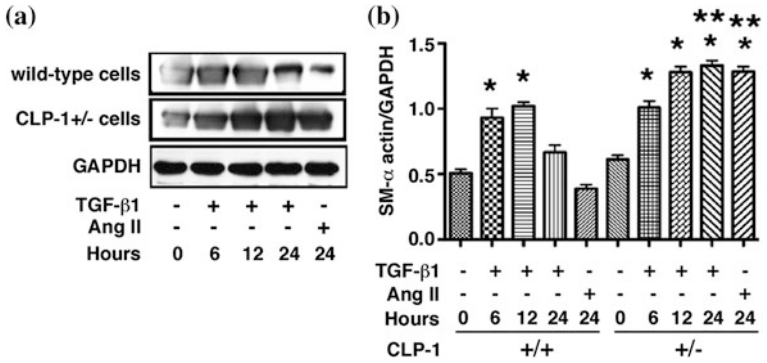


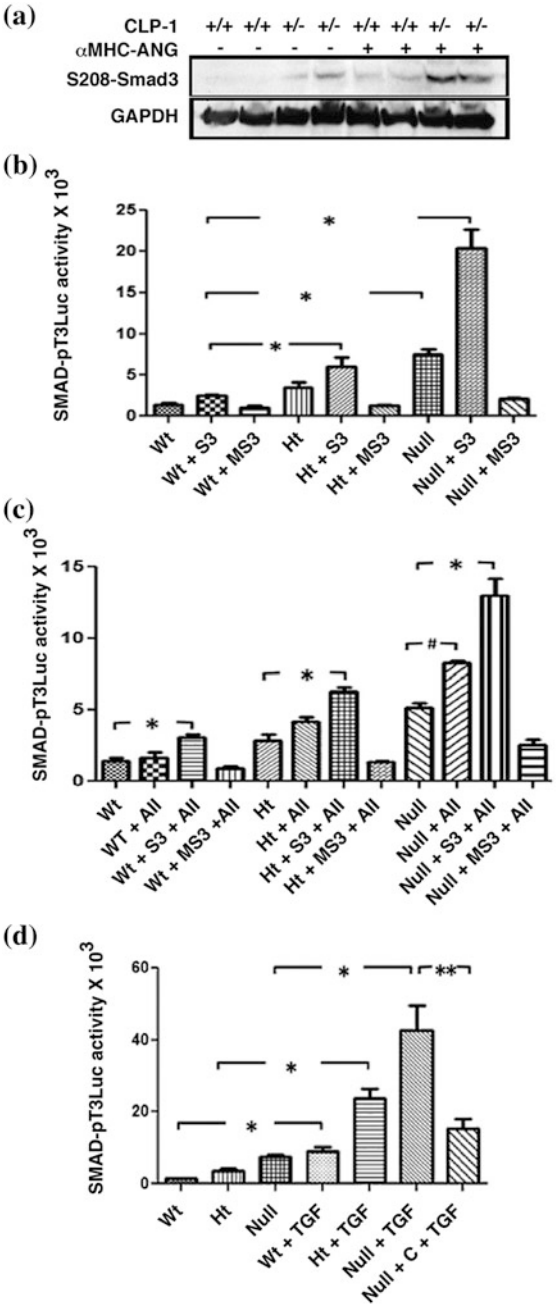
Fig. 18.3 SMAA and myofibroblast differentiation with Ang II or TGF-beta. **a** TGF-B1 or Ang II treatment of cardiac fibroblasts from wild-type or CLP-1+/− mice shows increased expression of the myofibroblast marker smooth muscle alpha actin (SMAA) in CLP-1+/− cells versus wild-type cells. **b** Quantitation of SMAA expression and normalization to GAPDH

on P-TEFb activity and P-TEFb activity on Ang II-dependent fibrotic gene expression. This possibility is suggested by the coordinate assembly model of CLP-1 function outlined above: increased P-TEFb activity resulting from decreased CLP-1 levels may be simultaneously activating nuclear transcription factors activated by the Ang II signal transduction pathway, e.g., smads, as well as P-TEFb. This synchronized activation would allow rapid expression of genes directing myofibroblast differentiation as well as collagen-encoding genes by coordinately assembling gene-specific transcription factors into the same transcriptional complex with phospho-RNA pol II activated into its elongation state. To begin to examine this possibility, we treated wild-type and CLP-1+/− cardiac fibroblasts with TGF-beta1 or Ang II, known activators of fibrosis [9, 64–66], and assessed myofibroblast formation by determining expression levels of the myofibroblast marker, smooth muscle alpha actin (SMAA). Results show that CLP-1+/− cardiac fibroblasts have a greater ability to differentiate into myofibroblasts than wild-type cells (Fig. 18.3). This suggests that the increased collagen deposition we see in the αMHC-ANG × CLP-1+/− myocardium in vivo is due to increased formation of cardiac myofibroblasts owing to the activation of the genes directing their fibrotic program of differentiation. Based on our model of CLP-1 function, we propose that reduced CLP-1 levels are promoting the fibrotic differentiation program by activating P-TEFb complexes to coordinately assemble activated gene-specific transcription factors (smads) with active RNA pol II to up-regulate the transcription and expression of fibrotic genes.

It is fairly well-established that Ang II induces fibrosis via its activation of the TGF-beta signal transduction pathway in cardiac fibroblasts. TGF-beta signals to the nucleus via activation of smad transcription factors and the involvement of smad factors in promoting myofibroblast formation and collagen production in hypertrophic heart has been well documented [15, 32, 64, 67, 68]. Smad 3 in particular has been shown to be activated by TGF-beta induced by cytokines

during the inflammatory and fibrotic processes that occur in Ang II-induced hypertensive hearts [69, 70]. Given our observation that the reduced levels of CLP-1 in αMHC-ANG × CLP-1+/− hearts are increasing the expression of fibrotic genes, we would predict based on our coordinate assembly model of P-TEFb gene transcription that this would involve coordinate activation of Ang II-activated transcription factors, the most likely candidate being Smad 3. When our observations on αMHC-ANG × CLP-1+/− hearts and CLP-1+/− cardiac fibroblasts were first made, it was unclear how or if P-TEFb with its cdk9 kinase activity could coordinately activate Smad 3 transcription factors, particularly since smads entered the nucleus already activated into transcriptionally competent dimers via their phosphorylation by TGF-beta and BMP receptors. Some indication that kinases can in fact regulate smad activity had come from studies showing that phosphorylation of the c-terminal of smads promotes their entry into the nucleus. The importation and subsequent nuclear transcriptional activity of smads can be further controlled by a second phosphorylation event directed to the linker region of smads that links the DNA-binding domain with the transcriptional activation domain. If the linker region is phosphorylated by cytoplasmic kinases, smads are retained in the cytoplasm or subjected to polyubiquitination-mediated degradation, both of which prevent smad transit into the nucleus [71, 72]. In the case of those smads that have successfully entered the nucleus, phosphorylation of the linker region by nuclear kinases promotes the full transcriptional activity of smads but also primes them for ubiquitin-mediated degradation, a possible mechanism to make smad activation more dependent on the presence of TGF-beta or BMP. Interestingly, the kinases responsible for this nuclear phosphorylation have been shown to be cdk 8 and 9 [36].

These findings, particularly with regard to the involvement of cdks 8 and 9 in regulating smads, provided support for our coordinate assembly model of P-TEFb, particularly as it related to Ang II and/or TGF-beta induced fibrosis in hypertrophic heart. We hypothesized that smad 3-dependent genes would be transcribed to a greater extent in CLP-1+/− fibroblasts treated with Ang II or TGF-beta because lowered CLP-1 levels would lead to greater cdk9 P-TEFb kinase activity and increased smad and RNA pol II activity. Based on this, we examined how smad3 transcriptional activity changes in response to changes in P-TEFb cdk9 kinase activity resulting from decreased CLP-1 gene dosage. From our model of CLP-1 function, decreasing CLP1 levels should lead to increasing de-repression of P-TEFb and increasing cdk9 kinase activity. In terms of smad3, this would mean increased phosphorylation of the smad3 linker region. To determine if this is the case in vivo, we compared the amount of smad 3 that is phosphorylated on serine 208, the linker region amino acid phosphorylated for full smad3 activation, in αMHC-ANG × CLP-1+/− versus αMHC-ANG hearts [73]. We found that smad3 S208 phosphorylation was greater in αMHC-ANG × CLP-1+/− hearts suggesting that lowered CLP-1 levels can induce greater cdk9 activity and smad3 linker phosphorylation (Fig. 18.4a). To determine if these events can lead to an increase in transcription of smad3-dependent genes, we performed in vitro experiments wherein we introduced smad3 along with a smad3-luciferase reporter construct


(a)
CLP-1 +/+ +/+ +/- +/- +/+ +/+ +/- +/-
αMHC-ANG - - - - + + + +
S208-Smad3
GAPDH
(b)
SMAD-pT3Luc activity X 10^3
25
20
15
10
5
0
Wt
Wt + S3
Wt + MS3
Ht
Ht + S3
Ht + MS3
Null
Null + S3
Null + MS3
(c)
SMAD-pT3Luc activity X 10^3
15
10
5
0
Wt
WT + All
Wt + S3 + All
Wt + MS3 +All
Ht
Ht + All
Ht + S3 + All
Ht + MS3 + All
Null
Null + All
Null + S3 + All
Null + MS3 + All
(d)
SMAD-pT3Luc activity X 10^3
60
40
20
0
Wt
Ht
Null
Wt + TGF
Ht + TGF
Null + TGF
Null + C + TGF

◀ **Fig. 18.4** Lowering CLP-1 gene dosage increases activation of a smad3-dependent luciferase reporter gene. **a** Western blots showing increased phosphorylation of serine 208-Smad3 in αMHC-ANG × CLP-1+/− hearts relative to hearts from wild-type, heterozygous CLP-1+/−, and αMHC-ANG mice. Null: CLP-1−/−; Ht: CLP-1+/−; Wt: wild-type; S3: wild-type smad linker region; MS3: smad linker region with mutated serine 208. **b** Transfection of a smad-dependent reporter construct into mouse embryonic fibroblasts with differing CLP-1 gene dosages shows increasing smad3-dependent transcriptional activity with decreasing CLP-1 levels. A substitution mutation at serine 208 rendered the smad-dependent reporter inactive confirming the importance of this serine as a phosphorylation site for cdk9. **c** Treatment of reporter-transfected embryonic fibroblasts with Ang II (AII) shows increasing smad-dependent transcriptional activity with decreasing CLP-1 gene dosage. Mutation of serine 208 has same negating effect as seen in **b**. **d** Treatment of reporter-transfected embryonic fibroblasts with TGF-β1 shows higher transcriptional activation relative to treatment with Ang II (panel **c**). Re-introducing CLP-1 into CLP-1 null fibroblasts using a CLP-1 expression vector, **c**, reduced transcription of the smad-dependent reporter construct

into fibroblasts of differing CLP-1 gene dosage: CLP-1 null, CLP-1 heteozygous, and CLP-1 wild-type embryonic fibroblasts (we used embryonic fibroblasts because CLP-1 nulls or knockout mice die in late fetal stages of development [46]. As shown in Fig. 18.4b, in the absence of any stimulation by Ang II or TGF-beta, decreasing CLP-1 gene dosage resulted in an increase in smad-luciferase reporter activity. Mutation of the target serine for cdk9 in the smad3 linker region, abolished the increase in smad3 transcription over all CLP-1 gene dosages. When the same experiment was performed in the presence of either Ang II or TGF-beta, similar results were obtained: decreasing CLP-1 gene dosage was associated with increasing smad3-dependent transcription (Fig. 18.4c). To further show that this increase in smad3-dependent transcription was actually a result of lowered CLP-1 levels, we introduced CLP-1 back into cells with the lowest CLP-1 levels possible, i.e., the CLP-1 null embryonic fibroblasts. Introduction of CLP-1 back into CLP-1−/− cells resulted in a reduction of smad3-transcriptional activity (Fig. 18.4d). Together, these experiments showed that by reducing or eliminating CLP-1, P-TEFb can form active complexes with de-repressed cdk9, phosphorylating the smad3 linker region to increase smad3 transcriptional competence. This regulatory intersection between P-TEFb and smad3 provides another way in which CLP-1 can regulate expression of fibrotic stress response genes via up-regulation of specific transcription factors such as smads and provides support for our coordinate assembly model of P-TEFb-regulated gene expression. These experiments also provide a cogent molecular rationale for why CLP-1+/− fibroblasts more readily differentiate into SMAA-expressing cardiac myofibroblasts than wild type cells: reduced CLP-1 levels increase smad3 linker phosphorylation, smad3 activity, and smad3-dependent gene expression critical for the differentiation of cardiac fibroblasts into myofibroblasts and the expression of fibrotic genes [65, 68].

18.9 Conclusion

The heart's response to hypertrophic stress requires the transcriptional apparatus of heart cells to effectively respond to stress-induced signals in order to mount the appropriate level and type of stress response. Our studies and those of others have shown that HEXIM1/CLP-1, the regulator of P-TEFb and RNA pol II-dependent gene transcription, plays a key role in translating hypertrophic stress signals into genomic stress response. To ensure a rapid response to stress, stress response genes reside in a state of "primed" readiness in which genes are "pre-loaded" with RNA pol II that has commenced transcription but then has "paused" to await receipt of stress signals. These signals then fully activate RNA pol II into an elongation state that allows completion of the paused gene transcript. Transition into the elongation state is dependent on phosphorylation of RNA pol II by cdk9 kinase, a critical feature of which is the "on–off" control of cdk9 kinase activity exerted by HEXIM1/CLP-1. More recent evidence suggests that in addition to controlling the basal transcriptional apparatus involving RNA pol II, cdk9 kinase can also control the activity of specific signal-responsive transcription factors such as the smads. By acting at the nexus of signal and transcriptional response for different transcriptional mechanisms, HEXIM1/CLP-1 provides a way of controlling important stress response programs such as metabolic regulation and fibrotic differentiation. Understanding how HEXIM1/CLP-1 controls these transcriptional mechanisms and stress response programs will be critical to understanding how the heart mounts a compensatory response to hypertrophic stimuli and how it could be maintained in order to avert progression to decompensatory hypertrophy.

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Chapter 19
Role of Sirtuins in Regulation of Cardiac Adaptation Associated with Hypertrophy

Vinodkumar B. Pillai and Mahesh P. Gupta

Abstract Lysine-acetylation within a protein is considered as a functionally relevant post-translation modification regulating activity of the target protein. While protein acetylation is regulated by acetyl-transferases, deacetylation is catalyzed by deacetylases. Recently a family of nicotinamide-adenine-dinucleotide (NAD)-dependent deacetylases, called sirtuins, has been identified, which are emerging as key regulators of many biological functions, spanning from cell growth to longevity. Among the seven sirtuin isoforms (SIRT1–SIRT7) expressed in mammalian cells, two isoforms SIRT1 and SIRT3 have been studied with some detail for their roles in regulating cardiac adaptation to increased workload. SIRT1 was found to regulate Akt signaling and plays an essential role for the development of cardiac hypertrophy. SIRT3 on the other hand was found to act as a negative regulator of cardiac hypertrophy, which also protects cardiomyocytes from oxidative stress-mediated apoptosis. The mechanism behind anti-growth and anti-apoptotic activity of SIRT3 seems to stem from its ability to deacetylate several mitochondrial target proteins and thereby promoting overall function of mitochondria to generate less reactive oxygen species (ROS) and more ATP synthesis. As both SIRT1 and SIRT3 are activated by physical exercise and calorie restriction, some of the cardiac benefits arising from these interventions are likely to be stemming from the activation of these two molecules. This chapter gives a brief overview of sirtuin biology and then focuses on the opposite roles of SIRT1 and SIRT3 to regulate cardiac remodeling associated with hypertrophy.

Keywords SIRT1 · SIRT3 · Cardiac hypertrophy · Heart failure

V. B. Pillai · M. P. Gupta (✉)
Department of Surgery, University of Chicago,
5841 South Maryland Avenue, Chicago, IL 60637, USA
e-mail: mgupta@surgery.bsd.uchicago.edu

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19.1 Introduction

Cardiovascular diseases are predicted to be the most common cause of mortality worldwide by year 2020 [1, 2]. Heart failure is already recognized as the most important cardiovascular disorder in the West from the perspective of health care resources utilization [1]. Incidence of cardiovascular diseases is increasing at an alarming rate throughout the world. In the United States alone heart failure is responsible for almost 1 million hospital admissions and 400,000–600,000 deaths annually, with $20–40 billion in yearly health care costs. It is well accepted that the major cause of this disturbing rise in CVD is our sedentary lifestyle and recent change in our dietary habits. Epidemiological studies have suggested that consumption of high-calorie-high-fat diets increases risk for atherosclerosis, diabetes, obesity, heart failure, and other cardiovascular associated diseases. In contrast to this, calorie restriction is considered beneficial for health and to extend life span by reducing aging associated diseases, such as heart failure, neurodegenerative diseases, diabetes, and cancer [3, 4]. In the heart, modest calorie restriction and exercise was shown to improve cardiac contractile dysfunction and myocardial remodeling in patients with myocardial infarction [5]. Because calorie restriction is practically hard to follow in today's busy lifestyle, considerable research has been done to delineate signaling pathways activated during calorie restriction. Once these signaling pathways are understood it may be possible to manipulate genes involved to mimic the effects of calorie restriction. Recent studies suggest that class-III group of histone deacetylases, also called sirtuins, play an important role in mediating the beneficial effects of calorie restrictions [3, 4]. Two analogs of sirtuins SIRT1 and SIRT3 which are activated in response to calorie restriction have been implicated in the regulation of cardiac hypertrophy and the development of heart failure and are the focus of this chapter.

19.2 Sirtuins in General

Sirtuins are conserved family of nicotinamide-adenine-dinucleotide (NAD) dependent enzyme which has deacetylation and ADP-ribosylation activities. Sirtuins have been shown to regulate life span extension in lower organisms as well as some aspects of physiological response of calorie restriction and fasting in mammals [6]. Because of dependency of sirtuins to NAD, activity of these enzymes is highly sensitive to fluctuations in cellular NAD/NADH ratio, which could happen during change in cellular metabolism and under stress conditions. It has been shown that increased cellular NAD content elevates the enzymatic activity of sirtuins, whereas high NADH and nicotinamide levels do the opposite [7]. Emerging evidence suggest that sirtuins are key regulators of myriad of biological functions. Dysregulation of sirtuins has been implicated in diseases like asthma, cancer, diabetes, inflammation, neuronal, and cardiovascular disorders [8].

Table 19.1 Subcellular localization and known cardiac effects of members of the sirtuin family

Sirtuin isoforms	Localization	Cardiac effects	References
SIRT1	Cytoplasm, nucleus, plasma membrane	Cell survival and pro-hypertrophic	[10, 15]
SIRT2	Cytoplasm, nucleus	–	[17]
SIRT3	Mostly mitochondria	Promoting stress resistance, anti-hypertrophic	[31, 32, 37, 41]
SIRT4	Mitochondria	–	[18, 21]
SIRT5	Mitochondria	–	[18, 21]
SIRT6	Nucleus	–	[19]
SIRT7	Nucleus	Deficiency leads to inflammatory cardiomyopathy	[20]

There are seven sirtuin (SIRT1–SIRT7) isoforms encoded by the mammalian genome (Table 19.1). All seven sirtuin isoforms are ubiquitously expressed and possess a highly conserved deacetylase domain, first identified in the yeast Sir2 protein [9]. SIRT1 is the prototype member of this family and the closest mammalian homolog of yeast Sir2. This isoform is the most widely studied sirtuin analog which is present both in the nucleus and cytoplasm. New evidence suggests that SIRT1 is also present at the levels of plasma membrane where it participates in activation of insulin and Akt signaling [10]. SIRT1 has been shown to deacetylate many non-histone proteins, including p53, FOXO, PGC1α, LKB1, HIF1α, Akt1, PDK1, and TORC2, which are shown to play important roles in cell survival, metabolism and stress-response [11–16]. Similar to SIRT1, SIRT2 is localized mostly in cytoplasm and in the nucleus. SIRT2 is known to deacetylate tubulin and regulate the tubulin polymerization and plays a role in regulating chromosomal stability during cell-division [17]. SIRT3, SIRT4, and SIRT5 are primarily localized into mitochondria and are referred as mitochondrial stress sensors that can modulate the activity of several mitochondrial proteins, involved in reactive oxygen species (ROS) and ATP synthesis [18]. SIRT6 is a chromatin-associated sirtuin isoform which is involved in regulating diverse cellular functions, including genome stability, glucose metabolism, and inflammation [19]. SIRT6 deficient mice develop complex degenerative phenotype with enhanced rate of aging and shortened life span and die at around 26 days of birth [19]. The last member of this family is SIRT7, which is associated with nucleoli and condensed chromosomes. SIRT7 deficient mice also display reduced life span and they develop inflammatory cardiomyopathy [20]. From the studies available to date, it is evident that sirtuins have considerable amount of redundancies to targets and to regulate cellular functions. The role of sirtuins in regulation of cardiac remodeling is just beginning to be understood and so far two sirtuin analogs have been studied in some detail for their roles in regulating development of cardiac hypertrophy and heart failure.

19.3 Role of SIRT1 in Cardiac Adaptation to Hypertrophy

SIRT1 is a ubiquitously expressed protein which was initially identified as a nuclear protein [21]. However, recent studies showed that the subcellular localization of SIRT1 varies depending on the cell type. While some cells showed nuclear localization of SIRT1, others expressed it either both in the nucleus and in the cytoplasm or in the cytoplasm alone. There are also reports showing that SIRT1 is localized at the level of plasma membrane [10]. In the heart, nuclear, and cytoplasmic localization of SIRT1 was found to be regulated developmentally and during stress of the heart [15]. In the mouse embryonic heart, high levels of SIRT1 was found in the nucleus of myocytes during development of the four chambered heart. Thereafter, expression of SIRT1 declines with organogenesis of the heart and it remain constant after birth up to 27 months of age [15]. In the adult heart of rodents, SIRT1 is localized mainly in the cytoplasm and moves to nucleus up on stress. Nuclear localization of SIRT1 in cardiomyocytes was inhibited by use of a PI3K inhibitor LY294002, which also blocked Akt activation, thus suggesting a possible role of PI3K/Akt-mediated phosphorylation in the nuclear translocation of SIRT1 [15]. Likewise, JNK1-mediated phosphorylation of SIRT1 also promotes its importation into the nucleus [22]. This importation of SIRT1 to the nucleus was found to be essential for its cytoprotective effects against oxidative stress through activation of MnSOD, suggesting that phosphorylation-mediated shuttling in and out of nucleus could be one mechanism by which SIRT1 activity is being regulated [15]. This study also reported the presence of nuclear SIRT1 in different models of heart failure, including failing hearts of hamsters, post myocardial infraction in rats and dilated cardiomyopathy in patients [15]. Because, the nuclear presence of SIRT1 is a feature of the fetal heart and SIRT1 localizes into nucleus under pathologic conditions, it is convenient to believe that nuclear translocation of SIRT1 could have a role in the activation of fetal gene program associated with the evolution of pathologic cardiac hypertrophy.

Recent studies from our group have shown that SIRT1 is also localized at the sarcolemma of cardiomyocytes. SIRT1 binds to Akt and PDK1 and deacetylate lysine at their Pleckstrin homology domain. This post-translational modification of pleckstrin homology domain by SIRT1 is essential for binding of Akt and PDK1 to PIP3 and for their membrane localization [10]. Because Akt signaling plays a central role in regulating development of physiologic cardiac hypertrophy, this study suggested that membrane localization of SIRT1 might have a role in regulating induction of physiologic hypertrophy of the heart under mild stress conditions.

A role of SIRT1 in Akt activation was also supported by initial overexpression studies which showed that SIRT1 protects cells from death in response to serum starvation, but at the same time causes an overall increase in size of cardiomyocytes [23]. This study also showed that blocking of SIRT1 activity with inhibitors increased the propensity of cardiomyocytes to death, but prevented hypertrophy of myocytes in response to stress stimuli, thus implying that though cytoprotective,

SIRT1 promotes cardiomyocyte growth under stress conditions by activating Akt signaling. These observations are supported by other reports where increased SIRT1 levels were found in hypertrophied and failing hearts [24, 25]. Studies performed with cardiac-specific SIRT1 transgenic mouse model showed that SIRT1 exhibits dual effects; that is, depending on the magnitude of SIRT1 expression; it can be beneficial or harmful. A low to moderate expression of SIRT1 (2–5-fold) was found to be protective against age-dependent increase in cardiac hypertrophy, apoptosis, and cardiac dysfunction; whereas robust (12-fold) overexpression of SIRT1-induced dilatation, hypertrophy, and cardiac failure [26]. A low level of SIRT1 overexpression was also shown to reduce infarct size and improve cardiac functions in a mouse model of myocardial infarction [27]. These observations strengthen the perception that SIRT1 is a pro-growth and pro-survival molecule for cardiomyocytes and hence its expression needs to be tightly controlled to obtain its desirable effects.

The data obtained by studying SIRT1-deficient mouse have also underscored a role of SIRT1 in the development and growth of the heart. SIRT1 homozygous knockout mice in an inbred genetic background exhibit severe developmental defects in the heart and they mostly die after birth [28, 29]. However, SIRT1 knockout mice developed on crossbred genetic backgrounds are alive [28, 30]. So far no studies have reported any cardiac phenotype in these mice. Similarly, cardiac-specific deletion of SIRT1 does not show any obvious phenotypes at basal conditions. These findings suggest that SIRT1 is dispensable for the survival of adult cardiomyocytes. However, these mice were more susceptible to cell death induced by ischemia/reperfusion injury [27]. Our data show that whole body SIRT1 knockout mice have smaller heart than their wild type littermates, and these hearts are resistant to develop cardiac hypertrophy induced by hypertrophic agonists [10]. These mice also show reduced activation of fetal gene program, lack of cardiomyocyte hypertrophy, and impaired Akt signaling following infusion of hypertrophic agonists, thus suggesting that SIRT1 plays a pivotal role for the induction of cardiac hypertrophic program [10].

19.4 Regulation of Cardiac Hypertrophic Program by SIRT3

Another sirtuin isoform which has gained credit for regulating cardiac hypertrophic program is SIRT3 [31]. SIRT3 is generally considered as a mitochondrial sirtuin. However, there are reports showing evidence against strictly mitochondrial localization of SIRT3 [32]. The full length human SIRT3 (hSIRT3) is a 44 kDa protein with an N-terminal mitochondrial localization sequence (MLS) [33]. Initial studies have suggested that the nuclear form of SIRT3 is enzymatically inert. Following import to mitochondria, 142 amino acids from the N-terminal of full length hSIRT3 are cleaved by matrix metallo-proteases (MMP) present in the mitochondrial matrix to generate an active 28KD short form [33]. This was confirmed by another study which showed mitochondrial cristae as the site of

localization of short form of SIRT3; whereas, overexpressed full length hSIRT3 was localized in the cytoplasm [34]. In contrast to this, two other studies reported the presence of full length hSIRT3 in the nucleus. Scher et al. reported that full length hSIRT3 is a nuclear protein that translocates to mitochondria upon stress; whereas, the second study reported the translocation of hSIRT3 to the nucleus upon co-expression with SIRT5 [35, 36]. These studies were later challenged by Cooper and Spelbrink, who demonstrated that endogenous hSIRT3 was present exclusively in mitochondria, whereas overexpression of hSIRT3 lacking MLS led to expression of the protein in the cytoplasm and in the nucleus [37].

Contrary to hSIRT3, the mouse SIRT3 (mSIRT3) has been shown to generate three different splice variants, designated as M1, M2, and M3 [38]. The translation of M1, M2, and M3 isoforms begins from Met1, Met15, and Met78, respectively. M1 and M2 isoforms were found to be localized in the mitochondria, whereas, M3 isoform which lacks MLS was absent from mitochondria, instead it was distributed in the cytoplasm, and upon high level of overexpression it was found to be expressed in the nucleus as well. All three isoforms of murine SIRT3 were reported to be equally active to deacetylase target proteins [39, 40]. Furthermore, like human SIRT3, M1 and M2 were found to be cleaved to generate identical shorter forms, similar in size to M3. We observed that in the heart, the endogenous long form of mSIRT3 was localized to mitochondria, cytoplasm, and to a lesser extend in the nucleus; whereas, the short form of mSIRT3 was detected only in mitochondria [41]. There is no other report currently available regarding the localization of endogenous SIRT3 in the heart. To date, it is also not known why these three alternatively spliced isoforms of murine SIRT3 are generated and whether they have compartment-specific effects. Initial studies done in our laboratory have demonstrated that SIRT3 levels are reduced during pressure overload cardiac hypertrophy and overexpression of SIRT3 isoform lacking in MLS is capable of blocking adverse cardiac remodeling associated with pressure overload and agonist-mediated hypertrophy [31]. We have also demonstrated that cardiac SIRT3 levels are increased in response to mild stress and overexpression of SIRT3 protects cardiomyocytes from oxidative stress-mediated cell-death [41]. Cardioprotective effects of SIRT3 were also observed from analyzing cardiac phenotype of SIRT3 deficient mice. These mice are born normally without a discernible phenotype at birth. However, as they reach adulthood, nearly 8 weeks of age, they develop symptoms of cardiac hypertrophy associated with interstitial fibrosis, and they become highly sensitive to infusion of hypertrophic agonists [31]. From these studies it is clear that SIRT3 is a negative regulator of cardiac remodeling associated with hypertrophy and it promotes stress-resistance capacity of the heart under adverse conditions.

At the cellular levels cardiac remodeling is comprised of transcriptional reprogramming of genes, which are otherwise expressed during fetal heart development. This change in gene expression also causes considerable metabolic transformation in the heart. A normal healthy heart oxidizes both fatty acids and glucose simultaneously to meet its high energy needs. But during cardiac hypertrophy, just like fetal heart, fatty acid oxidation becomes impaired, compromising

the ability of the heart to oxidize long chain fatty acids [42]. This causes switch in the myocardial energy substrate utilization from fatty acid β-oxidation to glycolysis [43]. During the initial adaptive phase of hypertrophy, this switch in substrate utilization increases efficiency of the heart to meet energy demands, but when hypertrophy progresses this metabolic adaptation becomes insufficient due to decreased capacity of the heart to oxidize glucose, leading to contractile dysfunction and eventually to heart failure. Decreased FFA (Free fatty acid) oxidation by the hypertrophied heart evoked the question as to whether by promoting FFA oxidation development of cardiac hypertrophy could be prevented [44]. However, data obtained from many studies have demonstrated that increasing activity of the regulatory program controlling FFA oxidation does not produce beneficial results. Rather it induces cardiomyopathy similar to diabetic heart [45]. These studies thus demonstrated that the utilization of FFA oxidation in the heart must be tightly controlled to meet energy demands, without excess fuel supply that could otherwise result in substrate-driven cardiomyopathy. Although, a role of SIRT3 in regulating cardiac metabolism has not been studied to date, findings reported from other organ systems of SIRT3-deficient mice suggest that it might play a role in regulating FFA oxidation in the hearts. Hirschey et al. observed that SIRT3 knockout mice had 50 % reduced oxidation of free fatty acids, which resulted in accumulation of long chain fatty acids in the liver [46]. This effect was more pronounced during fasting of the animal which is a well-known stimulus for SIRT3 activation. They also found 33 % reduction in fatty acid oxidation in the hearts of fasted mice. This defect in free fatty acid oxidation was correlated with increased acetylation of long chain acyl-CoA dehydrogenase (LCAD) in SIRT3-deficient mice, which led to decreased enzymatic activity of LCAD by 40 %. Other studies have recently confirmed a role of SIRT3 in upregulation of FFA oxidation under-calorie-restricted conditions [47]. Since cardiac hypertrophy is also associated with reduced free fatty acid oxidation, and SIRT3 levels are reduced during cardiac hypertrophy/heart failure [31], it may be logical to assume that a similar SIRT3-mediated mechanism could operate in the heart to regulate free fatty acid oxidation, though it has to be yet formally demonstrated.

One of the consequences of defective cardiac FFA oxidation is reduced ATP production. Several studies, including ours have shown a positive correlation between ATP depletion and cardiac hypertrophy [48, 49]. In a recent study where SIRT3 and ATP levels were simultaneously measured, a direct correlation between these two molecules was found in several tissues. Organs with lower ATP levels like pancreas, spleen, and skeletal muscle were shown to have reduced SIRT3 expression, while other organs with higher ATP levels like heart, liver, and kidney showed higher levels of SIRT3 expression [50]. In SIRT3-deficient hearts more than 50 % reduced levels of ATP, compared to wild type hearts, were reported [50]. Moreover, under stress conditions when ATP demands are high increased expression levels of SIRT3 were noticed. Taken together, these studies have demonstrated that SIRT3 is an important player in regulation of cellular ATP biosynthesis.

Although the mechanism behind the ability of SIRT3 to regulate ATP biosynthesis has not yet been fully understood, several proteins of the mitochondrial electron transport chain (ETC) and intermediates of TCA cycle have been found as substrates of SIRT3-mediated deacetylation. The first mitochondrial substrate identified as a target of SIRT3 was the enzyme acetyl-CoA synthase 2 (AceCS2), which is highly expressed in the heart [51, 52]. AceCS2 plays an important role in the ATP biosynthesis by generating acetyl-CoA for the TCA cycle. AceCS2 is inactivated by acetylation; SIRT3 deacetylates and reactivates this enzyme. This target of SIRT3 is particularly activated under fasting conditions and functions during ketogenic state to convert acetate to acetyl-CoA for ATP biosynthesis. There are three other mitochondrial matrix proteins which have been identified as substrates of SIRT3, whereby lysine acetylation inactivates them and SIRT3-mediated deacetylation enhances their activity [53]. Schlicker et al. have shown that SIRT3 can deacetylate and activate glutamate dehydrogenase which promotes oxidative deamination of glutamate to α-ketoglutarate and isocitrate dehydrogenase 2 [53]. Furthermore, another TCA intermediate, succinate dehydrogenase was found to be acetylated in SIRT3 knockout mice, which led to decreased activity of the enzyme [54]. There is also a report showing that SIRT3 regulates the activity of urea cycle enzyme ornithine transcarbomylase [47]. Other studies have shown that the proteins of complex 1 of ETC are hyperacetylated in SIRT3-deficient mice. This study also showed that SIRT3 directly interacts with and deacetylates NDFU9 subunit of complex 1 leading to increased activity of the protein [50]. In a separate study Kim et al. detected reduced activity of complex III in SIRT3-deficient Myc/Ras transformed cells, compared to wild type control cells [55], thus implying that SIRT3 plays a role in regulating the activity of ETC chain. Additionally, SIRT3 was shown to enhance the oxidative phosphorylation by modulating the activity of cyclophilin D [56]. Here in this case SIRT3-mediated deacetylation inhibits the enzymatic activity of cyclophilin D, thereby inducing its dissociation from ANT1 (adenine nucleotide translocator), which then promotes detachment of hexokinase-II from VDAC. This process caused redistribution of hexokinase-II from mitochondria to cytosol, resulting in increased mitochondrial oxidative phosphorylation [56]. Besides the role of cyclophilin D in regulating hexokinase association to mitochondria, it also play an essential role in regulating the activity of mitochondrial permeability transition (MPT) pore and concordance calcium flux and Ca-dependent mitochondrial enzyme activity. Because SIRT3-dependent deacetylation inhibits the activity of Cyclophilin D, it has been proposed that SIRT3 knockout mice might retain cyclophilin D activity and hence greater activity to MPT, and this could be one of the mechanisms why SIRT3 knockout mice possess higher susceptibility to develop pressure overload hypertrophy than their wild type counterparts [57]. As with the role of SIRT3 in regulating FFA oxidation, the direct evaluation of the role of SIRT3 in modulating the activity of TCA cycle and intermediates supplying substrates to this cycle for regulating cardiac metabolism has not been examined. Nevertheless, studies discussed above underscores a role of SIRT3 in regulating cellular ATP biosynthesis,

and suggest the immense possibility of finding other mitochondrial proteins whose activity could be regulated by SIRT3 to maintain energy demand of the heart.

In addition to above-mentioned targets, there are also reports showing that SIRT3 regulates energy metabolism by activating AMPK [49, 58, 59]. Activated AMPK can upregulate the catabolic pathways such as cellular glucose uptake and fatty acid oxidation resulting in the increased production of ATP, while switching off anabolic pathways that consume ATP. Since heart produces and consumes more energy than any other organ, it is highly relevant that AMPK may act as a key regulator of energy metabolism in the heart [60]. Consistent with this notion, activation of AMPK was shown to inhibit cardiac hypertrophy both in vitro and in vivo models [61, 62]. The upstream kinase of AMPK is LKB1 which phosphorylates the α-subunit of AMPK at threonine 172 (T172) leading to exposure of the site to AMP binding and hence activation. There are report showing that cardiac-specific deletion of LKB1 induces cardiac hypertrophy and heart dysfunction, thus again highlighting the role of LKB1 substrate AMPK in regulating the development of cardiac hypertrophy [63]. Recent work done in our laboratory has shown that SIRT3 can interact with and deacetylate LKB1 in the heart and this in turn activates AMPK. In this study, we also demonstrated that exogenous supplementation of NAD rescued the heart from ATP depletion and blocked the activation of pro-hypertrophic Akt signaling by activating SIRT3 [49]. These studies indicated that SIRT3-mediated AMPK activation could be another mechanism through which SIRT3 can maintain high cardiac ATP demands and protect the heart from developing pathologic hypertrophy.

As heart is an organ of high energy demand, it has more mitochondrial content per cell than any other organ system in the body. One undesirable side effects of high mitochondrial function is the production of high ROS during metabolism. These ROS levels if gone unchecked can cause serious damage to cells through oxidation of proteins, lipids, and nucleic acids, which could result unwanted post-translational modification of proteins leading to altered fluidity of the lipid membranes and could cause mutations in genomic and mitochondrial DNA [64]. Cells are protected from the deleterious effects of ROS by antioxidant enzymes, such as superoxide dismutases (SOD), catalase, and peroxidases. ROS generated from mitochondrial respiration are converted to hydrogen peroxide by SOD, whereas catalase and glutathione peroxidase helps to convert hydrogen peroxide to water. The expression of SOD, particularly MnSOD and catalase has been shown to be regulated by the members of the family of forkhead transcription factors (Foxos). Foxo3a binds to gene promoters and transcriptionally upregulates the expression of antioxidants, and thereby reducing the cellular content of ROS [65, 66]. Increased production of ROS has been strongly implicated in the development of cardiac hypertrophy [67]. Recent work done in our laboratory have shown that SIRT3 blocks cardiac hypertrophic response through activation of antioxidants gene expression in the heart [31]. Cardiomyocytes cultured from SIRT3-deficient hearts showed increased ROS levels, compared to their respective wild type controls; whereas, SIRT3 over expressing cells which were protected from hypertrophic stimuli had reduced levels of ROS, thus implying that SIRT3 prevented cardiac hypertrophic response by scavenging cellular ROS levels.

This observation was supported by additional findings showing reduced activation ROS targets like Ras oncoprotein and its downstream targets, namely MAPK/ERK and PI3K/Akt signaling pathways in SIRT3 transgenic mice infused with hypertrophic agonists. This study also showed that SIRT3 binds to and deacetylates Foxo3a and activation of Foxo3a resulted in the transcriptional upregulation of SOD2 and catalase. SIRT3-deficient mice showed reduced activity of SOD2 and catalase under basal conditions which was further reduced when challenged with hypertrophic agonists. On the other hand, SIRT3 transgenic mice had increased basal levels of both SOD2 and catalase, which remained unchanged when challenged with hypertrophic agonists, thus suggesting that SIRT3 elevates antioxidant defense mechanism of the heart. These observations were supported by more recent studies which showed a direct effect of SIRT3 in regulating the activity of antioxidants. It was shown that SIRT3 directly deacetylates and activates SOD2 and this mechanism was linked with the ability of SIRT3 to protect cells against radiation induced-stress and to suppress tumor progression. Kim et al. reported significantly increased ROS levels in SIRT3-deficient MEFs subjected to genotoxic and metabolic stress [55]. The same study also showed that increased levels of ROS in SIRT3-deficient MEFs partly contributed to the tumor permissive phenotype of cells. The tumor suppressive characteristic of SIRT3 observed in this study and anti-hypertrophic activity of this molecule reported by us suggest that SIRT3 might possess growth limiting activity in general to preserve cellular functions.

19.5 Conclusion

Given the intense interest in sirtuins and their roles in regulating cellular oxidative stress and longevity, it is surprising to see that relatively little efforts have been directed toward testing their effects in cardiac cell biology. Out of seven sirtuin isoforms only two SIRT1 and SIRT3 isoforms have been so far studied for their roles in regulating cardiac remodeling and hypertrophy (Fig. 19.1). With the discovery that SIRT1 binds to sarcolemma and regulates the activity of Akt and PDK1, it seems reasonable to believe that SIRT1 acts upstream of kinase signaling, regulating cell survival, and growth. As mentioned above, we as well as others have found that SIRT1 plays an essential role in regulating cardiomyocyte growth and survival and thereby acts as a pro-hypertrophic molecule [10]. On the other hand, SIRT3 has anti-growth activity as it has been shown to block cardiac hypertrophy as well as tumor cell growth. The primary site of action of SIRT3 is perhaps mitochondria as bulk of SIRT3 is localized in this organelle. But it should be noted that the shorter form of SIRT3 which lacks MLS also blocks cardiac hypertrophic response by targeting same targets like Foxo3 and Ku70 which are also targeted by SIRT1, yet SIRT1 does not show anti-hypertrophic activity as SIRT3 does [10, 31, 41]. These studies suggests that SIRT3, in addition to regulating mitochondrial function, might also have potential to exert a complex influence on the transcription of genes, which has not yet been examined. It is also

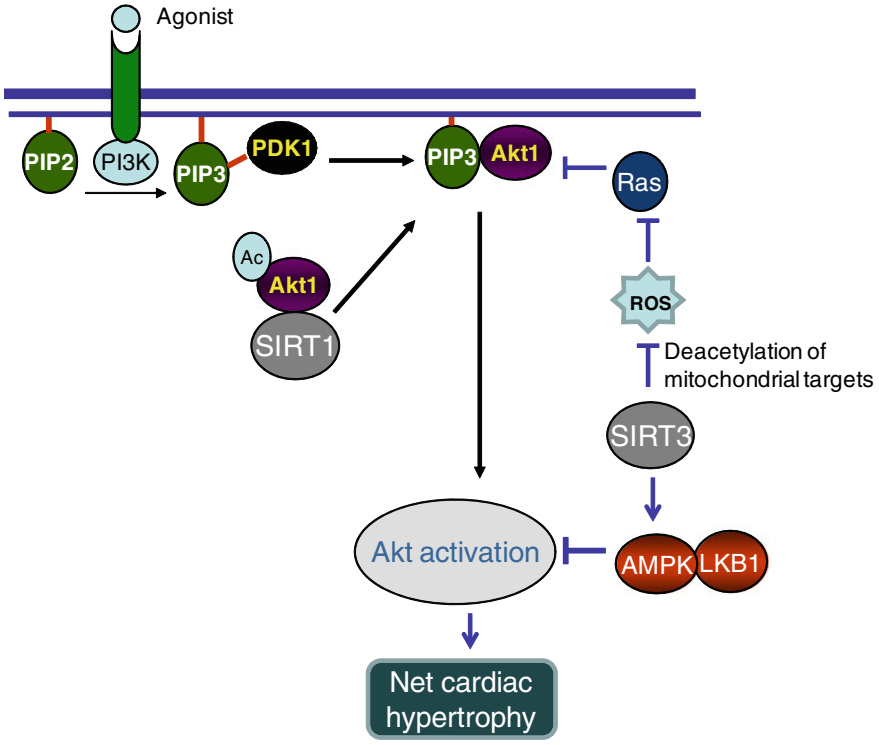

Fig. 19.1 Opposite effects of SIRT1 and SIRT3 to regulate cardiac hypertrophy. Under basal conditions Akt is acetylated leading to inhibition of Akt binding to *PIP*3 (phosphatidylinositol (3,4,5) triphosphate) and hence inactivation. After stimulation of cells with hypertrophic agonists, SIRT1 deacetylates Akt, enables it to bind to PIP3 which is generated from PIP2 by activation of PI3K. PIP3 binding promotes Akt localization to plasma membrane where it is activated by PDK1. On the other hand, SIRT3 activation promotes activity of antioxidants leading to reduced ROS levels and hence reduced activation of Ras and Ras-mediated Akt activation. SIRT3 can also activate AMPK which is known to block Akt activation. Thus, while SIRT1 promotes Akt1 activity, SIRT3 exerts opposite effects on this signaling molecule. At a given time cardiac hypertrophy could be an outcome of the activity of SIRT1 and SIRT3 both of which are activated during physical exercise, a known stimulus for the development of physiologic hypertrophy

not known how the activity of SIRT3 is regulated independently of gene transcription. By analyzing the SIRT3 sequences, several potential post-translational modification sites like glycosylation, phosphorylation, N-myristoylation, and amidation have been identified [34]. However, so far no post-translational modifications of SIRT3 have been reported. Future research in this area will help to identify modifications that regulate SIRT3 activity, which might enable us to develop agents that can modulate its activity. The pursuit of identifying SIRT3 activators is already underway and their roles is cardiac biology which may prove to be fruitful for future avenues of cardiac therapeutics.

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Chapter 20
Adaptation of Cardiac and Skeletal Muscle Mitochondria to Endurance Training: Implications for Cardiac Protection

Enn Seppet, Ehte Orlova, Teet Seene and Frank N. Gellerich

Abstract Endurance training is a widely practised physical exercise modality aimed at increasing the aerobic working capacity. This review discusses the current state-of-the-art in research on the mechanisms of the exercise-induced quantitative and qualitative alterations in mitochondrial biogenesis and intracellular energy transfer in cardiac and skeletal muscle cells. The data show that endurance training exerts a permissive effect on biogenesis of mitochondria through stimulating multiple pathways converging at activation of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). These pathways are mediated by stress hormones (glucocorticoids and catecholamines), p38 mitogen-activated protein kinase (MAPK), class III histone deacylases (SIRT1 and SIRT3), cyclic nucleotide regulatory binding protein (CREB), p53 tumor suppressor protein, and AMP-activated protein kinase (AMPK). As a result, oxidative capacity of cardiac and skeletal muscle cells increases to cope with enhanced ATP turnover. In parallel, exercise induces significant changes in intrinsic properties of mitochondria expressed as suppressed capacity to produce ROS and resistance to permeability transition and apoptotic

E. Seppet (✉) · E. Orlova
Faculty of Medicine, Department of Pathophysiology,
Institute of General and Molecular Pathology, University of Tartu, 19 Ravila Street,
50411 Tartu, Estonia
e-mail: enn.seppet@ut.ee

T. Seene
Faculty of Exercise and Sport Sciences, Laboratory of Functional Morphology,
University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia

F. N. Gellerich
Department for Behavioral Neurology, Leibniz Institute for Neurobiology
and Neurological Hospital, Magdeburg, Germany

F. N. Gellerich
Department of Neurology, Otto von Guericke University of Magdeburg, Magdeburg,
Germany

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signals. These effects of training enable to protect myocardium by attenuating the decay in cardiac function in conditions of ischemic heart disease, heart failure, diabetes, and obesity. The beneficial effects of endurance training disappear in conditions of application of excessive training volumes which results in overtraining syndrome (OTS) characterized by skeletal and cardiac muscle damage and suppression of oxidative energy metabolism. Therefore, establishing criteria for early detection of development of OTS to avoid associated harmful impact on organism is of ultimate importance.

Keywords Myocardium · Skeletal muscle · Endurance training · Overtraining · Mitochondria · Oxidative phosphorylation · Energy transfer

20.1 Introduction

Endurance training is a special type of chronic physical exercise aimed at increasing stamina and endurance through enhanced capacity of aerobic performance. It induces specific adaptive reactions in many systems including the cardiovascular and skeleto-muscular systems. Beneficial cardiovascular effects manifest as decreased heart rate, concurrent with increases in stroke volume, cardiac output, arteriovenous oxygen difference, and V_{O2} max [1]. In skeletal muscles, increased percentage of slow-twitch oxidative fibers, greater power per stroke, and resistance to fatigue is among the most characteristic adaptive changes [2–5]. Alterations in cellular energy metabolism play a central role in muscle adaptation to endurance training [6]. From a clinical perspective, endurance training is one of the most effective means for protecting body against pathological changes associated with disease conditions, such as ischemic disease, heart failure, metabolic syndrome, and hypertension. Regular exercising also postpones aging-related loss of skeletal muscle mass and increases the mean life span [7, 8]. However, prolonged and excessive (exhaustive) endurance training concurrent with insufficient recovery and performance decrements results in OTS. OTS is regarded as a complex of changes which do not support but restrict the exercise performance due to muscle damage, suppression of oxidative energy metabolism, and disruption of body's homeostasis [9–15].

Whereas the effects of endurance training on skeletal muscle have been extensively studied, much less is known about adaptation of cardiac muscle to that type of exercise, particularly in humans. Conceivably, the effects of endurance training on heart reflects the mutual interactions between the cardiovascular and musculoskeletal systems, with cardiac and skeletal muscles sharing mechanisms underlying the adaptive responses. This assumption lies behind the perception that myocytes from heart and skeletal muscle cover a continuum of phenotypic properties ranging from oxidative to glycolytic metabolic profiles, depending on fiber type and workload. Therefore, analysis of similarities and differences of cardiac and skeletal muscles in their response to exercise training might provide deeper insight into the nature of mechanisms contributing to increased physical working capacity [10, 11, 14].

The purpose of this review is to characterize the energy metabolism of heart and skeletal muscle in conditions of endurance training and overtraining. As a conceptual framework, it is considered that physical exercise represents a special type of stress governed by central and peripheral pathways with participation of a variety of stress hormones and metabolic effectors [16–18]. Another concept rests on the idea that in oxidative muscles (e.g. heart, m. soleus) energy metabolism is organized into functional complexes of mitochondria and ATPases termed as intracellular energy units (ICEUs) [19, 20]. Within the ICEUs, ATP produced in the reactions of oxidative phosphorylation (OXPHOS) is transferred to ATP-consuming enzymes by specialized energy transfer systems composed of creatine kinase (CK) and adenylate kinase (AK) isoforms [19, 20]. The function of ICEUs is strongly influenced by muscle contraction which, through eliciting structural changes in myocytes, modifies diffusion of adenine nucleotides through the mitochondrial outer membrane [21]. Based on the ICEUs-concept, effective adaptation of cardiac or oxidative skeletal muscle to exercise training is envisaged as activation of processes which enable to establish exact balance between the systems of energy utilization and supply in conditions of high ATP turnover. Accordingly, this review addresses the effects of endurance training and overtraining on mitochondria, ATPases, and the systems of energy transfer in relation to general adaptive processes in normal and diseased conditions.

20.2 Hormonal and Vegetative Mechanisms of Regulation of Striated Muscle Adaptation to Exercise

Physical endurance exercise represents a specific stress factor challenging the body to reach a new and optimum state of internal environment of the organism—a homeostasis—capable to support the higher level of body's endurance capacity [17]. Homeostasis is largely controlled by the hypothalamic–pituitary–adrenal (HPA) axis, which integrates metabolic, neural, and hormonal signals triggered by physical exercise [22, 23]. Glucocorticoids, the end-products of the HPA axis, favor endurance exercise by making the energy stores accessible for the body [24–26] and upregulating the OXPHOS through the genomic and nongenomic pathways [18]. The genomic effects are based on binding of the glycocorticoid-receptor complexes with the hormone response elements of genes regulating the nuclear respiratory factors and PGC-1α (see also below). The nongenomic effects are realized through increased intracellular Ca^{2+} concentration, followed by activation of calmoduline kinase IV that also stimulates the expression of PGC-1α gene [18]. As a consequence, a state of cellular bioenergetics, characterized by increased capacity of aerobic ATP production to meet enhanced energy requirements is achieved. To support this state, the HPA axis tightly co-operates with

autonomic nervous system (ANS), which exerts a direct control over heartbeat frequency and contractility of myocardium and skeletal muscles through the actions of catecholamines and acetylcholine [27–31]. Among many features, decreased heart rate at rest and during submaximal exercise represents a typical ANS-mediated adaptive response of body to regular endurance training [32–37]. Catecholamines can also potentiate biogenesis of mitochondria, through the mechanisms mediated by protein kinase A, CREB, and PGC-1α [16].

In adaptive mechanisms, the regulatory role of peripheral "tissue hormones", nitric oxide (NO) and adenosine, should be also considered. Exercise training promotes the expression and activity of endothelial NO synthase (eNOS) through increasing the levels of bradykinin, acetylcholine, and shear stress, all leading to increased NO levels in the vascular smooth muscle cells, thereby dilating arteries and increasing blood supply to muscles [27, 38, 39]. NO is also produced in cardiomyocytes and skeletal muscle cells by eNOS and neuronal NOS (nNOS) [40, 41]. These enzymes are activated by augmented Ca^{2+} influx into the myocytes during sustained stretch and increased contractility [40–43]. Once generated, NO not only promotes muscle relaxation but also stimulates mitochondrial biogenesis [8, 16, 44, 45] and affects the function of mitochondria [41]. It transiently reduces the activity of mitochondria by competing with O_2 on cytochrome oxidase [46] and combines with mitochondrial superoxide giving rise to toxic peroxynitrite ($ONOO^-$) causing nitrosylation of numerous mitochondrial enzymes thereby inducing irreversible damage of the respiratory chain and promoting mitochondrial permeability transition [38, 41, 47]. NO controls also the mechanical properties of the myocytes by inhibiting calpain-induced proteolysis, thus stabilizing the cytoskeleton [48]. By exerting an anti-adrenergic action, it favors rapid cardiac recovery from a single exercise bout [49]. Furthermore, NO inhibits the apoptosis [50], increases the expression of heat shock protein 70 (HSP70) [51, 52] and activates the K_{ATP} channels [27, 53–57], these processes providing protection of muscle cells against impairment and death (see below).

Adenosine, a purine nucleoside catabolite of ATP, is released from the myocytes during increased muscle activity. Adenosine augments the endurance capacity through a variety of means. As a potent vasodilator, it increases the blood flow in exercising muscles. In parallel, it stimulates glycogenolysis and erythropoietin production, which potentiates the insulin-mediated glucose uptake into muscle cells, and attenuates the metabolic and functional effects of β-adrenergic receptor-mediated pathways [27, 58–60], The adenosine receptors (A1, A2A, A2B, and A3) expressed on multiple types of cells including myocytes, initiate a variety of pathways mediated by protein kinase C, MAPK, and mitochondrial K_{ATP} channels, all of which help to protect the cardiac and skeletal muscle cells from death under conditions of intracellular accumulation of excess amounts of Ca^{2+} and ROS in pathological conditions [59, 60] (see below).

20.3 Bioenergetic Aspects of Adaptation of Muscle to Endurance Training

20.3.1 Relationships Between Muscle Growth, ATP Turnover, and Oxidative Capacity in Conditions of Endurance Training

It is generally believed that myocardium responds to exercise training by increasing its mass. However, rats trained voluntarily by running in free wheel do not exhibit cardiac hypertropy [61], whereas forced treadmill training is usually accompanied by increased heart to body weight ratio [62–64]. Clearly, there exists a threshold of intensity of work for induction of cardiac growth. An increase in cardiac mass under endurance training is regarded as physiological hypertrophy, in contrast to pathological hypertrophy occurring in disease conditions [65–68]. Physiological hypertrophy is characterized by normal or enhanced pumping function, absence of fibrosis, alterations in expression of genes characteristic of normal adult heart, proportional chamber enlargement, and increased capacity for using either carbohydrates or fatty acids with equal efficiency that enables cardiomyocytes to flexibly change the energy source in dependence on workload [69, 70]. These alterations, underlying a positive impact on body's working capacity, are largely mediated by activation of IGF-1 dependent signaling pathways promoting cardiac growth through downstream stimulation of p110α isoform of phosphatidylinositol-3 kinase and protein kinase B/Akt [65].

In contrast to cardiac muscle, hypertrophy of skeletal muscle is rarely observed in conditions of endurance training. This distinction stems from muscle fiber type specific nature of adaptation. (1) Skeletal muscles respond to exercise training by switching the myofibre composition toward increased proportion of type I oxidative slow-twitch fibers at the expense of proportion of type II glycolytic fast-twitch fibers [71, 72]. This change adds a little if any to overall muscle size as oxidative fibers are thinner than glycolytic ones [6]. (2) The proteasome-, lysosome- and Ca^{2+}-mediated protein degradation occurs at higher rates in oxidative than glycolytic fibers [70]. (3) The mechanisms stimulating either oxidative mechanisms or hypertrophy seem to exclude each other [6]. For example, increased mitochondrial biogenesis via AMPK may be accompanied by suppression of the myofibrillar protein synthesis through pathways mediated by MAPK and nuclear factor kappa B [6]. Further support for that assumption comes from the finding that exercise, though increasing oxidative metabolism, yet suppresses myofibre growth in myostatin knockout mice [73]. Taken together, these facts imply that cardiac and skeletal muscles possess distinct mechanisms for regulation of the balance between the capacities of oxidative potential and tissue growth in response to exercise training.

Interestingly, the muscle type specific differences in adaptation to endurance training can be revealed also at the level of ATP consumption. In cardiac muscle

the endurance training results in enhanced myosin ATPase activity along with increased contractility [74–76]. This change rests upon myosin isoenzyme shift toward increased fast V1 (α) isoform [75, 77, 78]. Also, alterations in regulation of myosin ATPase can be involved, as exercise results in increased myofilament sensitivity to Ca^{2+} [79], probably due to augmented expression of atrial myosin light chain-1 protein [80] that increases the capacity to consume ATP by myofibrils. Endurance training also promotes the expression of SR Ca^{2+}ATPase (SERCA2) together with increased Ca^{2+} transport into SR [81–83]. In addition, Ca^{2+} removal through transsarcolemmal route is facilitated due to activation of Ca^{2+}–ATPase localized in sarcolemma [76]. It appears, thus, that exercise increases the overall capacity of ATP consumption in cardiac cells. This contrasts with skeletal muscles, which respond to exercise training by shifting the myofibre profile toward increased proportion of type I slow twitch fibers characterized with markedly lower ATPase activity compared to type II fibers expressing the α-myosin [84, 85]. However, this change is beneficial as it increases the economy of ATP utilization for cross-bridge cycling [86, 87]. Also, endurance training stimulates the Na^{+}–K^{+}–ATPase activity in skeletal muscle [88] but not in myocardium [76].

20.3.2 Increased Mitochondrial Biogenesis and Underlying Mechanisms in Trained Muscle Cells

It is well documented that moderate endurance training stimulates mitochondrial biogenesis and improves the functional capacity of mitochondria in producing ATP in skeletal muscles [89–101]. Upregulated mitochondrial biogenesis manifests as increases in mitochondrial content per gram of tissue [90], mitochondrial volume relative to fiber area [102], and tissue activity of mitochondrial enzymes [92, 103]. These changes are considered to occur simultaneously in oxidative and glycolytic muscle fibers [90, 91, 102, 104]. Increased mitochondrial contribution to energy metabolism is associated with transition from carbohydrate utilization to preferably fat utilization, this change improving the endurance capacity [104].

The data describing responses of mitochondria to endurance exercise in cardiac cells are ambiguous. The 6–9 weeks running or swimming programs have resulted in increased tissue activities of mitochondrial enzymes suggesting enhanced oxidative capacity in heart of mice [105], dog [106] and rat [107]. However, endurance exercise is also known not to induce alterations in mitochondrial enzymes and yield in rat cardiac muscle [64, 67, 108–110]. According to Terblanche et al. [111] endurance training even decreased the oxidation rate of palmitoylcarnitine/malate without changes in pyruvate, 2-oxoglutarate, and succinate oxidation. The controversies regard also to morphometric studies. Whereas Bozner and Meessen [112] have reported increased mitochondria-to-myofibril ratio, others have found no change in this parameter [113]. Kayar et al. [68] have

shown that endurance training, though resulting in significant hypertrophy and increased oxidative capacity of rat heart, did not increase the volume density of mitochondria. Similarly, Paniagua et al. [114] demonstrated unaltered mitochondrial volume despite increased weight and size of the heart in rats subjected to swimming training. Thus, adaptative enhancement of total mitochondrial volume simply resulted from increased cardiac mass. However, this assumption is negated by the study assessing the effect of voluntary exercise in mouse which revealed that even though the heart weight did not change, the number of mitochondria per cardiomyocyte and total volume of mitochondria markedly increased [115]. The reasons for conflicting data on mitochondrial biogenesis are not clear. Variations in training intensity and volume (e.g. voluntary versus forced exercise) might lead to discordant results [61]. In addition, the age and sex differences could play a role. The older animals are less sensitive than young ones to stimulatory effects of exercise on mitochondria but more susceptible to unfavorable effects of higher workload [105, 116]. Concerning the sex differences, the female rodents exhibit greater exercise-induced cardiac hypertrophy than their male counterparts do [117]. Although confounding, most data seem to support a view that stimulation of mitochondrial biogenesis by endurance training is a common phenomenon in cardiac and skeletal muscle cells.

Notably, mitochondrial biogenesis may be stimulated prior to a detectable muscle growth, e.g., already after a single 90–120 min exercise bout [118]. On the other hand, as mentioned above, training-induced changes in oxidative capacity and size of the striated muscle fibers tend to mutually exclude each other through altered balance between the biosynthesis of contractile proteins and mitochondria [6]. What these data do suggest is that the mechanisms of mitochondrial biogenesis differ from those that underlie muscle hypertrophy. Indeed, as described below, the myocytes possess specific and powerful mechanisms capable to preferentially promote the biosynthesis of mitochondria in response to increased workload.

Most if not all of these mechanisms converge at PGC-1α, a "master regulator" of oxidative metabolism and mitochondrial content in muscle cells. While activated, PGC-1α binds to DNA-binding transcription factors, such as the nuclear respiratory factors NRF-1 and NFR-2, and trans-activates genes involved in the control over electron transport chain, mitochondrial protein import, and transcription factors Tfam, TFB1M, and TFB2M [119, 120]. Endurance training increases the activity and expression of PGC-1α in muscle cells through multiple mechanisms. (1) As mentioned above, stress hormones—glucocorticoids—activate PGC-1α through genomic and nongenomic effects [18]. (2) Exercise activates the p38 MAPK [121, 122] which phosphorylates the PGC-1α repressor protein $p160^{MBP}$ that relieves the inhibitory effect of repressor on PGC-1α, thereby permitting PGC-1α to interact with target proteins [123, 124]. p38 MAPK also increases the transcriptional activity of PGC-1α through phosphorylation [125]. (3) AMP produced in exercising muscle cells stimulates AMPK that, in turn, upregulates the expression of PGC-1α [126, 127]. (4) PGC-1α can be activated by reversible deacetylation carried out by SIRT1 (reviewed by [128]). The same

effect can be achieved or amplified through activation of AMPK which promotes the activity of SIRT1 followed by stimulation of PGC-1α [128]. SIRT1 can also upregulate the expression of PGC-1α through formation of the SIRT1–MyoD–PGC-1α complex on PGC-1α promoter [129]. Exercise-induced upregulation of SIRT1 occurs rapidly, as its mRNA level increases together with mRNAs for PGC-1α, cytochrome c, and citrate synthase in m. vastus lateralis already after a 3 h intensive cycling [130]. Besides SIRT1, SIRT3 can also activate PGC-1α [131]. A relevant mechanism appears to be mediated by stimulation of AMPK, as SIRT2 activates the liver kinase B1, a serine-threonine kinase that impels AMPK [131]. Most interestingly, it has been shown recently that in rat heart and skeletal muscle cells SIRT3 is localized exclusively within mitochondria and that the muscle SIRT3 protein content increases in parallel to elevations in citrate synthase activity and PGC-1α content among distinct muscle types (white ≪ red ≪ heart) [132, 133]. Chronic electrical stimulation (7 days) of rat muscle resulted in increases in SIRT3 protein and PGC-1α proteins in an AMPK-independent manner [132]. These data are in line with observations that exercise training increases SIRT3 and mitochondrial content in rat skeletal muscle [133, 134]. Given that SIRT3 activates also mitochondrial enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, glutamate dehydrogenase, NDUFA9 subunit of complex I of the respiratory chain, and acetyl-coenzyme A synthase, the targeted activation of SIRT3 may provide a means for shifting metabolism toward use of fatty acids thereby protecting failing heart [131]. (5) Exercise stimulates PGC-1α through activation of CREB, in association with upregulation of mitochondrial proteins in rat heart and muscle cells [63, 135]. As shown above, the CREB-mediated mechanism is specifically targeted by catecholamines, another class of stress hormones besides glucocorticoids.

Increasing evidence suggests also involvement of p53, a tumor suppressor protein, in mitochondrial biogenesis. p53 increases the expression of synthesis of cytochrome c oxidase 2 (SCO2), an important protein for assembling the cytochrome c oxidase complex and thereby controlling the rate of mitochondrial respiration [136]. p53 can translocate into mitochondria for interaction with and activation of the mitochondrial DNA polymerase γ [137]. p53 also interacts with Tfam [138, 139]. The importance of p53 in regulation of mitochondrial biogenesis was strictly confirmed in studies on p53 ablated mice [139, 140]. Saleem et al. [140] showed that these mice exhibited diminished mitochondrial content and lowered PGC-1α protein levels in gastrocnemius muscle as compared to wild-type controls. In association, reductions in the rates of state 3 respiration of mitochondria, working capacity during voluntary wheel running, and force generation under acute tetanic electrical stimulation were observed. Surprisingly, however, the running training did stimulate the cytochrome oxidase activity similarly in wild-type and p53 knockout muscles. Thus, lack of p53 did not limit the ability of mice to adapt to exercise training by stimulation of mitochondrial biogenesis. This finding was interpreted as factors other than p53 could induce mitochondrial biogenesis. Most probably, the alternative mechanisms realize through activation of p38 MAPK and AMPK, as both of these kinases were forced to phosphorylate

during in situ electrical stimulation of wild-type muscles. The study by Park et al. [139] confirms the results by Saleem et al. [140] in that depletion of p53 significantly attenuated the aerobic exercise capacity together with suppressed state 3 oxygen consumption and weaker succinate dehydrogenase staining in mitochondria. Similarly to observation by Saleem et al. [140], treadmill training did not increase the mitochondrial respiratory capacity in skeletal muscle of p53 knockout mice [139]. Interestingly, in contrast to skeletal muscles, the functional parameters of heart were similar in p53 deficient and wild-type animals which points to a muscle type specificity of the p53 effects [139]. The study by Qi et al. [141] sheds more light on p53-dependent mitochondrial biogenesis in the myocardium. They showed that expression of p53 and its translocation into mitochondria increased with aging, in association with diminished expression of COX subunits and PGC-1α. Endurance training (4 weeks treadmill) decreased the expression of p53 in cardiac mitochondria, but enhanced the p53 binding to SCO2 promoter along with increases in mtDNA content, expression of COX subunits and assembly proteins, and markers of mitochondrial biogenesis. As these changes occurred to larger extent in the hearts of older animals compared to young ones, it was concluded that age-related decay in COX can be postponed by exercise [141].

20.3.3 Effects of Endurance Training on Regulation of Mitochondrial Functions in Muscle Cells

20.3.3.1 Influence of Endurance Training on Intrinsic Capacity of Mitochondria to Synthesize ATP

It is possible that exercise training not only increases the content of mitochondria in the muscle cells, but also enhances the intrinsic capacity of mitochondria to convert the energy obtained by oxidation of substrates (carbohydrates, free fatty acids, amino acids, and lactate) into ATP. This effect can be detected as enhancement in maximum state 3 respiration over nonphosphorylating respiration normalized to protein content or citrate synthase activity in mitochondrial fraction isolated from the muscle cells. The assumption that mitochondrial intrinsic capacity to synthesize ATP increases under endurance training is supported by some studies which showed that 14–16 weeks endurance treadmill training of rats significantly increased the state 3 rates normalized to protein content of mitochondria isolated from the hearts. In the same experiments, the respiratory adenylate control ratio or the ADP/O ratio did not change, hence coupling of oxidation to phosphorylation was unaffected [62, 142]. However, in many studies no change in the rate of state 3 respiration, OXPHOS coupling, and activities of the respiratory chain complexes in rat cardiac mitochondria following the endurance treadmill training program was revealed [63, 64, 141, 143–145]. In study by Bo

et al. [145], increased ATPase synthase activity, normalized to mitochondrial protein, was the only change induced by a 6 weeks endurance training.

As regard the mitochondria in skeletal muscles, a pioneering study by John Holloszy showed that strenuous program of 12 weeks treadmill running, which increased the content of mitochondria in rat gastrocnemius muscle, augmented the intrinsic capacity of ADP-stimulated respiration in mitochondria as well [90]. Enhanced oxygen consumption together with elevated enzymes in mitochondria isolated from rat skeletal muscles was also observed after 6 weeks endurance treadmill training [146]. However, this effect was substrate specific, i.e., not observed in subsarcolemmal mitochondria with palmitoylcarnitine/malate or 2-oxoglutarate but detected with pyruvate/malate [146]. According to Venditti et al. [95], a 10 weeks swimming training of rats, even though stimulating an increase in mitochondrial content, did not affect the intrinsic state 3 respiration in m. gastrocnemius. A study on human muscle (vastus lateralis) has revealed increased rate of ATP synthesis normalized to mitochondrial mass already after 10-day cycling exercise program [147]. Much longer (6 weeks) endurance training of healthy subjects also increased the ADP-dependent respiration in mitochondria isolated from quadriceps muscle. However, when the latter data were normalized to mitochondrial citrate synthase content, the state 3 respiration remained unaltered, but the state 4 respiration decreased [148]. Controversies between the data registered in different laboratories may stem from artifacts due to impairment of mitochondrial membranes during isolation. Assessment of mitochondrial function in permeabilized muscle fibers enables to overcome these problems [19, 149]. Application of permeabilized fiber technique by Burelle and Hochachka [150] revealed that a 4 weeks endurance treadmill training of rats elicited no change in maximum ADP-stimulated respiration normalized to fiber weight in red part of m. gastrocnemius, but an increase in this parameter was observed in m. soleus. The latter finding is somewhat unexpected as it implies that mitochondrial biogenesis can be upregulated in a muscle fiber type-dependent manner, this challenging the original hypothesis by Baldwin et al. [108] that respiratory capacity increases in all types of muscle fibers in response to training (see above). Another study, applying the endurance training program in humans for 12 weeks, showed that training exerted no effect on ADP-dependent respiration normalized for mtDNA copy number in permeabilized fibers [151]. Altogether, these data show that, whereas there exists rather strong evidence indicating that in skeletal muscle cells increased oxidative capacity in response to long-term endurance training rests on enhanced cellular content of mitochondria. In contrast, myocardium appears to adapt to exercise by increased tissue mass, with unaltered density of mitochondria. At same time, due to discordant data available, it is still unclear whether endurance training improves the intrinsic capacity of mitochondria to generate ATP. As discussed below, resolving of that problem requires deeper insights into regulation of mitochondrial function in vitro and in vivo.

20.3.3.2 Influence of Endurance Training on Mechanisms of Energy Transfer and Feedback Between Mitochondria and ATPases

Since endurance training affects both mitochondrial mass and ATPases, an important issue arises regarding the nature and consequences of alterations in the mechanisms ensuring interaction between mitochondria and ATPases. The classic study by Chance and Williams [152], which demonstrated the rate dependence of OXPHOS on ADP concentration, predicted that the cytosolic ADP concentration must increase in order to regulate OXPHOS in muscle cells in vivo. However, the numerous experimental data have questioned the efficiency of cytosolic ADP in respiratory control. One set of studies has revealed that fluctuations in cytosolic free ADP concentrations are not common for all contracting muscles; while they are recorded in fast-twitch glycolytic muscle fibers, in slow-twitch fibers like heart the ADP concentration in cytosol stays rather constant, despite large changes in respiration associated with altered contractile activity [153]. Other studies have shown that in intact cells diffusion of ADP to ANT is strongly hindered that limits the ability of cytosolic ADP to regulate OXPHOS. It has been considered that the density of voltage dependent anion channels (VDAC) in the mitochondrial outer membrane is too low for permitting effective transmembrane exchange of adenine nucleotides [154]. Furthermore, oncotic pressure elicited by cytosolic proteins strongly reduces the permeability of VDAC for adenine nucleotides that results in establishment of transmembrane concentration gradients of ADP [155, 156]. It has been also demonstrated that in oxidative muscle cells diffusion of cytosolic ADP into mitochondria is limited by specific interaction of cytoskeletal protein, tubulin, with VDAC in the outer membrane, whereas such interaction is lacking in white glycolytic muscles [157, 158]. As a result, in permeabilized muscle cells the apparent Km for ADP in regulation of respiration varies from 8 μM in glycolytic to 350 μM in oxidative muscle cells [149]. There exists a strong evidence in favor of hypothesis that limitations in ADP diffusion in oxidative muscle cells can be overcome by coupling of mitochondrial kinases (CK and AK) to OXPHOS in the intermembrane space. In other words, in oxidative muscles OXPHOS is regulated by changes in local ADP generated by mitochondrial CK or AK near ANT, whereas in glycolytic muscles it is mainly controlled by fluctuations of cytosolic ADP [149, 153, 155–158].

Regarding the effects of exercise on ADP-dependent regulation of OXPHOS, the studies hitherto performed have produced discordant results. Dudley et al. [92], having trained the rats during 8 weeks, observed increased mitochondrial content in oxidative capacity of red gastrocnemius. Electrical stimulation of hindlimb muscles resulted in a 30-fold increase of oxygen consumption. Interestingly, this change was associated with much smaller increases in calculated cytosolic free ADP content in muscles of trained rats than of untrained rats. It was concluded that "variations in mitochondrial content will result in differences in the sensitivity of respiratory control" with ADP [92]. The following investigations on permeabilized muscle fibers have revisited the effect of exercise on ADP-dependent regulation of OXPHOS. According to Burelle and Hochachka [150], exercise training exerted no

effect on apparent affinity of mitochondria to ADP in regulation of OXPHOS in rat soleus muscle, but markedly increased it in red portion of gastrocnemius. In contrast, in human muscle the exercise training has been shown to result in decreased affinity to ADP (i.e.increased the Km^{ADP}) in regulation of OXPHOS [159, 160]. At present, it is difficult to explain this controversy. The observations made by Walsh et al. [159] and Zoll et al. [160] fairly conform to a concept that training leads to increased proportion of slow-twitch fibers in skeletal muscles [84, 85]. Along with other studies they also suggest that endurance training leads to changes in CK-mediated energy transfer systems. Indeed, it has been shown in canine heart that exercise that augmented mitochondrial oxidative capacity also increased the total myocardial CK activity and MB-CK isoform content in canine left ventricular myocardium [161]. In human skeletal muscle cells (m. gastrocnemius), enhanced activities of mitochondrial CK (mi-CK) and MB-CK isoforms without changes in total CK activity have been observed following a long-distance running training [162, 163]. Interestingly, in m. gastrocnemius biopsies taken from male marathon runners, the increases in MB-CK percentages of total CK activity strongly correlated with the increases in percentage of the slow-twitch muscle fibers [162]. Enhanced proportion of MB-CK is considered to favor ATP regeneration near ATPases due to higher affinity of that isoform toward ADP compared to MM-CK [164]. On the other hand, the training-induced increase in mi-CK facilitates conversion of ATP generated in the reactions of OXPHOS into PCr, a main molecule for energy transfer, due to functional coupling between ANT and mi-CK. In permeabilized cells, this type of coupling can be easily revealed from [ADP] versus respiration rate relationships measured in the absence and presence of creatine, where creatine-dependent decrease in apparent Km^{ADP} is indicative of coupling. Studies by Zoll et al. [160] showed that sedentary subjects lacked the coupling between ANT and mi-CK in m. vastus lateralis, but it became evident and strengthened along with increasing levels of physical activity, with maximum in well-trained athletic subjects. Collectively, these studies imply that exercise training, which stimulates mitochondrial biogenesis, also upregulates the capacity of the CK-mediated system of energy transfer, thereby ensuring effective feedback between mitochondria and ATPases. As a consequence, a new steady-state characterized by increased tissue PCr/ATP ratio but diminished Pi, ADP, and AMP concentrations in the cytoplasm is established [157, 165] which explains the earlier results by Dudley et al. [92] discussed above. The interesting questions are whether the changes in CK-mediated systems are strictly confined to slow-twitch muscle fibers, in this case merely reflecting increased proportion of these fibers in bulk muscle, or can training induce CK-system in glycolytic, especially, and oxidative glycolytic muscle fibers. Further studies are required to address these issues.

20.3.3.3 Endurance Training Suppresses the Mitochondrial ROS Production

Mitochondrial functions are not only regulated by ADP, but also by energization of the inner membrane which, in turn, depends on availability of substrates. As recently shown, the cytosolic formation of pyruvate, which is metabolically connected with the activity of malate-aspartate shuttle, is tightly controlled by cytosolic Ca^{2+} concentration (Ca^{2+}_{cyt}) at the level of glutamate/aspartate transporter, aralar. The activity of such "gas-pedal"—called mechanism increases in response to enhanced Ca^{2+}_{cyt} associated with enhanced-ATP turnover, and, vice versa, diminishes with decreasing Ca^{2+}_{cyt} in resting state [166–168]. Thus, through regulating the substrate supply, this mechanism exactly adjusts the mitochondrial redox pressure and $\Delta\psi$ to the energetic requirements of the cell. It is tempting to hypothesize that discordant data regarding the training-dependent alterations on intrinsic capacity of mitochondria to synthesize ATP discussed above may stem from variable state of activation of these systems that are sensitive to Ca^{2+}_{cyt}, including the "gas pedal". Further studies are required to test this idea, however. We also assume that one main advantage of the "gas pedal" is to diminish the unfavorable ROS formation [167, 168]. Nevertheless, it is well-known that a small fraction of oxygen (0.15 %) consumed by mitochondria is reduced into superoxide radical followed by dismutation to H_2O_2 intramitochondrially catalyzed by MnSOD [169, 170]. ROS can be produced on seven separate sites within the mitochondria [170] and it can induce damage of the respiratory chain [101]. On the other hand, it is known that ROS also plays an important role in regulation of physiological functions, such as induction of nutrient sensing within the hypothalamus [171], prevention of TNF-α-induced apoptosis through enhancing the NF-kB-mediated expression of anti-apoptotic proteins [172], and muscle differentiation [173]. These physiological tasks of ROS should not be neglected in attempts to pharmacologically limit the ROS formation.

It is well appreciated that endurance training attenuates the mitochondrial intrinsic capacity to produce ROS in cardiac and skeletal muscles [7, 8, 142]. This has been demonstrated in mitochondria isolated from rat heart after lifelong voluntary wheel running [144] or prolonged forced treadmill training [143, 145]. Exercise training blunted the mitochondrial H_2O_2 generation in rat m. gastrocnemius as well [95]. Suppressed ROS generation is likely beneficial as it is associated with reduced levels of oxidative stress markers [174] and prevents the age-associated decrease of antioxidant enzyme activities in mitochondrial membranes from mice heart [7]. There exists some evidence suggesting that exercise-induced mechanisms limiting mitochondrial ROS production are largely mediated through their effect on mitochondrial membrane potential, $\Delta\psi$, and redox state controlled by MnSOD. (1) It is known that the ROS formation depends on the $\Delta\psi$ [175, 176]. In state 3 conditions, when the transmembrane proton gradient is mainly consumed for ATP synthesis, the decrease in $\Delta\psi$ is associated with reduction in ROS generation. Vice versa, in state 4 conditions when ADP is

rephosphorylated, the $\Delta\psi$ regains its maximum value that also restores higher rates of ROS production. (2) It has been shown that a 14 weeks endurance treadmill training significantly increased the proportion of GDP-dependent inhibition of state 4 respiration, suggesting that a mild decrease in $\Delta\Psi$ through an UCP-related uncoupling mechanism could be induced [62]. In support of that suggestion, Bo et al. [145] has observed diminished $\Delta\Psi$ in cardiac mitochondria of endurance trained rats. These authors also demonstrated that pre-training of endurance type significantly modulated the mitochondrial ROS production in response to a single bout of treadmill exercise lasting for 150 min. In time scale, activation of expression of UCP2 was the earliest change, followed by dropping down of $\Delta\Psi$ and ROS levels, these changes suggesting that increased UCP2 controlled the ROS production through suppressed $\Delta\Psi$. Importantly, mitochondria from trained hearts exhibited attenuated upregulation of UCP2 in association with increased P/O ratio and diminished state 4 respiration; hence, in a course of 150 min exercise bout, the intrinsic efficiency of OXPHOS increased compared to untrained counterparts. (3) Both, a single exercise bout or chronical endurance training limit ROS production through upregulation of antioxidant enzymes, such as MnSOD, catalase, and glutathione peroxidase in cardiac and skeletal muscles [145, 177, 178]. The expression of MnSOD and catalase is upregulated by the members of the family of forkhead transcription factors (Foxo3), probably under SIRT3-dependent control [131]. It is expected that owing to intramitochondrial location MnSOD is of key importance in controlling the ROS production in mitochondria. Yet, it should be noted that the actual role of MnSOD in exercise-induced adaptation is far from being clear, because many studies have not revealed changes in MnSOD implemented by exercise training [178]. Therefore, the mechanisms of control over mitochondrial capacity to produce ROS still deserve further studies.

20.4 Exercise-Induced Cardioprotection

Exercise training has been proven to protect myocardium against injuries associated with ischemic cardiac disease, heart failure, diabetes, obesity, and use of cardiotoxic drugs for treatment of cancer [178–184]. As already discussed, protective effect of training can be related to suppression of mitochondrial ROS production. On the other hand, mitochondria isolated from hearts of trained rats exhibit decreased sensitivity to induction of PTP by Ca^{2+} or ROS [64, 182, 185]. As the opening of PTP is a key factor for initiating and promoting apoptosis, its suppression should attenuate or delay the apoptotic death of myocytes. Indeed, Siu et al. have shown that 8 weeks treadmill training of rats markedly increased the expression of anti-apoptotic proteins (Bcl-2) but decreased the pro-apoptotic proteins (Bax) in myocardium and m. soleus as compared to muscles of untrained animals [177]. Increased Bcl-2/Bax ratio in trained rat myocardium has been observed also by others [186]. Lee et al. [180] have demonstrated that 3-month treadmill training prevented the activation of Fas- and mitochondria-dependent apoptotic pathways in obese Zucker rats. In addition to

acquisition of anti-apoptotic properties the mitochondria also exhibit improved OXPHOS. For example, endurance treadmill training for 14 weeks, which increased the state 3 respiration in isolated mitochondria, also enhanced their resistance to in vitro anoxia-reoxygenation injury, as the trained group of mitochondria showed less attenuation in respiratory adenylate control and ADP/O ratios compared to untrained one [62]. Low-intensity treadmill training of spontaneously hypertensive rats suffering from cardiac failure resulted in increased content of cytochrome oxidase and cardiolipin in cardiac mitochondria [183]. Regular exercise also prevented the oxidative stress-induced oxidation and calpain-mediated degradation of L-type Ca^{2+} channels, phospholamban, and SR Ca^{2+} ATPase in conditions of ischemia/reperfusion injury [187]. These multiple beneficial effects of exercise were ascribed to upregulation of antioxidant and repair capacities, due to increased mitochondrial content of MnSOD and mitochondrial HSP60 and cytosolic HSP70 [177–179, 185, 187]. In conclusion, current evidence strongly suggests that through stimulating the mitochondrial adaptation endurance training induces a cardioprotective phenotype characterized by attenuation of pathological remodeling and postponement of functional decay of diseased myocardium [181–183].

20.5 Effects of Overtraining on Muscle Energy Metabolism

As already stated, prolonged and exhaustive endurance training associated with insufficient recovery leads to the OTS, characterized by chronic fatigue, decreased exercise performance, and disintegration of the muscle structure [9–12, 14, 188–192]. Muscle atrophy due to a prevalence of catabolism over synthesis of contractile proteins, is one of the typical signs of OTS in skeletal muscles [191, 193]. Activation of apoptosis may be partly responsible for induction of protein degradation and loss of muscle nuclei [194]. Our recent study showed that although the overtrained rats had lost their weight, their heart weights remained unchanged, giving rise to increased heart-to-body weight ratios. Thus, in contrast to skeletal muscles the anabolic responses prevailed in cardiac muscle even under conditions of overtraining [193].

Exhaustive endurance exercise impairs the bioenergetic systems in the muscle cells [195]. Impairment of structure and function of mitochondria is central to mechanisms of OTS. Even a single bout of rigorous exercise can cause mitochondrial swelling and cristae disruption in cardiomyocytes [196]. Long-term (10–15 months) enforced endurance training promotes degeneration of cardiac mitochondria evidenced as formation of unusual giant mitochondria characterized by disintegrated cristae and dense inclusions within the matrix [197]. Due to swelling of mitochondria their volume density increases in cardiac cells [198]. The molecular mechanisms underlying the mitochondrial alterations are poorly understood. It is possible that exhaustive exercise is associated with altered balance between fusion and fission of mitochondria, as increased duration of swimming load results not only in increased mitochondrial mass, but also in appearance

of the novel phenotype of mitochondria characterized by numerous invaginations suggesting the replication phenomenon [118]. Moreover, acute bout of treadmill exercise is known to suppress expression of mitofusins 1 and 2 but stimulate expression of fission protein Fis1 genes in rat hindlimb muscles. In parallel, the mitochondria functionally differed from their control (resting) counterparts by exhibiting decreased RCI and increased state 4 respiration, these changes emerging immediately after exercise and 24 h later [199]. Our study showed that overtraining resulted in suppression of OXPHOS and decreased respiratory control by ADP. Since these changes were accompanied by reduced tissue content of cytochrome c, its deficit could limit the mitochondrial state 3 respiration [193]. Based on a stress-linked concept of exercise, overtraining can be envisaged as a chronic distress that impairs mitochondrial capacities to produce ATP, increases their ROS production to the levels exerting harmful effect on mitochondria and other cellular structures, and suppresses mitochondrial biogenesis [16].

There exists some evidence that excessively vigorous endurance exercise or regular training affects the CK-mediated system of energy transfer. In athlete's blood, increased levels of MB-CK have been detected, which suggest injury of skeletal muscle and its adaptation to continuous cycles of degeneration and regeneration [200–208]. Relatively little is known about alterations in CK system in cardiomyocytes under exhaustive training. Whereas Chen et al. [209] have reported that swimming exercise resulted in release of myocardial BM-CK into circulation due to myocardial injury in rats, others have found no change in MB-CK in the myocardium of dogs undergoing exercise training [210]. We have observed marked structural impairment in overtrained rat heart cells, but did not find any change in total CK activity or its isoenzyme profile [193] that conforms to earlier data by [210]. Resistance of myocardial CK to overtraining was further supported by us in experimental settings which addressed coupling of mi-CK to ANT in permeabilized cardiac fibers, by monitoring the effect of 20 mM creatine on submaximally activated with 0.1 mM ADP respiration. As creatine stimulated similarly the mitochondria in untrained and overtrained heart fibers, it was concluded that overtraining did not impair the coupling. In contrast, AMP was found to stimulate the respiration in the presence of 50 μM ATP to a lesser extent in overtrained group than in control, which indicates impaired coupling of mitochondrial AK2 isoform to ANT in overtrained heart [193]. Based on the ICEUs concept, it was interesting to know as to whether overtraining can affect the sites of ATP utilization. For that purpose, the total activities of ATPases were assessed in homogenates of the control and overtrained cardiac muscle. However, no difference between these groups was observed [193], in agreement with earlier findings that actomyosin ATPase remains unaltered after heavy training [211]. It seems, thus, that in the cardiomyocytes of overtrained heart the CK- and AK-mediated energy transfer system weakens predominantly at the mitochondrial sites due to overall suppression of ATP synthesis and its coupling to mitochondrial AK2.

Since mitochondrial impairment in muscle cells progresses with increasing training volume [196, 212], it would be practical to differentiate between the beneficial and deteriorating effects of strenuous exercise. Studies on rat heart under

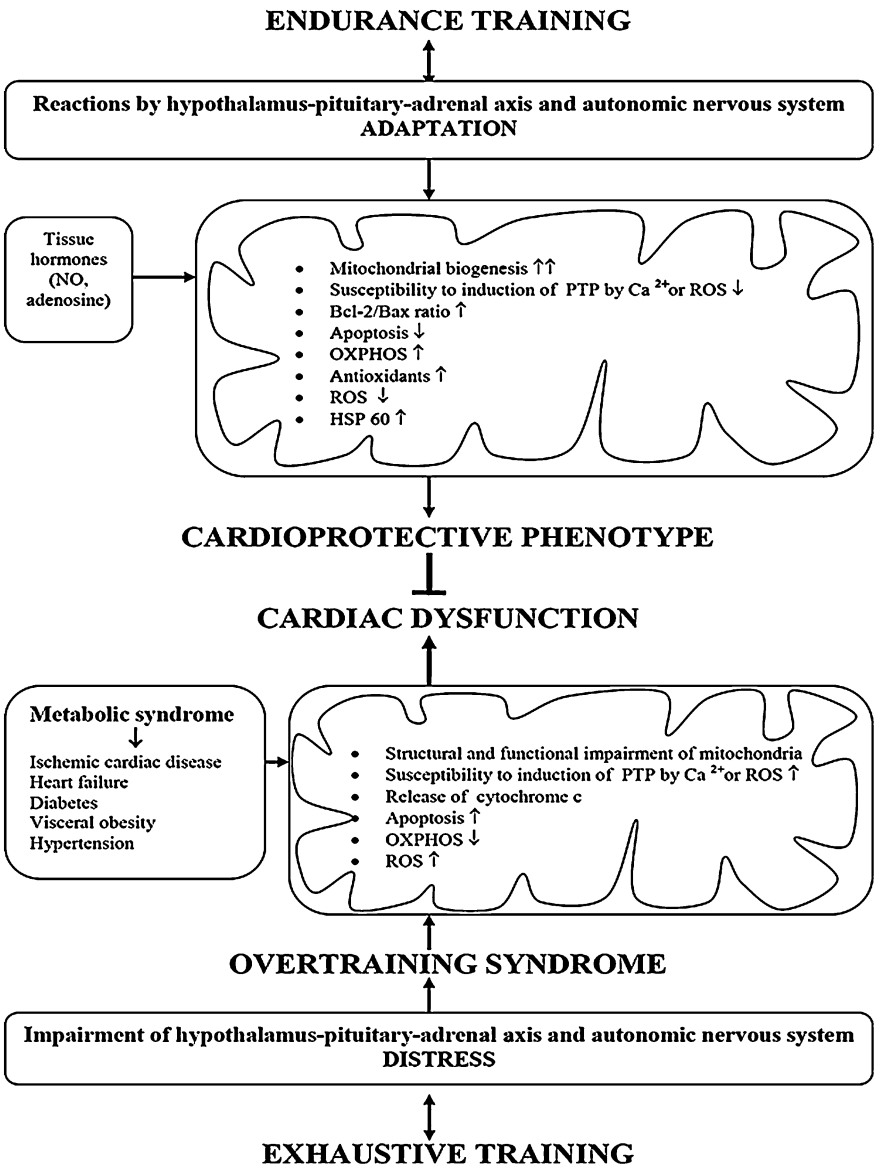

Fig. 20.1 Mechanisms of endurance training on heart. Endurance training induces the adaptive reactions which are coordinated by a network composed of hypothalamus–pituitary–adrenal axis system, autonomic nervous system, peripheral tissue hormones, and metabolic factors. Mitochondria are the main targets of the exercise-induced adaptive mechanisms which ensure increased aerobic working capacity in healthy organism and cardio protection in disease conditions. Exhaustive training, characterized by impairments of the general adaptation mechanisms and mitochondria, leads to cardiac dysfunction, an important component of the OTS. Abbreviations: *HSP* 60 heat shock protein 60; *PTP* permeability transition pore; *NO* nitric oxide; *OXPHOS* oxidative phosphorylation; *ROS* reactive oxygen species

various running intensities have shown that marked increase in expression of atrial natriuretic peptide demarcates transition from high-intensity exercise to exhaustive one when the cardiomyocytes lose their ability to increase the volume and numerical densities of mitochondria above the sedentary values and exhibit swelling of mitochondria [212]. Increased markers of oxidative stress, such as F_2-isoprostane in urine and reduced to oxidized glutathione ratio in blood, may represent another class of criteria for detecting physical overtraining [213]. These indicators could be helpful for selecting the optimal regimes for training to avoid damaging effects on muscle. Exact discrimination between the beneficial and harmful effects of exercise is necessary for studies of the mechanisms of mitochondrial biogenesis.

20.6 Conclusions

Regular endurance training is regarded as a popular exercise modality aimed at increasing the aerobic performance in healthy and diseased states. In general, endurance training can be viewed as a special type of stress, characterized with a complex of adaptive reactions coordinated by a network of hormonally and metabolically controlled pathways (Fig. 20.1). In the heart, these pathways stimulate mitochondrial biogenesis and modulate their properties in order to promote the capacity of OXPHOS and resistance to pathological factors, such as excess ROS and Ca^{2+}. As a result, a cardioprotective phenotype, characterized by attenuation of disease-induced functional decay, is induced. The beneficial effects of endurance training on heart muscle are lost in conditions of exhaustive training volumes, which result in an OTS characterized by damage of cardiomyocytes due to impairments of mitochondrial structure and function which promote apoptotic death. To avoid these unfavorable scenarios, selection of appropriate training intensities and volumes and early detection of muscle overload is of great practical importance.

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Chapter 21
Adenosine as an Endogenous Adaptive Cardiac Antihypertrophic and Antiremodelling Factor

Morris Karmazyn and Xiaohong Tracey Gan

Abstract Adenosine, a product of adenine nucleotide catabolism, has been demonstrated to exert numerous effects on the cardiovascular system which are mediated by activation of various receptor subtypes. The primary adenosine receptor in the myocardium is the A1 subtype which is linked to Gi-mediated inhibition of adenylate cyclase, although both A2a/b and A3 receptors have also been identified. There is increasing evidence that endogenously produced adenosine represents an important negative regulator of the hypertrophic and remodeling processes which contribute to heart failure. An important initial observation linking adenosine to the heart failure process was the report that plasma levels of the nucleoside are elevated in patients with heart failure irrespective of causative factor. Moreover, the degree of elevation was dependent on the severity of heart failure according to New York Heart Association (NYHA) classification with the greatest increases (more than fivefold) observed in NYHA class IV patients. Experimental observations have shown a direct antihypertrophic effect of adenosine receptor agonists on cardiomyocytes, which appears to be mediated by multiple adenosine receptor subtypes through yet to be determined processes. Further evidence obtained from in vivo studies also demonstrates a salutary effect of adenosine in reversing ventricular remodeling following aortic coarctation in rats. In addition to direct effects of adenosine receptor activation, deficiency in ecto-5′-nucleotidase which catalyzes the conversion of extracellular 5′-AMP to adenosine, thus increasing extracellular adenosine production, increases the degree of cardiac hypertrophy following aortic banding. Thus, when experimental evidence is taken together, it can be postulated that endogenous adenosine functions to limit the hypertrophic and remodeling processes which contribute to the development of heart failure.

M. Karmazyn (✉) · X. T. Gan
Department of Physiology and Pharmacology,
University of Western Ontario, London, ON N6A 5C1, Canada
e-mail: mkarm@uwo.ca

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21.1 Introduction

Adenosine, a product of adenine nucleotide catabolism, has been demonstrated to exert numerous effects on the cardiovascular system. For example, the vasodilating property of adenosine is well-established and it has been proposed that adenosine is an important regulator of coronary vascular resistance particularly under hypoxic situations; a phenomenon termed the "Berne hypothesis". Extensive evidence has been presented demonstrating a cardioprotective effect of adenosine, as well as adenosine analogos, against ischemic and reperfusion injury using a variety of both in vitro and in vivo approaches [1–9]. The primary adenosine receptor in the myocardium is the A1 subtype which is linked to Gi-mediated inhibition of adenylate cyclase, although both A2a/b and A3 receptors have also been identified [10–17]. Work from the authors' laboratory has demonstrated that the A1, A2a, and the A3 receptors are all upregulated in response to phenylephrine-induced cardiomyocyte hypertrophy [18]. Thus, overall, it appears that the mammalian cardiac cell expresses at least the three predominant adenosine receptor subtypes and that these receptors are upregulated, at least in response to one hypertrophic stimulus. It remains to be determined whether the upregulation of cardiac adenosine receptors occurs in response to other prohypertrophic stimuli in addition to phenylephrine.

The protective effects of adenosine A1 receptor activation are likely closely linked and may in fact occur secondary to activation of the ATP-sensitive potassium channels (K_{ATP}) and indeed we have previously provided evidence that K_{ATP} activation is also antihypertrophic ([19] and accompanying editorial). There is substantial evidence for activation of K_{ATP} protecting the reperfused ischemic myocardium, particularly with regard to myocardial stunning and development of arrhythmias [20–22]. Blockade of K_{ATP} with glibenclamide prevents the cardioprotective effects of preconditioning, thus suggesting the involvement of this channel in the cardioprotective phenomenon [23]. Although the mechanism by which K_{ATP} activation bestows protection is not known, adenosine A1 activation has been shown to stimulate K_{ATP} through a G protein-coupled mechanism [24, 25].

21.2 Cardiac Adenosine Synthesis, Metabolism, and Transport

The production of adenosine by the cardiac cell is summarized in Fig. 21.1 which also demonstrates the central role of ATP in adenosine production. Adenosine is generated through two primary pathways, namely dephosphorylation of 5′-AMP

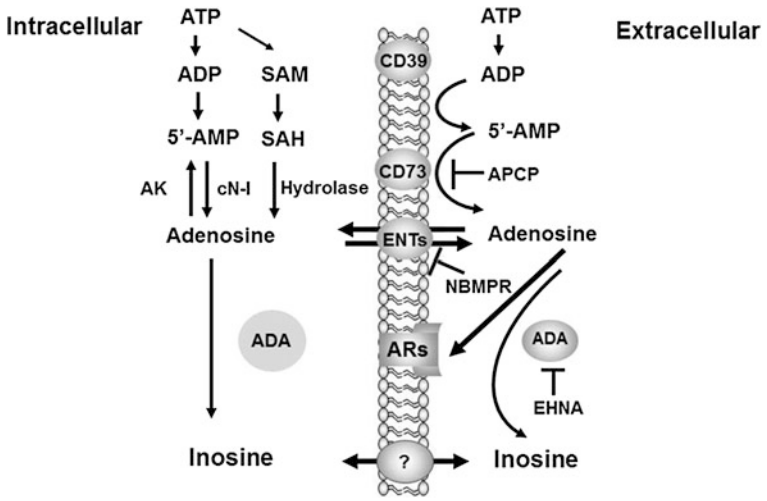

Fig. 21.1 Summary of primary sites of adenosine synthesis as well as its reuptake and metabolism. For additional details see text. Nonstandard abbreviations are as follows: *AK* adenosine kinase, *cN-I* cytosolic 5′-nucleotidase, *SAM* S-adenosyl-L-methionine, *SAH* S-adenosyl-L-homocysteine, *ADA* adenosine deaminase *CD*39 cluster of differentiation 39 or nucleoside triphosphate dephosphorylase, *CD*73 cluster of differentiation 73 or ecto 5′-nucleotidase, *ENTs* equilibrative nucleoside transporters, *ARs* adenosine receptors, *EHNA* erythro-9-(2-hydroxy-3-nonyl)-adenine, an ADA inhibitor, *NBMPR* nitrobenzylmercaptopurine riboside, an ENT inhibitor, *APCP* α,β-methylene ADP, a CD73 inhibitor

by 5′-nucleotidase and the hydrolysis of s-adenosylhomocysteine (SAH) by SAH hydrolase. The metabolism of 5′-AMP to adenosine via 5-nucleotidase can occur either intracellularly (by cytosolic 5′nucleotidase, also referred to as cN-I) -or via ecto-nucleotidases by highly regulated processes linked to the cell's bioenergetic status. In contrast, SAH hydrolase is localized exclusively intracellularly and transmethylation reactions contribute to adenosine production only within cells.

Under normoxic conditions, the salvage of adenosine by adenosine kinase and removal of homocysteine by both 5′-methyltetrahydrofolate-homocysteine methyltransferase, which yields methionine, and by cystathionine β-synthase which yield cystathionine, maintains net adenosine production by this pathway. Under these conditions, the transmethylation pathway serves as the principal source of intracellular adenosine production. However, under hypoxic or ischemic conditions, the degradation of the nucleotide is substantially increased and the production of adenosine from 5′-AMP becomes the more important source. Not only is the rate of AMP production increased, but it has been shown that the activity of cytosolic 5′-nucleotidase, which is normally relatively inhibited, is increased. Of the 2 nucleotidases 5′-ectonucleotidase (also known as CD73 for **C**luster of **D**ifferentiation 73) appear to be of particular in regulation of the cardiovascular system, especially as adenosine generated via this route is in close proximity to membrane adenosine receptors. Moreover, as will be discussed below, CD73

appears to play a critical role in tissue calcification which may potentially contribute to the development of heart failure.

Adenosine can be further metabolized to inosine by adenosine deaminase and also can be dephosphorylated to the nucleotides by the scavenging enzyme adenosine kinase. Adenosine deaminase appears not to be expressed in the cardiomyocyte, and thus adenosine is either scavenged or released. However, the deaminase is present in endothelial cells as are the enzymes responsible for the further degradation of inosine to hypoxanthine and uric acid, nucleoside phosphorylase, and xanthine oxidase, respectively.

Adenosine and other nucleosides are substrates for several nucleoside transporters which are widely distributed on mammalian cells. Indeed, inosine and adenosine also compete for nucleoside transport, thereby enhancing each other's extracellular levels and signaling capacity. The movement of adenosine into and out of cardiomyocytes occurs by facilitated diffusion via these transporters according to the prevailing gradients. The concentration of adenosine in the interstitium is, thus strongly influenced by the activity of these transporters. There is no evidence for Na^+ dependent nucleoside transporters on cardiomyocytes

21.3 Heart Failure: A Brief Synopsis

Heart failure is not a disease per se, but rather the final common pathway for numerous cellular and molecular defects caused by many instigating factors including myocardial infarction, genetic factors, diabetes, pulmonary hypertension, and so on. In the United States, more than 500,000 individuals are diagnosed yearly with heart failure. Although hospital admission rates are generally equally divided between males and females, mortality rate for women is markedly higher than those observed for men [26]. An important component of the heart failure process is ventricular remodeling in as manifested by alterations in the ventricular geometric architecture as a response to insult which eventually results in, or at least contributes to, defective ventricular function. As such, myocardial remodeling represents an important therapeutic target to mitigate deterioration of ventricular function after insult. Although remodeling can occur early after insult, it is generally considered to be a chronic event which progressively increases in severity (reviewed in [27–29]) over time. Remodeling is associated with various aspects of pathology such as hypertrophy, extracellular matrix deposition, as well as what may be considered as cellular remodeling which encompasses defective cell function including ionic regulation, generation of reactive oxygen species, energy production, and substrate utilization [27–29]. We have proposed that "mitochondrial remodelling" represents an important component of the remodeling program and one which may determine reversibility of remodeling and functional recovery following infarction [30]. As will be discussed below, the evidence points to adenosine as a key modulator (i.e. inhibitor) of the remodeling, and particularly, the hypertrophic process. Indeed, the early hypertrophic response

is one of the major compensatory responses to myocardial injury [31]. However, prolonged cardiac hypertrophy is, paradoxically, one of the major contributions to the myocardial remodeling processes leading up to heart failure.

Extensive research is ongoing which attempts to define the molecular mechanisms which contribute to or underlie cardiac hypertrophy and remodeling (reviewed in [32]). Prevention of hypertrophy, or as has become evident recently, *its reversal*, represents important therapeutic goals for the treatment of heart failure [33]. As such, it has been proposed that identifying endogenous *negative* regulators of hypertrophy may be of equal importance for developing antihypertrophic therapeutic strategies as the identification of endogenous prohypertrophic factors [34]. There is now compelling evidence that the adenosine system, including production, reuptake and degradation of the nucleoside as well as expression of adenosine receptors, represents the former category of hypertrophy regulators and that harnessing this complex system represents a potential effective approach toward mitigating the myocardial remodeling process.

21.4 Adenosine and Heart Failure: Current Knowledge

The potential role for adenosine, or more specifically the adenosine "system", in heart failure represents a novel and generally understudied area of investigation, although an association between plasma adenosine levels and the severity of heart failure has long been recognized. Thus, from a clinical perspective, an important observation linking adenosine to the heart failure process was the report of Hori's group in Japan that plasma levels of the nucleoside are elevated in patients with congestive heart failure irrespective of causative factor [35]. Moreover, the degree of elevation was dependent on the severity of heart failure according to New York Heart Association (NYHA) classification with the greatest increases (more than fivefold) observed in NYHA class IV patients. The levels of adenosine were significantly correlated with plasma norepinephrine levels [35]. These investigators also showed that the nucleoside transport inhibitors dipyridamole and dilazep (which increase adenosine levels) reduced the severity of heart failure, although the benefit reversed after drug discontinuation [36]. These results are of importance as they suggest that, although plasma adenosine levels are elevated in heart failure, this is insufficient to blunt the remodeling process. A major unknown entity at the present is how adenosine levels are regulated and the mechanism for increased adenosine plasma levels seen in heart failure. Further evidence obtained from in vivo studies also demonstrate a salutary effect of adenosine as the nucleoside has been shown to reverse ventricular remodeling following aortic coarctation in rats [37].

21.5 Adenosine and Myocardial Remodelling

Although the mechanisms underlying the anti-remodeling effects of adenosine are not known, a number of potential mechanisms have been proposed such as attenuation of norepinephrine release or reduction of endothelin production of angiotensin II or cytokines [38]. All these factors are known to be important contributors to cardiac hypertrophy and remodeling, and therefore contribute to heart failure [32]. Moreover, collagen and protein synthesis by isolated cardiac fibroblasts is inhibited by adenosine leading the authors to suggest that adenosine has an antifibrosis effect, possibly by activation of the adenosine A2B receptor in fibroblasts [39]. Indeed, long-term activation of these receptors (based on inhibiting the salutary effects of adenosine by selective adenosine A2B antagonist MRS1754 but not by the selective antagonists for other subtypes of adenosine receptors) reduces remodeling in the postinfarcted rat heart [40]. In the authors' studies, we have been unable to identify expression of the A2B in either neonatal or adult ventricular myocytes possibly suggesting that the salutary effects of A2B receptor activation may reflect actions on noncardiac myocyte components such as fibroblasts. Adenosine has also been proposed to regulate microtubule dynamics in hypertrophied hearts by attenuating microtubule content and decreasing microtubule stabilization which has been associated with myocardial hypertrophy [41].

The other interesting piece of evidence regarding the antihypertrophic effect of adenosine is that that the nucleoside *directly* modulates hypertrophy by its actions on the cardiac myocyte. Earlier studies by us [42] and others [43] reported that A1 receptor activation inhibits α_1 adrenoceptor-mediated responses in adult rat cardiac cells, although these studies were not related to the hypertrophic responses. However, both 2-chloroadenosine (an adenosine analogue acting on multiple receptor subtypes but not subjected to reuptake or metabolism as is adenosine) and the A1 receptor agonist cyclopentyladenosine-inhibited hypertrophy induced by phenylephrine, angiotensin II, or isoproterenol in cultured neonatal rat ventricular myocytes and reduced hypertrophy in a mouse aortic constriction model [44]. We have proposed that multiple receptor subtypes are involved, at least against phenylephrine-induced hypertrophy [45]. We have recent evidence that activation of multiple receptor subtypes inhibits the hypertrophic effects of both endothelin-1 and angiotensin II as well (unpublished data). These findings support the overall concept that adenosine serves as an endogenous antihypertrophic factor or, as suggested by Chen and Bache in their editorial, "general modulators of cardiac stress" [46]. It should be pointed out that not all studies have shown a salutary effect of adenosine receptor activation. In this regard, one study suggested that selective activation of the A1 receptor versus nonselective adenosine receptor activation may be deleterious since early constitutive overexpression of this receptor in mice produced cardiac dilatation, diminished ventricular function, and death, whereas when A1 receptor expression was delayed until 3 weeks of age the animals remained phenotypically normal [47]. Moreover, Lu et al. have recently reported an "unanticipated" (the authors' term) beneficial effect of A3 receptor deletion in mice subjected to thoracic

aorta coarctation [48]. In addition to the direct effects of adenosine receptor activation, it has also been recently demonstrated that a deficiency in CD73 which catalyzes the conversion (via dephosphorylation) of extracellular 5′-AMP to adenosine thus increasing extracellular adenosine production, increases the degree of cardiac hypertrophy following aortic banding [49]. Indeed, as will be discussed in the following section, we believe that CD73 represents a key regulator for adenosine production under pathological conditions, and by virtue of this ability, a key regulator of cardiac pathology especially in limiting myocardial calcification.

21.6 CD73: A Key Regulator of Myocardial Responses to Insult and its Potential Role in Myocardial Calcification

Myocardial calcification has been reported [50–59] for many decades and has been shown to be associated with various cardiovascular diseases including acute myocarditis [51], septic shock [52], myocardial infarction [53], myocardial fibrosis [56–59], as well as heart failure [54]. The onset and progression of myocardial calcification are not well understood and much of what we know today originates from vascular studies since arterial calcification is considered as an important manifestation of cardiovascular disease including atherosclerosis [60]. Calcification is an active tightly regulated process although underlying mechanisms are poorly understood. Evidence for the interrelationship between adenosine and myocardial calcification is somewhat serendipitous in that emerging evidence suggests calcification of joints and arteries is due to deficiency, or more appropriately, a nonfunctional CD73, encoded by the NT5E gene [61]. Overall, this suggests a significant contribution of CD73 in the control of calcification. CD73 is a glycosylphosphatidylinositol-anchored ubiquitous enzyme which is expressed widely in different tissues including the heart [62]. As already alluded to, in the heart CD73 has 5′-exonucleotidase activity that converts 5′-AMP to adenosine in several cell types, including endothelial cells, vascular smooth muscle cells, fibroblasts, and cardiac myocytes. This enzyme resides on the plasma membrane of the cells, supplying adenosine to receptors on the cell surface. CD73 may, therefore, represent a key regulator for adenosine production in the heart especially under pathological conditions. CD73 is, therefore, potentially a key factor contributing to insufficient adenosine production in the hypertrophied heart. Taken together, the finding of arterial calcification due to CD73 deficiency and suggesting that adenosine treatment can provide rescue therapy suggests the potential and beneficial roles of CD73-generated adenosine in the control of myocardial calcification in cardiac hypertrophy and heart failure. Indeed, CD73 deficiency has recently been shown to exacerbate heart failure in mice subjected to aortic coarctation [49].

One of the key enzymes in control of calcification in vitro and in vivo is the family of alkaline phosphatases. Alkaline phosphatases are dimeric enzymes which are expressed in a multitude of tissues including liver, bone, kidney, and heart [63–66] and are often used as a molecular marker for vascular calcification. Alkaline phosphatases have four isoforms: tissue nonspecific alkaline phosphatase (TNAP), intestinal alkaline phosphatase (IAP), placental alkaline phosphatase (PLAP), and germ cell alkaline phosphatase (GCAP). A key role of TNAP is to degrade inorganic pyrophosphate (PPi) to yield free inorganic phosphate (Pi) which can react with calcium to form hydroxyapatite while PPi is a potent inhibitor of hydroxyapatite formation at concentrations normally found in plasma and prevents calcification of rat aortas in culture [67–70]. Therefore, inhibition of TNAP will preserve PPi and suppress calcification. It is likely that there is a close association between cardiac hypertrophy and calcification which is closely regulated by the adenosine system. CD73 potentially plays a key role in directing traffic of this system.

21.7 Summary

Although there is now substantial and compelling evidence implicating adenosine as an endogenous cardioprotective agent, the potential role of the nucleoside as an adaptive endogenous inhibitor of the myocardial remodeling process is only just emerging. Yet the evidence for such a role of the nucleoside is impressive. For example, adenosine plasma levels are increased markedly in patients with heart failure which is related to the degree of heart failure severity. In experimental studies, cardiomyocyte adenosine receptor upregulation has been demonstrated in response to hypertrophic stimuli. Adenosine, as well as specific adenosine receptor agonists, has been shown to exert antihypertrophic effects in a number of experimental models, whereas inhibition of adenosine production exacerbates the hypertrophic process. Much still needs to be learned and one of the major challenges will be to identify the underlying mechanisms for the salutary effects of adenosine in limiting remodeling, particularly as this appears to be due to the activation of multiple adenosine receptor subtypes. Fully understanding the underlying processes is important and potentially rewarding as it offers the possibility of harnessing the adenosine system to limit the myocardial remodeling process, a potentially useful and novel therapeutic target for the treatment of heart failure.

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Chapter 22
Myocardial Adaptation and Autophagy

Dipak K. Das and Hannah R. Vasanthi

Abstract Cells are constantly exposed to adverse environmental conditions; but they possess remarkable ability to adapt them to the stress by up-regulating their own defense system. Adaptation has been widely studied in case of heart. Myocardial adaptation to stress occurs through up-regulating many stress proteins including oxidative stress-inducible proteins, antioxidants, and heat shock proteins. Recent studies have indicated that stress adaptation, especially adaptation due to nutritional deprivation occur via autophagy, a cellular degradative mechanism involved in the recycling and turnover of cytoplasmic constituents from these cells. This review discusses the molecular link between adaptation and autophagy, and establishes the fact that myocardial adaptation to stress may occur through diverse pathways including autophagy. Accumulating evidence supports the notion that autophagy and adaptation are intimately related. Autophagy due to nutritional deprivation is a unique example of cellular adaptation. Among a number of factors, redox signaling appears to be the most important link between adaptation and autophagy. SirT-FoxO network plays a key role in both the processes. Recent studies indicate that both adaptation and autophagy are under the control of miRNA and epigenetics.

Keywords Autophagy · Adaptation · Autophagosomes · Autophagosomes · Redox signaling · Redox signaling · Oxidative stress · Micro RNA · SirT-FoxO net work

D. K. Das (✉)
Cardiovascular Research Center, University of Connecticut School of Medicine, Farmington, CT 06030-1110, USA
e-mail: ddas054@gmail.com

H. R. Vasanthi
Department of Biotechnology, School of Life Sciences, Pondicherry University, Pondicherry, India

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22.1 Introduction

Recent years have witnessed the development of a novel concept for myocardial preservation that uses the cell's inherent propensity for self preservation by way of adaptation. The myocardium possesses a remarkable ability to readily adapt to a stressful situation by up-regulating its defense systems, which can defend it against subsequent insult. This phenomenon, known as ischemic preconditioning and myocardial adaptation so far, has been tested widely for ischemic heart disease. Myocardial protection by ischemic preconditioning has opened a new horizon; this concept is now believed to be one of the most powerful techniques for myocardial protection.

Autophagy, a cytoplasmic recycling process active from the early development throughout lifetime, especially aging has turned from largely a morphological phenomenon to a well described molecular mechanism in cardiovascular diseases. Autophagy has been well described during ischemia/reperfusion or pressure overload in the heart [1–8] as well as in atherosclerosis [9]. Diverse cellular factors and signaling pathways have been shown to contribute to autophagy induction in different cellular contexts, including reactive oxygen species (ROS) [10].

Any functional relationship between adaptation (adjustment against cellular stress) and autophagy (self-cannibalism) is a complex phenomenon; but autophagy constitutes a stress adaptation that avoids further cell death. The cellular and molecular link between autophagy and cellular stress response leading to adaptation share the common mechanism that both reduce subsequent cellular injury. Cellular stress response integrates into diverse signaling pathways, one of which is autophagy. However, all these pathways converge at some point, such as redox signaling, although mostly they run in parallel. Autophagy and adaptation are likely to be triggered by common upstream signals. It is possible that autophagy and adaptation share some common pathways that link the cellular responses. It is the intension of this review to discuss these common signaling pathways such as that mediated by ROS in response to autophagy and adaptation.

22.2 Myocardial Adaptation to Stress

Adaptation is the enhancement of the endogenous cellular defense system, thereby providing each cell with new protein synthesis that provides means to protect itself when it is more susceptible to injury. It has long been known that cells exhibit specific responses when confronted with sudden changes in their environmental conditions. The ability of the cells to acclimate to its new environment is the integral driving force for the adaptive modification of the cells. Such adaptation involves a number of cellular and biochemical alterations including metabolic homeostasis and reprogramming of gene expression. The changes in the metabolic pathways are generally short lived and reversible, while the consequences of gene

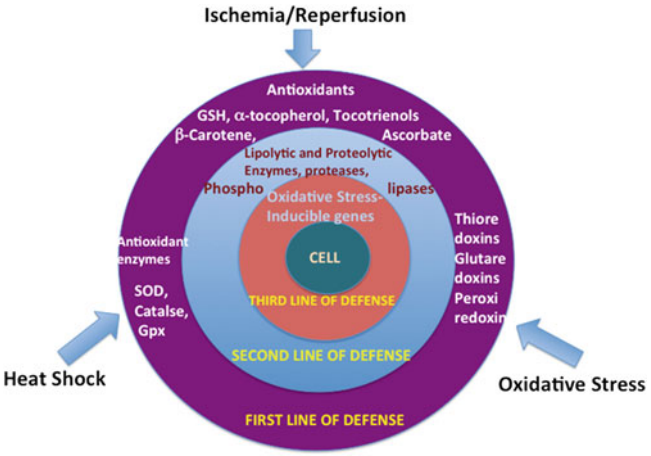

Fig. 22.1 A model demonstrating myocardial defense against ischemia/reperfusion, heat shock, and oxidative stress. Induction of gene expression is shown as the *third line of defense*

expression are a long-term process that may lead to the permanent alteration in the pattern of gene expression and subsequent protein synthesis (Fig. 22.1). Using this concept, it has been shown that preconditioning of heart by repeated short duration of ischemia and reperfusion can delay the onset of further irreversible injury [11], or even reduce the subsequent post-ischemic ventricular dysfunction [12], and incidence of ventricular arrhythmias [13]. It has been demonstrated that repeated ischemia, distinguished from a single ischemic insult, can reduce subsequent ischemia reperfusion injury [14] and post-ischemic ventricular fibrillation [15]. Such myocardial preservation by repeated short-term reversible ischemia leads to the development of the concept of stress adaptation. Consequently, new ideas of preconditioning have been developed, which include adenosine [16], potassium channel opening [17], α_1 receptor [18], hypoxia [19], oxidative stress [20], drug [21], and heat shock [22].

22.2.1 Adaptaion by Ischemic Preconditioning

The precise mechanism of ischemic preconditioning is still under considerable debate, despite many investigations. It is generally believed that ischemic preconditioning occurs in two different steps: (1) early effect (short-term adaptation) triggered in seconds to minutes, which is likely to be mediated by the release of some endogenous compounds such as catecholamines and adenosine, and may last up to several hours (*ischemic preconditioning*); and (2) late effect (long-term adaptation), which may occur after several hours and may last days (Fig. 22.2). The long-term adaptation is believed to be mediated by the transcription of genes

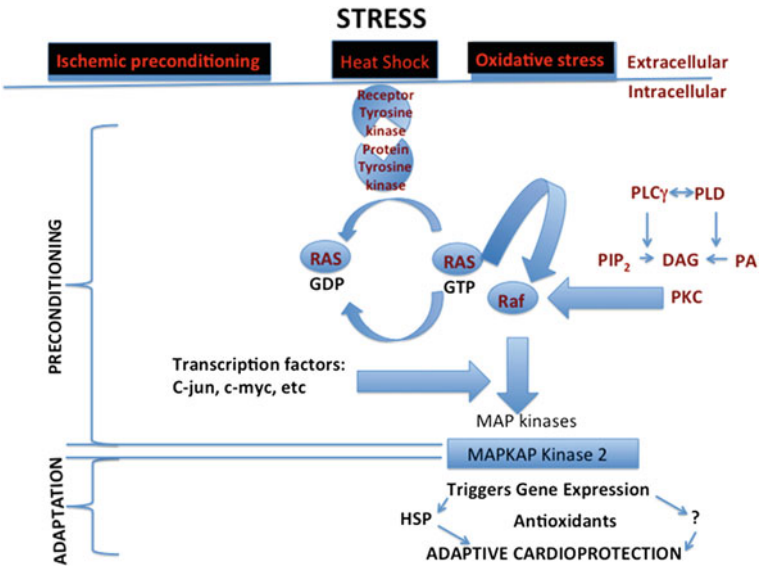

Fig. 22.2 A model supported from our work [Das DK, Maulik N, Yoshida T, Engelman RM, Zu Y-L. Annals New York Academy of Sciences]. The figure indicates the existence of tyrosine kinase-phospholipase D-MAPKAP kinase 2 signal transduction pathway in the preconditioned heart. MAPKAP kinase 2 is shown as the *molecular link* between the preconditioning and adaptation

and their subsequent translation into proteins, and has been termed as *myocardial adaptation to ischemia* [23].

Various methods are available to adapt the heart to ischemia. The one, which was originally used, consisted of subjecting the heart to repeated episodes of short durations of ischemia and reperfusion [24]. This technique is very reproducible, and has been valid in many animal models including dog, pig, rabbit, and rat [25]. We will describe here the method used for the rat heart. The hearts can be excised from the properly anesthetized rats, which are perfused for 10 min with noncirculating Krebs-Henseleit bicarbonate (KHB) buffer containing 3 % bovine serum albumin. The KHB buffer consists of the following concentrations (in mM) 119.0 NaCl, 25.0 $NaHCO_3$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 11.0 glucose. Coronary perfusion pressure and perfusate temperature are generally maintained at 100 cm H_2O and 37 °C, respectively. Hearts can be made globally ischemic by terminating the aortic flow for 5 min followed by 10 min of reperfusion (1XPC). Each preconditioning (1XPC or 4XPC) is followed by 60 min reperfusion. Experiments are terminated at various points, e.g., prior to PC (baseline), after PC, and after reperfusion. Heart biopsies are examined for gene and protein expression, signal transduction, stress proteins, and cardiac recovery.

22.2.2 Adaptation by Heat Shock

Hearts can be adapted by subjecting them to direct heat shock. Among many methods, the one that is most commonly used consists of allowing the animals to swim in warm water to raise their body temperature [26]. Studies are also known to exist where animals are heated using an electric blanket or heating pad. In the case of an isolated heart, the heart can be directly heated by perfusing with warm buffer or blood [27]. Recently, a novel approach was used in our laboratory to induce the heat stress [28]. A sympathomimetic drug, amphetamine, was used to raise the body temperature. Rats were injected with amphetamine sulfate (3 mg/kg, i.m.), and the rectal temperature was monitored. The control animals received saline injection only. The rectal temperature of the rats before amphetamine injection ranged from 37 to 38 °C. After 1 h of amphetamine injection, the rectal temperature increased markedly in the range of 41.5–42.5 °C. This hypothermic state lasted for up to 3 h., then it gradually returned to the basal temperature within another 3–4 h. Forty hours after the injection, the animals were anesthetized, isolated perfused heart was prepared as described previously. Both control and experimental hearts were subjected to 30 min ischemia followed by 60 min of reperfusion as described in IPC.

22.2.3 Adpataton by Oxidative stress

Similar to myocardial adaptation to ischemia and heat shock stress, the heart can be adapted to oxidative stress by a variety of methods. For example, animals can be subjected to oxidative stress by treating them to a low dose of endotoxin or its active component lipid A [21]. Oxidative stress can also be induced by cytokine treatment. Recently, oxidative stress was developed by injecting the rats with 30 μg/kg body weight of human recombinant IL-1α, 24 h before the isolation of the hearts [29]. The development of oxidative stress was confirmed by the increased amount of malonaldehyde formation in the heart. Control and experimental hearts were subjected to 30 min of ischemia followed by 60 min of reperfusion.

22.3 Molecular and Cellular Link Between Preconditioning and Adaptation

A large number of studies exist in the literature to suggest the role of protein kinase C in ischemic preconditioning. A short-term ischemia as well as ischemia followed by reperfusion can translocate and activate protein kinase C [30]. Furthermore, both α_1-receptor stimulation and Ca^{2+} ion translocate and activate protein kinase C [31]. Given the fact that both α_1-receptor activation and intracellular Ca^{2+} overloading are

the manifestations of ischemia/reperfusion injury, it was not surprising when ischemic preconditioning consisting of repeated ischemia and reperfusion was also found to translocate and activate protein kinase C [32]. Interestingly, it has long been known that protein kinase C can activate transcription of genes [33]. Indeed, many genes were found to be activated in the preconditioned myocardium [34]. Thus, protein kinase C, which is activated as a result of the events controlled by endogenous compounds (e.g., α_1-receptor, adenosine, diacylglycerol) can be instrumental for gene expression leading to translation into proteins. This uniqueness of protein kinase C led to believe that this kinase could be the molecular link between ischemic preconditioning and myocardial adaptation to ischemia [35].

The results of a number of recent studies now implicate that protein kinase C may not be the ultimate link between ischemic preconditioning and adaptation. These include the abundance of MAP kinase activated protein, MAPKAP kinase 2, in the heart, rapid activation of MAPKAP kinase 2 by diverse stresses including heat stress, oxidative stress, and ischemic stress; and most importantly, it is MAPKAP kinase 2, and not protein kinase C that can phosphorylate small heat shock proteins HSP 25/27, which are also activated by ischemic preconditioning [36]. Interestingly, activation of protein tyrosine kinase is coupled with the activation of phospholipase D in ischemic preconditioning, because inhibition of tyrosine kinase results not only on the inhibition of phospholipase D, but also abolishes preconditioning-mediated activation of protein kinase C, MAP kinase, and MAPKAP kinase 2.

22.4 Adaptaton and Autophagy

Autophagy is a cytoplasmic recycling process active from early development throughout lifetime and aging has turned from largely a morphological phenomenon to a well-defined molecular mechanism in health and disease. A previous study demonstrated that cardioprotection by adaptation to ischemia augmented autophagy in association of BAG-1 protein [37]. Another recent study showed that calorie restriction enhanced cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy [38]. A related study has described autophagy process as an adaptive response to nutrient deprivation [39]. Thus, it seems reasonable to assume that there is a link between adaptation and autophagy. The following sections will be devoted to describe autophagy and establish the molecular link between adaptation and autophagy.

22.5 Autophagy

Autophagy is an intracellular process in which a cell digests its own constituents via lysosomal degradative pathway. Although autophagy has been shown in several cardiac diseases such as heart failure, hypertrophy, and ischemic

cardiomyopathy, the role for the regulation of autophagy remains largely unknown. In normal conditions, autophagy occurs at low levels for the turnover of damaged or long-lived proteins, macromolecules, and organelles like mitochondria, ribosomes, endoplasmic reticulum, and peroxisomes [40]. Autophagy provides a necessary source of energy for the cardiac myocytes during early neonatal starvation period [41]. However, autophagy is shown to be the main mechanism causing cell death, leading to the progression from compensated hypertrophy to heart failure and left ventricular systolic dysfunction in pressure overloaded human heart [42]. Also, in dilated cardiomyopathy patients, autophagy is associated with the degradation of damaged intracellular organelles leading to the destruction of cardiomyocytes [43]. Moreover, basal level of autophagy is triggered in pressure-overloaded myocardium, a major risk factor for cardiac hypertrophy and heart failure [44]. In spite of this, autophagy is shown to protect the myocardium and cardiac cells against ischemia reperfusion injury [45]. Recently, we have shown that ischemic preconditioning, a state-of-the-art technique for the protection of myocardium induces cardiac autophagy [46].

There are three types of autophagy: macroautophagy, microautophagy, and chaperon-mediated autophagy. Macroautophagy is the most active form of autophagy and is known to play a major role in intracellular degradation [47]. Macroautophagy (hereafter referred to as autophagy) is initiated by the formation of an isolated membrane, a single-membrane structure possibly derived from the sarcoplasmic/endoplasmic reticulum [48]. Fusion of the tips of the isolated membrane results in the formation of a double-membrane structure known as the autophagosome, which surrounds portions of the cytoplasm and organelles such as mitochondria [49], selectively targeted to the toxic protein aggregates [50] or intracellular pathogens [51]. Autophagosomes undergo a series of maturation steps and finally fuse with the lysosome (this combined structure is known as the autophagolysosome), in which the sequestered contents and the inner membranes of autophagosomes are degraded by the lysosomal hydrolases [52]. The lifetime of autophagosomes is as short as about 8 min. In microautophagy, cytosolic macromolecules are locally taken into lysosome via invagination of the lysosomal membrane [53]. During chaperone-mediated autophagy, delivery of modified proteins to lysosome occurs via heat-shock protein 73 and receptor lysosome-associated membrane protein type 2a (LAMP 2a) [54].

22.5.1 Core machinery of Autophagy: Autophagosome Formation and its Relationship to Adaptation

Central process of autophagy is autophagosome formation, which is a double membrane vesicle responsible for delivering cytoplasmic material to lysosomes. Autophagosome formation is the hallmark morphologic feature of the dynamic process that with the help of other components allow the cells to adapt according

to the changing needs of the cells. Autophagosomes fuse with lysosomes, exposing the inner compartment to lysosomal hydrolases, the resulting macromolecules are then released into the cytosol through lysosomal membrane premises for recycling.

Recent studies clearly demonstrated that autophagy has diverse physiological and pathophysiological roles than expected, such as adaptation to starvation, clearance of intracellular proteins and organelles, development, anti-aging, cell death, and tumor suppression [55]. This review will restrict its discussion only to the adaptation process.

It has been known for some time that calorie restriction enhances cellular adaptation through SirT1-dependent autophagy [56]. Hypoxia caused by anti-angiogenic therapy induces tumor cell autophagy as a cytoprotective adaptive response [57]. A study from our laboratory showed that myocardial adaptation to ischemia occurs via autophagy and redox signaling plays a crucial role in this process [58]. Another related study demonstrated that cells respond to mechanical compressive stress by rapidly inducing autophagosome formation [59]. The authors showed that mechanical induction of autophagy is TOR independent and transient, lasting until the cells adapt to their new environment and recover their shape. The autophagic response appears to be part of an integrated response to mechanical challenge, allowing cells to cope with a continuously changing physical environment. Adaptation of neurons in autophagy may depend on sex [60].

22.5.2 Molecular Mechanisms of Autophagy

As mentioned earlier, autophagy is a self-clearing process to remove the damaged proteins and organelles, an alternate mechanism for proteasomal degradation, which can generate a survival signal, as in the case of myocardial ischemia [61]. Although autophagy was initially believed to be involved in a nonapoptotic form of programmed cell death, recent studies have changed this concept by demonstrating that autophagy can also cause cell survival. However, Matsui et al. [62] have demonstrated that in case of myocardial ischemic injury, autophagy causes cell survival, whereas the reperfusion injury causes the cell death. In our own study, we found that myocardial ischemic preconditioning induces autophagy for the protection of myocardium through the induction of BAG1 survival protein [63]. At low dose, resveratrol-mediated survival signaling is realized by its ability to induce pharmacological preconditioning [64]. Our results are consistent with our previous reports that resveratrol at low dose (2.5 mg/kg/day) protects the myocardium from IR injury by reducing myocardial infarct size via the activation of Akt [65]. Treatment with rapamycin alone or treatment with rapamycin followed by low dose (1 μM) resveratrol enhanced the autophagic puncta, and further enhanced the autophagosome formation.

22.5.3 Signaling Cascades

Although the physiological functions of many of the genes remain to be elucidated, some of these genes are specifically identified that are involved in signaling complexes including those in TOR signaling pathway, ATG complex, and Vps34/Class III PI3 complex [66]. TOR, a target of rapamycin and a serine/threonine kinase, mediates the initial signaling cascade in response to changes, especially for the changes due to nutritionally deprived conditions. TOR can be blocked by nutritional deprivation or by rapamycin treatment leads to the dephosphorylation of TAP42, which dissociates from PP2A that finally dephosphorylate its targets leading to induction of autophagy. Another signaling pathway that induces autophagy involves ATG1, which triggers CVT pathway. It has been suggested that ATG1 kinase activity is necessary for CVT pathway, but may not induce autophagy, which may be induced through any nonkinase structural role in autophagy induction. It is likely that during the nutritional stress, ATG1, ATG11, and ATG13 trigger a switch from CVT pathway to autophagy pathway [67]. A third autophagy signaling pathway includes VPS34/class III phosphatidylinositol 3-kinase (PI3 K) complex, which represent a family of enzymes responsible for diverse cellular responses. There are three classes of PI3 Ks, the class I PI3 K pathway participates in autophagy regulation, class III PI3 K plays an important role at the early stage of autophagosome formation, while class II PI3 K may not be involved in autophagy regulation [68].

22.5.4 Autophagosome Formation

There are at least three classes of proteins that involve in autophagosome formation: two ubiquitin-like (UBL) conjugates and a membrane complex containing ATG9. The UBL system associated with autophagy includes AT12–ATG5 conjugate and ATG8 protein. ATG12 is transferred to its target protein ATG5, which is then transferred to ATG10 that functions as E2 enzyme. ATG12–ATG5 conjugate interacts with ATG16, and this new complex ATG12–ATG5–ATG16 facilitates the formation of the outer side of the membrane followed by autophagosome formation. Once autophagosomes are formed, the conjugates start to dissociate from the membranes so that they are not present in the mature autophagosomes. The participation of ATG9 is also required for the preautophagosomal structure. However, this is absent in the matured autophagosomes, although a fraction of ATG9 can be found in the PAS together with other autophagy proteins [69].

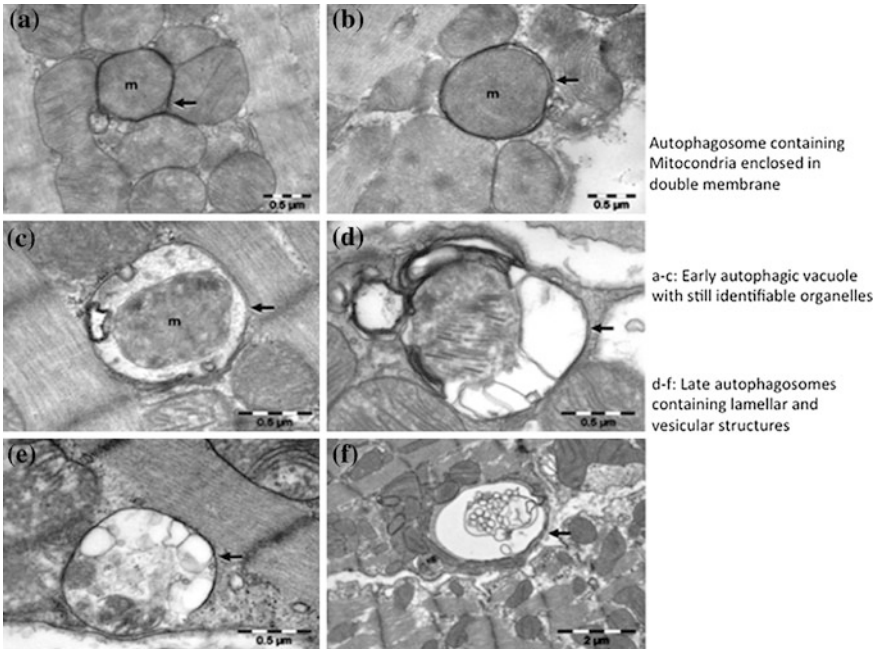

Fig. 22.3 zAutophagsomes containing mitochondria enclosed in a double membrane. **a–c** represent early autophagic vacuole with still identifiable organelle, **d–f** represent late autophagosomes containing lamellar and vesicular structures

22.5.5 Fusion of Autophagosome with the Lysosome

Fusion of autophagosome with lysosome is an essential step for the success of autophagic pathway. For example, human muscular disorders are sometimes caused due to the defective autophagosome-lysosome fusion. Such fusion requires a number of maturation steps prior to fusion, and RAB22 and RAB24 are required for correct autophagosome maturation. It is important that prior to fusion with lysosomes, autophagosomes must fuse with endosomes or endosome-derived vesicles [70]. A massive accumulation of nascent autophagosomes is observed in cardiac cells that protect the heart from injury (Fig. 22.3). The figure shows the involvement of cytoskeletal elements, which are required for the autophagosome maturation and autophagosome-lysosome fusion.

22.5.6 Function of Autophagy

Autophagy dysfunction has been cited for many diseases including neuronal, muscular, cardiovascular, infectious, and cancer [71]. Autophagy machinery is especially important for proper recycling of damaged organelles, thereby

maintaining cellular integrity. For example, autophagosomes sequester damaged mitochondria resulting from apoptosis, thus preventing the uncontrolled release of reactive oxygen species [72, 73]. Autophagy is an important step in maintaining cardiovascular health [74], eye disease [75], neuronal diseases [76], renal problems, infectious diseases, cancer, and aging [77]. In addition, autophagy is essential for several other tissue-specific functions such as erythroid maturation by eliminating ribosomes or mitochondria after removing the nucleus and neuromelanin biosynthesis in dopaminergic neurons via sequestration of cytoplasmic dopamine-quinone into autophagosomes.

22.6 Autophagy: A Stress Response Leading to Adaptation

During the exposure to any sublethal stress, eukaryotic cells undergo an adaptive response and protect themselves against subsequent lethal injury. Autophagy also leads to cellular adaptation, but through a different mechanism, i.e., by sequestering contents that are degraded in lysosomes, thereby allowing cells to eliminate the damaged components through catabolism and recycling to maintain energy homeostasis. Thus, adaptation and autophagy run in parallel, by many components falling in the crossroads of their cellular signaling as discussed below.

22.7 Converging Adaptation to Autophagy

From the foregoing discussion, it should be clear that intracellular adaptation requires upregulation of several stress proteins including oxidative stress inducible and antioxidant proteins and heat shock proteins, while oxidative and heat stresses are also important for autophagy. Several special proteins that are common for processes include BCL2, FOxO, and SirT. Oxidative stress and oxidative stress inducible genes and proteins appear to be one of the most important common parameters that are common to both adaptation and autophagy. In starvation-induced adaptation/autophagy, ROS levels are increased leading to damaging of electron transfer process [78–80]. Mitochondria generate ROS that are at low levels, play a role in redox signaling while at higher levels can cause damage. Autophagy removes the damaged proteins, and at the same time redox signaling induces many antioxidative enzymes including SOD, catalase and BCL2 [81]. An important redox signaling pathway involves Nrf2 [82, 83], since Nrf2-deficient cells exhibit a deficient stress response [84]. Another redox sensor of ROS that is intimately linked to both adaptation and autophagy is BCL2, which is increased due to redox signaling [85]. The co-chaperone protein BAG-1 (BCL-2associated athanogene) is also responsible for the cellular dependence on either proteosomal or autophagic activities [86], p53 is simultaneously modified, which activates AMPK, thereby inhibiting mTOR [87].

A recent study from our laboratory has indicated that mitophagy is controlled by PINK1 (PTEN phosphatase and tensin homolog deleted on chromosome (10)-

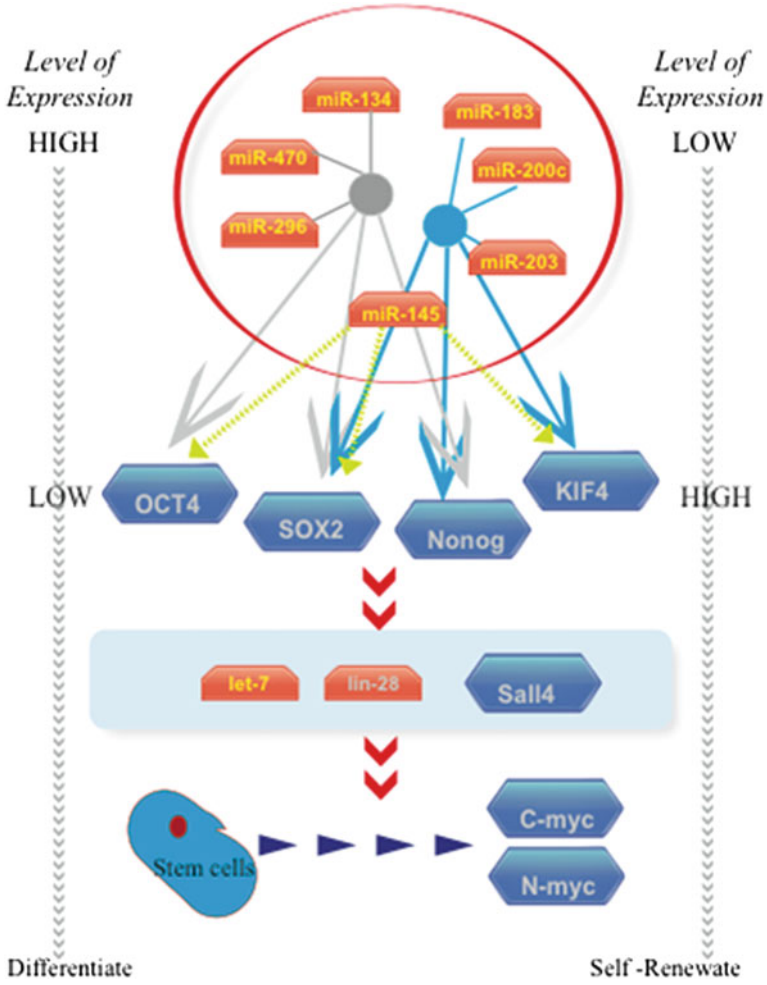

Fig. 22.4 Involvement of Micro RNA in Autophagy signaling. Regulation of the pluripotency factors-OCT4, SOX 2, and KL4- by various micro RNAs, and the effect on embryonic stem cell differentiation and self-renewal

induced kinase 1) and Parkin [88]. Mitochondrial fusion and fission are also intimately involved in this process [89]. Parkin is usually degraded, but when mitochondria are damaged, PINK 1 is rescued by recruiting and phosphorylating PARKIN, thereby initiating mitophagy [90]. It appears that PINK1 has duel functions: in one hand, it regulates mitophagy while on the other hand, it controls mitochondrial generation of ROS, being a likely target of redox signaling.

SirT and FoxO coordinately regulate autophagy and play an important role in adaptation. The FoxO-SirT network appears to be intimately involved in the redox regulation of autophagy homeostasis. SirT and AMPK pathways converge onto

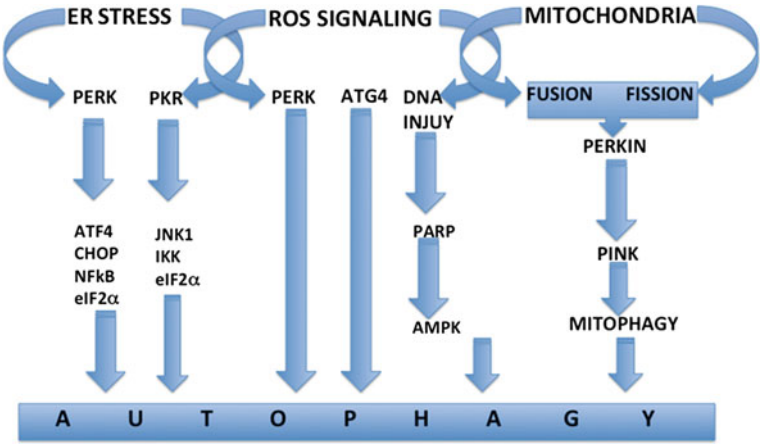

Fig. 22.5 Occurrence of Autophagy from three distinct stress signals, ER stress, ROS signaling, and mitochondrial fusion and fission. Three stress signals although distinct, they are overlapping

FoxO proteins regulating a variety of biological processes important for autophagy [91]. Several reports point out neuroprotective effects of SirT1-FoxO network in many models of neurodegenerative diseases. In addition to SirT, another target of FoxO is AMPK, a key regulator of cellular metabolism activated by a decrease in energy levels. Interestingly, many genes that control adaptation process such as Gadd45, Bmp4, and CXCR4 are controlled by FoxO, a molecular target of SirT [92].

22.8 Role of Micro RNA and Epigenetic Regulation

Finally, recent studies indicate that both autophagy and adaptation are under the epigenetic regulation. From the foregoing discussion, it should be clear that autophagy is triggered in response to intra- or extra- cellular stress and signals such as nutritional deprivation [93] and ER stress [94]. Many studies from our own laboratory have indicated that microRNAs (miRNAs) are involved in autophagy [95]. Some of these miRNAs are involved in epigenetic regulation by modification of RNA–RNA or RNA–protein interactions [96, 97]. Since miRNAs control numerous aspects of cellular functions, they are also responsible for controlling various steps in autophagy and adaptation (Fig. 22.4). Epigenetic regulation has been identified in a variety of disease processes including cancer, cardiovascular, neurodegenerative, and inherited disorders and in diverse health abnormalities that include antiinflammatory response, obesity, insulin resistance, diabetes, immune system, and stem cells. A recent study demonstrated the role of caveolin, a subcompartment of the plasma membrane that coordinates and regulates a variety of signaling processes, in epigenetic regulation of cardio-protection through ischemic adaptation [98]. In this study, caveolin 1 knockout mice (Cav-1 KO) abolished the acylation of histone (H3

and H4) and increased the methylation of histone in the preconditioned heart. The increased histone methylation was significantly correlated with the increased level of histone methyl transferase G9a protein and increased level of histone deacylase (HDAC) activity. Cav-1 KO mouse also reduced the translocation of FoxO3a to the nucleus and reduced the induction of the expression of SirT1 in the preconditioned heart. In another study, both SirT1 and some unidentified class I/II HDAC regulate the acylation levels of FoxO1 in cells. Loss of SirT1 induced an enhanced acylation of FoxO1 either under basal conditions or in the presence of the HAT p300, demonstrating that SirT1 is a crucial FoxO1 deacetylase in cells [99].

22.9 Future Directions and Concluding Remarks

Accumulating evidence supports the notion that autophagy and adaptation are intimately related. Autophagy due to nutritional deprivation is a unique example of cellular adaptation. Among a number of factors, redox signaling appears to be the most important link between adaptation and autophagy. SirT-FoxO network plays a key role in both the processes. Recent studies indicate that both adaptation and autophagy are under the control of miRNA and epigenetics (Fig. 22.5).

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Chapter 23
Modulatory Role of VEGF in Angiogenesis for Cell Survival

Dipti Gatne, Veeranjaneyulu Addepalli and Dev D. Santani

Abstract Vascular endothelial growth factor (VEGF) is established to play a crucial role in angiogenesis, cell survival, and thus appear to be important in many pathophysiological processes. VEGF and anti VEGF therapy have been used in treatment of cancer, diabetic retinopathy, cardiovascular disorders, psoriasis, wound healing, age-related macular degeneration, and so on. This review elaborates role of VEGF as cell survival factor in various diseases and several therapeutic strategies involving VEGFs and their receptors. The development of side effects and resistance to therapy involving VEGF needs to be considered while designing new molecules along with molecular modeling techniques.

Keywords VEGF · Angiogenesis · Cancer · Cell survival

23.1 Introduction

Tyrosine kinases play a crucial role in regulation of cell growth and differentiation and angiogenesis is related to vascular endothelial growth factor receptor (VEGFR), a type of tyrosine kinase. Angiogenesis is a highly regulated and dynamic

D. Gatne
Department of Pharmaceutical Chemistry, SPP School of Pharmacy and Technology Management, SVKM's NMIMS, V. L. Mehta Road, Mumbai 400056, India

V. Addepalli (✉)
Department of Pharmacology, SPP School of Pharmacy & Technology Management, SVKM's NMIMS, V. L. Mehta Road, Mumbai, Maharashtra 400056, India
e-mail: v.addepalli@nmims.edu; addepalliv@gmail.com

D. D. Santani
Department of Pharmacology, L.M. College of Pharmacy, Ahmedabad 380009, India

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process involving formation of new blood vessels from the pre-existing vasculature and members of the vascular endothelial growth factor (VEGF) family have been considered as one of the most important proangiogenic factors [1, 2].

Angiogenesis is related to the survival and functioning of cells as it delivers oxygen and nutrients to them. It involves several steps, such as detachment of pre-existing pericytes, degradation of extracellular matrix by endothelial proteases, migration and proliferation of endothelial cells, tube formation by endothelial cells, and reattachment of pericytes or vascular stabilization in sequential manner [3].

During angiogenesis, various factors are expressed by endothelial cells, such as transforming growth factors (TGF), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF), interleukins, members of the fibroblast growth factor (FGF) family, $\alpha_v\beta_3$-Integrin, and VE-cadherin apart from VEGFs [4].

This review highlights biology of VEGF signaling pathway, its role in cell survival and therapy involving angiogenic and anti-angiogenic agents in treatment of various diseases. Development of resistance and side effects has been the hurdles in the development of the therapy. Also, an overview of molecular modeling techniques which have supported the exploration of angiogenic and anti-angiogenic molecules is included in the chapter.

23.2 VEGF Signaling Pathway and Role in Cell Survival

One of the critical survival factor and regulator of endothelial cells is VEGF. It stimulates formation of capillaries and controls proliferation and permeability of endothelial cells. The mammalian VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor 1 (PIGF-1) as glycoproteins. $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ are the known isoforms of VEGF-A [5]. Expression of VEGF is regulated by various growth factors and cytokines, such as interleukin 1-β, PDGF, TGF-β, and TNF-α [6]. VEGF-A is reported to be endothelial cell mitogen, autocrine growth factor, and survival factor in endothelial cells [7]. Apart from angiogenic activity, VEGF affects neurons, pneumocytes and endothelial, neural, and hematopoietic progenitors [8, 9].

VEGF is significantly up-regulated in response to hypoxia by activation of the hypoxia-inducible transcription factors HIF-1α and HIF-2α which bind to the hypoxia-response element, leading to angiogenesis. Few studies have also reported survival and protection of cells against hypoxic condition independent of angiogenesis; for example, direct effect of VEGF on neuronal survival was observed by preventing motor neuron degeneration. VEGF has also been reported as autocrine survival factor for embryonic stem cells under hypoxic condition [8].

VEGF induces expression of antiapoptotic proteins Bcl-2 and A1 survivin acting as a survival factor for immature tumor blood vessels. It also promotes endothelial cells survival by activating phosphatidylinositol-3-kinase (PI3 K)/Akt pathway. Thus, VEGF plays a major role in survival of newly formed blood

vessels by affecting vascular permeability and endothelial cell migration and proliferation [10].

VEGF and TGF-β1 are involved in cell apoptosis and activation of $P38^{MAPK}$ by VEGF promotes endothelial cell survival [11].

VEGFs bind to three types of receptor tyrosine kinases i.e. VEGFR-1, VEGFR-2, and VEGFR-3. As VEGF binds to the receptor, it causes homodimerization of receptor leading to autophosphorylation of the tyrosine kinase domains. This activates downstream signal-transduction pathways [12]. VEGF is reported to act as survival factor in breast cancer cells by binding to VEGFR-1 [13].

VEGFR-1 binds to VEGF-A, VEGF-B, and PIGF, whereas VEGF-A, VEGF-C, VEGF-D ,and VEGF-E bind to VEGFR-2. VEGFR-1 regulates angiogenesis by ligand trapping, receptor homo-and hetero dimerization mechanisms [14]. VEGFR-2 is essential for mitosis in endothelial cells and recruits signaling proteins to stimulate downstream signaling pathways. VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells. Additionally, VEGFR-1 is expressed by monocytes, macrophages, and other non endothelial cells. VEGFR-2 is also expressed by hematopoietic stem cells [15].

VEGFR-2 is considered to be the most important receptor responsible for angiogenic activity of VEGF. Matsumoto et al. generated a phosphorylation sitemap of VEGFR-2 and identified Y951 as a major tyrosine phosphorylation site in kinase insert domain in addition to two known sites, Y1175 and Y1214 in C-terminal tail [16].

VEGFR-3 binds to VEGF-C and VEGF-D and has major role in development of the lymphatic vasculature. It is expressed by lymphatic endothelium and blood capillaries of few organs. VEGFR-3 pathway is also reported to contribute to tumor angiogenesis [17].

23.3 Role of VEGF in Various Diseases

Many diseases have been reported to involve angiogenesis. Insufficient angiogenesis is related to diseases like diabetes, atherosclerosis, coronary artery diseases, and chronic wounds. Diseases like cancer, psoriasis, arthritis, and obesity often involve excessive angiogenesis. Hence, understanding the role of growth factors responsible for angiogenesis gets a prime role to play in therapeutics [1].

Primary solid tumors are capable of growing only to 1–2 mm in diameter in the absence of blood supply. Beyond this minimum size, the growing tumor becomes necrotic and apoptotic. For further growth under hypoxic conditions, malignant cells secrete growth factor signals within their environment leading to an "angiogenic switch" that alter the balance of pro- and anti-angiogenic factors and trigger neovascularization, supporting further malignant growth. Physiologic and tumor angiogenesis is regulated by VEGF for endothelial cells. Inhibition of VEGF activity suppresses growth of tumor cells denoting its crucial role in cancer treatment.

Various anti-angiogenic therapies involving VEGF have been used for colorectal, breast, and lung cancer treatment [18, 19]. As human female reproductive tract is dependent on VEGF for endometrial proliferation and development of *corpus luteum*, therapy affecting VEGF activity has gained the importance for treatment of ovarian and cervical cancer [20]. Hematological malignancies like leukemia, lymphoma, and myeloma have been treated with angiogenesis inhibitors affecting the entire signaling pathway of angiogenesis [21]. Few new approaches for treatment of pancreatic cancer are under development along with use of predictive biomarkers [22].

Winkler et al. characterized the effect of monoclonal antibody against VEGFR-2 on morphological, functional, and molecular changes in the vasculature of glioblastoma. Vascular basement membrane of glioma vessels showed abnormalities related to VEGF signaling and treatment involving VEGFR-2 inhibitor restored the membrane along with temporary improvement in tumor oxygenation and response to radiation therapy. Thus, synergistic effect of multiple therapies should be optimized based on mechanism and timing of tumor response to the angiogenesis inhibitors [23]. A study showing different effects of VEGFRs inhibition depending on site of tumor was elaborated by Yoon-Jin et al. Treatment with VEGFR-1 antibody showed selective inhibition of liver metastasis, whereas lung metastasis was inhibited by VEGFR-2 antibody with no effect of VEGFR-1 antibody [24].

Many diseases affecting cardiovascular system involve endothelial dysfunction and ischemia. In such cases, the coronary revascularization therapy may get beneficial effects from therapeutic angiogenesis by supplying additional blood flow. VEGF along with bFGF has been reported to modulate capillary growth and facilitate expansion of large arterioles [25]. But, VEGF administration is related to few side effects such as hypotension due to nitric oxide release and arteriolar vasodilatation, plaque angiogenesis, occult malignancy, and proliferative retinopathy.

Zentilin et al. reported a study including role of VEGF in cardiovascular system. Apart from angiogenic properties, VEGFR-1 expression, activation, and signaling is established as an important mediator of cardiomyocyte function in rat experiments. Prolonged expression of VEGF-A and VEGF-B led to improvement in cardiac function after myocardial infarction and diminished fibrosis in rats [26].

Macular edema and abnormal retinal blood vessel growth lead to diabetic retinopathy. High blood glucose level in diabetes induces hypoxia in retinal tissues, leading to the production of VEGF-A. As hypoxia is a key regulator of VEGF-induced ocular neovascularization, therapeutic approach inhibiting VEGF activity is useful in therapeutics [27].

Angiogenesis is also the major cause of visual loss in age-related macular degeneration for which an approach of counteracting VEGF seems to be beneficial. VEGF is recognized as a key mediator of choroidal neovascularization [28] and VEGF-A has been reported as the survival factor for the developing and mature retina with stimulation of endothelial and neural cells. Treatment with pegaptanib is found to target VEGF165 only, the isoform responsible for ocular

neovascularization and so acts as an ideal target for treatment of age-related macular degeneration [29]. A study reported involvement of VEGF-A and autocrine VEGF-A/VEGFR-2/PI3K/Akt pathway in retinal pigment endothelial cell survival under oxidative stress [7].

Expression of VEGF is also considerable during wound healing which involves revascularization of damaged tissues. Platelets and monocytes on activation release VEGF, which assist in clot formation. Platelets release PDGF and TGF β in addition to VEGF, which in turn release TNF-β and bFGF, all leading to formation of new blood vessels in wounds [1].

VEGF inhibitors could be useful in treatment of rheumatoid arthritis by interfering with angiogenesis, hypoxia, oedema, and inflammatory vascular tissue generated by vascular permeability factor, VEGF [30]. Osteoarthritis is characterized by articular cartilage degradation, abnormal remodeling of subchondral bone, and synovial membrane inflammation. It includes angiogenesis and inflammation of synovial membrane leading to joint swelling and pain [31]. A study reported increased expression of VEGF by synovial fibroblast cells induced by interleukin in osteoarthritis patients [32].

Elevated levels of VEGF in psoriasis also suggest its possible role in vascular changes and inflammation seen in the disease [33]. A report of TNF-α-targeted therapy explains improvement in clinical signs and symptoms of psoriasis. Reduced VEGF expression, deactivation of vascular endothelium, and inhibition of vascularity were found to be responsible for decreased cell infiltration in skin and synovial tissues [34].

Lafuente et al. showed over expression of VEGFR-2 in astrocytes and neuronal somata after brain injury. Blood–brain barrier permeability is also affected by VEGFR-2 which can play a role in therapeutics [35]. Preeclampsia is a pregnancy-specific syndrome which involves onset of hypertension and proteinuria after 20 weeks of gestation as a consequence of endothelial dysfunction. The syndrome occurs due to imbalances between proangiogenic factors, such as VEGF, PIGF, and anti-angiogenic factors. The alteration in level of VEGFR-2 may act as a marker of endothelial dysfunction [36].

23.4 Therapeutic Strategies Involving VEGF

23.4.1 Anti-Angiogenic Therapy

Anti-angiogenic therapy involves matrix metalloproteinase (MMP) inhibitors, inhibition of endothelial cell proliferation, antibodies to growth factors, and their receptors, endothelial growth factor receptor (EGFR) inhibitors, calcium channel inhibitors, cyclooxygenase-2 (COX-2) inhibitors, integrin inhibitors, interferon, small molecules receptor tyrosine kinase (RTK) inhibitors, vascular targeting agents, endogenous inhibitors, and so on. [37].

The strategies used for anti-angiogenic approach with VEGF are as follows:

- VEGF-neutralizing antibodies: bevacizumab (colorectal, nonsmall cell lung and breast cancer) [21, 38], ranibizumab (for colic cancer and macular degeneration), pegaptanib (for macular degeneration) [28].
- VEGFR antibodies or VEGF trap, a soluble receptor with binding domain of VEGFR-1 and VEGFR-2 targeting VEGF-A, VEGF-B and placental growth factor.
- Antisense oligonucleotides targeting VEGF-A, C and D.
- Small molecule tyrosine kinase inhibitors: Specific VEGFR-2 inhibitors are small molecules which are able to pass through cell membrane, interact with intracellular domain of receptor and signaling molecules acting as anti-angiogenic agents [39, 40]. Substituted indolin-2-ones, phthalazines, anthranilamides, quinazolines, ureas, pyrimidine, triazines, thiazoles, oxazoles, imidazoles, indenopyrrolocarbazolones, pyridine, and indazoles derivatives have been reported as VEGFRs inhibitors [41].
- Prevent VEGF secretion: PlGF-1, a member of the VEGF family lack detectable angiogenic activity and forms intracellular hetero dimers preferentially with VEGF in cells co-expressing both factors. Lung carcinoma cells with retroviral vector containing PIGF-1 showed inhibition of tumor growth [42].
- VEGFR antagonist: A Peptoid antagonist of VEGFR-2 is reported to inhibit autophosphorylation of receptor by an allosteric mechanism and prevent subsequent downstream signaling pathway [43]. Udugamasooriya et al. developed a pharmacophore model with structural requirements for binding of the peptoid antagonist to VEGFR-2 which would help in improving potency of the antagonist [44].

A study involving structural analysis of binding sites of type I and II kinase inhibitors was performed by Liu et al. Type I kinase inhibitor binds to the ATP binding site through hydrogen bond in hinge region and show hydrophobic interactions in the region close to adenine ring of ATP. Type II inhibitors use the ATP and allosteric binding sites exhibiting hydrogen bonding and hydrophobic interactions with residues of the activation loop. This binding of molecules is characteristic of inactive conformation of kinases. Further detailed study may lead to generation of high affinity, potent, and selective kinase inhibitors [45].

Inhibition of VEGFR and EGFR pathways in combination with irradiation therapy observed additive anti-tumor effect on human head and neck cancer xenografts and such combination trials may provide better therapeutic effect [46]. Whereas EGFR and VEGF targeted antibodies together in the clinical trials did not show desirable effects due to failure of intracrine signaling pathways inhibition [47].

Sumariwalla et al. reported protease-activated kringles 1–5 as angiogenesis inhibitor for treatment of arthritis. Kringles 1–5 are related to angiostatin and contain the first five kringle domains of plasminogen which confer enhanced anti-angiogenic activity when compared with angiostatin [48].

Many types of cancer involve lymph node as the site of metastasis. Combination of anti-VEGFR-2 and anti-VEGFR-3 antibodies showed decreased lymph

Table 23.1 Physiological role of VEGF and side effect of anti VEGF therapy [39]

Physiological role of VEGF	Side effect of VEGF inhibition	Mechanism of side effects	Current treatment for side effects
Phosphorylation of endothelial nitric oxide synthase—production of nitric oxide— coronary artery relaxation, dilatation of small arterioles, and venules	Hypertension Reduced density of microvessels in tissues and organs (Rarefaction)	Vasoconstriction Proteinuria Cardiovascular and cerebrovascular thromboembolism	Use of vasodilators such as angiotensin converting enzyme inhibitors, angiotensin II blockers, and calcium antagonists Interruption and/or decreased dose of drugs of treatment
Endometrial recovery after menstrual bleeding, wound healing, and surgical adhesion formation	Bleeding (tumor dependant)	Local hypoxia, inferior quality of blood vessels in the vicinity of primary tumors or metastases	Interruption of drugs of treatment Exclude patients with known cerebral metastases and anticoagulant therapy
–	Gastrointestinal perforations (tumor dependant)	Local ischemia due to local perfusion	Contradiction of further treatment
Mucosal integrity	Voice changes (hoarseness)	Functional dysphonia, vocal cord paralysis, reflux laryngitis, Reincke's edema, laryngeal papillomas, and vocal nodes	Interruption of drugs of treatment
Maintenance of inflammatory response	Mucositis, gastrointestinal, and skin toxicity	Unclear	Prophylactic application of proangiogenic factors Interruption of drugs of treatment
Role in normal thyroid physiology	Fatigue (Dose dependant)	Hypothyroidism	Interruption and/or decreased dose of drugs of treatment

(continued)

Table 23.1 (continued)

Physiological role of VEGF	Side effect of VEGF inhibition	Mechanism of side effects	Current treatment for side effects
Structural and functional integrity of the liver	Transaminase elevations	Direct effect on hepatocytes	Interruption and/or decreased dose of drugs of treatment
Neuro protective	Neurological complications (seizures, dizziness, and ataxia)	Unclear	Interruption of drugs of treatment
Erythropoiesis and myelopoiesis	Myelosuppression, neutropenia, and thrombocytopenia	Kinase inhibition	Interruption of drugs of treatment

node and lung metastases than when each antibody was administered alone in breast cancer metastasis model [49]. Another study revealed reduction of blood vessel density and lymphatic vessels in tumor tissue exposed to anti VEGFR-3 antibody [17].

Several molecular modeling techniques have been used to assist development of potent VEGFR-2 inhibitors for anti-angiogenic activity. Docking and quantitative structure activity relationship (QSAR) studies were carried out in our laboratory for 1, 3-diarylpropenone analogos as VEGFR-2 inhibitors. The hydrophobic and hydrogen bonding interactions between ligands and the receptor illustrated requirement of aromatic ring and hydrogen bond acceptor groups in the structure [50].

Neaz et al. performed a QSAR study for 4-aryl-5-cyano-2-aminopyrimidines as VEGFR-2 inhibitors. Steric and hydrogen bond acceptor groups were shown to be important in pharmacophore and 3-D QSAR models [51].

23.4.2 Angiogenic Therapy

Therapeutic angiogenesis involve conventional VEGF based and cell-based strategies. The angiogenic therapy enhances revascularization process by improving ischemic condition. VEGF protein and gene delivery are the conventional ways, whereas cell-mediated gene transfer encoding for angiogenic factors is a new approach for the therapy [52].

23.5 Development of Side Effects and Resistance to Anti-VEGF Therapy

Variation in response of patients to anti-angiogenic therapies focused on issues like, development of side effects, resistance and individually varied response to these molecules. This also made it important to understand the mechanisms that mediate side effects and resistance to anti-angiogenic therapy.

Few side effects were observed for angiogenesis inhibitors though these molecules were expected to be nontoxic. Dose-dependant life threatening side effects common to cytotoxic agents were not observed, but clinical studies detailed about some of the drug-related unwanted effects mainly based on decreased VEGF activity (Table 23.1) [53].

Tumor and nontumor cells have been correlated to reduced response of patients to treatment of cancer and development of resistance [54]. Ellis and Hicklin explained diverse mechanisms of resistance against VEGF therapy depending on tumor type. Induction of compensatory angiogenic signaling pathways, role of microenvironment, and $CD11b^{+}Gr1^{+}$ myeloid cells have been responsible for the development of resistance for anti VEGF/VEGFR therapy [55].

Casanovas et al. reported a phenotypic resistance to treatment with VEGFR-2 inhibitor. The tumors displayed re-growth after an initial period of growth suppression during treatment. The resistance is believed to occur due to reactivation of tumor angiogenesis associated with hypoxia and stimulation of other proangiogenic factors like FGF family [56].

Researchers have attempted measurement of tumor angiogenic activity to provide prognostic information before treatment using angiogenesis inhibitors. Tumor microvessel density, intra-tumoral VEGF level, and circulating VEGF are determined to assess angiogenesis [57].

23.6 Conclusion

VEGF has a key role in the signaling pathways of angiogenesis and survival of cells. Therefore, development of a therapy involving angiogenic growth factors will provide a new alternative in future. Affecting VEGF signaling pathway represents a promising treatment in various diseases targeting survival, growth, and the development of cells which should be carefully combined with use of biomarkers and other suitable approaches like multi-target kinase inhibition.

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Chapter 24
The Role of CaM Kinase II in Cardiac Function in Health and Disease

Adriana Adameova, Adrian Szobi, Slavka Carnicka, Tanya Ravingerova and Tomas Rajtik

Abstract Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) has emerged as a critical molecule involved in the regulation of cell processes that are dependent on calcium, including excitation–contraction coupling (ECC), cell growth, and death. In addition to a generally accepted signaling pathway through beta-adrenergic receptors (ARs), oxidative stress has been suggested to promote CaMKII activation. Since many cardiac diseases, including those characterized by a phenomenon called as ischemia/reperfusion injury (IRI), are associated with oxidative stress, CaMKII is likely to be a crucial molecule underlying the phenotypes of this cardiac injury. In contrast, there is also evidence that CaMKII activation leading to phosphorylation of phospholamban and the subsequent decrease of calcium overload is important for attenuation of post-ischemic cardiac contracture, indicating that CaMKII may act as a double-edged sword depending on the actual conditions. In addition, CaMKII over-activation has been shown to destabilize the action potential and trigger early and delayed afterdepolarizations promoting arrhythmias. Experimental studies from our laboratory have revealed that CaMKII inhibition does not protect the heart against all types of IRI-induced ventricular arrhythmias, but it is capable to reduce the occurrence of the most life-threatening tachyarrhythmias. Moreover, the CaMKII inhibition appears to reduce oxidative stress and thus to increase the viability of cardiomyocytes upon IRI. In this manuscript, a dual role of CaMKII in IRI is reviewed and beneficial effects of the CaMKII inhibition are discussed with studies that have shown the opposite results.

A. Adameova (✉) · A. Szobi · T. Rajtik
Department of Pharmacology and Toxicology, Comenius University, Odbojarov 10, 832 32 Bratislava, Slovak Republic
e-mail: adameova@fpharm.uniba.sk

S. Carnicka · T. Ravingerova
Institute for Heart Research, Slovak Academy of Sciences and Centre of Excellence NOREG SAS, Bratislava, Slovak Republic

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We conclude that CaMKII activation either inhibition should be carefully considered in effort to mitigate cardiac IRI-induced injury.

Keywords Calcium/calmodulin-dependent protein kinase · Ischemia–reperfusion injury · Arrhythmias · Contractile dysfunction · Cell death · Oxidative stress

24.1 Introduction

In spite of considerable effort undertaken so far, exact mechanisms of the development of phenotypes of myocardial ischemia/reperfusion injury (IRI), such as arrhythmias, contractile dysfunction, and cell death, are not still satisfactorily elucidated. Impaired calcium homeostasis has emerged as a crucial event initiating IR-induced cardiac dysfunction and hence, many calcium handling proteins have been suggested to be a potential target to mitigate the outcomes of this type of cardiac injury. Recently, a role of calcium/calmodulin-dependent protein kinase (CaMKII), a protein kinase regulating intracellular calcium levels and activated by calcium itself, has raised interest of many investigators. In the heart, CaMKII, a multimer consisting of 8–12 subunits, is expressed in two isoforms, such as the delta and gamma (CaMKIIδ, CaMKIIγ); however, the CaMKIIδ is the predominant one [1]. CaMKIIδ has different splice variants, which are localized in the nucleus (δ_B) and the cytoplasm (δ_C), suggesting that different cellular processes can be regulated by this protein kinase. The cytoplasmic isoform regulates many of the key proteins of excitation–contraction coupling (ECC), while the nuclear isoform is likely to be important for the activation of various transcriptional factors regulating cellular growth and death [1, 2]. In addition, the expression of the splice variants of CaMKIIδ is age dependent. In fact, δ_B predominates in the adult heart, while δ_C is abundant in the embryonic heart [2].

24.2 Activation of CaMKIIδ in Physiological and Pathological Conditions

Under resting conditions CaMKIIδ is inactive; however, once the levels of intracellular Ca^{2+} are increased and Ca^{2+} is bound to calmodulin to form Ca^{2+}/CaM complex, which is subsequently bound to the regulatory domain of the kinase, the autoinhibitory effect of the domain is released and the kinase becomes activated. In conditions characterized by the sustained formation of Ca^{2+}/CaM, the catalytic domain of the kinase can activate a neighboring subunit at threonine 286/287 ($Thr^{286/287}$). This process referred to as autophosphorylation results in the persistent activation of CaMKIIδ, indicating that conditions that promote autophosphorylation increase CaMKII activation and thus may change the

physiological action of the kinase into the pathological effect [3]. One of the mechanisms leading to the significant elevation of intracellular Ca^{2+} is the stimulation of the beta-adrenergic receptors (ARs) that result in the activation of protein kinase A and subsequently in Ca^{2+} influx through the L-type calcium channels (LTCC) [4]. As the levels of catecholamines are increased in ischemic tissue, persistently elevated beta-ARs stimulation during IRI favors CaMKII autophosphorylation. Phosphorylation of CaMKII itself at $Thr^{286/287}$ has several important implications, such as the increase in the affinity of Ca^{2+}/CaM binding termed "CaM trapping", and maintaining catalytic activity of the kinase even in the absence of CaM binding [5, 6]. On the other hand, the phosphorylation of CaMKII at Thr^{306} prevents Ca^{2+}/CaM binding that results in the decreased CaMKII activity. This type of autophophosphorylation of CaMKII is considered to be the self-inhibitory effect, which provides a negative feedback regulation of the kinase activity, in particular, under resting/basal intracellular Ca^{2+} levels [7]. Another potential mechanism, which is assumed to participate in CaMKII activation during IRI is the production of reactive oxygen species (ROS). It has been shown that in the presence of oxidative stress, a pair of methionine residues within the regulator domain ($M^{281/282}$) become oxidized that results in the allosterical rearrangement of the domains of the kinase and consequently into the initiation/promotion of the Ca^{2+}/CaM-independent activation of CaMKII [8]. ROS leading to CaMKII activation have been found to be generated by NADPH oxidase upon the stimulation of angiotensin II [9]. Recently, Wagner et al. [10] have demonstrated that free calcium and a functional sarcoplasmic reticulum (SR) are required for ROS activation of CaMKII and that ROS-activated CaMKIIδ further enhances late Na current (I_{Na}), which in turn may lead to cellular Na^+ and Ca^{2+} overload [10]. Consequently, CaMKII-dependent changes in late I_{Na} have been suggested to be a major contributor to cellular arrhythmias [11]. In line, I_{Na} inhibition has been found to reverse arrhythmias in a transgenic Ca^{2+}/calmodulin-dependent protein kinase IIδ_C mice developing heart failure [12]. Hence, it appears that functional consequences of CaMKII activation under pro-oxidant conditions are initially dependent on Ca^{2+} content, but later on they may be propagated due to oxidative stress rather than due to Ca^{2+} overload (Fig. 24.1). This may also be of relevance in conditions associated with IRI, where ROS production is enhanced, indicating a link between oxidative stress and CaMKII-mediated cardiac injury.

24.3 Proteins Phosphorylated by CaMKIIδ

Early studies have revealed that the CaMKIIδ_C phosphorylates and thus activates LTCC, both the predominant pore-forming subunit LTCCα ($Ca_v1.2$) and the β-subunit ($Ca_v1.3$) and thus mediates gradation of I_{Ca}, a process termed I_{Ca} facilitation [13–15]. CaMKII phosphorylation sites are presently unknown on the LTCCα subunit, while the phosporylation of Thr 498 on the LTCCβ subunit is necessary for increases in LTCC opening and dynamic facilitation responses in

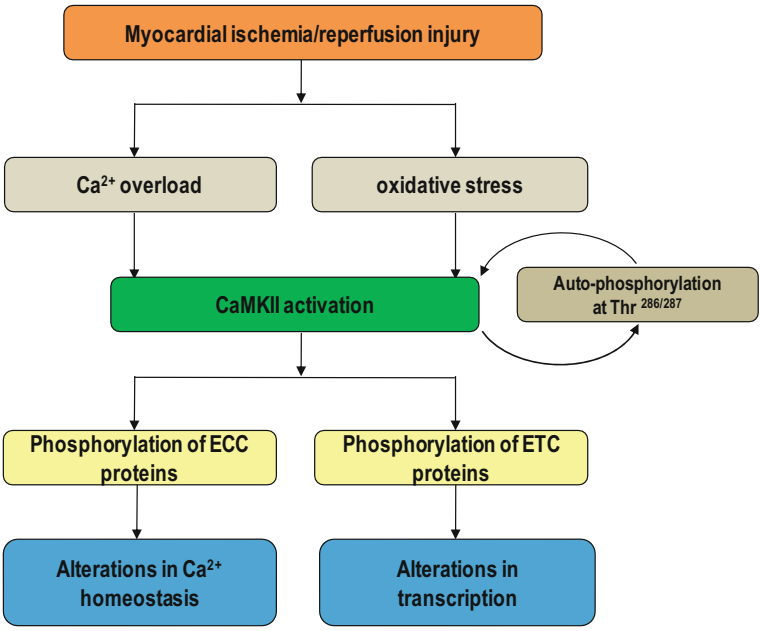

Fig. 24.1 Proposed myocardial ischemia/reperfusion-induced pathways leading to CaMKII activation, subsequent increase in phosphorylation of target proteins and its consequences. *ECC* excitation–contraction coupling, *ETC* excitation-transcription coupling, *Thr*$^{286/287}$ phosphorylation at the site of threonine 286/287

cardiomyocytes [13]. It is known that I_{Ca} in addition to increase in Na current increases net inward currents during the action potential plateau resulting in the action potential duration (APD) prolongation that is observed on electrocardiogram as the long QT interval. As consequence of excessive APD prolongation, the heart becomes more prone to the development of early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) that further give raise to various types of arrhythmias, including atrial fibrillation and ventricular tachyarrhythmias [16]. It has been shown that the CaMKII-induced hyperphosphorylation of the LTCC and of the ryanodine receptor (RyR), which induces Ca^{2+} leak from the SR and subsequently a net inward Na current via NCX, is associated with the induction of EADs and DADs, respectively [17, 18]. Association of CaMKII activation with APD prolongation and induction of EADs has also been shown upon the administration of clofilium, a K^+ channel antagonist drug that is along with other drugs of class III anti-arrhythmic class known to reduce repolarising currents [19].

Other Ca^{2+}-handling protein activated by CaMKII is the ryanodine receptor that upon Ca^{2+} entry through LTCC mediates release of Ca^{2+} from the SR. This process is referred to as Ca^{2+}-induced Ca^{2+} release. Although Ser^{2809} and Ser^{2815} have been shown to be phosphorylated by CaMKII, additional phosphorylation sites of

the RyR cannot be ruled out [20]. CaMKII action on Ca^{2+} release via the RyR is controversial. Cellular studies have revealed that the hyperphosphorylation of the RyR results in inappropriate diastolic Ca^{2+} release from the SR and thus contributes to impaired cardiac contractility and promotion of DADs. In mice, in which the S^{2814} Ca^{2+}/calmodulin-dependent protein kinase II site on RyR2 is constitutively activated, pathological SR Ca^{2+} release has been found to contribute to fatal pacing-induced arrhythmias, while genetic ablation of the CaMKII site on RyR2 protected mutant mice from arrhythmias [21]. These findings have supported an important role of CaMKII-mediated activation of RyR in arrhythmogenesis and sudden cardiac death. In addition to the increase, the decrease of Ca^{2+} release from the SR as a consequence of CaMKII overactivation has been shown [22, 23], supporting the view that CaMKII has a regulatory action on the RyR. This further seems to be determined by the presence/absence of the pathological state. Whether it is a CaMKII stimulatory or inhibitory action on the RyR function, and in which particular conditions, remains to be investigated in detail. In contrast to the above discussed Ca^{2+} handling proteins, which increase the intracellular Ca^{2+} levels, the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) acts oppositely and removes cytoplasmic Ca^{2+}. In humans, SERCA is responsible for about 70 % of cytoplasmic Ca^{2+} removal, while the remaining Ca^{2+} content is removed by the sodium/calcium exchanger (NCX) [24]. Under resting conditions, SERCA is inhibited by phospholamban (PLN) and its phosphorylation at Ser^{16} or at Thr^{17}, which is regulated by protein kinase A and CaMKII, respectively, relieves this inhibitory action. As consequence, this phosphorylation accelerates Ca^{2+} removal from the cytoplasm, and subsequently increases the SR Ca^{2+} content [25, 26]. With respect to SERCA regulation of frequency-dependent acceleration of relaxation (FDAR) determined by the Ca^{2+} removal, it has been shown that CaMKII is an important mediator of this property of cardiomyocytes, but it is unknown which key proteins underlie this process. It has been suggested that the modulatory effects of CaMKII on the PLN and secondary on the SERCA are more important in conditions characterized by slower, irregular frequencies, while they are likely to be less active under physiological, high steady-state frequencies [27].

24.4 Role of CaMKII in the Development of Phenotypes of Myocardial Ischemia/Reperfusion Injury

24.4.1 CaMKII as a Critical Component of Cascades Leading to IRI-Induced Ventricular Arrhythmias and Contractile Dysfunction

Based on findings that CaMKII overexpression and/or activity are increased in a mouse model of failing heart, which is known to have proarrhythmic remodeling, action potential prolongation, besides impaired contractile function, hypertrophy

and left ventricle chamber dilatation, CaMKII has been suggested to induce/promote electrical instability of the heart [28, 29]. Likewise, in the other mouse model of hypertrophied heart with the CaMKIV overexpression, which was associated with the increased CaMKII activity, the APD prolongation and the induction of EADs have been recorded [18]. In addition to these animal models, CaMKII has also been shown to be a proarrhythmic molecule in a model of catecholaminergic polymorphic ventricular tachycardia (CPVT). In this inherited arrhythmogenic disease characterized by the cardiac RyR2 mutation, which leads to abnormal diastolic Ca^{2+} leak from the SR upon adrenergic stimulation, CaMKII has been suggested to play an important role in the generation of the fatal arrhythmias [30]. CaMKII-dependent phosphorylation of the RyR, resulting in Ca^{2+} leak from the SR, has also been suggested to underlie mechanisms of digitalis-induced arrhythmias [31]. However, the CaMKII-mediated genesis of ventricular arrhythmias in conditions of IRI, which differ in several cellular and molecular mechanisms from all above-mentioned types of arrhythmias, is less known. We have clearly shown that CaMKII is involved in the genesis of R-induced ventricular arrhythmias. Although KN-93, an inhibitor which binds to the regulatory domain of the CaMKII, did not show any capability to modulate the incidence and duration of ventricular tachycardia, it protected the heart against the most life-threatening arrhythmia—ventricular fibrillation (VF) (Fig. 24.2a, b). Further analysis of the genesis of VF has revealed that the mean number and the duration of a single episode of VF were significantly decreased in the KN-93-treated group (Fig. 24.2c, d). In accordance with the study of Bell et al. [32], only one of eight KN-93 treated hearts exhibited ventricular tachycardia and/or fibrillation. Although in that study and ours, KN-93 was used to study CaMKII implication in IRI-induced arrhythmogenesis, the dose of the CaMKII inhibitor was different. In our studies, we use a dose of 0.5 μmol/l that is the fivefold lower than the dose used in other laboratories [32–34]. Pedersen et al. [35] have shown that increased CaMKII activity induces arrhythmias during metabolic acidification and that this effect is associated with spontaneous Ca^{2+} waves, which in turn are abolished by KN-93. Since acidosis is present during IRI, it is likely that this mechanism underlies the genesis of IRI-initiated arrhythmias. Interestingly, in our recent study, we have shown that the increased expression of the total CaMKIIδ in the hearts subjected to IRI co-exist with the higher NCX1 content, while there is such no co-existence with the expression of LTCCα [36]. These findings suggest that alterations in LTCCα protein content are unlikely to participate in CaMKII-depedendent phenotypes of heart injury, including arrhythmias. Thus, it can by hypothesized that the higher protein content of CaMKIIδ may trigger arrhythmias in conditions of IRI due to the increased NCX1-dependent promotion of DADs rather than due to LTCCα-induced EADs. Of note, the beta subunit of LTCC (LTCCβ) has been suggested to be crucial for CaMKII signaling promoting EADs and cardiomyopathy [37]. Said et al. [38] have suggested that CaMKII-dependent phosphorylation of SR proteins, in particular Ser^{2814} on RyR2 and Thr^{17} on PLN contribute to reperfusion-induced arrhythmias. Furthermore, in that study, no detectable EADs in transgenic mice with targeted inhibition of CaMKII at the

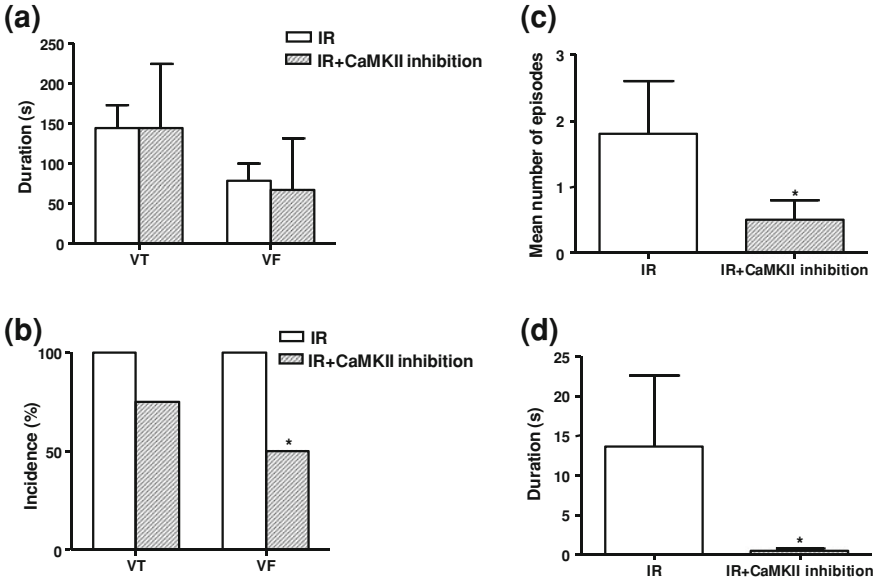

Fig. 24.2 Influence of CaMKII inhibition on reperfusion-induced ventricular arrhythmias after 30 min global ischemia in the isolated rat hearts. **a** The incidence and **b** total duration of ventricular tachyarrhythmias (ventricular tachycardia and fibrillation). **c** Mean number of episodes and **d** duration of a single episode of ventricular fibrillation (B). *$P < 0.05$ vs. non-treated control hearts

level of cardiac SR membranes, while there was the genesis of EADs-induced premature ventricular beats in wild type mice [38].

In addition to disturbances in cardiac rhythm associated with the increased CaMKII expression and/or activity, a role of the kinase in the development of contractile dysfunction, the other phenotype of IRI, has been extensively studied. Although excitation and contraction are a complex, CaMKII action in these two processes can produce the different effects that are independent of either one. In fact, Bell et al. [32] have reported that CaMKII mitigates R-induced ventricular tachyarrhythmias, however, this inhibition diminished contractile performance of the intact heart in the initial post-ischemic period. Moreover, CaMKII inhibition produced negative inotropic effects and increased coronary flow [32]. In contrast, in our recent study, we did not show these unwanted effects of KN-93 on coronary flow or cardiac contractility under baseline conditions. It is noteworthy that any effects of the drug observed during stabilization period can influence the final outcomes of IRI. In our hands, KN-93 administered 10 min prior to induction of I changed neither left ventricular systolic and end-diastolic pressure, nor maximal rates of pressure development and fall as the indexes of contraction and relaxation. On the other hand, CaMKII inhibition improved post-ischemic recovery of contractile function and attenuated diastolic contracture evidenced by a smaller rise of left ventricular end-diastolic pressure [36]. We further analyzed an overall heart

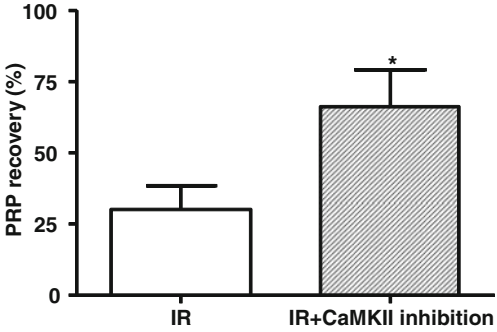

Fig. 24.3 Influence of CaMKII inhibition on the post-ischemic recovery of the pressure-rate product (*PRP*) after 30 min global ischemia and 40 min of reperfusion in isolated rat hearts. *P < 0.05 vs. non-treated control hearts

performance that in the Langendorff-perfused heart is determined as the pressure-rate product (PRP; LVDP x HR). Its better recovery in the KN-93-treated hearts also confirmed that CaMKII negatively affects contractile function of the heart subjected to IRI (Fig. 24.3).

24.4.2 CaMKII as a Mediator of Cellular Death Induced by Ischemia and Reperfusion Injury

It is generally known that the increased intracellular Ca^{2+} levels induce, besides disturbances in ECC, apoptotic and necrotic cell death. The two types of IRI-induced cell death are distinct. Necrotic cell death is characterized by membrane disruption, cell swelling, lysis, and inflammatory response, while apoptosis results in DNA fragmentation, prevents inflammation, and preserves membrane integrity [39]. Some studies have suggested that programmed death is induced by ischemia in the absence of reperfusion [40, 41], while other studies have demonstrated that reperfusion accelerates apoptosis initiated during ischemia [40, 42]. In addition, it has been reported that apoptosis is triggered during reperfusion and does not manifest during ischemia [43]. In the pathogenesis of programmed death, the mitochondria plays an important role, from which pro-apoptotic markers are released through the mitochondrial permeability transition pore (mPTP) and the mitochondrial apoptosis channel (mAC, or Bax channel). Their opening is dependent on the intracellular calcium levels; mAC/Bax channel may open in cells that are unloading calcium, while conditions associated with calcium overload cause and sustain mPTP opening [44, 45]. Of note, cells subjected to sustained mPTP opening are destined to die by necrosis; however, cells in which mPTP reverses before out-membrane rupture could either avoid necrosis and die by apoptosis or survive [46]. mPTP forming and opening is chiefly regulated by Bcl-2 family proteins; predominance of anti-apoptotic markers (Bcl-2, Bcl-xL) over the apoptotic ones (Bax, Bad) preserves mPTP opening and release of cytochrome C into the cytoplasm, where it is bound with ATP and caspase-9 to form apoptosome [47]. This complex further activates caspase-3 to

drive the execution phase of apoptosis. Since CaMKII regulates the cytoplasmic Ca^{2+} levels, it has been hypothesized that it is somehow involved in signaling pathway leading to IRI-induced cell death. Indeed, it has been shown that the mitigation of Ca^{2+} overload induced by the stimulation of beta-ARs is dependent on the CaMKII-mediated phosphorylation of PLN, thereby implicating PLN as an important regulator of anti-apoptotic action of CaMKII inhibition [17]. In our study, we showed that protein content of cytochrome C, caspase-9, and pro-caspase-3 were decreased by the CaMKII inhibitor (Fig. 24.4a–c). In addition, the pharmacological inhibition of CaMKII by KN-93 decreased the content of pro-apoptotic protein Bax and increased the levels of anti-apoptotic Bcl-2 as well as Bcl-2/Bax ratio (Fig. 24.4d–f). In line, Vila-Petroff et al. [34] have shown that the presence of KN-93 and the CaMKII-inhibitory peptide (AIP) in the perfusion medium are capable to decrease the extent of TUNEL-positive cells, and caspase-3 activity in the hearts subjected to IRI. Furthermore, the findings of that study established a cascade of CaMKII-dependent events integrating the NCX, the SR, and mitochondria that promote intrinsic cellular death. Likewise, Salas et al. [33] have confirmed that CaMKII is involved in the intrinsic apoptotic cellular death and showed that CaMKII does not participate in the extrinsic cascade of apoptosis. Both these studies have also shown that CaMKII is implicated into the regulation of necrotic death; CaMKII inhibition reduced the release of lactate dehydrogenase (LDH), a marker of necrosis [33, 34]. NCX in apoptosis in the hearts subjected to I/R also seems to play a role in our study; indeed, we showed that the increased NCX protein content in the ischemic hearts was downregulated by KN-93 [36]. However, it should be pointed out that the study of Salas et al. [33], Vila-Petroff et al. [34], and ours differ in some very important aspects. First, we used a protocol of reversible IRI, while those scientific groups induced irreversible IRI with a long-term R phase (120 min). Second, in our study, a dose of KN-93 used to inhibit CaMKII was fivefold lower than in the above-cited studies [33, 34]. Of note, higher doses of KN-93 has been reported to inhibit not only CaMKII but also other protein kinases such as, protein kinase C, A, and myosin light chain kinase [48].

Since it is known that oxidative stress is associated with CaMKII activation, and plays a crucial role in the pathogenesis of IRI as well as in the induction of intrinsic cellular death [49, 50], we analyzed protein content of pro- and anti-oxidant enzymes. Although the expression of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), which generates superoxide, was increased in the ischemic hearts, CaMKII inhibition normalized these changes (Fig. 24.5a). On the other hand, the protein levels of superoxide dismutase (SOD), which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, was not changed by the CaMKII inhibitor (Fig. 24.5b). Hydrogen peroxide per se is one of the main contributors of oxidative stress; however, by the action of the other anti-oxidant enzyme, catalase, it is converted into water and oxygen and thereby the damaging effects of hydrogen peroxide are ameliorated. In our hands, the protein content of catalase did not differ among the groups (Fig. 24.5c). Of note, SOD and CAT treatment of isolated hearts has been found to prevent the IRI-induced

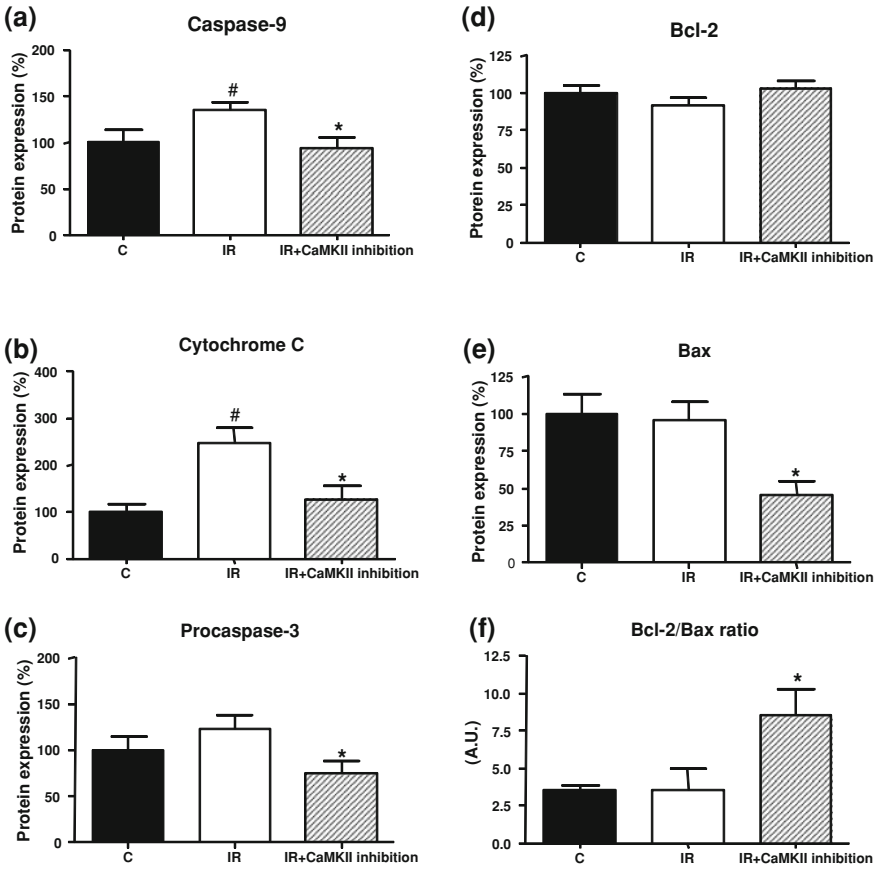

Fig. 24.4 Influence of CaMKII inhibition on extent of apoptosis in the hearts subjected to reversible ischemia/reperfusion injury. Protein content of **a** caspase-9, **b** cytochrome C, **c** procaspase-3, **d** bax, **e** bcl-2, and **f** bcl-2/bax ratio. *$P < 0.05$ vs. non-treated ischemic/reperfused hearts, #$P < 0.05$ vs. baseline pre-ischemic state

alterations in CaMKII phosphorylation of SERCA2a, PLN, and RyR and recover the depressed myocardial function in the hearts subjected to IRI [51, 52]. These findings have confirmed a proposed link between OS-mediated cardiac injury and CaMKII activation.

24.5 Conclusions

From the foregoing discussion it is apparent that CaMKII activation during IRI exhibits rather deleterious than beneficial effects. First, CaMKII is likely to be one of the molecules, at least partially, responsible for the proarrhythmic consequences

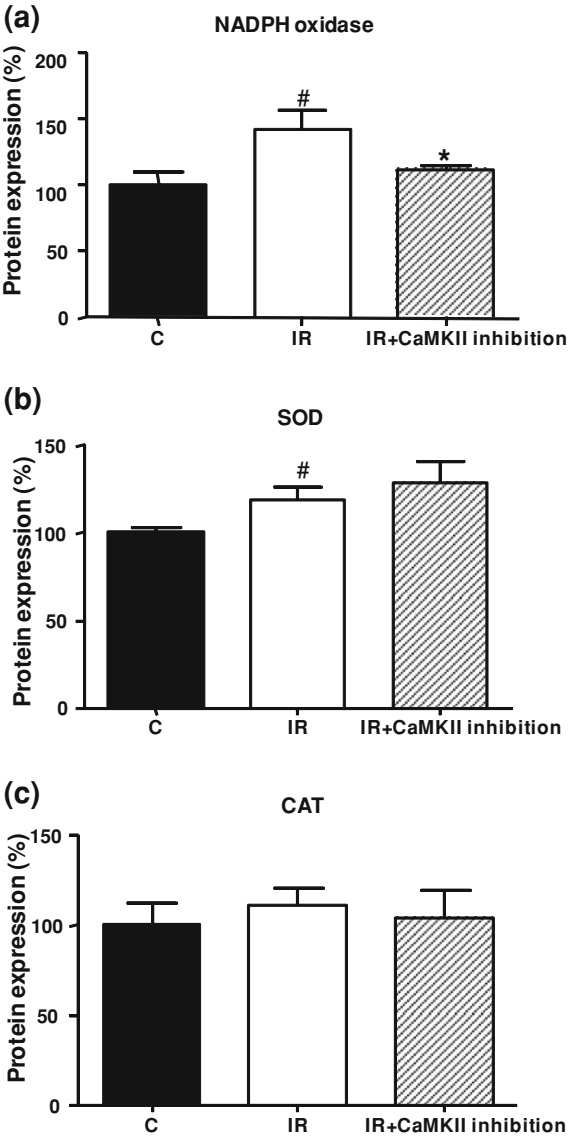

Fig. 24.5 Influence of CaMKII inhibition on protein content of the enzymes regulating oxidative stress in the hearts subjected to reversible ischemia/reperfusion injury. **a** NADPH oxidase, **b** superoxide dismutase (*SOD*), and **c** catalase (*CAT*). *P $<$ 0.05 vs. non-treated ischemic/reperfused hearts, #P $<$ 0.05 vs. baseline pre-ischemic state

of excessive QT interval prolongation. Second, due to Ca^{2+} mishandling it can participate in post-ischemic cardiac contracture. Third, it mediates the alterations in Ca^{2+}-dependent cascades that induce/promote cell death. And last but not the least, it is apparent that oxidative stress has an important role in all these CaMKII-dependent phenotypes of IRI. Based on this, strategies for CaMKII inhibition may represent an effective tool to protect the heart against injury and sudden cardiac death. On the other hand, as it has already been mentioned, Bell et al. [32] have

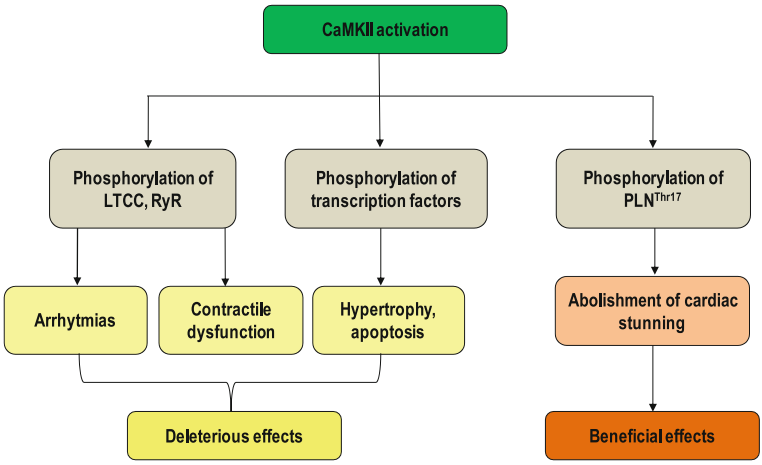

Fig. 24.6 CaMKII as a double-edged sword producing either beneficial or deleterious effects depending on the the conditions of myocardial ischemia/reperfusion injury. *LTCC* L-type calcium channel, *RyR* ryanodine receptors, *PLN* phospholamban

reported that CaMKII inhibition diminishes cardiac stunning of the intact heart in the initial post-ischemic period suggesting that CaMKII activation is important for the abolishment of cardiac contracture. Protective role of CaMKII activity on contractile function abolished by IRI has been suggested to be mainly mediated by the phosphorylation of the PLN. It has been shown that phosphorylation of the Thr^{17} site of the PLN at the onset of R is important for amelioration of Ca^{2+}-mishandling and recovery of cardiac function in the stunned heart [38, 53]. In line, Osada et al. [54] have shown that the IRI-induced depression in Ca^{2+}/calmodulin-dependent SR Ca^{2+}-uptake activity and the IRI-induced decrease in the CaMKII activity can be prevented by preconditioning (PC), whereas KN-93 blocks these effects of PC, suggesting that CaMKII plays an important role in PC-mediated cardio protection. Based on these reports, it is apparent that CaMKII activation in settings of IRI may also exhibit beneficial effects. Taken together, CaMKII may play a dual role in conditions of IRI and thus it may be termed as a double-edged sword (Fig. 24.6). Taken together, we believe that whether CaMKII activation is beneficial or deleterious in conditions of IRI should also be based on consideration of protocol details. It refers mainly to the duration of ischemia, and the dose of a CaMKII inhibitor. The negative effects of CaMKII overactivation have been observed in the case of long-term ischemia, while beneficial effects of CaMKII activation, which were associated with the phosphorylation of PLN, have been reported in a study with a short ischemic insult. The choice of dose of KN-93, which can directly influence a wide variety of target proteins, not only CaMKII alone, is the other point that needs to be taken into consideration while discussing the beneficial or deleterious effects of CaMKII activation. Although it is evident that alterations in CaMKII expression/activity produce direct/indirect changes in ECC during IRI, the exact mechanisms involved are still less known. It is

presumable that the consequences of CaMKII overactivation/expression are mainly linked with Ca^{2+}-mishandling; however, it cannot be ruled out that other proteins may also be influenced. In fact, there are some indications that CaMKII targets also K^+, Na^+ channels [55, 56]. Furthermore, utilizing more selective CaMKII inhibitors, such as CaMKII autoinhibitory peptide, can help to understand better the role of CaMKII in the heart subjected to IRI. Finally, we believe that CaMKII is a potential therapeutic target; however, in order to achieve protection of the heart against injury either by CaMKII inhibition or activation many aspects should be carefully considered.

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