Advances in Biochemistry in Health and Disease

Arunabha Ray
Kavita Gulati *Editors*

Nitric Oxide: From Research to Therapeutics

Springer

Advances in Biochemistry in Health and Disease

Volume 22

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Nitric Oxide: From Research to Therapeutics

Springer

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ISSN 2512-2142 ISSN 2512-2150 (electronic)
Advances in Biochemistry in Health and Disease
ISBN 978-3-031-24777-4 ISBN 978-3-031-24778-1 (eBook)
https://doi.org/10.1007/978-3-031-24778-1

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Nitric oxide (NO) is a ubiquitous signaling molecule that participates in virtually in every cellular and organ function in the body. NO has a fascinating and colorful scientific history, and its evolution from being an environmental pollutant, to a component of explosives (dynamite), and now a gasotransmitter was an outcome of several interesting observations spanning across three decades. The discovery of NO as a signaling molecule in the cardiovascular system was such that three distinguished scientists (Robert Furchgott, Louis Ignarro, and Ferid Murad) were jointly awarded the Nobel Prize in Physiology or Medicine in 1998 for "NO as a signaling molecule in the cardiovascular system." Since the discovery of NO's role in cell signaling, NO has become one of the most researched molecules in recent history. Nearly 100,000 scientific articles have been published on NO and its diverse physiologic effects, pathophysiological significance, and therapeutic applications. There is now an International Society for Nitric Oxide to promote research activity relating to NO, which publishes a well-read journal entitled *Nitric Oxide*. Soon after the identification of NO as a signaling molecule, it was reported that specific nitric oxide synthase (NOS) catalyzes an enzymatic reaction leading to NO formation from the substrates L-arginine and molecular oxygen under highly regulated conditions requiring several cofactors. Subsequently, an alternative NOS-independent pathway of NO synthesis was discovered, based on the simple reduction of nitrate and nitrite, the main oxidation products of NO. During this period, interest in the biological role of NO has led to a revolution in pharmacological and physiological research. In addition to its key role in regulating the cardiovascular function, NO has been reported to be involved in the pathological processes of a variety of human diseases, including, metabolic diseases, inflammatory and immunological diseases, cancer, and neurological diseases. Given the importance of NOSs in the pathophysiology of human diseases, these enzymes are considered potential therapeutic targets for the treatment of diverse human pathologies. Originally known for its regulatory role in cardiovascular physiology and pathophysiology, the domain of NO and NO signaling pathways have now vastly expanded to involve the central nervous system (synaptic plasticity, cognitive function, epilepsy, stress and anxiety, etc.), metabolic disorders, respiratory disease, infections and immunological pathology, critical care, and cancer.

The labeling of NO as a gasotransmitter (alongside CO and H_2S) has also opened up a completely new dimension in NO biology. Within the family of endogenous gasotransmitters, nitric oxide (NO) is the smallest gaseous intercellular messenger involved in the modulation of several physiological processes, such as blood flow and platelet aggregation control, essential to maintain vascular homeostasis. Further, their overlapping physiological roles/pathophysiological significance and interactions have given a new impetus to this field. Different roles of NO are now being advocated operating via distinctly varied signaling pathways. The classical GC-cGMP pathway has been reinforced by alternative signaling pathways which are unrelated to it. NO donors like nitrosoglutathione, NO-releasing nutraceuticals, inhaled NO, NO-releasing nanoparticles, etc., are exciting areas of contemporary translational NO research. Thus, NO is considered as an emerging molecular target for developing therapeutic strategies for a variety of disease states not necessarily restricted to the cardiovascular system. Several naturally derived compounds, such as polyphenols, are now proposed as modulators of NO-mediated pathways.

This book entitled *Nitric Oxide: From Research to Therapeutics* aims to comprehensively collate some of the most recent information about the role of NO in physiology, pathophysiology, and its translation to potential therapeutics. It is a state-of-the-art publication containing the most recent information on NO as a physiological regulator and its diverse potential as a therapeutic target for the treatment of various diseases, which are not necessarily restricted to the cardiovascular system. Specifically, an attempt has been made to include the most recent research developments with NO and its therapeutic implications in a variety of cardiovascular, respiratory, gastrointestinal, neuropsychiatric, metabolic, and infectious disorders. Its role in pregnancy and fetus-related situations like pre-eclampsia and teratogenesis has also received special attention. A special section of the book deals with the role of NO directed therapeutics for COVID-19, the most dreaded pandemic of the century. Newer drug delivery systems for delivering NO to therapeutic targets are also highlighted in this book.

The editors are vastly experienced and internationally reputed in the field of NO research. The authors contributing to the various chapters of the book are all globally established researchers and have made meaningful contributions and illuminated the expanding role of NO in biology and medicine. The book, with its balanced presentation of fundamental and clinically relevant information, will be a state-of-the-art publication and a comprehensive collection of the most recent information on NO and its diverse potential as a therapeutic target. This compilation will be of immense value to medical professionals, biomedical scientists, and students from both academia and the industry in the areas of basic biomedical and clinical sciences, and be a prized possession for the readers. The most recent information provided in this book will help in generating new ideas for further research and translation to rational therapeutics.

The editors are especially grateful to Prof. N. S. Dhalla, Series Editor, Springer-Nature, for providing them with this unique opportunity and for his constant encouragement and guidance at every stage during the realization of this project. The editors wish to express their sincere thanks to all the authors for their unstinted

cooperation and support and timely completion of the book in spite of COVID-19 pandemic situation which has had a major global impact on academic activity. The editors express their heartfelt thanks to Dr. Gonzalo Cordova, Ms. Sara Germans-Huisman, and Mr. Rajan Muthu (all from Springer-Nature), for their unstinted cooperation and support in putting together this book.

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Chapter 1
Translating Nitric Oxide Research to Therapeutics: A Critical Appraisal

Arunabha Ray, Kavita Gulati, and Sana Rehman

Abstract Nitric oxide (NO) is a signaling molecule with an extensive range of functions in both health and disease. Initially proposed as a regulator of cardiovascular function, the significance of NO has now been realized in the neurological, hematological and immune systems. Recent research has indicated complex roles for NO in skeletal muscle, myocardium, metabolic effects like insulin signaling, neurotransmission and cancer biology. Its emerging role as a gasotransmitter and interactions with other reactive molecules has been in the limelight and both pro and antioxidant effects are proposed. Regulation of NO production is determined by numerous factors including arginine bioavailability, co-factors and expression of endogenous regulators. Low (physiological) levels of NO are mostly protective, whereas high levels tend to be toxic. Mitochondria is also the site for the life-threatening deleterious effects arising from inflammation-related excessive NO levels. NO-deficient states are characterized by cell senescence, oxidative stress, inflammation, endothelial dysfunction and insulin resistance. In sepsis, NO synthesis is dysregulated leading to cardiovascular dysfunction, bioenergetic failure and cellular toxicity. NO-enriching therapy may be of benefit not only for its hemodynamic but also for its metabolic impact as well as other effects. In contrast, strategies are needed to curtail excessive NO in states such as septic shock. Thus, both lack and excess of NO production can have various important implications in which dietary factors can play a modulating role. Future research is needed to expand our understanding of the regulation of NO production at the organ level and by the different NOS isoforms. An innovative bench to bedside approach will facilitate the translation of these biological effects at cellular/tissue levels for clinical benefits. Both nutritional and pharmacological approaches are being increasingly adopted to device novel therapeutic strategies by modulating NO levels/activity for several critical pathophysiological conditions.

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_1

Keywords Nitric oxide · Physiological regulation · Therapeutic application · NO modulators

Introduction

Nitric Oxide (NO) is a simple yet complex molecule with a multidimensional physiological role and rapidly evolving pathophysiological significance. The unique concept that a gas synthesized in one cell diffuses into other cell to exert its biological actions revolutionized the field of biology and medicine. The journey of NO from an environmental pollutant to a vasoregulator (EDRF) and chemical messenger has opened up new vistas in disease biology and therapeutics. All these findings along with their therapeutic implications resulted in the Nobel Prize award in 1998 (to Furchgott, Ignarro and Murad) for NO research. NO is now recognized as a gasotransmitter with regulatory effects on cardiovascular functions (vascular tone and permeability, platelet adhesion), neural transmission, immune regulation and mitochondrial function. The complex role of NO is further highlighted by the fact that it is both protective viz antioxidant, inhibits leukocyte adhesion, contribute to antimicrobial defense, as well as harmful effects like suppression of enzyme function, facilitation of DNA damage and promotion of inflammation. Further concentration dependent effects of NO in physiological processes and pathophysiological states have also been documented. It is thus evident, dysregulation of NO mechanisms could precipitate wide range of disease processes which could involve multiple organ. Physiological factors in pharmacological agents that modulate NO levels are currently used to emphasize the role of NO in health and disease. NO modulatory strategies have been applied in cardiovascular, neurological, respiratory, gastrointestinal, endocrinal, reproductive, and inflammatory/immune disorders. In addition to older and newly developed drugs, dietary supplements to boost NO levels for beneficial effects are also being widely advocated [1–5].

NO Formation

The primary source of NO production in the body is by the action of NO synthase (NOS) enzyme on the precursor amino acid substrate, Arginine—commonly known as Arg-NOS-NO pathway. Alternatively, NO can be generated from nitrates and nitrites by the action of NO reductase (NOR) known as the nitrate-nitrite-NO pathway. The former pathway vizArg-NOS-NO pathway is direct and selective. However, in NOS compromised individuals NO deficiency can result into diseases. The nitrate-nitrite-NO pathway, however, is more stable as nitrates and nitrites can be obtained from diet. The enzyme NOS further, NOR is adequately present in all cells/tissues and depletion of NOR is rare. The deficiencies of the Arg-NOS-NO

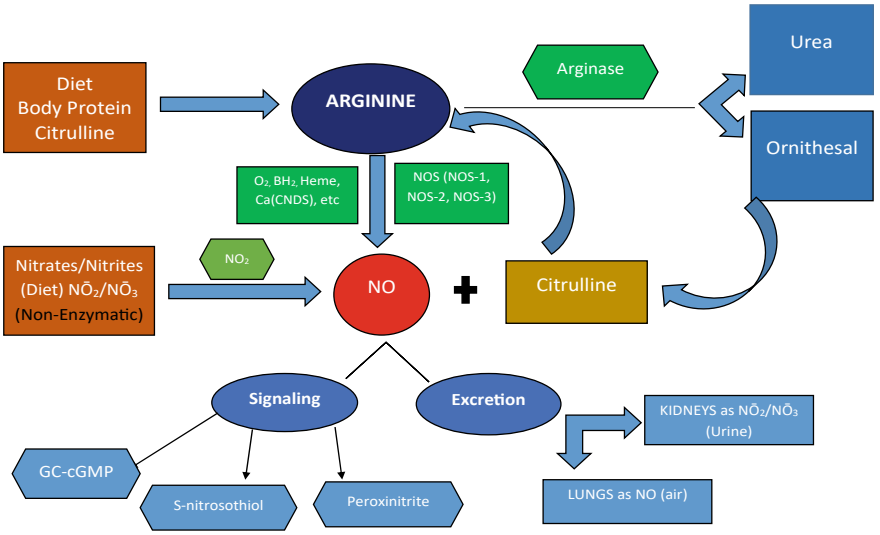

Fig. 1.1 Nitric oxide (NO) generation and signalling NOS: Nitric oxide synthase; NOR: Nitrate reductase; BH4: Tetrahydrobiopterin

pathway ae therefore compensated by the nitrate-nitrite-NO pathway and both pathways complement each other to ensure the continuous availability of NO in the biological system. The role of the Arg-NOS-NO pathway has been implicated in the cardiovascular, nervous (central and peripheral) and immune systems. NOS enzyme exists in three isoforms all of which—use L-arginine and oxygen as substrates to form NO and citrulline throughout the body. This reaction requires cofactors like NADPH, FMN, FAD, and BH4. In addition to NO, citrulline is also formed which is reconverted back to NO (Fig. 1.1).

Arginine is derived from both exogenous (Diet) and endogenous sources (body protein breakdown). Endogenous arginine is dependent on the availability of citrulline which is also the limiting factor for arginine formation. Unlike arginine which undergoes first pass elimination in the gastrointestinal tract, citrulline has a better pharmacokinetic profile. Arginine metabolism is dependent on the degrading enzymes which are differentially present in various tissues. The enzyme NOS accounts for small proportion arginine breakdown and Tetrahydrobiopterin (BH4) is a rate limiting cofactor for such NO production. Oxidative stress results in BH4 deficiency and contribute to NOS3 uncoupling, and increased production of superoxide in place of NO. The fate of arginine is also dependent on intracellular transport, degradation and synthesis. Elevated arginase activity can also contribute to low arginine bioavailability which has been shown in acute and chronic stress situations. Thus it is evident that NO production is not only dependent on arginine availability but also on other factors like cofactors/enzymes. The role of differential arginine metabolism is exemplified in diseases atherosclerosis, hypertension

diabetes, hypercholesterolemia, and ischemic heart disease where NOS3 uncoupling is evident. Strategies to influence endogenous arginine and NO metabolism include enhanced substrate availability, targeting specific metabolic pathways and blocking of NOS3 uncoupling. This can be achieved by dietary supplements of arginine/citrulline, NO donors, NOS3 modulators or interfering with endogenous NOS inhibitor eg asymmetric dimethylarginine [6, 7].

Factors Affecting NO Production

Arginase activity is significant determinant of NO formation. It competes with NOS enzyme for the substrate L-arginine and influences NO bioavailability. Arginase exists in two forms vizarginase-1 (liver) and arginase-2 (kidney, small intestine and endothelial cells). In addition to reduction in NO production, arginase can enhance reactive oxygen species formation and endothelial dysfunction. Overexpression of arginase has been shown in disease conditions like atherosclerosis, hypertension, myocardial ischemia, congestive heart failure, and diabetes mellitus induced complications. Inhibition of arginase activity results in enhanced NO production, lowered oxidative stress and provides cardiovascular protection. Therefore, arginase is the potential therapeutic target for cardio-metabolic targets by modulating interactions between NO and reactive oxygen species. Recent studies have also shown that arginase2 can also contribute to the bioavailability of NO, endothelial dysfunction, atherosclerosis and other disorders. Further, pathogen induced inflammation up regulates arginase1 and iNOS in macrophages which also results in reduced arginine availability and NOS3 dysfunction (via eNOS). Since attenuation of arginase activity increases NO production, pharmacological modulation of this enzyme may be an exciting option where there is NOS3 dependent reductions in NO formation eg sepsis [8–10].

Asymmetric dimethyl arginine (ADMA), an arginine analog/variant and endogenous, competitive NOS inhibitor, can also influence NO bioavailability. Arginine and ADMA use the same transporter for cellular transport and since both competes for NOS, Arginine-ADMA balance determine NO levels in the body. Dysregulation of the ADMA degrading enzyme dimethyl arginine dimethylaminohydrolase (DDAH) is also a contributory factor for cardiovascular morbidity and mortality via elevated ADMA levels. The cardiovascular effects of ADMA is also due to its inhibitory effects on endothelial cell motility and angiogenesis. Clinically, Arginine-ADMA ratio is a good predictor of NO availability and atherosclerosis. This ratio is also a more consistent indicator of mortality from any cause in the geriatric agr group, as compared to ADMA alone. Thus, exogenous arginine by normalizing Arginine-ADMA ratio can restore NO levels and provide therapeutic benefits [11–16].

Recent studies have shown that NOS3 (eNOS) uncoupling and superoxide formation during stress are important factors in cardiovascular and pulmonary pathophysiology. Arginase or ADMA determined arginine deficiency could result in such NOS3

uncoupling. Thus, targeting NOS uncoupling and the resultant oxidative stress could be important therapeutic approaches. Interestingly arginine may play a dual role as a NOS substrate and a free radical scavenger. As endogenous arginine availability is inconsistent, citrulline appears to be a good alternative/better source of NO. Further, it is possible that the availability of citrulline to attenuate oxidative stress could inhibit NOS uncoupling. Specific arginase inhibitors or cofactors (BH4) may also increase NO formation. In view of the complexity of the factors involved in arginine synthesis and availability, a multipronged approach to address the issue of optimizing NO bioavailability could be the focus of future research for conditions with dysregulated NO production in a variety of acute and chronic disorders [17–21].

Nitric Oxide Dynamics

Nitric oxide (NO) is a ubiquitous, multi-dimensional, gasotransmitter which regulates a wide array of physiological functions by activating multiple intracellular signaling pathways. These signaling events are made possible by its lipophilicity and the capacity to signal in both an autocrine and paracrine manner. Importantly, cells have evolved exquisite mechanisms to regulate NO biosynthesis, NO diffusion and cell responses in order to shape these physiological responses. Classically, NO biosynthesis is regulated via L-arginine-dependent mechanisms (i.e. via neuronal, inducible and endothelial nitric oxide synthases) or through L-arginine-independent mechanisms (i.e. nitrite reduction by NOR enyme). Though a plethora of NO effects, sometimes contradictory, have been revealed by research, less is known about the dynamics or mechanisms by which these effects occur. Some molecular targets have been firmly established (such as the binding of NO to heme groups in guanyl cyclase), whereas other potential targets for NO are less clearly elucidated. NO and its metabolites interact with and modify protein targets to generate intracellular signals that can affect cellular function and potentially differential gene expression. Following its synthesis, the surrounding environment can modulate NO diffusion through reactions with other free radicals including superoxide or heme proteins such as hemoglobin and cytoglobin. Finally, target receptors for NO (and its derivatives) can activate both cGMP-dependent (i.e. soluble guanyl cyclase) or cGMP-independent mechanisms [7, 17].

NO signaling via the sGC-cGMP pathway is characterized by sGC activation, cGMP formation, and lowering of intracellular calcium concentrations—all of which collectively result in the vasodilatory effects of NO. In addition to stimulating sGC, NO can interact with cysteine thiols to give s-Nitrosothiols, which also reduce intracellular calcium and induce vasorelaxation, Such S-nitrosylation also prevents beta adrenoceptor down regulation/internalization and facilitate vasodilation. Interestingly, when NO is produced in excess (via iNOS) it results in the formation of a toxic moiety and reactive nitrogen species, OONO (peroxynitrite) after combining with superoxide. Recent studies have indicated that the various forms of NOS viz. nNOS (neural), iNOS (immune) and eNOS (vascular), may not necessarily be

organ/tissue specific. For example, NO containing neurons may regulate cardiovascular (blood pressure) and reproductive (erectile dysfunction) effects. Atherosclerosis and hypotension seen in septic shock may involve iNOS. eNOS, which is normally cardioprotective, has been identified in extra-vascular tissues and some cardiovascular risk factors like oxidative stress and endothelial dysfunction have been correlated with eNOS (NOS3) uncoupling. Lipid lowering agents like statins and drugs modulating renin–angiotensin–aldosterone axis have been shown to facilitate eNOS coupling and block endothelial dysfunction [7, 8, 17, 18].

While NO is mainly generated from L-arginine by nitric oxide synthase (NOS) enzymes, there is a growing realization that additional NO-generating pathways do exist. The most common idea is that nitrite can be reduced back to NO via the nitrite reductase (NOR) activity of some proteins and enzymes. While this is generally an activity that is associated with prokaryotes, several mammalian proteins and enzymes have currently been reported to have a low levels of NOR activity. These include xanthine oxidoreductase, cytochrome c oxidase, and, strangely enough, NOS itself. Interestingly, deoxygenated hemoglobin also possesses NOR activity. Recently, it has been demonstrated that this (NOR) activity represents a mechanism by which plasma nitrite can be converted back to vasoactive NO under deoxygenated conditions and so represents a hypoxically activated vasodilatory mechanism. Independent of the source of NO, the association of NO deficiency (L-arginine dependent or independent) with cardiovascular conditions (hypertension, atherosclerosis, ischemic heart disease, heart failure, stroke) respiratory/allergic conditions (bronchial asthma) and septic shock has now been documented. In view of the significance of various NOSs in human health and disease, this enzyme is considered a potential target for developing therapeutic strategies in a variety of pathophysiological states where NO homeostasis is dysregulated. Further, in depth understanding of complex NO signaling pathways has resulted in development of pharmacological agents for rational therapeutics viz. organic nitrates (NO releasers), arginine/citrulline (NO precursors), PDE-5 inhibitors (NO potentiators), dietary supplements and inhaled NO. The term "gasotransmitter" is now used to describe NO (besides CO and H_2S), and their complex interactions are the subject matter to extensive contemporary NO research [6, 8, 22–25].

Sources of Nitric Oxide

Nutraceutical research has revealed that NO could be derived from dietary sources as well. High levels of NO can be achieved by (a) food rich in the amino acid precursor, L-arginine, viz. poultry, dairy products, soya bean, peanuts, lentils, chickpeas etc., and, (b) Nitrate-rich food matter viz. spinach, red beetroot, lettuce, celery, etc. which are converted by salivary bacteria to NO. Arginine supplements are being considered as a therapeutic strategy for endothelial dysfunction induced atherosclosis, and dyslipidemia, with beneficial results. Consumption of nitrate rich veggies (beetroot, spinach, lettuce etc.), which are transformed to NO by saliva) improves cardiac performance during exercise. Activation of BH4 (co-factor in NO biosynthesis) by

ascorbic acid is also considered as a strategy for enhancing NO levels. In case of arginine deficiency, citrulline can be considered as a viable alternative due to its improved pharmacokinetic profile (minimal first pass elimination). High doses of cholecalciferol (Vitamin D3) have been reported to stabilize NO homeostasis by influencing eNOS and endothelial functions, which in turn could be helpful in several cardiovascular conditions. Aerobic and/or anaerobic exercise can also improve NO bioavailability and help in endothelial dysfunction [20, 26–30].

Therapeutic Potential of NO

The pleotropic effects of NO signaling pathways have been revisited in cardiovascular disease biology and all three forms of NOS have been implicated. Most recent studies have implicated NO in the reversal of genetic factors in cardiovascular hemodynamics via the eNOS pathway [22–25]. However, it is the complex regulatory effects of NO outside of the cardiovascular system which has been the focus of recent research worldwide. Over expressed or dysregulated iNOS and its complex functioning has been proposed in inflammatory disorders like sepsis, cancer, epilepsy, neurodegenerative disorders etc. and iNOS inhibitors could be promising therapeutic agents. In fact, High levels of NO via iNOS are now known to be crucial in infectious diseases whereas, low (non-toxic) levels of NO may induce biofilm dispersal—thus making some highly pathogenic bacteria more antibiotic susceptible. The role of NO in cancer biology is another emerging field of research. Specific roles for NO in tumor cells and microenvironment, cancer progression, angiogenesis, and effectivity of treatment and prognosis have been shown. NO and reactive nitrogen species (RNS) via iNOS from macrophages are required for cancer immunotherapy. The equivocal role of NO is also highlighted by the eNOS (and VEGF) dependent angiogenesis. Both NO mimetics and NOS inhibitors could thus impact cancer in different ways. The photodynamic therapy against cancer involving NO release (iNOS—NO) and concentration dependent effects of NO on cytoprotection and cytotoxicity are emerging areas for NO in cancer therapeutics [8, 11, 31–34]. The role of NO in the brain and neuromodulation has also received considerable attention and its role in stress regulation, stress related pathophysiology and cognitive disorders has been proposed [5, 35].

From a therapeutic perspective, in addition to the established uses of NO mimetics in ischemic heart disease, hypertensive crisis, heart failure and erectile dysfunction, inhaled NO gas is rapidly emerging as a effective therapeutic option in pulmonary hypertension, acute respiratory distress syndrome (ARDS), high altitude pulmonary edema, and lung transplant. NO inhalation therapy is best used in invasively ventilated patients. However, NO can also be administered by face mask or nasal cannulae. Inhaled NO diffuses through alveolar membrane to reach smooth muscle cells and increase cGMP which results in the reduction of vascular tone. NO then diffuses into the blood stream and is inactivated. Safety issues are paramount for inhaled

NO as there is risk of NO_2 formation with oxygen. Pulmonary toxicity and methemoglobinemia can be minimized when NO is given at doses of 40 ppm for up to 6 months. Careful monitoring of NO levels are crucial as concentrations >100 ppm can induce toxicity like pulmonary edema, methemoglobinemia and bleeding tendencies (due to inhibition of platelet aggregation), and treatment with methylene blue attenuates most such situations [4, 6–8, 17, 22].

The pathophysiological role of NO in cardiovascular hemodynamics in the critically ill has received a lot of attention, viz. sepsis, cardiogenic shock, acute lung injury, etc. The involvement of mitochondrial NOS in sepsis and the correlation between enhanced ROS production and compromised mitochondrial respiration has been suggested. On the other hand, the vital role for NO in providing protection in microcirculation and improving microcirculatory hemodynamics with specific reference to organ failure in septic shock. Indications are that NO (or NO modulators) may play a crucial role in critical care patients, both as a biomarker as well as a therapeutic agent, and improve resuscitation. Clinical studies are being conducted on efficacy of inhaled NO in patients with compromised end organ function and impending failure [8, 36, 37].

A potential role for inhaled NO in the COVID-19 pandemic has also been suggested. Inhaled NO gas is considered as a potentially useful prophylactic measure. In acute respiratory distress syndrome (ARDS), a fatal complication of the viral infection, inhaled NO, influenced cardiorespiratory physiology by improving arterial oxygenation, and showed beneficial effects. Earlier, during the SARS epidemic (2002–2003), inhaled NO was found to be beneficial by improving arterial oxygenation, lowering pulmonary hypertension and reduction of pulmonary infiltrates. Lung oxygenation increased while duration of ventilator support was minimized. An anti-viral effect for NO was also shown via the modification of viral proteins and nucleic acids as well as modulation of ACE-2/S-protein interactions. An additional pulmonary vasodilatory effect could also have contributed to the beneficial effects in COVID-19 patients. NO is produced in the paranasal sinuses and nasopharynx epithelium by the L-arginine-NOS pathway. Such low concentration (approx. 10 ppm) diffuses into the adjoining bronchii and lung tissue to induce bonchodilation and vasodilation. The facilitatory effects of NO on mucus secretions and ciliary movement compliment its respiratory effects. Higher basal levels of exhaled NO are associated with fewer common cold symptoms—further indicating an anti-microbial role of endogenous NO against airway viruses. In view of the above, it is possible that inhaled NO or NO donors may be of potential benefit in COVID-19 infections. The improvement in lung oxygenation and bronchodilation may reduce the requirement of ventilators. On the contrary, lowered NO availability in the respiratory tract may actually promote COVID-19 infection development and progress. In fact, NO levels are lower in Caucasians and people with history of habitual intake of tobacco/alcohol/caffeine/corticosteroids. In addition, modifying life style factors (like moth breathing and smoking) to improve the lowered endogenous NO levels may also help by reducing viral load and manifestations of pneumonia. All of these, could be considered as viable treatment alternatives in view of the lack of appropriate specific strategies for COVID-19 infections [38–43] (Table 1.1).

Table 1.1 Nitric oxide (NO) and therapeutic applications

Type of therapy	Mode of action	Indication (References in brackets)
Physiological/natural		
Diet eg. Polyphenols (in tea, red wine, etc.), Resveratrol, Quercetin Green leafy vegetables (Flavonoids)	↑eNOS expression; ↑NO production; ↑Sirt 1; ↑Nitrates	NO deficient states For reduction of cardio-metabolic risk [4, 20, 26–28]
Exercise (mild to moderate) (skeletal muscle, cardiac muscle, vasculature)	↑endothelial eNOS expression ↑ blood flow ↑ NO signaling ↓Oxidative stress and pro-inflammatory signaling	Hypertension, Coronary Artery Disease, Heart failure [4, 30]
Pharmacological		
NO inhalation (controlled NO gas delivery via ventilator)	Pulmonary vasodilation Anti-viral effect Bronchodilation	Pulmonary hypertension in neonates and adults ARDS (post COVID) High altitude pulmonary edema Lung transplant [38–43]
NO precursor (Arginine)	NO generation (via enzymatic pathway)	Hypertension [7, 20, 26–29] CAD Erectile dysfunction Peripheral arterial disease
NO donors (Nitroprusside, organic nitrates, s-nitrosoglutathione, Deta-NO NO ates, molsidomine)	Stable delivery of NO	Angina, Heart failure, Pulmonary hypertension, Hypertensive crises [4, 20, 26–29]
ACE inhibitors, ARBs	Increase NO bioavailability and downstream signaling ↑Endothelial NO production ↑eNOS expression ↓Oxidant inactivation of NO ↓ADMA formation	↓Atherosclerosis ↓Stroke ↑insulin sensitivity, glucose toleranceand ↓T2DM onset Hypertension, CAD [4]
Beta adrenergic blocking agents (3rd gen)-Nebivolol, Celiprolol, Carvedilol	β-1 blocker, β-2 stimul. (celiprolol), ↑eNOS activity, ↑NO bioavailability, ↑endothelial function ↓oxidative stress, ↑adiponectin levels ↓insulin resistance	CAD, Stroke, insulin resistance (T2DM) [4]

(continued)

Table 1.1 (continued)

Type of therapy	Mode of action	Indication (References in brackets)
Phosphodiesterase 5 (PDE-5) Inhibitors (sildenafil, verdenafil, tadalafil)	Vasodilation (corpora caver-nosa, pulmonary), ↓cGMPdegrad. (↑cGMP), ↑eNOS expression, ↑Adenosine, bradykinin,↑NO release, Antioxidant, ↑Insulin sensitivity	Erectile dysfunction Pulmonary hypertension Chronic heart failure CAD T2DM [4, 8, 22–26]
HMGCoA Inhibitors (Statins)	↑eNOS expression/activity ↓eNOS down regulation by LDL ↓Caveolin induced eNOS inhib	Hyperlipidemia CAD [4, 8]
iNOS (NOS-2) inhibitors (aminoguanidine)	↓Excess NO levels	Sepsis (and organ dysfunction); anaphylactic or cardiogenic shock; transplant organ rejection [4]

Conclusion

In summary, after more than three decades of arrival of NO in medicine and biology, it continues to play a rapidly evolving role not only in cardiovascular regulation but also in other body systems. The role of NO has now extended from a vascular regulator to neurotransmitter (gasotransmitter) and immune modulator, with newer signaling pathways being identified. Advances in research has led to widespread and diverse potential applications of NO and NO modulators in therapeutics. The fact that the subtypes of the enzyme NOS are not necessarily localized at specific targets have considerably expanded the scope of NO research. Targeting alternative pathway by arginase inhibitors to enhance arginine bioavailability is considered a promising therapeutic approach. NO signaling pathways other than the conventional GC-cGMP pathway are also being explored to identify therapeutic avenues. NO and NO modulators are now being considered as treatment moieties in shock, sepsis (in critical care), allergy and immunology, gastrointestinal disorders, neuropsychiatric/neurodegenerative disorders and cancer. Newer concepts involving NO and its translation to novel therapeutic targets may thus play a major role in redefining future therapeutics.

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Chapter 2
Nitric Oxide and Cardiovascular Health

Yuan Li, Ashok K. Srivastava, and Madhu B. Anand-Srivastava

Abstract Nitric oxide (NO) is a diffusible free radical and universal messenger that is produced from L-arginine by three different isoforms of nitric oxide synthases (NOS), neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). NO plays an important role in the regulation of variety of physiological functions including myocardial contractility, vascular tone, blood pressure, cell growth, proliferation and platelet aggregation. Most of the effects of NO are mediated through the activation of soluble guanylate cyclase–cGMP system, however, cGMP-independent pathways have also been shown to be responsible in mediating its effects. The levels of NO are regulated by several factors and cofactors required for the activation of NOS, however, reduced bioavailability of these factors results in the decreased levels of NO and thereby endothelial dysfunction leading to the pathogenesis of cardiovascular diseases including hypertension, diabetes, atherosclerosis etc. This review will focus on the role of NO in physiology and pathophysiology of cardiovascular system including vascular remodeling, hypertension and the underlying molecular mechanisms contributing to these functions.

Keywords Nitric oxide · Signaling · Nitroxidative stress · Vascular remodeling · Hypertension · Atherosclerosis

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_2

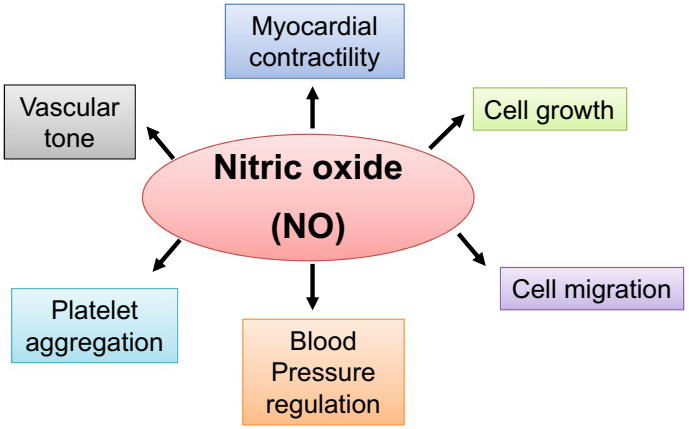

Fig. 2.1 Role of nitric oxide (NO) in the regulation of physiological functions

Introduction

Cardiovascular disease that affect the heart and/or the blood vessels is considered as leading cause of morbidity and mortality worldwide [1]. Several risk factors including hypertension, vascular remodeling, insulin resistance, endothelial dysfunction, reduced cardiovascular nitric oxide (NO) bioavailability, cardiac hypertrophy [2], and alterations in the circulating lipids are implicated in pathophysiology of cardiovascular disease [3]. Nitric oxide (NO) is a ubiquitous intracellular messenger that acts as an important biological signaling molecule involved in the regulation of variety of physiological functions including myocardial contractility, vascular tone, blood pressure regulation, cell growth, proliferation, platelet aggregation etc. [4] (Fig. 2.1). Under physiological conditions, NO exerts cardiovascular protection, however, dysregulation of NO production contributes to endothelial dysfunction leading to the pathogenesis and progression of cardiovascular diseases including hypertension, diabetes, atherosclerosis etc.

Nitric Oxide Synthesis

NO is short lived free radical generated by the oxidation of L-arginine to L-citruline, a reaction catalyzed by nitric oxide synthase (NOS) [5, 6] and requires the presence of several cofactors including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH, tetra-hydrobiopterin (BH4), heme prosthetic group as well as the redox cofactor (Fig. 2.2) [7]. Three isoforms of NO synthases are identified named neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). In addition, a novel constitutively active mitochondrial NOS (mtNOS) has also been identified in mitochondria from liver

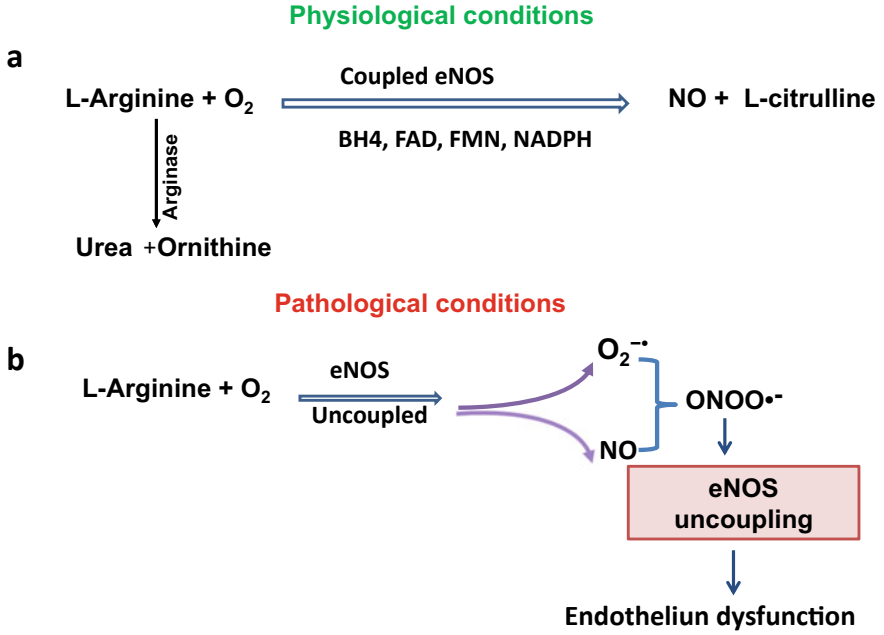

Fig. 2.2 Nitric oxide synthesis **a** Under physiological conditions, nitric oxide is synthesized by the oxidation of L-arginine to L-citrulline, a reaction catalyzed by nitric oxide synthase (NOS) and requires the presence of several cofactors including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH, tetra-hydrobiopterin (BH4), heme prosthetic group as well as the redox cofactor **b** Under pathphysiological conditions the absence or limited availability of either substrate or cofactor BH4 and augmented levels of oxidative stress result in the uncoupled eNOS that produces O2- instead of NO and forms ONOO-that further promotes eNOS uncoupling leading to endothelial dysfunction and the pathogenesis of cardiovascular diseases

that appears to be involved in Ca^{2+} regulation [8]. nNOS and eNOS are constitutive enzymes and predominantly expressed in neuronal and endothelial cells (EC) as well as in other cell types including vascular smooth muscle cells (VSMC) [9]. Both nNOS and eNOS are regulated by intracellular Ca^{2+}/ calmodulin (CaM) whereas iNOS is inducible at the level of gene transcription, Ca^{2+}—independent and expressed in macrophages and other tissues in response to inflammatory mediators including cytokines and endotoxins [10–12]. eNOS is also activated independently of Ca^{2+} upon phosphorylation by Akt in response to shear stress, estrogens and insulin [13]. In addition, nNOS and iNOS are cytosolic enzymes whereas eNOS is associated with the membranes of EC [14, 15]. These two isoforms of NOS are crucial regulators of cardiovascular homeostasis and regulate vascular tone and blood flow, inhibit platelet aggregation and adhesion, modulate cardiac contractility and inhibit VSMC proliferation [4]. The structures of iNOS, eNOS and nNOS have been determined [16, 17]. All three NOS isoforms are dimers and contain two major functional domains fused into a single polypeptide. The N-terminal catalytic domain possesses the binding

sites for heme, redox cofactor, BH4 and CaM. The C-terminal reductase domain has binding sites for FMN, FAD and NADPH. NOS activation relies on the presence of the cofactor BH4 and the substrate L-arginine to couple the oxidation of molecular oxygen to produce NO [18]. Two molecules of BH4 bind to each eNOS dimer and facilitate electron transfer for the oxidation of L-arginine. BH4 thus maintains eNOS in a dimeric state and preserves the endothelial function [19].

Regulation of Intracellular Levels of Nitric Oxide

Several factors which are involved in the activation of NOS play a key role in the regulation of intracellular levels of NO. These include the substrate L-arginine, cofactors including BH4, asymmetrical dimethylarginine (ADMA), N^G -mono-methyl-L-arginine (L-NMMA), naturally occurring metabolites that circulate in the plasma [20]. The absence or limited availability of either substrate or cofactor BH4 and increased circulating levels of arginase, a hydrolytic enzyme that converts L-arginine to urea and L-ornithine, ADMA and L-NMMA, inhibitors of NOS, result in the decreased production of NO leading to endothelial dysfunction and the pathogenesis of cardiovascular diseases (Fig. 3a) [20–24]. In addition, oxidative stress also contributes to the decreased levels of NO. The augmented oxidative stress oxidizes BH4 to BH2 resulting in the destabilization of eNOS dimer which becomes uncoupled and leads to the production of superoxide anion (O_{2^-}) instead of NO, the process is referred to as eNOS uncoupling (Fig. 2.3a) [25]. O_{2^-} reacts with NO forming peroxynitrite ($ONOO^-$), a strong cytotoxic oxidant and reactive nitrogen species (RNS) that induces nitrosative stress. $ONOO^-$ in turn oxidizes BH4 to BH2 and further promotes eNOS uncoupling [26]. $ONOO^-$ reacts with lipids, DNA, and proteins, causing damage to these macromolecule, interferes with important vascular signaling pathways and contributes to various cardiovascular dysfunction [27] (Fig. 2.3).

On the other hand, several hormonal factors are also implicated in the regulation of intracellular levels of NO. Angiotensin II (Ang II) that promotes vascular remodeling, increases oxidative stress and blood pressure, has been shown to decrease the levels of eNOS and NO in VSMC [28]. On the other hand, NO donors, antioxidants, adiponectin, AT1 receptor blocker, ACE inhibitor, Statins, C-$ANP_{4\text{-}23}$, a specific agonist of natriuretic peptide receptor-C (NPR-C) and resveratrol were shown to increase the intracellular levels of NO and exert vascular protection and ameliorate hypertension (Fig. 2.3b) [29–39].

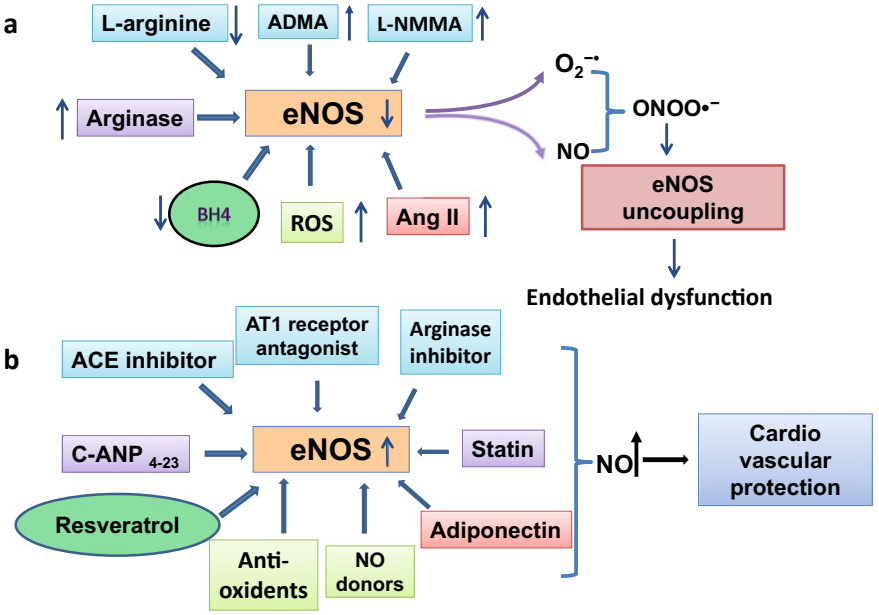

Fig. 2.3 **a** Factors that may contribute to reduced bioavailability of nitric oxide leading to endothelial dysfunction and pathogenesis of cardiovascular diseases. Several factors that could reduce NO availability are; the reduced levels of substrate L-arginine, BH4, an essential cofactor and augmented levels of circulating inhibitors such as asymmetrical dimethylarginine (ADMA), N^G -mono-methyl-L-arginine (L-NMMA), arginase, Ang II and destruction of NO by reactive oxygen species (ROS). **b** Factors that increase the expression of eNOS and NO levels and exert vascular protection. These factors include NO donors, antioxidants, adiponectin, AT1 receptor blocker, ACE inhibitor, Statins, C-$ANP_{4\text{-}23}$, a specific agonist of natriuretic peptide receptor-C (NPR-C) and resveratrol

Cross-Talk Between Oxidative Stress and Nitrosative Stress

Oxidative stress has been shown to play an important role in the pathogenesis of cardiovascular diseases [40–43]. Oxidative stress is caused by the overproduction of reactive oxygen species (ROS) and a decreased elimination of these ROS by antioxidants. ROS are produced by a wide array of enzymes that include NADPH oxidases, xanthine oxidase, peroxidases, lipoxygenases, cyclooxygenases and complex I and III of mitochondrial respiratory chain and eNOS uncoupling [44]. NADPH oxidases and xanthine oxidase catalyze the formation of (O_2^-) by single electron reduction of molecular oxygen which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Several studies have demonstrated a reciprocal relationship between ROS and RNS. The augmented levels of ROS decrease the levels of NO, whereas decreased levels of ROS have been shown to increase NO synthesis. For example, the antioxidant ascorbate that decreases the levels of (O_2^-) resulted in increased NO synthesis in EC by improving the binding of BH4 to NOS and stabilizing the dimeric structure of NOS [31, 32]. In addition, Huang et al. have also

demonstrated that ascorbate augmented the activity of NOS in endothelial cells by increasing the levels of BH4 [39]. Modulation of O_2^- by NO through the regulation of SOD-1 in VSMC has also been reported [45]. In addition, in VSMC from spontaneously hypertensive rats (SHR), increased levels of (O_{2^-}) are associated with decreased levels of NO and augmented levels of $ONOO^-$. Furthermore, elevating the intracellular levels of NO by NO donors decreased the augmented levels of (O_{2^-}) and $ONOO^-$ in these cells [29, 46]. Similarly, C-$ANP_{4\text{-}23}$ (natriuretic peptide receptor-C) agonist- induced suppression of enhanced levels of (O_{2^-}) provoked by Ang II was associated with augmented levels of NO in VSMC [28]. The increase in ROS and subsequent increased ONOO—formation reduces the bioavailability of NO and results in endothelial dysfunction. Thus the imbalance between the formation of RNS and ROS plays a critical role in the pathogenesis of cardiovascular diseases [40, 47–50].

Nitric Oxide Signaling

The canonical signaling mechanism by which NO exerts most of its biological effects is through the activation of soluble guanylate cyclase (sGC), The sGC is a heterodimeric protein composed of two subunits, α and β, of which the β subunit contains a heme moiety that confers the NO-sensitivity of the enzyme [51, 52]. Binding of NO to heme results in a conformational change of cGC and activation of the catalytic domain [52] that converts intracellular GTP into the second messenger cyclic guanosine 3′5′-monophosphate (cGMP) [52, 53], cGMP interacts with a variety of effector proteins including cGMP-dependent protein kinases (PKGs) [53], cGMP-regulated phosphodiesterases (PDEs) and ion channels. Two different types of PKGs, type I (PKG-1) and type II (PKG-2) are expressed in mammalian tissues, however, their relative distribution is tissue- and species- dependent [54, 55]. In cardiovascular tissues, a predominant expression of PKG-1 has been reported that mediates the anti-proliferatve effect of cGMP [54, 56–58]. PKG-1 is a serine/threonine kinase and elicits its effects through the phosphorylation of multiple targets which include IP_3 receptor, phospholamban, troponin, myosin light chain phosphatase, c-raf kinase, Ca^{2+} and K^+ channels. All these signaling targets are implicated in the reduction of intracellular levels of Ca^{2+} or in decreasing the Ca^{2+} sensitivity of contraction or both, resulting in the vasorelaxation (Fig. 2.4) [53, 59–63]. In addition, NO has also been shown to mediate some of its effects through cGMP-independent pathways because 1H- [1, 2, 4] oxadiazolol [4,3-α]quinoxalin-1-one, ODQ, a selective inhibitor of sGC was unable to inhibit these NO-mediated effects. For example, NO decreases the levels of Giα proteins as well as proliferation of VSMC by cGMP-independent pathway [29]. The cGMP-independent pathways implicated in NO-mediated effects include Ras, MAP kinase [46, 64], cyclin dependent kinase inhibitor P21, [65, 66] and cAMP\PKA signaling pathway [67–69]. In addition, posttranslational modification such as S-nitrosylation [70, 71], eNOS S-glutathionylation [72] and tyrosine nitration [73] mediated effects of NO are also

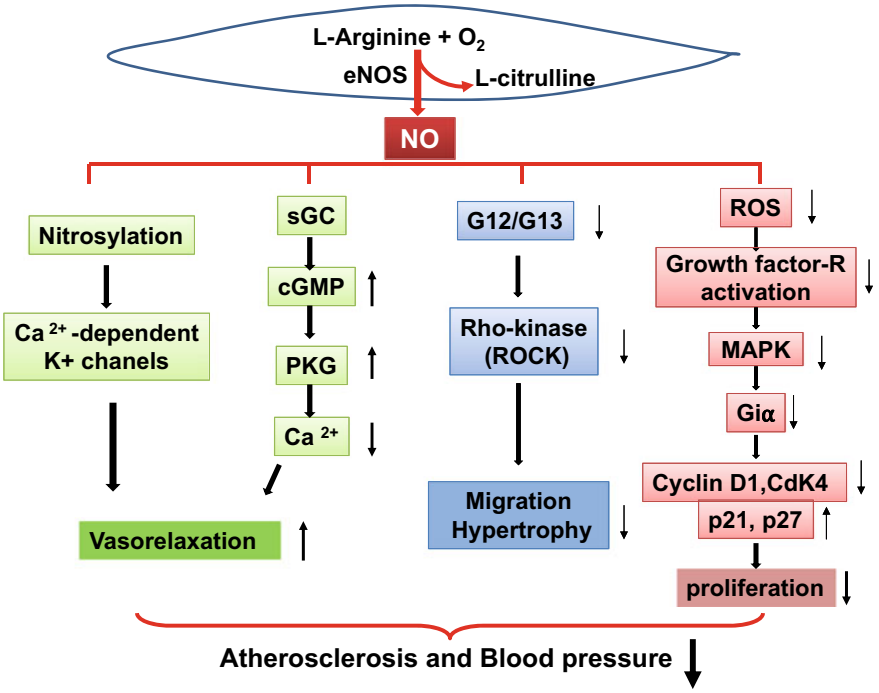

Fig. 2.4 Signaling mechanisms implicated in NO-mediated cardiovascular protection. NO induces vasorelaxation through S-nitrosylation-induced activation of Ca^{2+}-dependent K^+ channels as well as via sGC/cGMP/protein kinase G-dependent pathway. The beneficial effects of NO on remodeling of the vessels include the attenuation of signaling pathways involved in the activation of the hypertrophic, migratory and proliferative program in the cardiovascular tissues. NO through the inhibition of G12/G13-Rho/ROCK pathway exerts antimigratory and antihypertrophic effects. In addition, NO decreases ROS which through the inhibition of growth factor receptor activation and MAPK signaling inhibits Giα protein expression and modulates cell cycle regulatory proteins leading to decreased proliferation

independent of sCG and cGMP and regulate downstream pathways contributing to cell proliferation [74–77]. Furthermore, S-nitrosylation-induced activation of VSMC Ca^{2+}-dependent K^+ channels has also been shown as a potential mechanism of cGMP-independent vasorelaxation (Fig. 2.4) [78].

Nitric Oxide and Cardiovascular Diseases

NO plays an important role in the protection against the onset and progression of cardiovascular disease that include regulation of blood pressure and vascular tone, inhibition of platelet aggregation and smooth muscle cell proliferation [4]. Endothelial dysfunction due to the decreased availability of NO is the contributing factor in

the pathogenesis of cardiovascular diseases. In the following section, the protective role of NO will be discussed in different pathologies.

Vascular Remodeling and Molecular Mechanisms

Vascular remodeling refers to alterations in the structure of resistance vessels and contributes to the pathophysiology of vascular diseases, such as atherosclerosis, restenosis, and hypertension [79] and is associated with alteration in VSMC growth, hypertrophy, migration etc. [80, 81]. Vascular remodeling is influenced by dynamic interactions between local growth factors, vasoactive substances, and hemodynamic stimuli [82]. Several intracellular signaling pathways that regulate the expression of upstream and downstream target genes are involved in the proliferation, hypertrophy and migration of VSMC. Vasoactive peptides such as Ang II and endothelin-1(ET-1) as well as growth factors receptors such as epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) all contribute to VSMC hypertrophy, proliferation and migration through the activation of several signaling pathways including $G_i\alpha$/Gqα, MAP kinase and Rho-kinase (ROCK), an effector of a small G protein [83–94]. In addition, enhanced oxidative stress induced by Ang II is also implicated in the enhanced expression of Giα proteins and proliferation of VSMC through the transactivation of EGF-R and MAP kinase signaling [91]. Furthermore, the augmented levels of endogenous vasoactive peptides including Ang II and ET-1 have also been shown to contribute to hyperproliferation as well as hypertrophy of VSMC from SHR through ROS and ROS-mediated transactivation of EGF-R/PDGF-R and MAP kinase signaling pathways [95–99]. Hyperproliferation of VSMC is associated with accelerated entry of cells from G_0/G_1 phase of cell cycle to the synthetic phase [100]. Ang II- and FBS-induced exaggerated growth of VSMC from SHR is associated with progression from G1 to S phase [98, 101]. The role of enhanced expression of Giα proteins in the overexpression of cell cycle proteins including cyclin D1, cyclin D1-dependent kinase (Cdk)4 and phosphoretinoblastoma protein (pRb) and resultant hyper-proliferation of VSMC from SHR has been demonstrated [102, 103]. Furthermore, we and others have demonstrated that several distinct signal transduction pathways including c-Src, reactive oxygen species (ROS), growth factor receptor transactivation, MAP kinase, PI3Kinase, that are implicated in the overexpression of Giα proteins, also contribute to the overexpression of the cell cycle proteins and vascular remodeling by promoting VSMC proliferation [48, 92, 103–106].

Nitric Oxide and Vascular Remodeling

A multitude of studies using in vitro and in vivo models have shown that an increase in the cellular levels of NO, either by direct delivery of NO donors or gene transfer of eNOS or iNOS, potently suppressed proliferation, migration and hypertrophy in VSMC and, neointimal growth [107–112]. Although the precise molecular events that provoke these responses remains elusive, accumulated evidence has suggested that modulation of key components of cell cycle regulatory proteins and signaling pathways responsible to drive these events play an important role. For example,

eNOS overexpression in VSMC isolated from pig coronary arteries resulted in attenuation of PDGF-induced proliferation that was accompanied by reduced levels of cell cycle regulatory proteins cyclin A, and a delayed expression of cyclin E [113]. Similarly, a reduction in the expression levels of cyclin A and cyclin-dependent kinase (cdk) 2 was associated with diethylenetriamine NONOate (DETNONOate)-induced reduction of fetal calf serum (FCS)-induced cell proliferation in human VSMC [114]. S-nitroso-N-acetylpenicillamine (SNAP) was also reported to inhibit FCS- and FGF-induced cell cycle progression in VSMC via inhibition of Cdk2 and upregulation of p21 [115]. In addition, SNAP was also shown to inhibit the overexpression of Giα proteins and hyperproliferation of VSMC from SHR by cGMP-independent mechanism and involves ROS and ROS-mediated transactivation of EGF-R/PDGF-R and MAP kinase signaling pathways [46]. More recent in vivo studies have demonstrated that NO donor, sodium nitroprusside (SNP), reduced the increased BP in SHR and reduced the heightened expression of Giα, cyclin D1, Cdk 4 and pRb and augmented the reduced levels of cdk inhibitors p27 and p21 (Fig. 2.4) [116]. Interestingly, NO has been shown to upregulate p21 levels by preventing its degradation in rat aortic VSMC and in pulmonary VSMC [65, 66]. The antihypertensive effect of SNP in SHR was associated with a reduction in the overexpression of AT1R, growth factor receptor phosphorylation, ERK1/2 activation an Giα protein expression in VSMC [116]. SNP as well as 8-bromo cyclic GMP were also reported to block ET-1 and EGF-induced Ras/ MEK/ ERK1/2 pathway while suppressing DNA and protein synthesis in VSMC [117, 118]. Thus, inhibition of the signaling events involved in cell cycle progression appears to be a key mechanism for the antiproliferative effects of NO. Furthermore, the implication of both cGMP—dependent and—independent pathways have been suggested to elicit this response [46, 109, 119, 120].

Several NO donors including SNP, SNAP, DETNONOate, spermineNONOate and S-nirosoglutathione have also been shown to reduce Ang II-evoked VSMC migration [108, 121, 122]. In addition, increasing the intracellular levels of NO by eNOS gene transfer also suppressed the migration of VSMC induced by Ang II or PDGF [112, 123, 124]. The molecular mechanism implicated in these events appear to be mediated through the inhibition of matrix metalloproteases (MMPs) 2 and 3 as well as the Ras family of small G- protein, Rho A and its effector, RhoA kinase (ROCK) [112]. However, these effects were shown to be independent of growth factor receptor transactivation and ERK signaling pathway [112]. An involvement of RhoA/ROCK pathway in mediating the antihypertrophic action of adiponectin and NO was also reported (Fig. 2.4) [35]. Interestingly, this inhibitory response was associated with a reduction in Ang II-induced phosphorylation of cofilin and actin cytoskeletal remodeling as judged by altered F-actin/G-actin ratio [35].

Consistent with the antiproliferative, antimigratory and antihypertrophic effects of NO, several studies have reported protective effects of NO donors and NOS overexpression on neointimal growth and hyperplasia in animal models. Among the first reports to implicate NO in blocking neointimal hyperplasia in a rabbit model of vascular injury, utilized L-arginine, a substrate of NOS to raise tissue levels of NO [125]. These studies demonstrated that administration of L-arginine by gavage reduced the intimal hyperplasia by about 39% in balloon catheter-injured rabbit

thoracic aorta and co-administration of L-NAME reversed the protective effect of L–arginine and suggest that NO generation was responsible for this effect [125]. This observation was quickly confirmed, and showed that as compared to systemic delivery, the topical application of L-arginine was slightly more effective in inhibiting neointimal growth in a rat carotid artery injury model [126]. These studies prompted several investigators to further explore the usefulness of NO donors in conferring beneficial effects in rat, rabbit or porcine models of vascular injury [127–131]. For example, intravenous infusion of 4-hydoxymethy-furazone-3 carboxylic acid-2 oxide, an organic NO donor not only reduced neointimal thickening in injured rat carotid artery but also inhibited the proliferation of VSMC [127, 129]. In addition, continuous chronic inhalation of NO also resulted in a similar response and decreased the intimal growth by about 43% after 14 days of therapy [129]. Moreover, perivascular, topical delivery of short and long acting NO donors, 1-[2-(carboxylato)pyrrolidin-1-yl]dazen-1-ium-1,2-diolate (PROLI/NO) (short half-life) and diazeniumdiolated poly(acrylonitrile) (PAN/NO) (long half-life) was also found to suppress neointimal hyperplasia in rat carotid artery model [132, 133]. This group also reported that systemic administration of S-nitrosylated(S-NITROSYL(SNO))-targeted nanofibre suppressed neointimal hyperplasia in rat model of carotid artery injury [45]. Similar to NO donors, local delivery of eNOS or iNOS genes were also reported to suppress the neointimal growth in both rodent and porcine models of vessel injury [134–141]. Thus, there is ample evidence to support that NO donors or gene transfer of NOS exert beneficial effects in suppressing vascular remodeling and inhibiting neointimal hyperplasia in cellular and animal model systems. However, because of the labile nature of NO and, confounding factors of targeted gene delivery and appropriate transfer vectors, some limitations for its translational use have been noted [142]. To overcome these issues, several groups are engaged in developing nanofiber and stent-based delivery systems for NO production and gene delivery for therapy of vasculopathies [143, 144].

Hypertension and Molecular Mechanisms

Hypertension is a multifactorial disease where the interplay between neuronal, hormonal and cellular signaling processes contributes to the pathogenesis. Several factors including vasoactive peptides, the renin–angiotensin–aldosterone system (RAAS), activation of the sympathetic nervous system, abnormalities in G protein-coupled receptor (GPCR) signaling, oxidative and nitrosative stress and inflammation are implicated in the pathophysiology of hypertension.

Ang II, a dominant player of renin–angiotensin system plays an important role in the development of blood pressure through the activation of downstream signaling pathways including oxidative stress. Ang II has been shown to increase the levels of ROS, ONOO- as well as of Giα proteins and decrease the levels of eNOS and NO in aortic VSMC [28], which appear to be important contributing factors in the development of hypertension [145, 146]. Furthermore, NO has also been shown to decrease the expression of Giα proteins in aortic VSMC [64] which may be one of the molecular pathways responsible for NO-induced reduction in blood pressure in SHR [29]. Consistent with this notion, the enhanced oxidative stress, decreased levels of eNOS

and NO have been shown to be associated with the overexpression of Giα proteins and downstream signaling pathways including growth factor receptor transactivation and MAP kinase and PI3Kinase in VSMC from SHR [147]. Studies showing that reduction in ROS generation by C-ANP_{4-23} and resveratrol attenuated increased blood pressure through the inhibition of exaggerated levels of Gi α proteins provide additional evidence for the role of this pathway in the pathogenesis of hypertension [148, 149].

Nitric Oxide and Hypertension

Accumulating evidence demonstrates that NO produced by the endothelial nitric oxide synthase (eNOS) in the vascular endothelium, plays a critical role in the regulation of blood pressure [150, 151]. NO stimulates guanylyl cyclase to increase cGMP production, which promotes vasodilatation of VSMC [152, 153], prevents platelet adhesion and aggregation, exerts antiproliferative and antimigratory effects on EC and VSMC [154, 155]. Reduction in NO bioavailability is the hallmark of endothelial dysfunction and contributes to the development of hypertension and other vascular diseases [156–158]. This has been demonstrated by several studies using knockout mice as well as hypertensive patients and rat models. eNOS knockout mice develop high blood pressure and display decreased vasodilation, whereas nNOS or iNOS deficient mice did not show any changes in the blood pressure [159, 160]. In addition, the role of sGC and PKG1, the downstream signaling molecules of NO in NO-mediated vasorelaxation and blood pressure regulation has also been demonstrated by using knockout mice. sGC deficient mice ($sGC\beta1^{-/-}$) as well as smooth muscle cell specific $sGC\beta1^{-/-}$ mice exhibit higher blood pressure than wild type mice [161, 162], however,in these mice, NO donor was ineffective in reducing the blood pressure and the vasodilatation of isolated aortic rings [162]. These results suggest that NO-inducible sGC activity is required for NO in mediating vasorelaxation in these vessels. Similarly, PKG1 deficient mice also developed hypertension and elicited an impaired dilation of large conductance and small resistance arteries in response to NO-cGMP signaling [163, 164]. In addition, the inhibition of eNOS by Nω-nitro-l-arginine methyl ester (L-NAME) was also shown to result in the development of hypertension in rats and was associated with increased levels of Giα proteins, decreased cGMP levels and increased levels of Ang II [165, 166]. The decreased levels of NO due to increased oxidative stress has also been shown to contribute to high blood pressure in other models of hypertensive rats. In SHR, the levels of AT1 receptor, Giα proteins, (O_{2^-}) and ONOO- were increased whereas the levels of eNOS and NO were decreased [116, 148]. Similarly, the expression of eNOS mRNA was downregulated in mesenteric arterioles of high-salt treated Dahl hypertensive rats [167]. Deoxycorticosterone acetate-salt hypertensive rats (DOCA-Salt HR) exhibited reduced eNOS phosphorylation that resulted in decreased NO/cGMP signaling in mesenteric arteries [168]. In addition, NO-mediated relaxation was depressed in mesenteric arteries of hypertensive rats with reduced renal mass, due to decreased bioavailabilty of NO [169, 170]. An impairment of NO-mediated vasodilatation in patients with essential hypertension has also been demonstrated [171]. On the other hand, several studies have demonstrated that the elevation of intracellular NO by NO

donors ameliorates the development of hypertension in different models of hypertensive rats (HR). SNP was shown to attenuate high blood pressure in SHR through the inhibition of oxidative stress, overexpression of AT1 receptor, Giα proteins and $ONOO^-$ levels [116]. In addition, supplementation of exogenous nitrite that augments the intracellular levels of NO [172] also attenuated blood pressure [173] and endothelium-dependent relaxation in isolated aortae of SHR through activating the eNOS-NO-soluble guanylyl cyclase (sGC)-cGMP pathway [174, 175]. The attenuation of hypertension and NADPH oxidase activity by nitrite\nitrate treatment has also been demonstrated in two-kidney one-clip (2K1C) HR, DOCA-Salt HR and Ang II-induced HR [176–178]. In addition, a cohort study of European ancestry also showed that genetic predisposition to enhanced NO signaling is associated with decreased blood pressure and reduced risks of coronary artery and peripheral arterial disease [179]. Furthermore, $C\text{-}ANP_{4\text{-}23}$ and resveratrol that possess antioxidant property were also shown to attenuate hypertension in SHR through the inhibition of enhanced levels of Giα proteins, (O_{2^-}) and $ONOO^-$ [148]. In addition, several studies have shown that antihypertensive drugs including ACE inhibitors and Ang II AT1 receptor blocker mediate their effects through the release of NO [33, 34, 38].

Role of eNOS uncoupling in hypertension

eNOS uncoupling occurs when eNOS produces (O_{2^-}) instead of NO resulting in the decreased bioavailability of NO and increased oxidative stress causing endothelial dysfunction leading to the pathogenesis and progression of hypertension. The decreased levels of L-arginine\cofactors required to activate eNOS and NO synthesis, increased NO inactivation by (O_{2^-}) and increased levels of circulating ADMA, NMMA and arginase contribute to eNOS uncoupling and endothelial dysfunction resulting in the development of hypertension (Fig. 3a) [23, 24, 180–184]. This was supported by the study showing that a defect in L-arginine transport exists in hypertensive and genetically predisposed normotensive subjects [183]. In addition, offsprings of essential hypertensive patients display a reduced vasodilatory response to acetylcholine linked to a defect in the L-arginine-nitric oxide pathway [182]. These studies suggest a role of decreased levels of L-arginine in the pathogenesis of hypertension. This was supported by the study showing that the intravenous administration of L-arginine decreased the mean arterial pressure as well as total peripheral resistance in hypertensive patients [185]. In addition, L-arginine was also reported to reduce blood pressure in animal models of hypertension including salt–sensitive hypertensive rats [186]. Furthermore, perinatal dietary supplementation of L-arginine with antioxidants including vitamin C, vitamin E and taurine was also shown to attenuate the development of hypertension in aging SHR [187]. In addition, the inhibition of arginase that is upregulated in hypertension and decreases the intracellular levels of L-arginine also attenuated blood pressure, vascular function and cardiac fibrosis in SHR and suggests a link between L-arginine and development of hypertension [188].

The cofactor BH4 is an important regulator of eNOS activation and NO generation. Numerous studies have demonstrated that reduced bioavailability of BH4 is associated with endothelial dysfunction contributing to the pathogenesis of vascular disease states including hypertension. Inhibition of BH4 biosynthesis has been shown

to impair endothelium-dependent relaxations in canine basilar artery [22]. In addition, BH4 oxidation-induced eNOS uncoupling has also been demonstrated in endothelial cells from DOCA-Salt hypertension [189]. Furthermore, in SHR, the supplementation of BH4 was shown to diminish the eNOS-dependent generation of (O_{2^-}) associated with increased production of NO [190]. Coronary endothelial cells from the diabetic BB rats also exhibited BH4 deficiency which was attributed to the decreased expression of GTP cyclohydrolase, the rate-limiting enzyme for de novo synthesis of BH4 [191]. Furthermore, the hypertensive and diabetic patients also displayed the reduced levels of BH4 and eNOS uncoupling [184, 192] and supplementation of BH4 improved endothelial cell function in patients with diabetes, coronary artery disease and hypertension [192–196]. The antioxidant ascorbate was shown to increase NO synthesis in endothelial cells by increasing the levels of BH4 and improving its binding to eNOS [31, 32, 39]. This was further supported by the study showing that both ascorbate and BH4 prevented the $ONOO^-$—induced uncoupling of eNOS in bovine aortic endothelial cells [26].

In addition to L-arginine and cofactor BH4, ADMA an inhibitor of eNOS also plays a role in the regulation of NO synthesis [197]. An upregulation of ADMA was shown to impair the bioavailability of NO leading to eNOS uncoupling and vascular dysfunction [198]. In support of this, several studies showed that increased levels of ADMA were associated with the pathogenesis and progression of vascular diseases including hypertension and diabetes mellitus [199]. However, exogenous supplementation of L-arginine was shown to relieve the inhibitory effect of ADMA on NO synthesis and NO-mediated vascular functions [200]. Thus, strategies to maintain the physiologically relevant levels of these cofactors is essential to prevent eNOS uncoupling associated pathologies.

Nitric oxide and Atherosclerosis

Atherosclerosis is a chronic vascular disease that leads to myocardial infarction and ischemic stroke due to thrombotic occlusion and stenosis of blood vessels. The precise sequence of events responsible for the initiation and progression of atherosclerosis remains currently elusive, however, studies done during the last decade have demonstrated an important role of dyslipidemia and associated changes in the milieu of the vessel wall as crucial mediators of this process [201, 202]. Exaggerated levels of oxidized form of low density lipoproteins (ox-LDL) and endothelial dysfunction, along with the activation of pro-inflammatory pathway are among the key contributors of atherogenesis [201–204]. Enhanced adhesion, migration, accumulation and proliferation of immune and non-immune cells such as monocytes, VSMC, macrophages, foam cells, leucocytes have also been associated with thrombogenesis [205, 206]. A decreased bioavailability of NO has been suggested as a hall mark of endothelial dysfunction associated with atherosclerotic vascular disease [207]. As alluded earlier, eNOS ‘uncoupling’ appears to be one of the prominent mechanisms resulting in reduced NO generation in the vessel wall. Ox-LDL has been shown to suppress NO levels in EC via excessive production of ROS through lectin-like ox-LDL receptor-1(LOX-1) [208]. Suboptimal concentrations of L-arginine or BH4 or

higher levels of ADMA may also limit catalytic activity of eNOS to generate sufficient amount of NO in EC. Studies showing that L-arginine supplementation in LDL receptor knockout (KO) mice or hypercholesterolemic rabbit models of atherosclerosis resulted in a reduction in the lesion surface area in aorta, support a role of NO as an antiatherogenic molecule [209, 210]. Additional support for a role of NO in inhibiting the progression of atherosclerotic disease was provided by the observations that treatment of either apolipoprotein E (apo-E) KO mice or cholesterol clamped rabbits with L-NAME accelerated the plaque formation [211, 212].

The molecular mechanisms by which NO exerts its atheroprotective role include its ability to increase vasodilation, inhibit platelet aggregation and monocyte adhesion to endothelium [213, 214]. NO also suppresses the expression of key mediators of cell adhesion including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant-1(MCP-1), [215, 216] as well as reduces the hypertrophic, proliferative and migratory responses in VSMC [46, 107–112, 116]. Thus, modulation of these key cellular pathways by NO appear to be responsible for the atheroprotective properties of NO. Consistent with this notion, lipid lowering HMG CoA reductase inhibitors of the statin family in addition to lowering plasma LDL levels and atherosclerotic plaque stability also increased eNOS expression and improved endothelial functions [217].

Conclusions

Nitric oxide (NO) is an important vasoprotective molecule that serves as a vasodilator and is a key regulator of endothelial functions. A dysfunctional NO generating system causes oxidative and nitrosative stress due to eNOS uncoupling resulting in impaired endothelial functions as well as remodeling of the vessels. NO donors or gene transfer of NOS exert beneficial effects in improving endothelial functions, lowering hypertension and suppressing vascular remodeling and neointimal hyperplasia in cellular and animal model systems. The potential mechanisms by which NO exerts these beneficial effects include the attenuation of signaling pathways responsible for inducing the hypertrophic, migratory and proliferative cellular responses that are often upregulated in cardiovascular pathologies. Thus, NO remains a promising therapeutic molecule for the treatment of cardiovascular diseases, however, the labile nature of NO and confounding factors of targeted gene delivery, limit its translational use. To overcome these issues, several groups are engaged in developing nanofiber and stent-based delivery systems for NO production and gene delivery for eventual use in cardiovascular therapy.

Acknowledgements Original work contributing to this chapter was supported by grants from the Heart Stroke Foundation of Canada and Canadian Institutes of Health Research to M.B.A.-S. and A.K.S.

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Chapter 3
Nitric Oxide and Cardiovascular Diseases: Cardioprotection, Complications and Therapeutics

Gaurav Kumar, Sanjay Kumar Dey, and Suman Kundu

Abstract Perpetually increasing cardiovascular complications significantly contribute to economic slow-down in developing nations. Indeed, adverse cardiovascular events are among the world's greatest mortality factors. The underlying cause behind these events is hypertension, which in advance stages, manifests with the development of multifactorial outcomes ultimately leading to organ damage and subsequent death of the individual. One of the major reasons behind the onset of hypertension is endothelial dysfunction, a physiological and clinical situation where normal functions of vascular endothelium are altered. This alteration results in a lack of proper production as well as the distribution of nitric oxide, which is a potent vasorelaxant. Efforts to maintain adequate NO signaling are always in practice. One of such approaches is targeting cytochrome b5 reductase3 at the myoendothelial junction, an anatomical location between endothelial cells and vascular smooth muscle cells. This chapter highlights the production and distribution of NO by nitric oxide synthases and cytochrome b5 reductase3, respectively, its contribution in various cascades of vascular homeostasis and its established role in cardiovascular disorders followed by different strategies and a glimpse of the clinical studies considered to improve NO signaling in vivo.

Keywords Nitric oxide · Endothelium · Hypertension · Cardiovascular homeostasis · Cytochrome b5 reductase3 · Nitric oxide synthase

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_3

Introduction

Cardiovascular diseases (CVDs) are responsible for significantly high socio-economic burden in a population. Global incidences of CVDs are ever increasing which leads to premature mortalities and disabilities in humans [1]. It is interesting to note that underlying causes and pathologies of most of CVDs, including coronary artery diseases, peripheral vascular disease, myocardial infarction, cardiac arrhythmia, stroke, atherosclerosis and venous thromboembolism, are of vascular origin [1]. Of note, the etiological risk factors of all these CVDs include hyperlipidemia, diabetes, obesity, sedentary lifestyle, hypertension and dysfunctional nitric oxide (NO) balance in the vasculature [1, 2]. Although, even after high fatalities among CVDs, the recognition and attentive anticipation of underlying risk factors may reduce the world-wide rising toll of CVDs. However, besides currently available effective anti-hypertensive therapeutics, the overall control of CVDs still remains sub-optimum [1, 3–6]. In such a scenario, NO signaling offers ample opportunities to curb CVDs and related vascular disorders.

NO is a pleiotropic molecule with crucial roles in cardiovascular equilibrium. Significant evidences have been secured regarding the roles and mechanisms of NO signaling in governing the cardiovascular functions. However, it still remains the focus of active research as to how this gaseous molecules precisely regulates critical cardiovascular events [7]. Uncovering the multiple roles of NO in maintaining the wide array of vascular functionalities has led to an appreciable shift in cardiovascular therapeutics. While the dilemmas involving the intricacies of NO have thrived, NO still remains one of the under-estimated and under-studied molecules [8]. Since NO is an extremely crucial component of the vascular system, efforts in regulating the bioavailability of NO to modulate cardiovascular status in humans are always in practice. However, the fact that early attempts to regulate NO bioavailability by therapeutic means have enormously failed in clinical trials, cannot be neglected. Therefore, enhanced empathy has come up with a plethora of novel therapeutic tactics to be recognized [9]. While uncovering the complex regulation of NO signaling, we first summarize the distribution of the enzymes and their isoforms involved in the synthesis and distribution of NO in the microvasculature, followed by the downstream effector molecules of NO. We next summarize the involvement of NO in the maintenance of vascular homeostasis while linking it with existing CVDs along with the novel therapies involved in modulation of NO bioavailability in CVDs. Finally, we provide a glimpse of therapeutic modulation in NO signaling and clinical trials accompanying NO signaling.

Nitric Oxide (NO) and NO Synthases (NOS)

NO, a free radical, is generated by natural electrical discharges, for example, lightning [10, 11]. Additionally, it is also released by automobiles and fuel power plants as an air pollutant [12]. At industrial scales, NO is produced as a key chemical intermediate [13]. In 1986, the endogenous synthesis of NO as the endothelium-derived relaxing factor was initially suggested by Robert Furchgott and Louis Ignarro [14, 15]. Later on, NO was accepted as a gaseous signaling mediator in organisms [16]. Since the abnormalities in production and distribution of NO are linked with several CVDs including but not limited to hypertension, it was soon realized that NO plays a major part in the homeostasis of the entire cardiovascular system [17–19]. In the human body, NO is endogenously synthesized by NOSs; encoded by distinct genes, namely NOS1 which forms neuronal NOS (nNOS), NOS 2 which forms inducible NOS (iNOS), and NOS 3 which encodes for endothelial NOS (eNOS) [7]. It is noteworthy that nNOS and eNOS are constitutively expressing isoforms which can be controlled by numerous post-translational events including acetylation, phosphorylation, S-nitrosylation, S-glutathionylation and even by direct or indirect protein–protein interactions. In contrast, iNOS, which is the inducible isoform, is majorly under the control of gene transcription governed by the pro-inflammatory and oxidative environment [7]. Since NOSs are the key producers of NO in the human body, it becomes important to emphasize these enzymes in cardioprotection and cardiac impairment. The following few sections are briefly focused on NOSs in the context of their genetic location, structural aspects, physiological location and functional roles.

Neuronal Nitric Oxide Synthase (nNOS)

nNOS, a 1434 amino acid protein, having molecular mass of 161 kDa, is the product of NOS 1 gene located on chromosome 12 (12q24.2) in humans [20]. This constitutively expressing enzyme is found in autonomous cardiac neurons [21], in vascular smooth muscle cells (VSMCs) [22, 23] and on sarcoplasmic reticulum present in cardiomyocytes [24]. Although, nNOS exists in monomeric and dimeric mixtures, only the dimeric form of nNOS is functional. Monomeric nNOS is composed of N-terminal and C-terminal oxygenase and reductase domains, respectively. The N-terminal catalytic domain of nNOS binds with tetrahydrobiopterin (BH_4), which acts as a redox-co-factor for this enzyme. Apart from BH_4, the catalytic domain of nNOS also binds with L-arginine (a substrate for NO generation) and zinc ions. In contrast, the C-terminal domain in nNOS binds with FMN, FAD and NADH [25–27]. Similar to the other forms of NOS, nNOS is also regulated by several intrinsic and extrinsic factors, such as availability of co-factors and substrates, interacting partners and a plethora of post-translational modifications [28].

Inducible Nitric Oxide Synthase (iNOS)

iNOS, a 1,153 amino acid protein, with a molecular mass of 130 kDa, is encoded by a gene, i.e., NOS 2, located over chromosome 17 (17q11.2-q12) in humans. Structurally, iNOS contains an N-terminal and C-terminal oxygenase and reductase domain, respectively. The N-terminal domain of iNOS has heme and binds with L-arginine, BH_4 and calmodulin. Whereas, the reductase domain joins with NADPH and is engaged in transferring electron from NADPH to FAD and finally to FMN [29]. iNOS mediated NO production is dependent on transcriptionally regulated cytosolic expression of the enzyme, which in turn, is dependent on several oxidative and pro-inflammatory environment in the cells. iNOS is expressed in several cells including, cardiomyocytes, nerves cells, leukocytes, ECs, fibroblasts and VSMSs [30–33]. As compared to the other isoforms, iNOS is a calcium-independent enzyme [34]. The V_{max} of iNOS is surprisingly higher as compared to nNOS and eNOS. Therefore, iNOS maintains a higher NO production until the complete expenditure of substrates and co-factors or enzyme degradation [35]. Hence, the expression of iNOS is majorly linked with the pathological remodeling in cardiovascular biology [36]. Apart from this, iNOS-mediated increased NO production following the exposure of pro-inflammatory environment is also a major reason behind hypotension, cardiodepression and lowered reactivity in vasculature with retarded vascular tone [37–41].

Endothelial Nitric Oxide Synthase (eNOS)

eNOS, a 1203 amino acid protein, with a molecular mass of 133 kDa, is encoded by NOS 3 gene located over chromosome 7 (7q35-7q36) in humans [42]. In the vasculature, eNOS is majorly prevalent in ECs. However, occasional expression of eNOS can also be seen in cardiac myocytes [42, 43], platelets [44] and erythrocytes [45, 46]. Similar to nNOS and iNOS, eNOS is also a dimeric enzyme containing oxygenase and alpha (α) reductase domains. The α-reductase domain of eNOS transfers the electrons from NADPH to FAD and FMN while recruiting calmodulin. Calmodulin binding with the α-reductase domain facilitates the transfer of electrons [47]. In the presence of heme and BH_4, the oxygenase domain in eNOS attaches to α-reductase rendering the eNOS efficient in NO synthesis [48, 49]. The expression of eNOS can be activated by shear stress and stretch. Both of these events lead to stabilization of eNOS mRNA, which leads to nuclear factor-κB (NF-κB) and Krüppel-like factor 2 (KLF2) aided expression of eNOS [50]. On the other hand, eNOS expression can also be activated by reactive oxygen species (ROS) via several oxidant-responsive kinases, including p38 mitogen activated protein kinase as an event generated by receptor directed or physical stimulus. Furthermore, eNOS expression can be regulated by several genetic polymorphisms, epigenetic modifications, post-translational modifications and some cardiovascular therapeutics [7].

NO Synthesis and Distribution

Among the above-mentioned NOSs, eNOS has special importance in the vascular system. eNOS reacts with a variety of cellular partners while producing NO. eNOS mediated NO formation is a two-step reaction. In the initiation phase, L-arginine is hydrolyzed to N^{ω} -hydroxy-L-arginine in presence of BH_4 and calmodulin. This is followed by a second step, which comprises the oxidation of N^{ω} -hydroxy-L-arginine to L-citrulline and NO as shown below in Fig. 3.1 [47, 51, 52].

Since NO is a lipophilic signaling molecule, it can execute paracrine signaling through its rapid diffusion from ECs to VSMCs. Apart from paracrine signaling, NO also executes autocrine signaling in cardiac myocytes [42]. Once diffused to adjacent VSMCs, NO can bind with α-subunit of hemoglobin (α-globin) located at the MEJ, which is a "sandwiched" region between ECs and SMCs. Of note, the redox state of α-globin is the chief deciding factor for binding with NO. The ferrous (Fe^{2+}) state of α-globin tightly binds with NO creating a hindrance in its diffusion from ECs to VSMCs, which leads to vasoconstriction and subsequently result in hypertension. Whereas, the ferric (Fe^{3+}) state of α-globin binds with NO transiently and weakly allowing it to diffuse into VSMCs, consequently resulting in vasorelaxation. The interplay between these two redox states of α-globin is managed by CYB5R3. In addition to α-globin, CYB5R3 is also located at MEJ. Active CYB5R3 is capable of converting Fe^{3+}α-globin to Fe^{2+}α-globin, which leads to vasoconstriction executed by the above-mentioned mechanisms (Fig. 3.2) [53]. Indeed, pharmacological inhibition of CYB5R3 is an emerging strategy to control hypertension and thus associated CVDs. The authors of this chapter are working in this direction with initial preliminary success.

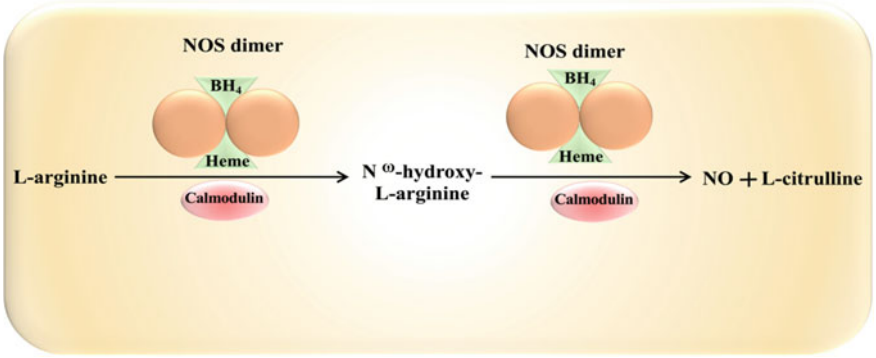

Fig. 3.1 NOS dependent secretion of NO in the vasculature. Dimeric eNOS efficiently utilizes L-arginine as a substrate in the presence of BH_4 and heme while producing NO and L-citrulline

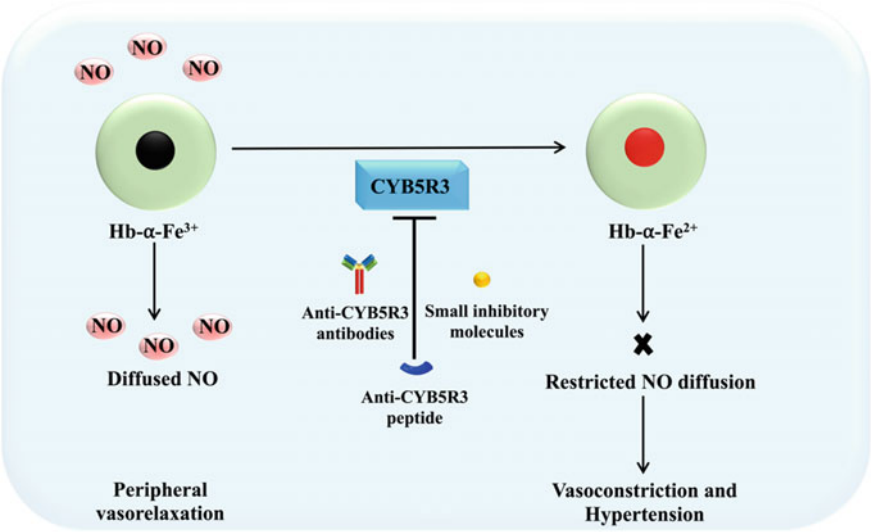

Fig. 3.2 Role of CYB5R3 in managing vascular tone. CYB5R3 controls the oxidation states of α-globin localized heme. This results in the modulation of NO diffusion across ECs and SMCs via MEJ, leading to vasoconstriction or vasodilation

NO and Vaso-Modulation

There are two different pathways by which NO modulate the vascular tone and hence, the cardiovascular functions. One of these is an indirect pathway, which involves the stimulation in soluble guanylate cyclase (sGC) activity, followed by cGMP mediated activation of protein kinase (PK) G in VSMCs, which is a major regulator of several post-translational events in the sarcoplasmic reticulum [54, 55]. Activated PKG blocks the entry of calcium ions via voltage-gated calcium channels as well as calcium discharge aggravated by inositol 1,4,5-triphosphate receptor (integrated in sarcoplasmic reticulum) leading to vasodilation (Fig. 3.3). Furthermore, PKG also modulates sarco/endoplasmic reticulum embedded calcium dependent ATPase, which endorses the *capture* of cytoplasmic calcium, and discharges it to cell exterior [15, 56–59]. This results in a net shortage of available intracellular calcium ions subsequently leading to calmodulin inactivation along with the suppression of calmodulin-dependent myosin light chain kinase (MLCK). Moreover, intracellular calcium deficiency also leads to an upsurge in myosin light chain phosphatase (MLCP) activity. The activation and inactivation of MLCP and MLCK, respectively, results in breakage of the actin-myosin-cross bridge ensuring the relaxation of SMCs resulting in vasodilation [12].

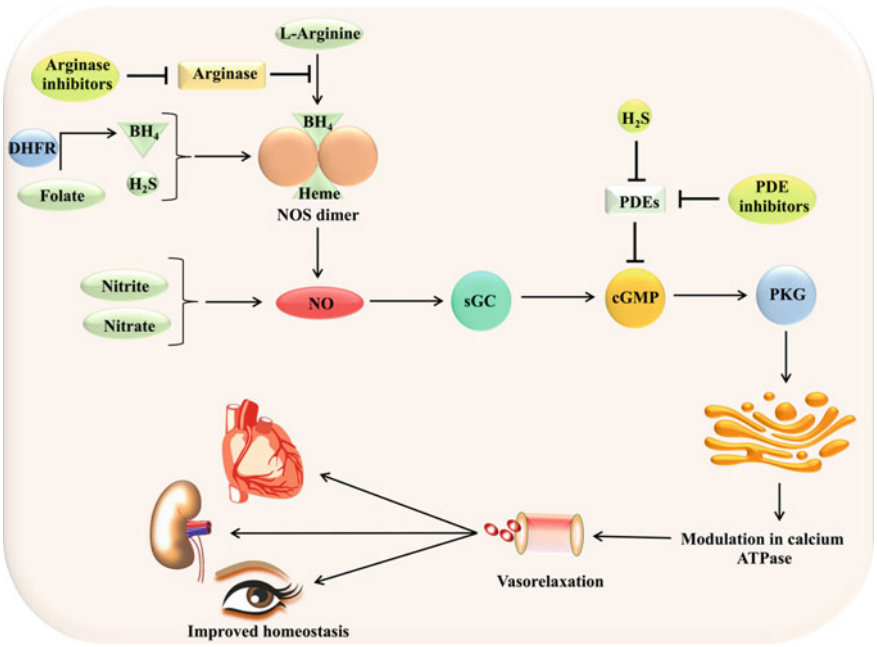

Fig. 3.3 Role of NO in homeostasis and different therapeutic approaches and potential targets in cardiovascular complications. Several inhibitors of arginase and phosphodiesterases (PDEs) contribute to efficient production and distribution of NO in the vasculature. Additionally, supplementation with dietary nitrate, nitrite and folate also results in proper production of NO. Endothelial NO activates sGC, which ultimately leads to vasodilation followed by improved homeostasis in vivo

The Link Between NO and Hydrogen Sulfide (H_2S) Signaling

Although the NO signaling is itself self-sufficient mechanism of vasodilation, H_2S signaling is also being sought by the scientific community as a "balancing" vasodilatory mechanism in cardiovascular biology. In the last 10 years, H_2S has established itself as a potent vasodilatory factor [60]. H_2S enhances and complements NO signaling [61, 62]. In the human body, H_2S is produced by several enzymes including cystathionine β-synthase, 3-mercaptopyruvate sulfurtransferase and cystathionine γ-lyase. These three enzymes are known to express in vascular walls [63]. While complementing with NO signaling, H_2S increases the phosphorylation of eNOS at Ser1177, which is an activating post-translational modification, and results in increased synthesis of eNOS mediated NO [62]. Apart from phosphorylating eNOS at Ser1177, H_2S is also considered to prevent the oxidation of Fe^{2+} heme, an obligatory factor required to dimerize eNOS subunits [64]. Moreover, H_2S also inhibits the activity of PDEs [65], the enzymes accountable for the indirect breakdown of NO. Furthermore, H_2S also activates PKG through S-sulfhydration at Cys42 [66]. All of these cooperative events synchronously reinforce NO mediated cardioprotection [67].

Cardiovascular Homeostasis Maintenance by NO-NOS

Homeostasis in Blood Vessels

eNOS derived NO regulates the vasorelaxation via its paracrine diffusion to VSMCs and subsequently activating PKG [68]. Additionally, in coronary microcirculation, paracrine NO also modulates left ventricle dependent hemodynamic events [69, 70]. As discussed earlier, CYB5R3 is the major regulatory factor at MEJ as it regulates the redox state of α-globin, and thus the paracrine diffusion of NO from ECs to VSMCs [71]. In addition to α-globin, cytoglobin (Cygb), an oxygen-dependent NO dioxygenase, is also a chief regulator of NO concentration in the microcirculation. Cygb is expressed in fibroblasts and VSMCs and it converts NO to nitrates and thus, it lowers the available NO, which is otherwise used in the activation of sGC dependent PKG [72]. In support of this, Cygb knockout mice have demonstrated greatly prolonged NO decay, increased vascular relaxation, lowering in blood pressure and systemic vascular resistance [72]. Interestingly, the NO-dependent dioxygenase functionality of Cygb is also controlled by CYB5R3.

In addition to eNOS, blood pressure is also regulated in numerous ways by nNOS. In nitrergic nerve fibers, nNOS controls cerebral and renal blood fluidics [73–76]. It has been seen that in NOS 3 null mice lacking eNOS expression, flow-induced expansion of coronary arteries is partially compensated by nNOS [77]. Likewise, in human peripheral and coronary vascular beds, operational nNOS might be seen [78, 79]. This is supported by the effect of systemic infusion of S-methyl-l-thiocitrulline, which is an nNOS preferential inhibitor [80]. The hypertensive outcomes of S-methyl-l-thiocitrulline were supposed to be dependent on nNOS inhibition in skeletal myocytes. In fact, by inhibiting α-adrenergic vasoconstriction, nNOS is considered to augment the perfusion of skeletal muscle occurring during contraction [81, 82].

Homeostasis in Perivascular Adipose Tissue (PVAT)

Certain adipocytes, different from those usually found in white or brown adipose tissue, forms PVAT. PVAT down-regulates the contractile response of vessels residing in close vicinity by releasing adipokines [83]. While controlling the contractile response, a variety of mediators including, adiponectin [84], H_2S [85, 86], eNOS derived NO [87], palmitic acid methyl ester [88], angiotensin (1-7) [89] and H_2O_2 [90] are involved, which in turn, depends on the composition, physiological condition and localization of PVAT [91]. Many of these cascades converge at eNOS mediated NO synthesis in ECs and its diffusion into VSMCs or adipocytes. Apart from this, adiponectin itself provokes NO release from adipocytes and ECs, so as to relax VSMCs [92–94].

Homeostasis in Cardiac Myocytes

The functional aspects of the heart are greatly dependent on eNOS and nNOS. Studies with isolated cardiac myocytes have shown that stretched and induced eNOS derived NO induces a gentle increase (over 5–10 min) in the shortening of sarcomeres, along with calcium transient amplitude by enhancing the secretion of calcium out of sarcoplasmic reticulum. This is a positive Anrep effect, as well as an autoregulation process where the myocardial contractility intensifies with afterload. This effect is facilitated by phosphatidylinositol 3-kinase (PI3K)-AKT dependent phosphorylation at Ser1177 in eNOS, which is a permissive post-translational modification, leading to an increase in NO release. In support of this, NOS 3 null mice demonstrated an abolished Anrep effect, which was restored later by the administration of NO donor [95]. NOS also regulates basal contractility and β-adrenergic responsiveness in cardiac myocytes by altering calcium ion homeostasis or calcium sensitivity of myofilaments [96].

Homeostasis in Cardiovascular Nerve Cells

As mentioned earlier, nNOS is expressed in never cells. Additionally, the expression of nNOS is also prevalent in cholinergic (vagal) neurons as well as sympathetic ganglia reaching the sinoatrial node of the heart. nNOS derived NO executes sGC-cGMP-dependent PDE3 inhibition, consequently resulting into increase in cAMP-PKA dependent phosphorylating events in N-type calcium channels of cholinergic neurons. On the other hand, in sympathetic ganglia, nNOS derived NO regulates the activation of sGC-cGMP-PDE2 axis. This results in calcium induced release of acetylcholine by exocytosis, which leads to a reduction in cAMP-PKA-dependent calcium influx, as well as inhibition in calcium-dependent discharge of norepinephrine by exocytosis. All these events result in nNOS derived shift in autonomic balance towards a parasympathetic shift, leading to a decrease in heart beat [97, 98].

Altogether, nNOS diverts the autonomic equilibrium towards a greater parasympathetic effect, thereby decreasing the heart rate. Increase in eNOS or nNOS expression levels or activity may be fruitful in certain scenarios of increased adrenergic rush, for instance in heart failure [99, 100].

NOS, NO and Cardiovascular Disease

Endothelial Dysfunction

Endothelial dysfunction is a pathophysiological event where its vasoprotective state changes to deleterious one. Indeed, endothelial dysfunction is the initial step towards atherogenesis [101, 102], which later on results in the development of hypertension [103, 104], and associated CVDs. Of note, endothelial dysfunction initiates from oxidative stress resulting in rapid uncoupling, and thus the inactivation of dimeric eNOS [105]. In addition to a significant decrement in NO bioavailability due to dysfunctional endothelium, it is also responsible for the secretion of a variety of factors, which are further unfavorable to the vascular intima [106].

Fortunately, endothelial dysfunction can be reversed by improving BH_4 availability in hypertension, and atherosclerosis [107–109]. Apart from improving BH_4 availability, endothelial dysfunction can also be reversed by understanding the functional regulation of enzymes, such as by the use of statins to reduce caveolin 1, which is a negative regulator of eNOS. Apart from this, co-existence and association of NADPH oxidase in caveolae, increase in sirtuin 1 levels [110, 111], supplementation with L-arginine [112], use of statins [113], antioxidants [114, 115], Mediterranean diet, increased physical activity, body weight management and conventional antihypertensive therapeutics, including but not limited to, calcium channel blockers and their combination with renin inhibitors [116–118], some β-blockers, (for instance, Nebivolol) and their combination with angiotensin converting enzyme inhibitors [119–122], can improve or revert endothelial dysfunction [119].

NOS and NO in Cardiac Remodeling

Loss of eNOS or nNOS function is associated with myocardial damage occurring due to infarction or ischemia–reperfusion [123–126]. Conversely, over-expression of eNOS or nNOS reduces the infarct size as well as improves left ventricular function [127–131]. Of note, ischemia–reperfusion activates protein-tyrosine kinase 2β, which provokes the inhibition of eNOS through inhibitory phosphorylating events, for instance, phosphorylation of Tyr657 [67]. NOS shields the ischemia–reperfusion mediated myocardial damage through a variety of mechanisms, including the inhibition of ROS generation by mitochondrial cytochrome c oxidase, decreasing calcium overloads by inhibiting L-type calcium channels in the sarcolemma, inhibition of mitochondrial K^+-ATP channels and reducing the oxidative stress by inhibition of xanthine oxidoreductase [123, 125, 127, 132, 133].

Contribution of NOS and NO in Common CVDs

Hypertension

In hypertension, the endothelium-dependent relaxations are significantly reduced [134]. This reduction in response to endothelial vasodilators, including NO, may be due to higher circulating profiles of asymmetric dimethylarginine (ADMA) [135], which performs as an intrinsic and intracellular competitive eNOS inhibitor. ADMA also participates in oxidative stress [136–138], leading to endothelial dysfunction, and thus hypertension. Similarly, chronic hypoxia in pulmonary hypertension also leads to a significant decrement in endothelium-dependent vasorelaxation in pulmonary arteries. This may be due to the overproduction of ROS, which reduces eNOS activity as a consequence of the tight coupling of caveolin 1 [139, 140].

Diabetes

Though diabetes is itself not a CVD, being a hallmark of many CVDs, it may contribute to their onset [106]. Chronic exposure to increased glucose levels, which is a common factor in insulin resistance and diabetes, may impair arterial endothelium-dependent relaxation [141–143]. Furthermore, in type 2 diabetes, endothelial dysfunction may be dependent on genetic predisposition [143]. Impaired NO-dependent vasodilation in diabetes may be dependent on: (1) reduced availability of BH_4 leading to uncoupling of eNOS [144], (2) overactivity of arginases which compete with eNOS for their common substrate [145, 146], (3) elevated ADMA levels in circulation [147, 148], (4) NO scavenging by superoxide and increased peroxynitrite [143, 149, 150], (5) alteration in the responsiveness of VSMCs [151, 152] and many more [19].

Heart Failure and Ventricular Hypertrophy

Reduced endothelium-dependent relaxations can be seen in coronary and peripheral arteries in vivo in conjugation with heart failure and/or ventricular hypertrophy. It may be due to exaggerated oxidative stress budding from under-perfusion of tissues, which leads to eNOS downregulation and hence, a reduced NO bioavailability [153–155]. Furthermore, impaired responsiveness of VSMCs is also responsible for eNOS dysfunction [156]. Interestingly, the extent of endothelium-dependent vasodilation forecasts the outcomes of chronic heart failure [157]. It is noteworthy that endothelial dysfunction accompanying heart failure can be reversed temporarily by heart transplantation. Additionally, low and calculated doses of ouabain may also improve endothelial NO production in vivo [155, 158].

Coronary Artery Disease (CAD)

Subjects with a profound risk of CAD are characterized by hampered peripheral vasodilation [159]. In coronary circulation, the endothelial dysfunction is represented by CAD [160–165]. Further, it is also associated with mitochondrial dysfunction resulting from low physical activity [166] and increased ADMA levels [167]. In animals and humans, the occurrence of myocardial infarction and stroke are the consequences of endothelial dysfunction [154, 168–171], followed by defective eNOS-NO signaling.

Atherosclerosis

Atherosclerosis is the manifestation of cellular, biochemical and hemodynamic events in blood vessels. It causes vascular injury, which is manifested by endothelial dysfunction, cell proliferation, accumulation of oxidized LDL and recruitment of inflammatory cells [172]. Endothelial NO suppresses the proliferation of VSMCs in response to vascular injury [173]. Additionally, NO usually suppresses the development of atherosclerotic plaques by modulating several events, including the suppression of platelet aggregation and decrement of leukocyte recruitment [174, 175]. Therefore, deficiency of vascular NO, endothelial dysfunction, or dysfunctional eNOS, results in the predisposition of atherosclerosis.

NO Signaling and Therapeutics and Pitfalls

Physiological NO signaling can be restored, and NO production and its bioavailability can be altered by targeting downstream elements in NO signaling using therapeutics (Fig. 3.3). Here we discuss the emerging therapeutic approaches to manage NO bioavailability which are applied in CVDs and related vascular disorders.

SGC Stimulators or Activators

sGC activators or stimulators augment the production of cGMP by sGC [176] (Fig. 3.3). sGC activators, including ataciguat and cinaciguat, are known to activate sGC in either oxidized or heme free state [177]. On the other hand, sGC stimulators, including riociguat and vericiguat bind with sGC in heme bound state and amplify the effects endogenous NO. Indeed, cinaciguat has previously been used in heart failure. However, trials were terminated owing to exaggerated hypotensive effects [178]. Whereas, riociguat has been shown to stimulate favorable events in

pulmonary atrial hypertension and chronic-embolic pulmonary hypertension with an overall improvement in the quality of life and exercise tolerance [179].

BH_4 or Folate Supplementation

Since BH_4 is a natural coupling agent involved in the dimerization of eNOS, therapeutic administration of BH_4 can be considered as among emerging therapeutic approaches. Using BH_4 supplementation, initial clinical trials done with the diabetic and hypertensive subjects have shown promising outcomes [180, 181]. Unfortunately, later trials with BH_4 as a therapeutic linage (NCT00532844 and NCT00325962) [182] have been disappointing, which have been attributed to rapid oxidation and clearance of BH_4, leading to lowering in its sufficient bioavailability to NOSs. Similarly, folic acid and its products are distributed in plasma in a dose-dependent manner, followed by dihydrofolate reductase (DHFR) dependent reduction [183]. Increased DHFR activity has been attributed to protective events in vascular endothelium as a consequence of augmented reductive recycling of BH_4 (Fig. 3.3) as well as direct ROS quenching effects of folate, especially superoxide [184, 185]. Folic acid supplementation has been shown promising in improving CAD and an overall decreased the risk of CVDs [186–188].

Nitroxyl (HNO)

HNO is generated as a result of either NO reduction or amine oxidation [189]. HNO donors viz. Angeli's salt increases the myocardial contractility in vivo [190–192]. Additionally, HNO increases the myofilament responsiveness to calcium ions in a cAMP/cGMP independent manner [193]. HNO donors are also known for vasodilating events owing to sGC activation [194]. Although, HNO donors, for example, CXL-1020, have the distinctive advantage, with their preserved efficacy even in oxidative stress, which justifies their use in CVDs, but are known to cause inflammatory irritation at the site of infusion [195].

Nitrate and Nitrite Supplementation

Nitrate and nitrite supplementation is beneficial in in vivo models of CVDs including, ischemia reperfusion and hypertension [195]. Moreover, treatment considering the use of nitrate or nitrite could also be beneficial in pulmonary hypertension (Fig. 3.3) [196]. However, a chief drawback of this approach is the difficulty in the accurate determination of nitrate or nitrite absorption and reduction to NO especially due to the impact of dietary components or concomitant therapeutics. Indeed, treatment with

proton pump inhibitors may abolish nitrate mediated reduction in blood pressure [197]. Similarly, the simultaneous consumption of linoleic acid can also alter the metabolic outcome of nitrite, which may abrogate its vasodilatory effects [198].

PDE Inhibitors

Inhibitors of PDEs, especially PDE5, used to be initially developed for the treatment of CAD. PDE5 is overexpressed in lung vasculature and its activity is increased in pulmonary atrial hypertension. Hence, PDE5 inhibitors are mainly used in the treatment of different subtypes of pulmonary hypertension (Fig. 3.3). Some of these PDE5 inhibitors are avanafil, sildenafil, tadalafil, vardenafil, sildenafil and tadalafil. Of note, sildenafil and tadalafil have been approved for the treatment of pulmonary atrial hypertension, as they efficiently curb pulmonary artery pressure and vascular remodeling [199, 200]. To a greater extent, the beneficial effects of PDE5 inhibitors are limited to animal models and have not been translated to the clinic straightforward [201–203].

Experiences Form Clinical Studies

Since cardiovascular complications impart a significant burden on the economy of any country, efforts to lower down the mortality rates due to CVDs are always in practice. Scientists and clinicians conduct enormous effort worldwide demanding clinical studies with the ultimate aim of human welfare [195]. Some of the clinical studies considering the use of above-mentioned therapeutic approaches are given below in Table 3.1.

Conclusion and Future Perspectives

Certainly, NO is a crucial molecule in maintaining the homeostasis in cardiovascular biology and any adverse alteration in its bioavailability may lead to the development of critical clinical situations manifested with severe CVDs. Targeting NOSs in the clinical setting of a plethora of CVDs, offers an emerging therapeutic approach since these enzymes are directly involved in the production of NO and NO production and distribution is the “branching point” in the onset of CVDs and associated pathologies. Since eNOS is a regulatory enzyme, alteration in its activity, enhancing its coupling

Table 3.1 Clinical studies considering emerging therapeutic approaches in the modulation of NO signaling in CVDs (Adapted and modified from Farah et al. [7])

Category/molecule	Phase	Duration of treatment	Pathological condition	Trial Identity
sGC stimulators or activators				
Riociguat	II	6 months	Heart failure with preserved ejection fraction (HFpEF,)	NCT0274439
	II	4 months	Pulmonary hypertension, left ventricular systolic dysfunction	NCT01065454
Vericiguat	II b	3 months	Heart failure with reduced ejection fraction (HFrEF)	NCT01951625
	III	~3.5 years	HFrEF	NCT02861534
	II b	3 months	HFpEF	NCT01951638
Ataciguat	II	12 months	Aortic valve calcination	NCT02481258
Nitroxyl				
Infused BMS-986231	II	6 h	HFrEF	NCT02157506
Infused BMS-986231	II	48 h	HFrEF	
Nitrate and nitrite supplementation				
Nitrite	II	2 months	HFpEF (>70 years)	NCT02918552
	II	3 months	Pulmonary hypertension- HFpEF	NCT03015402
Nebulized nitrite	II	1 month	HFpEF	NCT02742129
Inhaled nitrite	II	3 months	HFpEF	NCT02713126
	II	Acute	HFpEF	NCT02262078
Infused nitrite	II	Acute	HFpEF	NCT01932606
PDE5 inhibitors				
Tadalafil	III	3 years	Congenital systemic right ventricle	NCT03049540
	IV	6 months	Aortic stenosis	NCT01275339
Sildenafil	III	6 months	Chronic heart failure	NCT01616381
	III	3 months	Pulmonary hypertension-systolic heart failure	NCT01913847
	III	3 months	Diastolic heart failure	NCT00763867

and regulating its expression in the vasculature either therapeutically or by post-translation modification also governs the cardiovascular status of a subject. Furthermore, eNOS enhancers, along with antioxidant response enhancers, may ameliorate the therapeutic efficacy of current strategies against several CVDs. The clinical trials considering the different therapeutic strategies of CVDs have failed enormously. Under such circumstances, advanced drug-delivering strategies involving the virus encapsulation, coupling with nanoparticles, using the novel and small peptides against the protein of interest in NO signaling, may also give an efficient troubleshooting and therapeutic sideline approach in CVDs [204]. Furthermore, modulating the distribution of NO by CYB5R3-α-globin-axis is also one of the approaches, which is advantageously involved in the alteration of NO signaling within human vasculature. Of note, currently available therapeutics for CVDs have a plethora of adverse effects. In such situations, targeting NO bioavailability by CYB5R3 may prove itself as a boon.

Acknowledgements All authors of this chapter are grateful to the Department of Biochemistry, University of Delhi, South Campus, New Delhi, India, for supporting this study. The authors are thankful to Pixabay for supporting the artwork of Figure 3.3. SK acknowledges the Department of Biochemistry (DBT), Government of India, for the financial assistance (vide Grant IDs: BT/PR13531/MED/30/1523/2015, BT/PR8391/BRB/10/1231/2013 and BT/01/COE/07/UDSC/2008). SK is thankful to the University of Delhi (R&D; Institution of Eminence) and University Grants Commission (UGC-SAP) for financial support. SK also acknowledges Department of Science and Technology (DST-PURSE) and Defense Research and Development Organization (DRDO), Government of India for providing the necessary funding. Council of Scientific and Industrial Research (CSIR) is acknowledged by GK for providing research fellowship. SKD acknowledges Dr. B.R. Ambedkar Center for Biomedical Research (ACBR), University of Delhi, India for various help to complete this work.

Declaration of Competing Interest Declared none.

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Chapter 4
Nitric Oxide and the Heart Autonomic Nervous System

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Abstract Nitric oxide (NO) is important in physiological cardiovascular regulation and is involved in the pathophysiology of cardiac disorders. This chapter describes the role of NO in the structure and function of the cardiac nervous system, with special focus on the intrinsic ganglia and nerves of the heart. Neuronal nitric oxide synthase (nNOS) is the NOS isoform expressed in somata and intracardiac nerve fibers, mainly associated with the postganglionic parasympathetic nervous system. Intrinsic cardiac ganglia contain parasympathetic postganglionic neurons that are nNOS positive or co-localize nNOS with choline acetyltransferase (ChAT). Scarce postganglionic sympathetic nitrergic fibers and parasympathetic afferent fibers are also observed. NO is a strong modulator of parasympathetic/sympathetic interaction and autonomic action on myocardial function, and of the cardiac conduction system and coronary circulation. Loss of endogenous NO modulation predisposes to dysautonomia and impaired cardiac function.

Keywords Parasympathetic nervous system · Sympathetic nervous system · Cardiac function · Cardiac disease · Nitric oxide synthase · Dysautonomia

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_4

Nitric Oxide in the Heart

Nitric oxide (NO) is involved in the regulation of multiple cardiovascular functions, both under physiological and pathological conditions. The literature on the subject is extensive, having become increasingly relevant, and important advances have been made in the knowledge of the biology of NO in the last decades. However, many aspects of the nature and exact site of action of NO in the heart and the role that each nitric oxide synthase (NOS) isoform performs are not yet fully understood.

NO is synthesized by three isoforms of NOS: endothelial (eNOS or NOS III), neuronal (nNOS or NOS I) and inducible NOS (iNOS or NOS II). All three isoforms are present within the heart and are localized in a subcellular compartment. Under physiological conditions, the production of NO—stimulated by mechanisms that involve NOS-receptor activity—is mediated by the eNOS and nNOS isoforms, while the stress response is mediated by iNOS [1]. eNOS is located primarily in the plasma membrane and caveolae of myocyte T-tubules of the cardiac myocytes and endothelial cells [2, 3]. nNOS was well identified in intracardiac somata and nerve fibers, with a great predominance in the parasympathetic system [4]. Furthermore, at the level of myocytes, nNOS is found within the sarcoplasmic reticulum and mitochondria [5, 6]. Additionally its expression in smooth muscle cells of the coronary walls was also demonstrated [7].

nNOS is the only NOS isoform that is expressed in fibers and cardiac neuronal somata in the heart, and, therefore, it is the one isoform that will be expanded upon the most throughout this chapter. NO also plays a crucial role in the central regulation of cardiovascular autonomic function, but the objective of this chapter will be to describe its function within the intrinsic cardiac nervous system.

Nitric Oxide and Neuronal Nitric Oxide Synthase in the Intrinsic Cardiac Nervous System

The neurochemistry of the intrinsic cardiac nervous system is complex and is being investigated in different mammalian species [8]. The presence of nitrergic fibers and neurons in the heart has been well documented in recent decades [9–16]. Specifically, the nNOS enzyme was found in cardiac plexi in rats [11], rabbits [17], guinea pigs [11], mice [18] and humans [19].

Studies in guinea pig atria showed that 44% of the neurons contained in the cardiac ganglia are positive for nNOS [20]. Intrinsic neurons labeled for nNOS also showed immunoreactivity for choline acetyltransferase (ChAT), suggesting that postganglionic parasympathetic neurons are the ones expressing nNOS [20]. Furthermore, these intrinsic nitrergic neurons have a long axonal extension that enters a nerve bundle and traverses long distances within the heart without making connections with other neighboring neurons of the originating ganglion. Although the intrinsic nitrergic fibers move away from the ganglion where the nNOS-expressing

neuronal somata are located, immunohistochemical studies demonstrate the presence of numerous extrinsic fibers [20]. The studies by Calupca et al. suggest that these extrinsic fibers labeled for nNOS belong almost completely to parasympathetic visceral afferents inputs whose neuronal bodies are located in the inferior ganglion of the vagus nerve (*ganglion nodosum*) [20, 21]. Recently, Navickaite et al. showed in rats that the most abundant population of purely nNOS-immunoreactive neuronal somata was observed in the nodose ganglia, and a high number of nitrergic nerve fibers spread along the vagal nerve and entered its cardiac branches [22] (Fig. 4.1).

Another potential origin of extrinsic nitrergic nerve fibers are the parasympathetic preganglionic neurons, located in the vagal cardiovascular regulatory nuclei of the medulla, such as the nucleus ambiguus and dorsal motor nucleus of the vagus nerve [23–28]. However, the small size and location of the medullary NOS immunoreactive neurons suggested they were probably interneurons and not preganglionic neurons whose fibers reach the heart [20]. In this same regard, the lack of co-localization of nNOS labelling with ChAT suggests that these neurons are not preganglionic parasympathetic [29–31].

Finally, extrinsic nitrergic fibers of the heart may also belong to the sympathetic system, with its afferent component located in the sensory neurons of the dorsal root ganglion, and its efferent component provided by postganglionic neurons originating mainly from the stellate ganglion. Despite the presence of nitrergic fibers being thoroughly demonstrated in the stellate ganglion and in dorsal root ganglia [12, 13, 32–35], the works of Calupca et al. suggest that there are no extrinsic sympathetic fibers that express NOS in the cardiac ganglia in guinea pigs [20]. This is also consistent with the absence of intrinsic nerve fibers that co-localize nNOS with tyrosine hydroxylase (TH) in rats and guinea pigs [11]. Conversely, NO donors or viral gene transfer of nNOS reduces noradrenaline release via a cGMP-PDE2-dependent pathway to reduce the tachycardia in response to sympathetic nerve stimulation of the right stellate ganglion [36–39]. These studies demonstrate a participation of nNOS as a neuromodulator of postganglionic sympathetic activity in the heart, which has become the most widely accepted paradigm at present.

Valuable anatomical contributions were made by Dr. Pauza and his group regarding the presence and cardiac distribution of pure nitrergic, or biphenotypic cholinergic/nitrergic neurons and fibers [40]. Their studies in rabbits discovered an important nervous co-localization of nNOS and ChAT in the ventricles. Furthermore, these biphenotypic fibers are distributed in similar amounts in the endocardium and epicardium of the left ventricle, but with a predominance at the base and a progressive reduction towards the apex. Interestingly, they observed a clear predominant innervation of nitrergic fibers over ChAT positive fibers in the mid-myocardium [40]. The presence of a high number of nitrergic or cholinergic/nitrergic neuronal populations was also proven in the rabbit sinoatrial node [41]. This vast majority of biphenotypic and nitric neurons were also validated by Hoover et al. in nerve ganglia of human atria. It is interesting to note that the nitrergic fibers not only behave as afferents or efferents of the intracardiac ganglion, but also, many end up within the same ganglion as if they were interneurons [19]. This shows that NO possibly plays a critical role in the intrinsic local connections between different intraganglionic neurons as well.

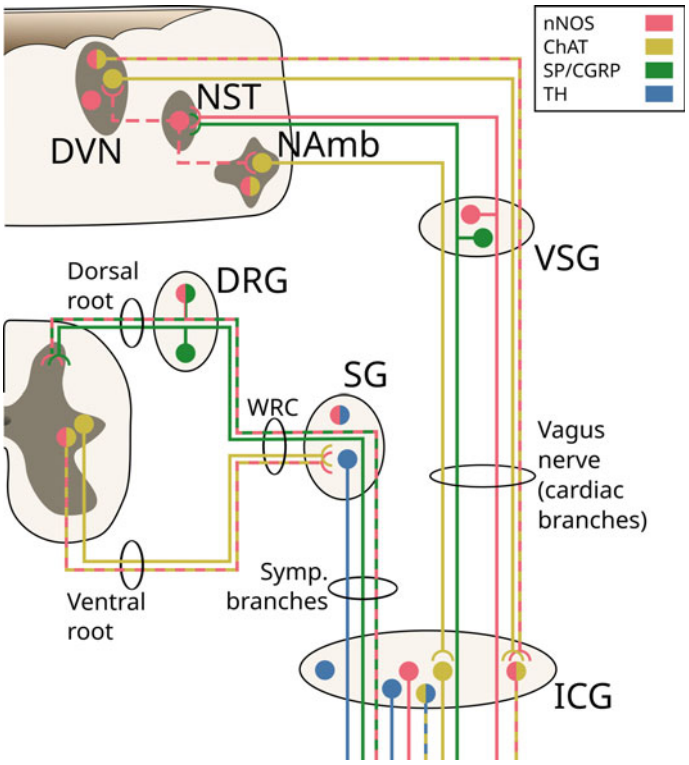

Fig. 4.1 Illustration of the distribution of neuronal somata and nerve fibers immunoreactive for diverse chemical phenotypes in the cardiac nervous system. The most frequent source of nitrergic fibers of the vagus nerve and cardiac nerves comes from neurons located in the sensory ganglia of the vagus (VSG). 37% of the somata in the VSG and a significant number of nitrergic neurons located within the nuclei of the solitary tract (NST) express neuronal nitric oxide synthase (nNOS). Also worth noting are about 3% of the dorsal vagal nucleus (DVN) which express nNOS as well but appear to be small interneurons. About 90% of neuronal somata in the DVN and about 94% of neuronal somata in the nucleus ambiguus (NAmb) express only choline acetyltransferase (ChAT). Only 7% of the somata of the DVN and 6% of the NAmb simultaneously express both ChAT and nNOS. Preganglionic sympathetic neurons of the intermediolateral nucleus of the spinal cord (Th_1-Th_5) are predominantly biphenotypic by nNOS and tyrosine hydroxylase (TH); whereas postganglionic neurons in the stellate ganglion (SG) poorly express TH and nNOS. Similarly, nitrergic neurons are found in small numbers in the dorsal root ganglion (DRG). WRC: white ramus communicants; ICG: intrinsic cardiac ganglia. Substance P (SP) or calcitonin gene-related peptide (CGRP)

Consistent with the foregoing, recent electrophysiological studies suggest an important role of nitrergic fibers in the regional regulation of the intrinsic cardiac nervous system [42].

The co-localization of nNOS with ChAT and the predominance of neurons with a nitrergic phenotype are correlated with functional studies that demonstrate that NO acts as a co-transmitter in postganglionic parasympathetic neurons, having a

critical role in heart rate modulation, and other cardiac functions following vagal activation [43, 44]. NO can act as a facilitator of acetylcholine (Ach) release from cardiac cholinergic fibers, or directly on the cardiac myocyte through guanylate cyclase. That is why, in the face of parasympathetic deterioration in cardiac disease, both the decrease in ACh and the loss of NO could be poor prognostic factors for patients [19].

In summary, the heart contains a considerable number of phenotypically nitrergic neurons and fibers, which can be intrinsic or extrinsic, and all express the nNOS subtype. Both the neuronal somata and the intrinsic nitrergic fibers of the cardiac nervous system belong to the parasympathetic system. Intrinsic cardiac ganglia contain parasympathetic postganglionic neurons that are nNOS positive or co-localize nNOS with ChAT. The intrinsic nitrergic cardiac nerve fibers are parasympathetic postganglionic axons. On the other hand, the extrinsic fibers are sensory afferents whose somata are located within the nodose ganglion of the vagus, or sympathetic postganglionic efferents originating from the stellate ganglion, although the latter are sparse. Furthermore, an additional integrative role is played by the positive nNOS interneurons located in intrinsic ganglia and in the vagal nuclei of the brain.

Role of Nitric Oxide as a Mediator of Cardiac Parasympathetic Effects

Abnormal autonomic activity is a strong prognostic indicator of mortality in patients with heart failure [45] or ischemic heart disease [46]. This so-called *dysautonomia*, consisting of an increased sympathetic tone and a reduced parasympathetic tone, predisposes patients to sudden death due to lethal arrhythmias such as ventricular fibrillation.

Decreased heart rate variability and decreased baroreflex sensitivity are indicators of loss of vagal tone associated with poor prognosis in patients with heart disease. For this reason, electrical stimulation of the vagus nerve has garnered great interest in recent years as a technique to increase parasympathetic activity in patients with heart failure [47]. Although the presence of parasympathetic nerve fibers in the ventricles has been well verified [48], the direct effects of vagal nerve stimulation (VNS) on ventricular function have not yet been observed [49] or, at most, have been found to be controversial [50] for a long time. However, there is consistent evidence demonstrating the benefits of increased vagal tone in cardiovascular disease.

Consistent experimental evidence demonstrates the benefits of VNS on infarct size [51, 52], heart failure [53], and arrhythmias [54, 55]. Ng et al. observed that vagus nerve stimulation affects the effective refractory period, the ventricular fibrillation (VF) threshold and the electrical restitution in the absence of background sympathetic tone [56]. They show that the vagal anti-fibrillatory action in isolated ventricles of rabbits with preserved innervation occurs via postganglionic efferent nerve fibers, independent of activation of muscarinic receptors, the vasoactive intestinal peptide

(VIP), and the endothelium [57]. Interestingly, the effects of VNS were blocked by nonspecific NOS inhibitors. These indirect data include NO for the first time as a potential mediator of vagal activity in the ventricles. Subsequent fluorescence measurements confirmed this finding through increased myocardial NO levels by cervical VNS, which was blocked by a nonspecific NO synthase inhibitor [58].

Although increases in NO during VNS were attributed to production by nNOS, there is a constitutive production of NO that depends on eNOS [59]. This basal production of NO in the myocardium was also studied by measuring its metabolites, by chemiluminescence or measurements in the coronary effluent [1, 60–66]. While the participation of eNOS in VNS-induced NO production could not be ruled out, the work of Brack et al. strongly suggests exclusive activation of nNOS [58]. Firstly, selective nNOS blocking completely interferes with NO increases during vagal activation. Secondly, the increase in NO during VNS does not cause changes in the coronary perfusion pressure, as can be seen with the release of endothelial NO. As mentioned before, nNOS is located in the nerve terminals of the heart, therefore they should be the main source of NO induced by VNS. The administration of the selective nNOS blocker, 1-(2-trifluoromethylphenyl) imidazole (TRIM), did not modify the basal production of NO in the myocardium, which shows that the non-neuronal locations of this isoform do not participate in vagal activation [58]. The physiological role of NO produced in neurons on the function of the ventricular myocardium is not well known. However, perfusion of isolated hearts with TRIM reduced left ventricular pressure, suggesting a role in inotropic regulation [58]. These data are consistent with previous studies in smooth muscle that demonstrate the role of nNOS in the regulation of muscle tension and calcium homeostasis [63].

In summary, the studies published by Brack et al. strongly suggest that ACh and NO released by the vagus nerve can act through parallel independent signaling pathways [64]. At the same time, NO released from the vagal terminals is produced by nNOS, independently of the coronary endothelium and regardless of the nicotinic and muscarinic cholinergic action of ACh and its co-transmitter, VIP. In addition to serving as a physiological regulator of the heart, the NO produced by nNOS modulates the presence of severe ventricular arrhythmias. Based on this, Ng et al. proposed an interesting theory about the existence of a separate network of parasympathetic nitrergic antifibrillatory neurons within the ventricle [64]. Despite lacking demonstration with histochemical and electrophysiological studies, the hypothesis proposed by Ng et al. has morphological support given by the presence of parasympathetic neurons in intracardiac ganglia of different mammalian species, as many of these neurons and fibers that run through the ventricular myocardium are positive for nNOS, as described in the previous section [65, 66].

Unlike the aforementioned results, in vivo studies indicate that vagal protection against arrhythmias [67–69] and its reduction of infarct size in acute myocardial infarction [51, 52, 70] is blocked by atropine, implying muscarinic receptors are necessary steps for parasympathetic protection. Furthermore, NO induced by nNOS facilitates both ACh release in vagal terminals, and bradycardia activated by VNS [71]. According to Herring and Paterson the neuronal NO generated by nNOS facilitates ACh release from release sites of the ganglionic projections

via a cGMP-phosphodiesterase-3-dependent pathway increasing protein-kinase-A-dependent phosphorylation of N-type calcium channels [71]. Along these same lines, using an isolated heart model from rats treated with the ACh analogue carbamylcholine, Kalla et al. demonstrate a protective effect of carbamylcholine on the VF threshold that depends upon both muscarinic and nicotinic receptor stimulation, where the generation of NO is likely to be via a neuronal nNOS-sGC dependent pathway [72].

Supporting the concept of vagal presynaptic action of NO, in vitro and in vivo studies demonstrated that gene transfer of nNOS in cardiac autonomic ganglia promotes and facilitates vagal cholinergic transmission and bradycardia by acting presynaptically. Furthermore, dysautonomia after myocardial infarction or hypertension is partially restored by increasing expression of nNOS in cholinergic neurons of the heart, with a tendency to improve survival assessed in the short term [73–76]. In the same way, studies in mice showed that there is an increase in the expression of nNOS in physically trained animals, thus involving NOS in increasing vagal tone with physical activity [77]. Taken together, these studies demonstrate that a significant component of cardiac vagal impairment in cardiac diseases resides at the end organ level because of abnormal NO-cGMP signaling in intracardiac ganglia [75]. Restoring the neuronal phenotype of nNOS in cardiac neurons facilitates ACh release and restores parasympathetic tone in cardiovascular disease, opening up a potential opportunity for future treatment through this pathway.

Over the years, several studies have shed light on the difference between the two theories proposed to try to explain the role of muscarinic receptors in nNOS-mediated parasympathetic cardiovascular regulation. Firstly, and given that the vagus nerve contains a higher proportion of afferent than efferent fibers [78], it is possible that retrograde afferent stimulation could explain the local generation of nNOS-produced NO [72] observed by Brack et al. [57]. On the other hand, nNOS was also found in myocytes [5], fulfilling numerous physiological functions in the heart, such as the regulation of the myocardial calcium cycle [79]. However, a direct link between cholinergic receptors and myocyte nNOS has not been found. Therefore, this pathway does not appear to be involved in the effects of NO-mediated vagal activation [80]. Finally, variations in the experimental model and the species studied could be responsible for the differences between the works of Brack and Herring. In this sense, the facilitating role of nNOS in cardiac cholinergic regulation was demonstrated in rats, dogs and ferrets, but it could not be proven in in vivo studies in rabbits or guinea pigs [81], which resonates with Brack's rabbit heart studies. Conversely, the presynaptic regulation of ACh release by NO was demonstrated in in vitro studies with guinea pigs [82]. Further work is necessary to determine whether NO of neuronal origin acts as a self-contained pathway, independent of the muscarinic receptor, or whether it is only a presynaptic facilitator of cardiac parasympathetic muscarinic regulation.

Role of Nitric Oxide as a Modulator of Cardiac Sympathetic Effects

The role of NO in sympathetic regulation at the heart level can occur in the sympathetic postganglionic terminals that come from the sympathetic trunk or at the cardiomyocyte level, modulating the activity of the β-adrenergic receptor.

An inhibitory effect of NO on the release of catecholamines at the cardiac sympathetic terminals was suggested by gene transfer studies of nNOS in postganglionic stellate ganglion neurons [36–39]. The modulating capacity of NO on the increase in heart rate before sympathetic stimulation was also demonstrated with the use of both nonspecific and nNOS-specific inhibitors in the cardiac sympathectomized and vagotomized anesthetized rabbit, and in isolated guinea pig atria with intact vagus nerves [81]. In pathological conditions with dysautonomia such as arterial hypertension, results suggest that the increase in sympathetic tone may be due to a loss of the inhibitory effect of the NO-cGMP pathway on the release of norepinephrine in the cardiac postganglionic sympathetic terminals. In hypertension models, increased nNOS gene expression in sympathetic neurons restored autonomic balance both acutely and in the long term, suggesting future therapeutic potential in the management of cardiovascular dysautonomias [76, 83].

At the cellular level, many reports have shown that inhibition of NOS induces an increase in the chronotropic response to β-agonists at the cellular, myocardial tissue, and whole-animal levels [84, 85]. Similarly, experiments using eNOS−/− mice in vivo [86] or Langendorff-perfused hearts demonstrated augmented cardiac contractility responses to β-agonists [87]. In contrast, the chronotropic response to β-adrenergic stimulation was observed in mice with eNOS gene over-expression in cardiomyocytes [88]. Some discrepancies in the characteristics of the models and experimental conditions used could justify these differences in the role of NO in cardiovascular sympathetic modulation.

Interestingly, in isolated myocytes, NO has been shown to play a role in both muscarinic-cholinergic slowing of heart rate and attenuation of the contractile response to β-adrenergic stimulation [89]. Additionally, NO also participated in the vagal inhibition of the increase of inotropism mediated by β-adrenergic activation in in vivo studies [90]. This shows that NO also plays an important role in modulating the sympathetic-parasympathetic interaction, specifically in modulating the muscarinic cholinergic inhibition of β-adrenergic cardiac responses.

As can be seen in previous studies, nNOS and eNOS can mediate independent effects on cardiac sympathetic physiological responses. NO inhibits L-type Ca^{2+} channels, but stimulates the release of calcium from the sarcoplasmic reticulum, which is why it can generate seemingly contradictory responses on myocardial contractility. In other words, the effects of NO will depend on the spatial location of specific NOS isoforms [86]. eNOS is located in caveolae, where it compartmentalizes with L-type Ca^{2+} channels and β-adrenergic receptors and, thus, NO inhibits sympathetic-induced inotropism. On the other hand, nNOS is related to the sarcoplasmic reticulum. There, NO stimulates the release of calcium in the

sarcoplasmic reticulum via the ryanodine receptor, suggesting that nNOS has a facilitating effect on inotropic action, having an opposite effect to that of eNOS [86].

NO as a Mediator in Autonomic Regulation of the Cardiac Conduction System

In addition to the NO-dependent action of parasympathetic activation on ventricular myocardial function, vagal mechanisms dependent on neuronal NO participate in the regulation of the conduction system, such as the control of heart rate [91] and atrioventricular conduction [92]. The presence of nitrergic and cholinergic/nitrergic neurons in the area of the sinoatrial node [41] correlates with the functional capacity of NO from nNOS to modulate the vagal response on heart rate [71]. The importance of NO in chronotropic function is evidenced by the presence of a greater number of nNOS-labeled neurons compared to those that only mark with ChAT, in samples of rabbit hearts [41]. Similarly, NO plays a facilitating role for vagal action in reducing dromotropism, acting directly on the AV node and independent of heart rate [92].

NO as a Mediator in Autonomic Regulation of Coronary Flow

Outside the myocardium and the conduction system, possibly the most studied relationship between the NO system and the cholinergic pathways is that of the coronary vascular endothelium, a substantial source of NO in the heart. Administration of ACh to the coronary arteries has a biphasic effect on the vascular response. At low doses, ACh generates vasodilation by induction of NO from the eNOS of the endothelium. In contrast, high doses of ACh produce a paradoxical effect of vasoconstriction by independent direct action of NO on the smooth muscle of the coronary wall [93–96]. Interestingly, ACh perfusion-induced vasodilation is blocked by nonspecific NOS inhibitors, but not by the specific nNOS inhibitor. Taken together, these data suggest that both eNOS and nNOS may be regulated by the cholinergic system, but have different origins. ACh perfusion produces NO of endothelial origin (eNOS), whereas VS produces NO of nervous origin (nNOS) and independent of endothelial function [97, 98].

More recent studies have shown that nNOS is expressed in the walls of human coronary arteries, more specifically in smooth muscle cells [7], and plays an important role in maintaining basal blood flow [99]. Other in vivo studies have also confirmed the role of local NO production by nNOS in regulating coronary flow and, furthermore, have shown that coronary vasodilation in response to a pacing-induced increase in cardiac workload is exclusively mediated by eNOS-derived NO [100]. Therefore,

eNOS and nNOS have different local physiological functions in regulating coronary vascular tone. While local production of NO by nNOS intervenes in the microvascular regulation of basal coronary flow, eNOS responds to ACh and substance P, causing vasodilation and increasing coronary flow, which mainly intervenes in response to increased demand in situations of stress such as physical exercise or other situations that lead to an increase in metabolic demand.

Conclusion

Over the past decades, NO has been shown to play an essential role as a mediator and modulator of the physiological and pathophysiological neural control of the heart. The endogenous neuronal NO of the cardiac parasympathetic system acts in a paracrine way on the myocytes or as a facilitator of ACh release in the vagal terminals. On the other hand, NO acts as a sympathetic modulator, reducing the β-adrenergic effect on the increase in heart rate. The loss of endogenous modulation of NO on the cardiac nervous system observed in various cardiovascular pathologies facilitates the development of dysautonomias with sympathetic hyperactivity and loss of parasympathetic tone, which is deleterious for the evolution of the patient. Experimental studies showed that the increased expression of NOS in pathological hearts favors the restoration of autonomic balance. In turn, NO participates in the protection mediated by techniques that increase parasympathetic tone, such as remote conditioning or vagal stimulation.

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Chapter 5
Exercise Induced NO Modulation in Prevention and Treatment of Cardiovascular Diseases

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Abstract Many aspects of modern life, including lack of physical activity, contribute to the primacy of cardiovascular disease as a cause of death worldwide. Endothelial dysfunction is recognized as initial step in occurrence of a variety of cardiovascular disorders, wherein the reduced production of nitric oxide (NO) represents the pathogenetic basis of endothelial dysfunction. Various therapeutic approaches aim to increase NO production or to enhance some of the downstream NO signaling cascades. Exercise training is found to be effective modulator in NO synthesis, and thus potential tool in prevention and treatment of cardiovascular diseases. Results from many authors indicated that exercise training could be protective procedure in altering cardiovascular risk factors such as hypertension and atherosclerosis, and therapeutic maneuver in cardiovascular diseases such as coronary artery disease, stroke and heart failure. Exercise training increases NO production through several mechanisms, including increase in nitric oxide synthase (NOS) expression, phosphorylation and activity, reduction of oxidative stress, which take place in different cells and tissues. We aimed to present some of the mechanisms involved in increased NO

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_5

bioavailability by exercise training, as well as NO mechanisms of action in cardiovascular protection. Physical activity, applied as non-pharmacological therapeutic approach, may be useful in many ways in the prevention and treatment of cardiovascular disorders, if adequate modality of physical activity is used and if it is dosed correctly.

Keywords Nitric oxide · Exercise training · Nitric oxide synthase · Cardiovascular disease · Endothelium

Nitric Oxide and Exercise

Cardiovascular diseases (CVDs) remain the leading cause of morbidity and mortality worldwide regardless of the economic status and development of the country [1, 2]. Given that human genome has not been significantly changed in the last 50 millennia, the basic anatomical and physiological constants remained unchanged [3]. Dramatic changes in life habits in the last century, and even more in last decades, reduced physical activity and favored sedentary lifestyle. Body functions and overall metabolism are adapted for preserving energy due to limited sources of food in the history, and further to physical activity needed for food seeking [4]. Due to above fact, there is large gap between energy intake (food) and energy consumption (physical activity) nowadays. Sedentary lifestyle, characterized by low level of physical activity, is recognized as one of the leading contributors of development and progression of CVD. On the other hand, it was shown that physical activity and proper exercise dramatically reduces predisposition for CVD occurrence and increases overall well-being [5]. Evan slight increase in cardiorespiratory fitness is associated with significant reduction of CVD mortality [6], while the sedentary behavior was related to reduced cardiorespiratory fitness, defined as ability of cardiovascular and respiratory systems to supply enough oxygen to skeletal muscles during physical activity.

Many aspects of cardiovascular homeostasis may be altered in occurrence of CVD. Systolic and diastolic function of the heart refers to myocardial contractility and relaxation, while the vascular function mostly relay to the endothelial function and arterial stiffness [5]. Endothelium is one of the key determinants of vascular homeostasis due to regulation of vasodilation and subsequent reduction of mechanical damage due to shear stress, decrease of vascular permeability and limitation of leukocyte interaction to the walls of the blood vessels [7]. Despite the confirmed and obvious beneficial effects of physical activity on cardiovascular health, the precise mechanisms underlining CVD prevention by frequent exercise are not fully understood. But research data by others and us highlighted nitric oxide (NO) as one of the key mediators in regulation and maintaining of cardiovascular homeostasis [8, 9].

Somewhat contradictory research results concerning the impact of physical activity on NO production and cardiovascular health could be assigned to modality and intensity of physical activity. We showed that NO production (measured through

nitrite (NO_2^-) outflow) increases at the beginning of the exercise training in professional athletes [10]. But increase of exercise intensity led to decrease of plasma level of NO_2^- and superoxide anion radical (O_2^-), suggesting the role of ROS in NO inactivation. Furthermore, NO bioavailability seems to be in positive correlation with maximal oxygen consumption (VO_{2max}) [11]. Shunting between aerobic and anaerobic metabolism appears to be important determinant in signal transduction related to O_2^- and NO. Another study showed significant increase of NO_2^- outflow after professional handball training, compared to the graded exercise test [12]. Comparing the NO production in athletes and non-athletes upon exercise training, we showed that athletes had significantly higher levels of NO and lower levels of O_2^- [13]. Increased capacity to inactivate ROS, mainly due to increased SOD activity, in athletes appears to be important contributor in NO signaling.

We aimed to review the effects of changes of NO production in CVD occurrence as well as to reveal the possibility of altering NO synthesis by exercise training. Exercise training, in accordance with the carefully planned intensity and type, could be crucial non-pharmacological method in prevention and treatment of CVD.

Nitric Oxide Synthases

By its chemical characteristics NO belongs to the free radicals and reactive species. Compared to the other reactive species, NO is more stable, moderately reactive, with longer half-life in cellular surrounding [14]. NO is small and hydrophobic molecule, thus easily diffusible through cellular membranes [15]. Given that it may interact with plethora of molecules resulting in production of variety bioactive compounds, NO affect various signaling pathways and produce many biological effects. Considering all properties of NO, it can be appraised as one of the most versatile signaling molecules [14, 16]. Three decades of investigations, since discovery of endogenous NO and its effects in vascular regulation, showed that biological effects of NO can be exerted by its reactions with specific target molecules or through the generation of secondary reactive species formed downstream from NO. Beside the role of NO as a second messenger in three systems of the body – cardiovascular, immune and neuronal, now it is known that NO is involved in regulation of various functions such as orchestration of gastrointestinal peristaltic activity, skeletal muscle metabolism, insulin secretion, etc. [17–20].

Most of the endogenous NO is created through catalytic activity of three isozymes of nitric oxide (NO) synthase (NOS) (EC 1.14.13.39) – neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) or NOS1, NOS2 and NOS3, respectively [21, 22]. Initially nNOS and eNOS were considered as constitutive forms of NOS involved in the synthesis of small (physiological) amounts of NO due to Ca^{2+} dependent regulation. On the other hand, it was believed that iNOS activity and NO production is not Ca^{2+} regulated and depends on induction by endotoxins or pro-inflammatory cytokines [23]. More recent data indicated that expression of the constitutive forms

of NOS, nNOS and eNOS, may be affected by various factors, as well as that iNOS may be constitutively expressed in various tissues [24–26].

All NOS contain haem and bind calmodulin. Also, all three NOS utilize L-arginine as the substrate, and molecular oxygen and NADPH (reduced nicotinamide-adenine-dinucleotide phosphate) as co-substrates [26]. Further, FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide) and BH_4 ((6R-)5,6,7,8-tetrahydro-L-biopterin or tetrahydrobiopterin) serve as cofactors for all NOS isoform [21]. The process of NO synthesis involves two-step oxidation of L-arginine to L-citrulline. In the first reaction L-arginine is hydroxylated to N^{ω}-hydroxy-L-arginine (NOHA), an intermediate compound which remains largely bound to the NOS, while in the second reaction NOHA is oxidated to L-citruline and NO [27] (Fig. 5.1). All NOS isoenzymes bind calmodulin. It was shown that Ca^{2+} increase in the cytoplasm and subsequent binding of calmodulin to NOS, changes conformational state of these NOS isoforms and facilitate their catalytic activity [28, 29]. In the case of iNOS, calmodulin controls post-translational assembly of the enzyme, enabling formation its stable and active form [30].

NO, generated through NOS activity, appears to be the regulator of NOS activity. Binding of NO to NOS amino acid residues and consequent S-nitrosylation leads to reversible NOS inhibition [31]. Availability of L-arginine appears to be one of the key determinants in limiting NOS activity [32, 33]. There are several suggested mechanisms involved in activation and inactivation of NOS. It is well documented that exercise may increase expression of NOS and thus increase production of NO. Physical activity mediates in increased NO secretion through several mechanisms, involving mechanical, humoral, and metabolic factors, and thus affects many aspects

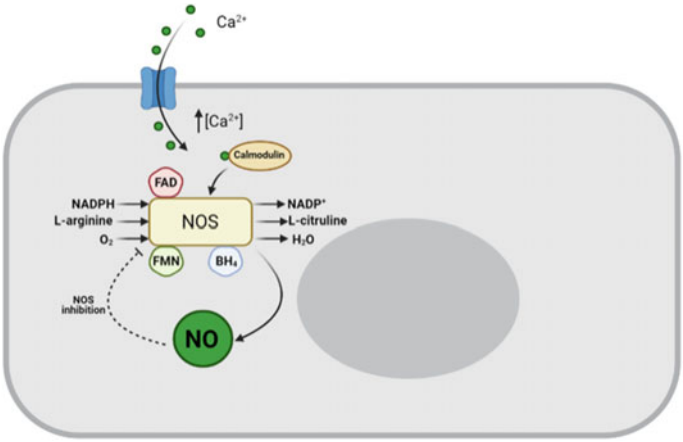

Fig. 5.1 Synthesis of nitric oxide and related products. Legend: BH_4—tetrahydrobiopterin; FAD—flavin adenine dinucleotide; FMN—flavin mononucleotide; $NADP^+$—nicotinamide adenine dinucleotide phosphate; NADP – reduced nicotinamide adenine dinucleotide phosphate; NO—nitric oxide; NOS—nitric oxide synthase. Figure was created using BioRender.com

of cardiovascular homeostasis [34, 35]. One of the molecular links between exercise and increased production of NO is hydrogen peroxide (H_2O_2) [36]. Recently it was shown that enzyme myeloperoxidase can activate eNOS via phospholipase C-dependent Ca^{2+} signaling and consequent changes in eNOS phosphorylation status [37]. Phosphorilated form of eNOS (1177 serine residue) is active form of enzyme and assessment of eNOS phosphorylation is usually used as indicator of eNOS activation [38, 39]. Exercise induced production of NO represents one of the possible explanations or part of the mosaic of exercise induced cardiovascular protection.

Increased production of NO in pro-oxidant environment could result in formation of various reactive nitrogen species (RNS), thus provoking damaging of biological structures. The reaction between NO and superoxide anion radical (O_2^-) to produce peroxynitrite ($ONOO^-$) is one of the best known reactions in RNS formation [40]. Participation of $ONOO^-$ in direct and indirect oxidation reactions result in protein tyrosine nitration and consequent changes in protein structure, DNA damage, generation of secondary reactive species, creating all together nitrosative stress.

Nitric Oxide—Mechanism of Action

NO synthesized in endothelial cells through NOS metabolic activity diffuses into tunica media and vascular smooth muscle cells where it activates soluble guanylyl cyclase (sGC). Binding of NO to the heme segment of sGC increases production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) which mediates relaxation [41]. The level of cGMP and tone of vascular smooth muscles depend on precise balance between cGMP production by sGC and cGMP degradation by phosphodiestrase (PDE). cGMP activates protein kinase G (PKG), which catalyzes phosphorylation of different proteins involved in vasodilatation and regulation of smooth muscle tone, such as myosin light chain and ion channels. cGMP may also activate cyclic-nucleotide gated ion channels [42]. Overall changes enhance relaxation of vascular smooth muscles and increment of vascular velocity (Fig. 5.2).

Nitric Oxide and Cardiovascular System

Most of the NO synthesized in the cardiovascular system (CVS) originates from catalytic activity of eNOS given this form of NOS is predominantly expressed in endothelial cells and some other cell types important for maintaining cardiovascular homeostasis, such as cardiomyocytes, erythrocytes, platelets, kidney tubular epithelial cells [21, 43]. Activation of eNOS in the vasculature and consequent production of NO can be enhanced by several stimuli such as shear stress, acetylcholine, bradykinin or histamine [44–47] (Fig. 5.3).

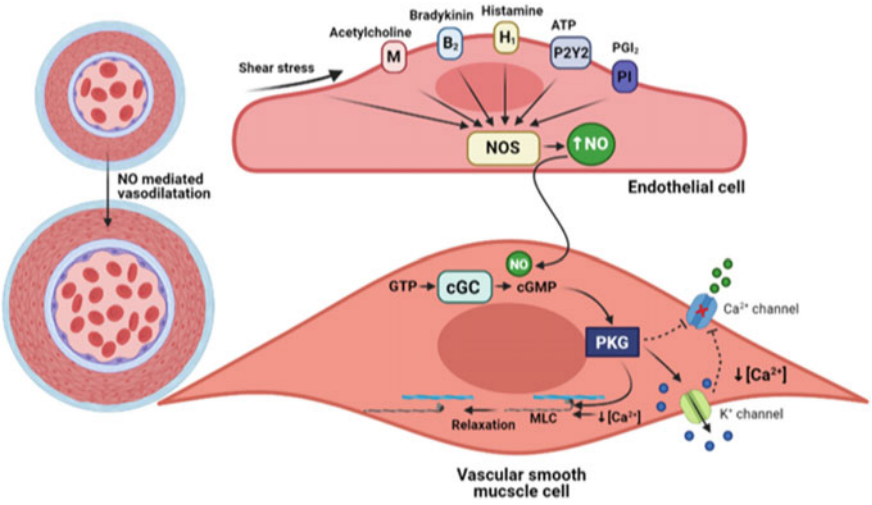

Fig. 5.2 Regulation of vascular smooth muscle tone by endothelium-derived NO. Various factors, including increased shear stress and neurohumoral mediators, via specific receptors located on endothelial cell membrane activate endothelial nitric oxide synthase and increase production of nitric oxide. Legend: ATP—adenosine triphosphate; B—bradykinin receptor; GTP—guanosine triphosphate; H_1—histaminergic 1 receptor; NO—nitric oxide; NOS—nitric oxide synthase; M—muscarinic receptor; MLC—myosin light chain; PGI_2—prostacyclin; PKG—protein kinase G; PI—prostacyclin receptor; P2Y2—purinergic P2Y2 receptors; sGMP—cyclic guanosine monophosphate; sGC—soluble guanylyl cyclase. Figure was created using BioRender.com

Shear stress induces eNOS activation through several endothelial surface molecules such as glypican-1, syndecan-1 or heparan sulfate which are probably anchored near eNOS residues [48]. Integrin-linked kinase (ILK) as mechanosensor sensitive to shear stress regulates eNOS activity and NO production. ILK deletion induced eNOS uncoupling followed by decreased bioavailability of NO and increased production of O_2^- [49]. Namely, although synthesized as monomers, eNOS molecules has to create homodimers to produce NO, in monomeric form they can only produce O_2^-. It is also showed that shear stress mediates increase of erythrocyte NO production [50]. Exposure of erythrocytes to shear stress resulted in increase of intracellular Ca^{2+}, NOS phosphorylation and enhancement of NO production. Physical activity, regardless vasodilatation, significantly increases shear stress. It is estimated that increased cardiac output and blood flow through the arteries induced by moderate exercise result in tenfold increase of shear stress in abdominal aorta [51]. There are several postulated pathways involved in augmentation of erythrocyte NO synthesis due to increased shear stress induced by exercise including pannexin-1, mechanosensitive non-selective cation channels Piezo1 and cytoskeletal protein spectrin [52]. Physical activity is recognized as important mechanism for CVD risk reduction in advanced age, due to improving of NO synthesis by regular exercise and enhanced endothelium-dependent dilation in elderly [53]. Shear stress and physical activity increases production of ROS in endothelial cells, which in small concentrations act

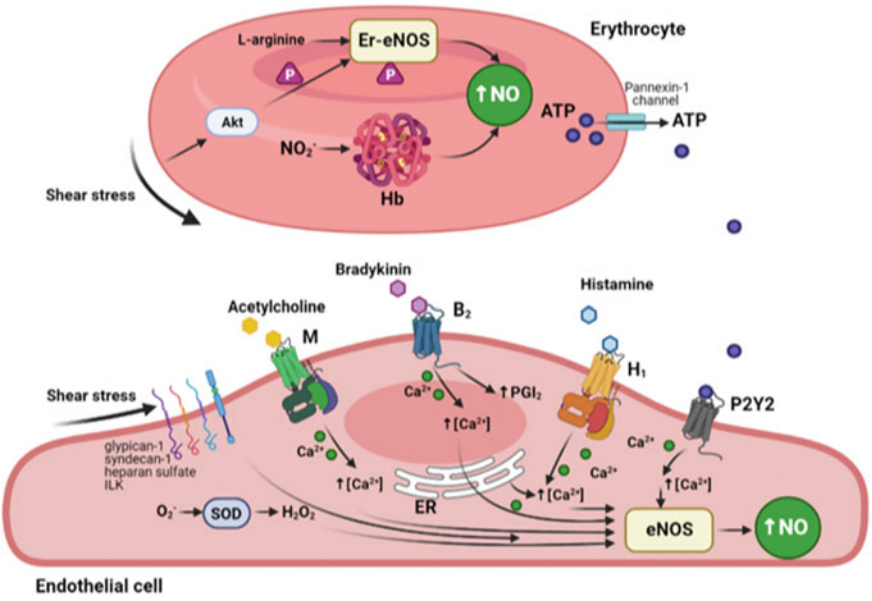

Fig. 5.3 Increase of nitric oxide production in endothelial cells and erythrocytes. Many factors mediate increased synthesis of nitric oxide creating complex milieu which can be differently affected by physical activity. Legend: ATP—adenosine triphosphate; B—bradykinin receptor; ER—endoplasmic reticulum; H_1—histamine 1 receptor; Hb—hemoglobin; H_2O_2—hydrogen peroxide; NO—nitric oxide; NO_2^-—nitrites; eNOS—endothelial nitric oxide synthase; Er-eNOS—erythrocyte endothelial nitric oxide synthase; M—muscarinic receptor; O_2^-—superoxide anion radical; P—phosphate; PGI_2—prostacyclin; P2Y2—purinergic P2Y2 receptors; SOD—superoxide dismutase. Figure was created using BioRender.com

as signaling molecules which further increase NO production. Underlining mechanism includes exercise-induced activation of superoxide dismutase (SOD) which catalyses dismutation of O_2^- to H_2O_2. H_2O_2 diffuses through the vascular wall and increases production of NO by activation of eNOS [54, 55].

Acetylcholine binds to G-protein coupled muscarinic receptors resulting in transient increase of intracellular Ca^{2+} which increases production of NO (Fig. 5.3), endothelium-derived hyperpolarizing factor (EDHF) and eicosanoids [56]. Comparing the functional and structural features of the femoral arteries from trained and sedentary rats it was shown that training increases acetylcholine-induced relaxation and bioavailability of NO [57]. Increased NO production aroused as a consequence of higher eNOS expression, decreased microRNA (miRNA)-124a and miRNA-155 and the posttranslational eNOS phosphorylation. Furthermore, other study shown that exercise significantly improves acetylcholine-induced vasodilatation in ovariectomized hypertensive rats [58]. Vasodilatation was absent due to sodium nitroprusside stimulation, suggesting that vasodilatation was endothelium dependent, and NOS inhibition, implying the acetylcholine and NO interaction. Results of the study assessing the effects of 2 week immobilization of one leg

on microvascular function showed that acetylcholine-induced change of vascular conductance was significantly reduced compared to control, not immobilized, leg [59]. Interestingly, eNOS protein level in skeletal muscles of immobilized leg did not change significantly, suggesting variations in NO secretion rather than in eNOS expression. Investigation of the effects of exercise in experimental model of familial hypercholesterolemia (LDL receptor (LDLr) deficient mice) showed that acetylcholine derived endothelium-dependent relaxation was preserved in trained $LDLr^{(-/-)}$ mice compared to the sedentary [60]. Such results indicate prophylactic effects of physical activity in the prevention of early endothelial dysfunction due to hypercholesterolemia, as one of the most common risk factor in CVD occurrence.

Bradykinin is derived from larger molecule kininogen by proteolytic enzymatic action of kallikrein in plasma [61]. Vascular effects of bradykinin are mediated through two G-protein-coupled receptors, B_1 and B_2 (Fig. 5.3). Binding of bradykinin to its receptors results in NOS activation and enhanced NO production [61]. Training enhanced bradykinin mediated coronary vasodilatation of healthy and ischemic hearts of Yucatan pigs [62]. Also, isolated coronary arteries from both control and ischemic hearts of trained animals showed prolonged vasodilatation induced by bradykinin, which was abolished by NOS inhibition. Thus, physical activity improves endothelium-mediated bradykinin vascular relaxation via increased Ca^{2+} signaling and NO production. Another, similar study showed that exercise significantly increases NO production triggered by bradykinin [63]. Bradykinin also induced increased secretion of prostacyclin (PGI_2), as potent vasodilatory mediator.

Binding of histamine to endothelial histamine 1 (H_1) receptors, which belong to Gq-protein coupled receptors, results in activation of phospholipase C and mobilization of intracellular Ca^{2+} [64] (Fig. 5.3). Increase of intracellular Ca^{2+} induces eNOS activation and NO production. Blocking of H_1 receptors or histidine decarboxylase (HDC), an enzyme that catalyzes histamine synthesis, reduced endurance in mice [65]. Application of H_1 receptors in the same time reduced NO availability. On the other hand, exercise in mice provoked increased amount of HDC mRNA, as well as increased activity of HDC in quadriceps femoris muscles.

Erythrocyte Derived NO and Exercise

NO also may be produced in erythrocytes through non-enzymatic reaction from nitrites (NO_2^-) under catalytic orchestration of deoxyhemoglobin (the nitrite reductase hypothesis) especially under hypoxic conditions [52]. Erythrocytes may be considered as reservoir of NO-associated metabolites which can provide NO mediated vasodilatation in hypoxia when eNOS activity is compromised [66, 67]. Decreased saturation of hemoglobin with oxygen between 40 and 60% provides maximal NO production rate from NO_2^- [68] (Fig. 5.3). S-nitrosohemoglobin (SNO-Hb), compound aroused from reaction between NO and hemoglobin was first recognized as bioactive NO-metabolite in non-enzymatic NO production [69]. Measurement of NO metabolites in plasma and erythrocytes from cerebral and leg arteries

and veins at resting, hypoxia and physical activity showed increased consumption of nitrites [70]. The authors indicated that increased NO_2^- consumption during exercise was accompanied with increased formation of iron nitrosyl hemoglobin (HbFeNO), but the increased formation SNO-Hb was not observed. Precise mechanisms mediating non-enzymatic NO synthesis in erythrocytes remain speculative due to variations in understanding of hemoglobin chemistry and allostery.

Another mediating mechanism in erythrocyte control of blood flow implies the release of ATP from erythrocytes into the blood. Although lacking in mitochondria, ATP synthesis occurs in erythrocytes through glycolytic pathways [71]. Erythrocyte ATP release occurs upon increased mechanical deformation of erythrocytes, acidity, CO_2 concentration or temperature [72–75]. Released ATP binds to purinergic receptors (probably P2Y2) on the surface of the endothelial cells and initiating Ca^{2+} release and eNOS activation [76, 77] (Fig. 5.3). Increased shear stress induced ATP release from erythrocytes in significantly higher amount in comparison to the endothelial cells [78]. Increased shear stress induced by perfusion of blood vessels with full blood enabled sufficient Ca^{2+} influx into the endothelial cells to increase enough NO production. Conversely, shear stress of endothelial cells induced by perfusion with erythrocyte-free solutions increased endothelial NO production, but failed in endothelial Ca^{2+} increase. Within the same research it was shown that shear stress-induced Ca^{2+} influx into endothelial cells was mediated through erythrocytes ATP release via pannexin-1 channel [78].

It also known that erythrocytes contain functional eNOS (Er-eNOS) localized in the plasma membrane and the cytoplasm [79]. The Er-eNOS activity is conditioned by availability of L-arginine as a substrate, by Ca^{2+} concentration and phosphorylation status. Er-eNOS activity is important in regulation of erythrocyte deformability and functionality, as well as in inhibition of platelets activation [80]. Supporting the role of Er-eNOS in regulation of vascular homeostasis it was shown that moderate physical activity increases phosphorylation of Akt kinase and Er-eNOS, suggesting their increased activity and Er-eNOS activation via Akt kinase [81] (Fig. 5.3). The NO release by Er-eNOS was also increased after moderate exercise, as well as downstream NO/cGMP signaling cascade. Erythrocytes conditioned with shear stress preserved deformability and Er-eNOS phosphorylation upon exposure to O_2^- [82]. Increased oxidative stress decreases erythrocyte deformability and Er-eNOS activity due to impaired Akt kinase activity. Such results suggest protective mechanism of exercise and increased shear stress. Still, there are some inconsistent conclusions regarding the effects of intensive training on Er-eNOS activity. Suhr and colleagues showed that intensive physical activity induces down-regulation of Er-eNOS and subsequent decrease in NO production and erythrocyte deformability [83]. Koliamitra and coauthors showed that only high intensity training, in comparison to the high volume training and moderate intensity training, was able to increase Er-eNOS mediated NO production [84].

Taking altogether it can be concluded that erythrocytes are important sources, carriers and scavengers of NO thus affecting blood rheology and physiological features of vasculature. Exercise induced changes of rheological properties of the blood strongly suggest the important role erythrocyte-mediated secretion of NO in

vascular regulation and complex relationship between erythrocytes, endothelial cells, NO production and modality of physical activity.

Neuronal Nitric Oxide Synthase in Cardiovascular System and Physical Activity

Neuronal NOS (nNOS), first discovered in 1990 upon its neuronal isolation, poses many important roles in regulation of various neurological functions [85]. Beside nervous system nNOS appears to be important mediator in regulation of non-neural cells and tissues including CVS [86]. Cardiac nNOS appears to be important regulator of Ca^{2+} handling and basal cardiac contractility [87]. Deletion of nNOS, as well as its pharmacological inhibition, resulted in increased Ca^{2+} influx and basal contraction in isolated cardiomyocytes and myocardium in vivo. Similarly, contraction of $nNOS^{(-/-)}$ cardiomyocytes were higher in basal state and due to beta-adrenergic stimulation with isoproterenol compared to wild type (WT) [88]. Underlining mechanism implies enhancing effects of NO generated from nNOS on Na^+/K^+ ATP-ase activity and thus indirectly advanced activity on Na^+/Ca^{2+} exchanger located on sarcoplasmic reticulum membrane [89]. Furthermore, nNOS derived NO activates guanylate cyclase and cGMP/protein kinase G (PKG) cascade resulting in inhibition of L-type Ca^{2+} channels and decrease of Ca^{2+} influx [90, 91].

Beside predominant role of eNOS in NO production in the vasculature, nNOS is also present in various cells present in blood vessels. nNOS significantly contributes to regulation of vascular tone, vasodilatation, vascular conductance and blood flow [92–94]. Seddon and coauthors implicated divergent roles of eNOS and nNOS derived NO in regulation of human microvascular tone [95]. Application of selective nNOS inhibitor S-methyl-l-thiocitrulline (SMTC) resulted in significant reduction in basal blood measured by venous occlusion plethysmography. Interestingly, SMTC did not affect eNOS mediated arterial vasodilatation induced by acetylcholine. Similarly, nNOS blockade by SMTC also induced significant reduction of coronary blood flow in patients subjected to cardiac catheterization [96, 97]. This selective inhibition of coronary nNOS and reduction in blood flow, measured by intracoronary Doppler and angiography, had no effects on coronary vasodilatation induced by substance P. Production of NO by nNOS in blood vessels is also important in regulation of newborn kidneys circulation [98]. Administration of SMTC into renal artery of new born piglets induced significant reduction of blood flow and glomerular filtration rate combined with increase of vascular resistance in renal vascular bed. The effects of eNOS blockade by L-nitro-arginine methyl ester (L-NAME) were similar, but in the adults only L-NAME had effect on circulatory dynamics. SMTC also induced increase of mean arterial pressure and decrease of skeletal muscle and renal blood flow in rats [99].

Physical activity, as well as application of tempol, metal-independent superoxide dismutase (SOD) mimetic, induced increased expression of eNOS and nNOS in the

aorta and kidney of the spontaneously hypertensive rats (SHR) and their normotensive controls [36]. Exercise induced increase of SOD activity and increased production of H_2O_2 stimulated augmentation of NO production. Aerobic exercise induced increased expression of β_3-adrenergic receptors (β_3AR), activation of cardiac nNOS and NO outflow in experimental model of transverse aortic constriction induced heart failure [100]. Protective role of β_3AR-NOS interaction in cardiovascular pathophysiology was previously shown on transgenic mouse model expressing the human β_3AR in cardiomyocytes [101]. Increased activation of nNOS by β_3AR results in cGMP increase and PCG signaling with pleiotropic protective effects on CVS [102]. Furthermore, aerobic exercise resulted in increased activity of cardiac SOD and decreased production of heart ROS. Such results imply the involvement of β_3AR-nNOS-NO signaling cascade in exercise mediated cardioprotection. It was also shown that low-intensity resistance training also induced increase of eNOS and nNOS protein expression in mesenteric artery of trained rats [103]. Exercise mediated nNOS protection in cardiovascular system is unquestionably at least partly based on augmentation of antioxidative potential [104]. Physical activity induced nNOS driven positive shift in the nitroso-redox balance. Altogether, nNOS/NO aroused as complex and important mediator in maintaining of cardiovascular homeostasis which may be altered by physical activity. Assessing the changes in nNOS/NO signaling due to different models modalities of physical activity could further clarify the interconnection between nNOS and exercise in cardioprotection.

NO, Exercise and Cardiovascular Diseases

Hypertension

Hypertension is the most common chronic non-communicable disease which significantly increases risk for development of serious cardiovascular disorders including coronary artery disease, congestive heart failure, stroke, kidney insufficiency. Several clinical and experimental investigations dealt with the effects of exercise on NO synthesis and reduction of blood pressure in hypertensive patients. Comparing the two modalities of exercise, moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT) it was shown that patients in both groups showed increased NO release and improvement in flow-mediated dilation, but these benefits were more pronounced in HIIT group [105]. Similarly, it was shown that HIIT increases nitrite/nitrate followed by decrease in blood pressure of hypertensive older individuals [106]. Contrary to these results, experimental study conducted on SHR indicated beneficial effects of MICT compared to the HIIT [107]. Assessing the capacity of MICT and HIIT in treatment of detrimental changes in endothelial ultrastructure and function induced by hypertension it was shown that MICT increased NO bioavailability and decreased oxidative damage. Such effects of MICT reversed remodeling of endothelial function and ultrastructure. HIIT had opposite effects due

to increased production of ROS and decreased synthesis of NO. Another clinical investigation showed that submaximal isometric exercise session involving large muscle mass induced significant increase of NO outflow and reduction of blood pressure in hypertensive patients [108]. These effects on NO and blood pressure were followed by decrease in TBARS values as measurement of oxidative stress, and increase in catalase (CAT) activity as measurement of antioxidative capacity. Regular exercise increases expression of eNOS, tissue inhibitor of matrix metalloproteinases 2 (MMP-2), connexin 43 (Cx43) and extracellular superoxide dismutase-3 (SOD-3) in hypertensive patients [109] (Fig. 5.4). Hypertensive individuals improved eNOS expression and antioxidative capacity by regular physical activity. Assessment of the effects of heated water-based exercise on resistant hypertension showed that exercise succeeded to decrease systolic and diastolic blood pressures [110]. Simultaneously, NO level was increased while endothelin-1, rennin and norepinephrine were decreased.

Mediation of melatonin through MT2 melatonin receptor in exercise induced NO increase indicated possibly new mechanism of increasing of NO bioavailability [111]. Exercise induced reduction of blood pressure in SHR was diminished by application of melatonin receptor antagonist. Furthermore, hypertension induced decrease of MT2 and eNOS co-localization in endothelial cells, but exercise induced restoration of their co-localization. Such results indicate potential activation of eNOS via MT2. Another study showed that melatonin was able to decrease blood pressure in rats with metabolic syndrome, while eNOS and nNOS protein expressions were unchanged in heart and aorta, but increased in brain cortex and cerebellum [112].

Exercise training applied in various protocols exerted beneficial effects on menopausal hypertension through changes in NO handling [113]. One of the studies

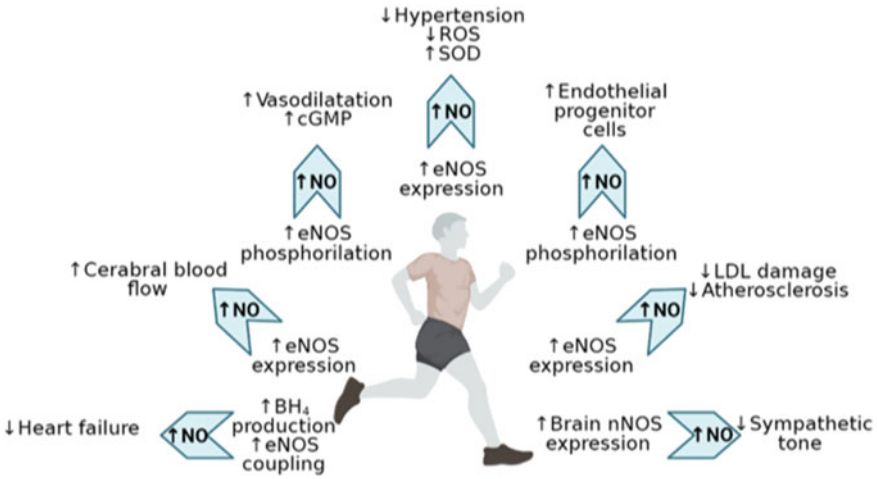

Fig. 5.4 Protective effects of exercise training in prevention and treatment of cardiovascular diseases. Figure was created using BioRender.com

showed aerobic exercise in duration of eight weeks induced significant increase in plasma nitrite/nitrate, as measurement of NO outflow, and decrease in of systolic blood pressure [114]. Another study assessing the effects of the same training process indicated similar effects [115]. The plasma values of nitrite oxide, reflecting NO synthesis, and cGMP were significantly improved by physical activity. Combination of aerobic and resistance exercise training also increased NO production and decreased systolic blood pressure, but such combination of exercise modalities seems to be less efficient compared to the aerobic exercise in postmenopausal hypertensive women [116]. Comparing the effects of resistance training and power training in older women showed that both estimated types of physical activity induced increased NO outflow combined with hypotensive and bradicardic effects, respectively [117].

Preeclampsia (PE) represents complex hypertensive disorder of pregnancy in which hypertension develops suddenly, de novo after 20 weeks of gestation and is usually (but not necessarily) combined with proteinuria [118]. The pathogenesis and etiology of PE are not fully understood, but several mechanisms involved in PE onset are recognized. The main cause of abnormal placentation and endothelial dysfunction, underlining the PE development, is reduced bioavailability of NO [118]. Decreased availability of NO disrupts sinthesis of vascular endothelial growth factor (VEGF) resulting in disturbed maternal-placental circulation [119]. One of the dominant mechanisms in reduction of NO availability is oxidative stress due to augmented production of ROS in PE [120]. Increased ROS results in eNOS uncoupling and reduced NO synthesis combined with increased O_2^- production. In the same time, increased O_2^- production additionally reduces NO through the formation of $ONOO^-$ [40]. Assessing the effects of combined aerobic and resistance exercise training during pregnancy it was shown that such physical activity lead to increased placental expression of eNOS, and even more robust increase in NO availability [121]. Such increase in NO production was accompanied with decrease of O_2^- and H_2O_2 production rate in the placental mitochondria. Meta-analysis of 17 trials showed that 30–60 min of aerobic exercise two to seven times per week applied during pregnancy significantly reduced risk of gestational hypertension and cesarean delivery [122]. Another meta-analysis confirmed such conclusion suggesting that exercise training could reduce the occurrence of gestational hypertension in overweight pregnant women [123]. Conversely, other authors showed that changes in diet and increased exercise failed to reduce the risk of PE in overweight or obese pregnant women [124]. There lot of unknowns and doubts in pathogenesis of gestational hypertension and PE, but the importance of nitric oxide in maintaining physiological pregnancy is unquestionable, and physical activity can only be beneficial.

Regular physical activity and downstream increase in NO bioavailability represents powerful tool in the treatment of hypertension and related cardiovascular disease (Fig. 5.4). Future investigations will probably focus on individual approach in exercise treatment of hypertension, in search of a model of physical activity that will achieve the most desirable effect in a particular patient.

Atherosclerosis, Coronary Artery Disease, Stroke

Atherosclerosis is characterized by chronic inflammation of the tunica intima and tunica media of the medium and large arteries. Endothelial dysfunction is recognized as one of the first steps in atherosclerosis development. Atherosclerotic complications and related cardiovascular disorders, such as coronary artery disease (CAD) and stroke, remain the leading causes of death worldwide. Enormous scientific effort has been dedicated to finding novel therapeutic advantages in treatment of these conditions and improvement of disease outcome. Due to proved beneficial effects of exercise on NO synthesis and consequent reduction of CVD risk factors, several experimental and clinical investigations dealt with possible application of physical activity, as non-pharmacological healing maneuver, in reduction of atherosclerotic complications.

Results of the clinical study aimed to assess the effects of exercise on eNOS expression and Akt-dependent eNOS phosphorylation in patients with sable CAD showed exercise mediated improvement of vasodilatory capacity [125] (Fig. 5.4). The changes were investigated in left internal mammary artery due to response to acetylcholine, and exercise was followed with significant improvement of acetylcholine-induced vasodilatation compared to control, sedentary patients with stable CAD. Furthermore, changes in vasodilatative response were mediated by exercise induced increase of shear stress was followed with higher eNOS expression, Akt and eNOS (Ser1177) phosphorylation. Exercise was also able to increase the pool of circulating endothelial progenitor cells in patients with stable CAD and reduce their apoptosis [126]. Experimental part of the same study showed that training-induced increase of endothelial progenitor cells in mice was NO mediated. Namely, the increase of circulating endothelial progenitor cells after physical activity was significantly attenuated in eNOS deficient mice, as well as after application of L-NAME as eNOS inhibitor. Augmentation of NO production was followed by increased production of VEGF and decreased apoptosis of endothelial progenitor cells in trained mice. Similar results were obtained in patients with metabolic syndrome [127]. Eight week of exercise training improved endothelium-dependent vasodilation and NO availability, as well as capacity of endothelial progenitor cells in NO production. Transplanted endothelial progenitor cells of trained subjects to into nude mice with carotid endothelial injury showed increased endothelial repair capacity. Physical activity significantly improved endothelial recovery in $LDLr^{(-/-)}$ mice fed for six weeks with a high-fat diet [128]. The exercise training conducted two weeks before and four weeks after arterial injury exhibited the most protective effects, including increase of eNOS expression, promoting endothelial cell growth and inflammation decrease.

The measurement of plasma NO_2^-, as major oxidation product of NO, showed significant increase in patients suffering from peripheral arterial disease (PAD) after three months of exercise training [129]. Increase in NO_2^- flux was accompanied with reduction of disease symptoms such as claudication onset pain time and peak walking time. Another study, including 442 patients with stable intermittent claudication, showed that treatment with NO-donor drug for 6 months significantly

reduced progression of atherosclerosis in patients [130]. Studies of similar design showed that supplementation with inorganic nitrate, as NO donor, lasting for eight weeks significantly improved NO production, blood flow and pressor response due to moderate exercise in PAD patients [131, 132]. Acute resistance exercise also increased production of NO, blood flow and reactive hyperemia in patients with PAD [133].

Diabetes mellitus type 2 (DM2) could be the limiting factor in desirable effects of exercise training in patients with PAD [134]. In patients suffering from the DM2 and PAD physical activity failed to achieve improvements in endothelial functions and NO production in comparison to the patients suffering from PAD only. Such results indicate possibility of reduced ability of endothelium to increase production of NO outflow during exercise in DM2 patients. On the other hand, it was shown that low volume HIIT lasting for 12 weeks significantly increased the plasma nitrite/nitrate level and flow-mediated dilatation in DM2 patients [135].

The intensity of physical activity could be important seesaw factor that determines the effects on CVS. Graduated and moderate swimming exercise induced increase of NO production and reduction of atherosclerotic lesions in hypercholesterolemic mice [136]. L-arginine supplementation combined with training elicited the most pronounced protective effect. Results of another study also showed that swimming training significantly reduced formation of atherosclerotic plaques via NO in experimentally induced atherosclerosis [137]. Apolipoprotein-E (apoE)-deficient mice fed with high-fat diet underwent swimming exercise training, 3 times per weeks for 8 weeks. Exercise decreased formation of fatty streak plaque lesions and increased expression of eNOS. The involvement of NO in protective effects of swimming training against development of atherosclerosis was confirmed by application of L-NAME in trained animals. Trained mice treated with eNOS blocker L-NAME developed atherosclerotic lesions, and expression of eNOS was also suppressed. Study of similar methodological approach showed that attenuation of atherosclerosis in $apoE^{(-/-)}$ mice by swimming training was also mediated by reduction of oxidative damage and production of ROS which were mediated by increased NO production [138]. Another study indicated possibility that swimming exercise increases the sensitivity to acetylcholine induced vasodilatation and extends the signaling actions of NO [139].

Experimental data indicated that aerobic exercise improves collateral circulation of the brain and prevents loss of pial collaterals due to increased expression of eNOS in aged mice [140]. Enhanced brain collateral circulation reduced infarct size induced by ligation of middle cerebral artery. Similarly, exercise significantly improved cerebral blood flow, endothelium-dependent vasorelaxation ad reduced infarct size in trained mice [141]. In the $eNOS^{(-/-)}$ mice neuroprotective effects of physical activity were absent. Furthermore, trained mice had more endothelial progenitor cells in circulation and exhibited improved functional outcome after stroke induction [142]. Again, protective effects of exercise training were diminished by eNOS inhibition or eNOS gene deletion.

It can be concluded that exercise training significantly reduces progression of atherosclerosis and related complications, certainly in part (and perhaps mostly),

through NO mediated mechanisms (Fig. 5.4). Intensity and type of physical activity has to be adjusted to specific characteristics of the patients and severity of the disease in order to achieve the most pronounced protective effect.

Heart Failure

Heart failure (HF) is deadly and disabling disease with major share in population. One of the key determinants in HF pathophysiology is decreased NO bioavailability. Several authors investigated the possibilities exercise training in improvement of endothelial function and reduction of HF symptoms. Assessment of the endothelial function through noninvasive methods, such as flow-mediated (FMD) and nitrate-mediated dilation (NMD) in HF patients indicated the relation between the severity of diastolic and endothelial dysfunction [143]. Rehabilitation strategies for HF should be directed toward enhancement of endothelial function and attenuation of endothelial dysfunction.

Results of the study by Cuoto and coauthors pointed out one of the possible molecular mechanisms underlining the beneficial effects of exercise training in HF [144]. In experimentally induced HF in rats by coronary artery ligation exercise training induced restoration of acetylcholine and sodium nitroprusside-provoked vasodilatation, and increased expression of eNOS and sGC. Increased production of NO in trained HF rats was exerted through increased expression of BH_4 secreting enzyme, GTP cyclohydrolase 1, and augmented availability of BH_4. Increase in BH_4 production induced eNOS coupling and increased NO synthesis. L-arginine supplementation, in trained rats after experimentally induced myocardial infarction and consequent HF, enhanced hemodynamic responses, decreased oxidative stress and pro-inflammatory cytokines [145]. Experimentally induced HF showed dramatic changes in endothelial function [146]. In HF Dahl salt-sensitive rats expression of eNOS in aorta was reduced by half, while the expression of MMP-2 and MMP-9 were extremely increased. All these detrimental modifications were diminished by HIIT. Conversely, in SHR model of hypertension induced HF MICT elicited protective effects reflected through decrease of myocardial fibrosis, increased angiogenesis and eNOS expression [147]. In this model HIIT exerted mostly detrimental effects.

Another NO mediated molecular mechanism involved in the development of HF that could be altered by exercise training is NO signaling in sympathetic regions in the medulla and hypothalamus. Reduction of NO availability in these regions was shown in experimental models of HF [148, 149]. Reduction of NO bioavailability occurs partly due to a decrease in NOS protein expression and partly due to increased production of O_2^- and further reaction to NO [150–152]. Exercise training increases expression of nNOS in the paraventricular nucleus of the hypothalamus of the rats with HF and restores NO-mediated changes [153].

Moderate-intensity treadmill exercise in patients with HF significantly improved activity of platelet NOS activity, followed by reduction of platelet aggregation and reduction of risk of thrombotic events [154]. Augmented NOS activity was combined

with increase in SOD and CAT activity, and decrease of inflammation in trained HF patients. Furthermore, comparing the effects several exercise modalities in patients with stable HF it was shown that swimming induced the most significant increase of plasma NO_2^-, while every single training program improved the cardiorespiratory capacity of patients [155]. Assessing the effects of various models of swimming training in rats we showed that if the intensity of the training stays within the moderate range there are no adverse effects [156]. The ability of the coronary endothelium to significantly enhance synthesis of NO is also observed.

Measurement of L-arginine consumption revealed increased L-arginine clearance in trained HF patients [157]. Such results justified the attempts of modulation NOS/NO system in treatment of HF [158, 159]. It was shown that inhaled NO_2^- reduced pressures in ventricles during exercise and rest, as well as pulmonary blood pressure [160]. Similarly, acute infusion of sodium nitrite or orally applied inorganic nitrate mitigated hemodynamic disturbances in HF patients induced by exercise and augmented exercise capacity [161, 162].

Beyond all therapeutic approaches in HF, physical activity remains the oldest, but unsurpassed curing method in HF treatment (Fig. 5.4). Exercise training should be considered as adjuvant therapeutic modality for HF, able to improve the quality of life, work capability and longevity in HF patients. Future investigations in this area should be directed to finding the most adequate type of physical activity for each patient as well as the degree of load that brings the greatest benefits.

Concluding Remarks

Endothelial dysfunction plays a capital role in the initiation, progression and adverse outcome in many CVD. Ability of endothelium to produce NO appears to be key determinant of healthy blood vessels. Thus, decreased NO bioavailability and impaired NO mediated vasodilatation assembles the main pathophysiological feature of endothelial dysfunction. Variety of pharmacological approaches in modern medicine targeted endothelial NO synthesis and downstream NO cascades in preventing and treatment of CVD with varying degrees of success. Most often the effects of these approaches do not meet expectations. On the other hand, exercise training and different models of physical activity has emerged as a non-pharmacological therapy, effective in both prevention and treatment of CVD. Taking into account the variety of signaling cascades in which NO participates, as well as complex changes initiated by exercise training into vasculature, the mutual conditioning of NO, physical activity and achieved effects are not fully understood. The future scientific attempts in this field should be directed toward discovering the most satisfying and the most beneficial type of physical activity for each CVD, as well as to the most benevolent degree of physical load depending on the functional state of the patient.

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Chapter 6
Nitric Oxide-cGMP-PKG Signaling in the Cardioprotective Effects of Phosphodiesterase 5 Inhibitors

Rakesh C. Kukreja, Anindita Das, Saisudha Koka, Arun Samidurai, and Lei Xi

Abstract Phosphodiesterase 5 (PDE5) is an enzyme that catalyzes the degradation of cGMP to its inactive form, 5′-GMP. The inhibition of PDE5 leads to the increase in bioavailability of cGMP which exerts its downstream signaling effects through the activation of protein kinase G (PKG). The dysregulation of cGMP-PKG signaling cascade plays a critical role in the pathology of several cardiovascular disorders. PDE5 inhibitors including sildenafil and tadalafil are widely prescribed drugs for the treatment of erectile dysfunction and pulmonary hypertension in patients. In the pre-clinical setting, treatment with PDE5 inhibitors protect against several cardiovascular pathologies including ischemia/reperfusion (I/R) injury, heart failure, pressure overload-induced hypertrophy, and cardiomyopathy associated with type 2 diabetes and metabolic syndrome. Mechanistic studies reveal that nitric oxide (NO)-cGMP-PKG signaling driven multiple signaling pathways are involved in protection against most of these pathologies. Moreover, the PDE5 inhibitors generate other gasotransmitters including hydrogen sulfide, carbon monoxide in addition to NO that may play a critical role in cardioprotection.

Keywords Nitric oxide · cGMP · Hydrogen sulfide · Phosphodiesterase · Ischemia · Reperfusion injury

Introduction

Ischemic heart disease is the leading cause of death in the world. During ischemia, the sudden occlusion of coronary artery reduces or eliminates the flow of oxygenated blood to the myocardium. As a result, the supply of oxygen trapped in the tissue is depleted within seconds of ischemia resulting in the rapid changes in metabolism as

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_6

well as contractile function of the heart. If ischemia persists, many of the cardiomyocytes become irreversibly injured and die even if ischemia were eliminated. Therefore, the goal is to re-establish blood flow to the ischemic area as quickly as possible to rescue cardiomyocytes that would be permanently damaged by ischemia. While reperfusion is necessary for tissue survival, a major caveat is that reperfusion itself can also cause tissue damage, termed "reperfusion injury" [1]. As more tissue is irreversibly injured, the prognosis becomes worse because terminally differentiated cardiomyocytes have very limited or no regenerative potential [2]. Thus, the loss of contractile muscle exerts extraordinary load on the surviving tissue, which becomes hypertrophic resulting in adverse remodeling of the left ventricle (LV) leading to heart failure. Therapeutic strategies that would make the cardiomyocytes resistant to death from ischemia/reperfusion (I/R) injury would greatly improve the chances of survival following acute myocardial infarction (AMI) in patients with coronary artery disease.

In the 1980s, a cardioprotective strategy was discovered where multiple bouts of brief ischemia and reperfusion were shown to reduce infarct size following sustained I/R injury [3]. This unique phenomenon, called ischemic preconditioning (PC) was found to occur in two phases: an early phase which occurred immediately after short bursts of ischemia and disappeared within 2–3 h [4]. A second phase of PC was observed after 24 h of the initial stimulus which was called "delayed PC" or "second window of PC" [3–5]. The protective effect of the delayed phase lasted up to 96 h after the initial stimulus of brief episodes of ischemia. The possible cellular and molecular mechanisms of PC have been extensively studied and a number of mechanistic pathways have been suggested (see [5, 6] for reviews).

While the clinical application of PC is difficult, searching for pharmacological agents or drugs that could mimic early and delayed PC has been the subject of interest because of their potential direct clinical application in lieu of PC. In fact, the identification of the cellular and molecular basis of PC has provided an excellent conceptual framework for developing several novel therapeutic strategies aimed at mimicking the cardioprotective effects with pharmacological agents. For example, endogenously released agents were identified during PC, which included adenosine [7], norepinephrine [8], opioids [9], free radicals [10] and bradykinin [11, 12]. In addition, a number of pharmacological activators of the pathways of PC including adenosine receptor agonists [7, 13–16], nitric oxide (NO) donors [17–19], bradykinin [12, 20, 21], p38 MAP kinase activator, anisomycin [22], chemical inducer of hypoxia-like responses, cobalt chloride [23], hypoxia-inducible factor-1 [24, 25], ATP-sensitive potassium channel (K_{ATP}) opener, diazoxide [26, 27] and Ca^{2+}-activated potassium channel opener NS1619 [28, 29], have been shown to induce PC-like cardioprotective effects. Thus, the discovery of PC had huge impact in identifying previously unknown new pathways of cardioprotection.

Nitric Oxide (NO)—A Key Signaling Molecule in Cardioprotection

NO is a short-lived intracellular messenger, which has been shown to regulate blood pressure, platelet adhesion, neutrophil aggregation, as well as synaptic plasticity in brain [30–32]. NO and its secondary oxidants including the peroxynitrite (ONOO–) can also form major cytotoxic agents produced by activated macrophages and neutrophils. Peroxynitrite protonates and decomposes by homolytic fission to generate the hydroxyl radical (•OH) or some other potent oxidant with similar reactivity. NO also reacts with lipophilic peroxyl radicals to generate alkyl peroxynitrates (LOONO) [33]. NO is produced by the oxidation of L-arginine by nitric oxide synthase (NOS) which has three isoforms including endothelial NOS (eNOS), neuronal (or brain) NOS (nNOS), and an inducible isoform (iNOS) [34, 35]. eNOS produces NO via a complex reaction that is stimulated by calcium and requires NADPH, among other co-factors [34]. Inducible NOS was the first isozyme of NOS identified as a source of NO during the second phase of PC [36, 37]. NO was shown to increase the resistance against I/R injury through inhibition of calcium influx, blockade of β-adrenergic stimulation, reduction in myocardial oxygen demands, opening of sarcolemmal and/or mitochondrial K_{ATP} (mitoK_{ATP}), activation of COX-2 which increased synthesis of prostaglandins. The eNOS-derived NO triggered signaling cascade which included activation of protein kinase C, and MAP kinases [38, 39], various transcription factors including NF-kappa B, STAT1/3 resulting in the increased synthesis of cardioprotective genes such as iNOS, COX-2, and HO-1, which contributed to the long-lasting protection of the heart following PC [40–43].

Cyclic Nucleotide Phosphodiesterases

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are important intracellular second messenger that mediate multiple tissue and cellular responses [44]. Their levels are maintained by a family of enzymes named cyclic nucleotide phosphodiesterases (PDEs) [41, 45, 46]. The PDEs degrade the phosphodiester bond of 3′-5′cAMP and 3′-5′cGMP and convert them to their inactive forms: 5′AMP and 5′GMP, respectively [47]. PDEs have 11 families (PDE1–PDE11) with more than 80 enzyme variants generated from multiple promoters and as a consequence of alternative splicing [46]. PDE5 is the primary enzyme with cGMP-hydrolyzing activity in human corpus cavernosal tissue which plays an important role in the mechanism of penile erection. Sexual stimulation either physically or psychologically induces release of NO from non-cholinergic, non-adrenergic neurons in the penis, as well as from endothelial cells [48]. NO diffuses into smooth muscle cells and activates soluble guanylate cyclase (sGC) which converts GTP to cGMP and initiates protein phosphorylation cascade. This decreases intracellular calcium levels which results in the dilation of the arteries bringing blood to the penis thereby

leading to the compression of spongy corpus cavernosum. PDE5 inhibitors help in erection by blocking the enzymatic hydrolysis of cGMP in the corpus cavernosum.

PDE5 as Therapeutic Target for Cardiac Protection

We hypothesized that PDE5 inhibitors may trigger NO-cGMP pathway in the heart which could lead to protection against I/R injury. In support of this notion, for the first time we showed that treatment of rabbits with sildenafil prior to prolonged (index) ischemia significantly reduced myocardial infarct size which was blocked by 5-hydroxydecanoate, a blocker of mitoK$_{ATP}$ [49]. Vardenafil is 20-fold more potent than sildenafil for inhibiting PDE5 [50] and as a result, 50-fold lower concentration of this drug (compared to sildenafil) had similar protective effect against I/R injury in the rabbit model of myocardial I/R injury [51]. Tadalafil is a long-acting PDE5 inhibitor with a half-life of 17.5 h [52] and is effective for erectile function up to 36 h. Like other PDE5 inhibitors, tadalafil also reduced infarct size and improved cardiac function following I/R in mice [53]. We confirmed these findings in our in vivo as well as isolated perfused hearts models of I/R injury [54–56] and in the in vitro cell culture model in which the isolated cardiomyocytes were subjected to simulated ischemia and reoxygenation [57]. In the mouse heart and isolated primary cardiomyocytes, we demonstrated that treatment with sildenafil increased iNOS and eNOS protein [55]. Moreover, the activation of PKG, phosphorylation of extracellular signal-regulated kinase (ERK) as well as glycogen synthase kinase-3β (GSK-3β) led to the increased Bcl-2 expression and inhibition of cardiomyocyte apoptosis [58]. PKG has also its own independent effect on I/R injury as it may directly open the mitoK$_{ATP}$ channel and limit infarct size through preserving ATP and decreasing the calcium (Ca^{2+}) influx into the mitochondria [59, 60] as outlined in Fig. 6.1.

Myocardial infarction and other stimuli, including pressure and volume overload, trigger a complex process of myocardial remodeling, including myocyte hypertrophy and loss, ventricular wall thinning and dilatation, and fibrosis [61]. These pathophysiological changes lead to maladaptive remodeling with a progressive contractile dysfunction and heart failure. To demonstrate whether sildenafil prevents remodeling, we treated mice immediately or 72 h following coronary artery occlusion [62]. The results showed reduced infarct size as well as improved cardiac function, survival rate and decrease in cell death in the border zone of the infarcted myocardium. Nitric oxide generated following sildenafil treatment appeared to be the key molecule in reducing damage of the myocardium after infarction because eNOS and iNOS isozymes were increased in the hearts. Also, treatment with the non-specific NOS inhibitor, blocked the protective effect of sildenafil in this model of heart failure [62, 63]. These studies suggested that PDE5 inhibition with subsequent generation of NO may potentially have beneficial effect in patients with heart failure [63].

In a mouse model of transaortic constriction induced pressure overload, the chronic administration of sildenafil also prevented and reversed cardiac hypertrophy [64]. In these studies, sildenafil treatment suppressed myocyte hypertrophy,

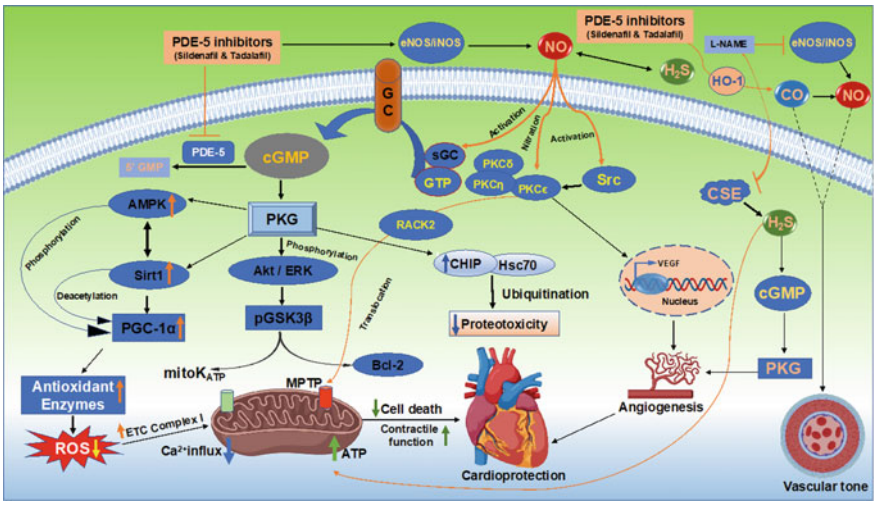

Fig. 6.1 Nitric oxide (NO) triggered signaling pathways in cardioprotection with PDE5 inhibitors. Regulation of cGMP by PDE5 inhibition activates PKG which causes phosphorylation of ERK 1/2 and phosphorylated glycogen synthase kinase-3β (pGSK3β) in conjunction with an increase in Bcl-2, and inhibition of apoptosis as well as MPTP. PKG opens mitochondrial ATP-sensitive mitoK$_{ATP}$ channels, which limits I/R injury through preservation of ATP and a decrease in Ca^{2+} influx in the mitochondria. In the diabetic heart, PKG can also increase in PGC-1α through AMPK phosphorylation and deacetylation of Sirt1 leading to decreased ROS production, improved mitochondrial biogenesis/function through preservation of ETC complex I. PKG activation also enhances post-translational protein quality control via carboxyl terminus of CHIP leading to decreased cell death and ischemic injury. NO may also activate the novel isoforms of PKC including ε, α, δ, and θ, which may translocate to the particulate fractions including the nuclear fractions to increase gene expression of VEGF and angiogenesis. PKC α-Src module enhances PKCε-associated Src enzymatic activity which is linked to cardioprotection. NO derived from PDE5 inhibitors may also activate HO-1 and CSE to produce CO and H_2S which may reduce ischemia injury through increased angiogenesis or improvement of vascular tone. See text for further details. *Abbreviations* AMPK—5′AMP-activated protein kinase; CHIP—carboxyl terminus of Hsc70-interacting protein; CO—carbon monoxide; CSE—cystathionine γ lyase; ERK—extracellular signal-regulated kinase; ETC—electron transport chain; GTP—guanosine triphosphate; H_2S—hydrogen sulfide; I/R—ischemia/reperfusion; PGC-1α—peroxisome proliferator activated receptor γ coactivator 1α; PKC—protein kinase C; PKG—protein kinase G; pGSK3β—phosphorylated glycogen synthase kinase-3β; ROS—reactive oxygen species; sGC—soluble guanylate cyclase; mitoK$_{ATP}$—mitochondrial ATP-sensitive K^+; MPTP—mitochondrial permeability transition pore

improved contractile dysfunction and decreased fibrosis. Sildenafil also reversed pre-established hypertrophy induced by pressure overload while restoring LV function to normal. PDE5 expression increased in pressure-loaded hearts which was associated with increased cGMP catabolism. PDE5 inhibition also led to restoration of cGMP signaling and activation of PKG. The anti-hypertrophic effect was associated with activation of PKG, and its targets included regulator of G protein-coupled signaling-2, as well as calcineurin-NFAT and transient receptor potential channel 6,

one of the nonselective and non-voltage-gated ion channels that convey signaling information linked to a broad range of sensory inputs [65]. Another study suggested that chronic treatment with sildenafil attenuated LV remodeling and exercise intolerance following chronic mitral regurgitation [66]. This benefit was associated with the anti-apoptotic and anti-inflammatory effects of sildenafil.

Cardioprotective Signaling in Ischemia/Reperfusion Injury—Role of Gasotransmitters It has been recognized that NO may interact with other two other major gasotransmitters in cellular signaling, i.e. hydrogen sulfide (H_2S) and carbon monoxide (CO), either by inhibiting or potentiating the level and activity of the other, depending on their physiological milieu in various organs and tissues [67]. These signaling molecules possess significant differences in physiological half-lives, i.e. CO is more stable and effective distal to the site of its production, whereas NO and H_2S are short lived and act only close to sites of their production [67]. H_2S is produced enzymatically on a continuous basis at micromolar levels in mammalian organs including the cardiovascular system. Early studies showed the cardioprotective effect of H_2S via administration of a donor, sodium hydrosulfide (NaHS) which was mediated through opening of K_{ATP} channel [68]. Treatment with H_2S donor (NaHS, 40 μM) in isolated rat hearts improved I/R-induced cardiac dysfunction and tissue injury markers, while increased tissue NO production [69]. The H_2S-producing enzyme, cystathionine-γ-lyase (CSE), is expressed in the heart. Interestingly, the cardiac expression of CSE was suppressed by L-NAME, a pan inhibitor of NOS suggesting that H_2S and NO interact to cooperate in protection against myocardial I/R injury [69]. Also, H_2S is capable of regulating the generation of NO [70] and to facilitate release of NO in vascular tissues [71]. H_2S can regulate the availability of NO by increasing its release from nitrosothiols [72]. It was also suggested that the cooperative action of NO and H_2S were essential in increasing and maintenance of intracellular levels of cGMP as well as activation of PKG, angiogenesis, and vasorelaxation [73]. The H_2S-induced wound healing and microvessel growth are suppressed by pharmacological inhibition or genetic ablation of eNOS [73].

We first reported the cardioprotective effect of PDE5 inhibitor tadalafil in mice. Mice treated with tadalafil had reduced infarct size and improved cardiac function [53]. These studies further showed an essential role of H_2S producing enzyme, CSE because the cardioprotective effect of tadalafil was abolished by the enzyme inhibitor, dl-propargylglycine (PAG) as well as in CSE-knockout mice. Interestingly, a previous study also showed that sildenafil enhanced production of both NO and CO, by stimulating expression of iNOS and heme oxygenase (HO-1), a CO-producing enzyme in vascular smooth muscle cells [74]. These studies suggested that sildenafil stimulated the expression of iNOS and HO-1 likely via soluble guanylate-cGMP pathway. Also, it was shown that CO inhibited NOS activity [75] or directly stimulated NO formation [76] in vitro. CO also acts as a tonic regulator of NO-dependent vasodilation in the rat brain [77]. Thus, PDE5 inhibitors may have therapeutic benefit where the gasotransmitters, NO, H_2S or CO may act alone or in concert in cardioprotective signaling (Fig. 6.1).

Protein Kinase C in Cardioprotection One of the major intracellular signal transduction pathways of preconditioning involves NO mediated activation of PKC [78]. The PKC family, which has 12 members, is comprised primarily of three sub-families: the conventional, the novel isoforms which are calcium dependent (α, βI, βII, and γ) and the calcium independent isoforms including the δ, ϵ, η, θ, and μ. The activity of these isoforms is determined by their translocation from the cytosolic to the particulate fraction (sarcolemmal, mitochondrial, nuclear fractions) which subsequently bind to the specific receptors of activated C kinase (RACK) localized in membranes [79]. The translocated-specific PKC isoforms have been linked to the opening of mitoK_{ATP} channels as well as gene expression [80]. The PKC-mediated cardioprotection is isoform specific: the ϵ- and η-isoforms have been shown to be the mediators of ischemic and pharmacological PC in the heart and cardiomyocytes [16, 81–84]. The NO-PKC (especially the novel isoforms, PKCε and PKCδ) signaling pathway has been connected to a number of cardioprotective modalities such as pharmacological PC with acetylcholine [85], mitoK_{ATP} channel opener, diazoxide [27], δ-opioid receptor agonist BW373U86 [86], K_{ATP} channel opener, nicorandil [87], bradykinin [88], oxytocin [89], as well as several mechanical/physiological approaches, e.g. delayed ischemic PC [39], chronic continuous normobaric hypoxia [90] and short-term mild exercise [38].

It has been shown that NO-induced late preconditioning forms PKCε-Src module which results in enhanced PKCε-associated Src enzymatic activity. Inhibition of PKC blocked cardioprotection, the PKCε-Src module formation, and PKCε-associated Src activity [91]. It was also demonstrated that NO donors promoted translocation and activation of PKCε in an NO- and peroxynitrite-dependent fashion (Fig. 6.1) [92]. NO/peroxynitrite-mediated tyrosine nitration of PKCε was observed in both rabbit cardiomyocytes in vitro and NO donor-preconditioned rabbit myocardium in vivo [92]. There was also a peroxynitrite-dependent increase in PKCε-RACK2 interactions in NO donor-treated cardiomyocytes, indicating post-translational modification (nitration) of PKCε by NO donors would facilitate interaction with RACK2 and promote translocation and activation of PKCε [92]. We demonstrated that sildenafil induced cardioprotection in the rabbit was also mediated by PKC because the protective effect was abolished by the inhibitor, chelerythrine [93]. However, in contrast to PKCε, we observed selective translocation of PKC α, δ, and θ isoforms from cytosol to membrane fractions suggesting their potential role in sildenafil-induced cardioprotection. Further studies are required to identify whether α, δ, and θ isoforms also have the ability to form module with Src during sildenafil-induced protection.

Improvement of Protein Quality Control Another potential mechanism of cardioprotection could be the improvement in protein quality control by sildenafil-triggered cGMP-PKG pathway for quality control during ischemic stress. Proteotoxicity from insufficient clearance of misfolded or the damaged proteins is the cause for many diseases [94]. The carboxyl terminus of Hsc70-interacting protein (CHIP) functions as an E3-ligase and co-chaperone that facilitates protein degradation (Fig. 6.1) [95]. CHIP mediates protein degradation via the proteasome, as well as by autophagy-lysosome-dependent pathways [96]. Genetic loss of CHIP causes worsening of

hemodynamic or ischemic stress [97, 98]. Conversely, upregulation of CHIP caused angiogenic effect, attenuated inflammation, improved cardiac function as well as survival post MI [99]. More recent work has shown that PKG activation enhanced post-translational protein quality control via carboxyl terminus of CHIP [100]. Downregulation of PKG activity decreased CHIP-S20 phosphorylation and protein, exacerbated proteotoxicity and worsened heart function following ischemia. On the other hand, CHIP-S20E knock-in mice demonstrated improved clearance of ubiquitinated proteins and showed protection against ischemic injury. These studies suggested a new role of PKG activation in providing post-translational enhancement of protein quality control via CHIP.

Cardioprotection in Diabetes and Metabolic Syndrome

Endothelial dysfunction is common in vessels of diabetic patients than in the non-diabetic population. With the progression of disease, there is increase of macrovascular and microvascular complications which is one of the main causes of increased mortality in patients with diabetes mellitus [101]. Reduced levels of NO within the vascular endothelium contributes to impaired insulin utilization in patients with insulin resistance [102]. Vascular NO is critical for normal vasodilatation and endothelial function, and impairment of NO bioavailability and the NO-cGMP signaling. Epidemiologic studies demonstrate that eNOS mutations are associated with hypertension, increased atherosclerosis, and worse outcomes from MI, cardiac arrest, and stroke [103]. An epidemiological study provided evidence of a strong correlation between the risk factors associated with metabolic syndrome (i.e. obesity, elevated fasting glucose levels, dyslipidemia, hypertension) and urinary cGMP excretion, suggesting that a reduction of NO bioactivity concurs with these CV risk factors [104]. In streptozotocin-induced diabetic rats, sildenafil improved vasorelaxation through enhanced endogenous NO signaling [105]. Another major issue is that the diabetic myocardium is more vulnerable to I/R injury [106, 107] and refractory to many cardioprotective modalities, such as PC [108] and ischemic postconditioning [109]. In our studies, we found that chronic treatment with tadalafil significantly reduced myocardial infarct size following I/R injury in mice with Type 2 diabetes (T2D) [110, 111]. There was significant reduction in fasting glucose and triglyceride levels with tadalafil treatment although body weight remained unaltered. Treatment with tadalafil also enhanced plasma levels of NO. NO can activate SIRT1 which regulates peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), which is a key regulator of mitochondrial biogenesis and co-activator of transcription factors impacting energy homeostasis. We found that myocardial SIRT1 and PGC-1α expression and phosphorylation of Akt as well as AMPK were increased in the diabetic hearts. Interestingly, these signaling changes were associated with attenuated mitochondrial dysfunction as shown by improved mitochondrial glutamate state 3 respiration rates and reduced ROS production from complex I [112] as outlined in Fig. 6.1.

Metabolic syndrome (MetS) is a cluster of risk factors characterized by abdominal obesity, dyslipidemia, hypertension, and insulin resistance [113, 114]. The MetS is associated with increased risk of multiple chronic diseases, including the cardiovascular, T2D, arthritis, chronic kidney disease, cancer, and all-cause mortality [115–117]. NO plays a crucial role in the pathogenesis of MetS because its reduced bioavailability may be a contributing factor in this condition [118]. Interestingly, chronic treatment with tadalafil enhanced NO production in the db/db diabetic as well as MetS mice in addition to the positive effect on metabolic health status by improving insulin sensitivity, lowering circulating lipids, and protecting the heart against I/R injury [119]. More importantly, treatment with tadalafil improved LV diastolic dysfunction in MetS mice. These studies suggested that tadalafil treatment in MetS mice could clinically benefit MetS patients who are at high risk for cardiovascular diseases. Thus, tadalafil may turn out to be promising therapeutic strategy providing dual benefit of treating ED as well as reducing cardiovascular injury in MetS patients.

Conclusions

Over the past 5 decades, enormous progress has been made in our understanding of the basic cellular and molecular mechanisms underlying the development of I/R injury and the protective actions of therapeutic interventions including PC and several pharmacological agents. The role of NO elicited from NOS, particularly the eNOS or iNOS isoforms has been well established in cardioprotection in a variety of therapeutic modalities including PC and pharmacological agents. Preclinical studies have shown that PDE5 inhibitors have powerful cardioprotective effects against I/R injury and doxorubicin-induced cardiomyopathy [120–122], post-infarction heart failure [62], pressure overload hypertrophy [64], T2D and MetS [110, 111, 119]. As summarized in Fig. 6.1, a large body of work has provided insights into the critical role of NO-cGMP signaling in cardioprotection. Therefore, drugs such as PDE5 inhibitors which trigger NO-dependent signaling pathway could be promising therapeutics against I/R injury and several other cardiovascular disorders.

Acknowledgements This work was supported by Grants from the National Institutes of Health RO1HL134366 (RCK & AD), R37 HL-51045, RO1HL118808, R01CA221813, R01DK120866 (RCK).

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Chapter 7
Role of Nitric Oxide Synthases in Doxorubicin-Induced Cardiomyopathy

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Abstract Since the discovery of Doxorubicin (Dox), its safe use is under intense discussion due to its cardiotoxic side effects manifested in patients at different times during the treatment or even after years of treatment. Several therapeutic approaches to replace conventional Dox or use of other drugs in combination have exhausted the clinicians and researchers without much success. When the replacement strategies failed to show any rigor, a better understanding of Doxorubicin's mechanism of action seemed like the only gateway to the discovery of a new targeted therapeutic approach. An increase in reactive oxygen species and the resultant oxidative stress as the mechanism of Dox-induced cardiomyopathy, proposed by us as well as others has gained some traction. However, this explanation has not been enough to alleviate concerns with Dox and we are still in search for a solution for its safe use. More recently, our laboratory and others have also shown the importance of nitrosative stress. Furthermore, we have shown that Vitamin C not only mitigates nitrosative stress but it also modulates Dox-induced cardiotoxic changes in isolated cardiomyocytes as well as in whole animals exposed to Dox. The present review chapter focusses on the mechanism of Dox-induced nitrosative stress and the role of Vitamin C in mitigating the cardiotoxic effects of Dox.

Keywords Doxorubicin induced cardiomyopathy · Oxidative stress · Nitrosative stress · Vitamin C

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_7

Introduction

Doxorubicin (Dox), also known as Adriamycin, is an antitumor antibiotic isolated from bacterium *Streptomyces peucetius var caesius* [1]. Dox belongs to the family of anthracyclines which also includes other antitumor antibiotics such as daunomycin, rubidomycin, idarubicin, and epirubicin. Dox is the primary chemotherapeutic drug used for a wide range of cancers, including lymphomas, soft-tissue sarcomas, solid tumors, and hematological cancers.

Dox-Induced Cardiac Effects

The usage of Dox is limited by its acute as well as chronic side effects. Acute effects such as nausea, vomiting, fatigue, bone marrow suppression, alopecia, arrhythmia, and injury to non-targeted tissues are generally reversible and/or clinically manageable. However, chronic use of Dox is mainly limited by its cardiac toxicity seen during or even years after the end of treatment. These adverse cardiac effects of Dox are irreversible and are generally life-threatening. These are manifested in different forms, including loss of myofibrils, dilation of the sarcotubular system, aberrant arrhythmias, ventricular dysfunction, dilated cardiomyopathy, congestive heart failure which is generally refractory to most cardiotonic drugs and ventricular assist devices approaches and is the most serious side effect [2–5].

Mitigation of Dox-Induced Cardiac Effects

Over time, several attempts have been made to address Dox-induced cardiotoxicity. The most common strategy attempted has been to reduce the cumulative dose and minimize the peak plasma concentration of the drug by delivering intravenously over 2448 h but this has not reduced the risk of cardiac toxicity in patients [6]. Even though Dox can be administered as a single agent, however, in order to reduce the total amount used oncologists have tried combining it with other antitumor drugs such as vincristine, cyclophosphamide to form ‘cocktails’ which had shown lower cytotoxic effects on malignant cells [7]. Another approach has been to synthesize different analogs with the goal of reducing or eliminating the life threatening cardiotoxic side effects while keeping the antitumor efficacy [8]. In this regard, more than 2000 analogs have been synthesized without any notable success. Only some had received approval for usage such as, daunorubicin, 5-imino, 13-deoxydoxorubicin (GPX-150) even though it had lower oncologic efficacy but did not confirm lower cardiotoxicity than conventional Dox [9]. Packaging of the drug and targeting delivery to tissues was also proposed to have improved safety profile and better efficacy. While the FDA approved PEGylated liposomal doxorubicin formulation such as Doxil®, it

is restricted only for ovarian cancer and AIDS-related Kaposi sarcoma [10, 11]. Moreover, several solution-based materials, nanoparticles or nanoparticles embedded in a matrix were studied but did not achieve the targeted goal [12, 13].

The Challenge

Despite the above mentioned approaches, nothing has replaced the use of conventional Dox in cancer treatment even after the risk of developing cardiomyopathy and this risk remains high even many years after treatment [3, 14]. Thus a complete understanding of the biology of Dox-induced cardiomyopathy appears to be the only answer.

An understanding of the subcellular basis of the cardiotoxic effects of Dox, remains under intense debate and have not been completely settled even after 60 years of extensive research. In this regard, several different possible pathways have been put forward to explain the complex pathophysiology of Dox-induced cardiomyopathy. This list includes, oxidative stress (OS), nitrosative stress (NS), inflammation, iron metabolism, calcium dysregulation, structure/function changes in the mitochondria and the sarcoplasmic reticulum, and cell apoptosis [3, 4, 15]. Among these different mechanisms for Dox-induced cardiotoxicity, OS has been the most accepted mechanism of action and has been the subject of many high quality reviews [3, 4, 16]. The focus of this chapter is nitrosative stress subsequent to Dox exposure and its mitigation.

Nitrosative Stress

As a molecular basis of the pathogenesis of Dox-induced cardiotoxicity, increasing evidence also suggests involvement of the nitrosative stress. Nitrosative stress which is implied in the pathogenesis of various conditions including heart failure, myocardial infarction, diabetes, acute ischemia, sepsis and cancer is brought about by an increase in the generation of reactive nitrogen species (RNS) [17–21]. It is characterized by an increase in peroxynitrite leading to protein nitration and nitrosylation. The nitrosative stress is exacerbated by the oxidant environment in the cellular milieu. In myocardium, nitrosative stress adversely affects cardiac performance by disruption of nitric oxide (NO) mediated signaling of Ca^{2+} channels responsible for normal systolic and diastolic functions [22]. It also enhances loss of cardiomyocytes via peroxynitrite mediated activation of apoptosis [20, 21, 23]. It also upregulates proinflammatory cytokines such as TNF-α, IL-/1B and IL-6 to promote inflammation [20, 21].

There is a long list of RNS such as peroxynitrite ($ONOO^-$), nitrogen dioxide ($\bullet NO_2$), peroxynitrous acid (HNO_3), dinitrogen trioxide (N_2O_3), nitroxyl (HNO), peroxynitrous acid (ONOOH), peroxynitrate (O_2NOO^-), peroxynitric acid

(O_2NOOH), nitrosonium cation (NO^+), nitrate ($NO3^-$), nitrite ($NO2^-$) and nitroxyl anion (NO^-) [24]. Despite their short half-life, these RNS are able to diffuse into the intracellular organelles and can still react at extremely higher rates [20, 21, 23, 25].

Peroxynitrite ($ONOO^-$)

Cytokine mediated upregulation of inducible nitric oxide synthase (iNOS) expression promotes formation of NO and thereby results in NO/redox disequilibrium. Near diffusion limited reaction of NO with superoxide anion results in the formation of $ONOO^-$ which is a strong biological oxidant (Fig. 7.1). Increased generation of $ONOO^-$ is a major mechanism associated with pathogenesis of a number of cardiovascular pathologies such as Dox-induced cardiomyopathy, myocardial infarction, chronic HF and diabetes [17, 20, 21, 23, 26]. Although $ONOO^-$ exerts its deleterious effects via targeting multiple signaling pathways in the cell, direct oxidation of cellular biomolecules such as lipid, protein and DNA are also the basis for its cytotoxicity (Fig. 7.1). Peroxynitrite mediated nitration of proteins at their tyrosine residues can result in either inactivation or hyperactivation of their activity [27]. Furthermore, nitration can prevent subsequent phosphorylation, alternatively enhance phosphorylation of proteins, or alter degradation of proteins. Nitration is involved in the initiation and progression of a number of diseases [28].

It has also been reported that, $ONOO^-$ acts as a major effector of apoptosis in cardiomyocytes via the activation of caspase-3 and PARP. Reduction in $ONOO^-$ suppressed protein nitration and apoptosis in H9C2 cells [29]. Peroxynitrite induced oxidation of sulfhydryl groups inhibited mitochondrial respiratory chain enzymes and irreversibly damaged mitochondrial membranes resulting in excess generation of ROS [17, 27, 30]. Furthermore, $ONOO^-$ impaired the antioxidant defense by inhibiting activities of SOD and GPx [31] as well as decrease the levels of other endogenous antioxidants such as Vit C and plasma thiols via enhanced nitration resulting in increased generation of ROS [32].

Peroxynitrite impairs cardiac contractility in two ways: (i) by enhanced nitration of various proteins such as myofibrillar creatine kinase [33], Ca^{2+} handling proteins (Ryanodine receptor, Phospholamban) [34] and (ii) by causing loss of bioactive NO via uncoupling of eNOS. Peroxynitrite can trigger eNOS uncoupling via nitration of eNOS and/or oxidation of its cofactor BH4 or alternatively via disrupting endothelial caveolae [34, 35]. Functional alteration of proteins through enhanced nitration has significant impact on the pathogenesis of cardiovascular diseases.

Nitric Oxide (NO)

NO or nitrogen monoxide is one of the oxides of nitrogen along with nitrous oxide (N_2O) and nitrogen dioxide (NO_2). N_2O, commonly known as laughing gas is popular

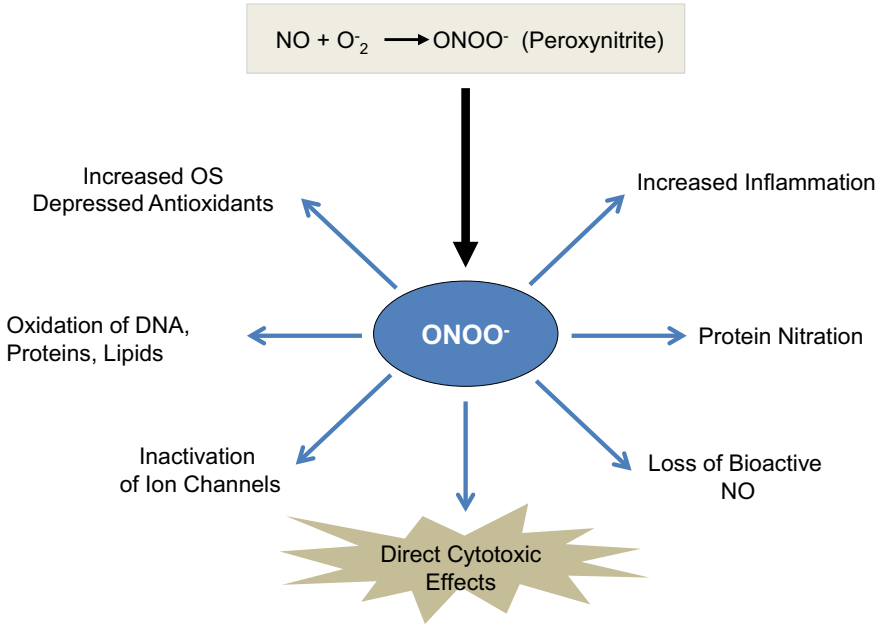

Fig. 7.1 *Formation of peroxynitrite and its cytotoxic effects.* Superoxide (O_2^-) together with Nitric Oxide (NO) leads to Peroxynitrite ($ONOO^-$) formation which alters cellular functions by targeting several biomolecules. Peroxynitrite can cause direct or indirect cytotoxic effects by protein nitration, loss of bioactive NO, oxidation of DNA, proteins and lipids. It also leads to an escalation of oxidative stress (OS), through an overproduction of ROS and decrease in antioxidant enzymes, increases inflammation and causes inactivation of ion channels

for its medicinal use due to its anesthetic properties. In contrast, NO_2 is an air pollutant also used as an oxidizer in rocket fuel as well as nitrating agent in chemical explosives. NO is a free radical and considered as an environmental pollutant at high concentrations, whereas at low concentration it is a crucial signaling molecule with a key role in various physiological as well as pathological functions in mammalian systems [36, 37].

Although initially recognized as endothelium derived relaxing factor (EDRF) involved in vascular functions, NO also plays an important role in cardiomyocyte contraction. It is produced in a variety of cell types such as endothelial cells, smooth muscle cell (SMC), cardiomyocytes, skeletal muscle, neuronal cells as well as inflammatory cells such as macrophages and monocytes [36, 38]. Alterations in NO concentration are associated with progression of many conditions such as diabetes, cancer, atherosclerosis, hypertension, arthritis and myocarditis [38].

Although the physiological importance of NO was recognized in 1998, medical use of nitrate containing compounds such as nitroglycerin has been practiced since 1895 for relief from angina pectoris [39]. In 1977, Murad's laboratory demonstrated that NO mediated the activation of soluble Guanylate Cyclase (sGC) and the upregulation of cyclic guanosine monophosphate (cGMP) as being the mechanisms involved

in the vasodilatory effect exerted by nitroglycerin and nitroprusside in vascular SMC relaxation [40]. Furchgott and Zawakzki (1980) later identified that NO is same as EDRF [41]. Three individuals, Robert Furchgott, Louis Ignarro and Ferid Murad were honored with Nobel Prize in Medicine or Physiology in 1998 for their contributions for the identification of crucial role of NO and NO was declared as "molecule of the year".

At physiological concentrations in the range of pico molar (pM) to low nano molar (nM) amounts, NO provides beneficial effects; whereas at higher concentration i.e. in the μM range, it can be toxic and proinflammatory resulting in deleterious effects [36, 42–44]. NO is a transient free radical with a half-life of about 5 s. This could be a result of its high reactivity with proteins, lipids and DNA or reaction with ROS or interaction with sGC [36]. Given such a short half-life, spatially regulating NO production closer to its target molecule is critical to facilitate its precise signal transduction and specific targeting as well as reducing its deleterious reactions [45]. Endogenously, this is achieved by the action of various NOS isoforms to produce NO as discussed in the later section.

Although the function of NO is less defined in the heart, it is a key regulator of excitation–contraction and hence myocardial contractility [22, 46, 47]. Ballingad and associates (1993) first demonstrated the role of endogenous NO in influencing β adrenergic receptor (β-AR) mediated signaling. Low concentration of NO is produced in cardiac myocytes, in a pulsatile manner, in phase with the cardiac excitation–contraction cycle [22, 46, 47].

Nitric Oxide Synthases (NOS)

There are different isoforms of NOS: neuronal NOS (nNOS) or NOS1, inducible NOS (iNOS) or NOS2 and endothelial NOS (eNOS) or NOS3 [22, 38, 43]. The names of the isoforms do not indicate exclusive localization of the isozyme in a particular cell type, but is indicative of the cell type where they were first discovered. NOS isoforms are encoded by different genes on separate chromosomes [38] and demonstrate about 50–60% homology in their sequence with respect to cofactor binding regions [48]. Although all three isoforms have similar enzymatic reactions and cofactor requirement, each of the isoform has distinct expression pattern, regulation of their activity and subcellular localization thereby possessing distinct catalytic activity.

All the isoforms of NOS require L-arginine as the substrate and molecular oxygen (O_2) as well as NADPH as co-substrate. The catalytic activity of the enzyme also requires binding of other cofactors such as FAD, FMN, (6R) 5, 6, 7, 8-tetrahydro-L-biopterin (BH4) and adenosine to the enzyme. Catalytically active enzyme converts L-Arginine to L-citrulline and NO [49]. NOS enzymes are usually present as two monomeric proteins bound together by BH4 and heme [50]. Oxidation of BH4 results in the dissociation of NOS dimers leading to uncoupling of NOS and the monomeric

forms are unable to produce NO [51–53]. BH4 thereby has a crucial role in maintaining functionally active forms of NOS [52, 53]. All of the NOS isoforms have a reductase and an oxygenase domain. While the substrate L-arginine, O_2 and BH4 bind to the oxygenase domain, transfer of electrons occurs from NADPH in the carboxy reductase domain to the heme in oxygenase domain of the enzyme [49, 50]. The flow of electron is facilitated by conformational changes in NOS as a result of binding of Ca^{2+} to calmodulin in the enzyme. However, the requirement for the concentration of Ca^{2+}, facilitating the binding of calmodulin and the concentration of NO produced by different NOS isoforms varies [43, 49].

Endothelial Nitric Oxide Synthase (eNOS)

eNOS is a constitutively expressed, Ca^{2+}/Calmodulin dependent enzyme generating NO in pM-nM concentrations for a short period of time [54]. Hence upon stimulation it is produced rapidly causing direct and short acting effects [48]. Even though initially thought to be exclusively present in endothelial cells, eNOS is found in a number of cell types such as cardiomyocytes, platelets, SMC and certain neuronal cells [20, 21, 38, 49]. eNOS is involved in several cellular functions such as vasodilation, modulation of platelet aggregation, cardiomyocyte and SMC contraction, leukocyte-endothelial cell interaction as well as inhibition of SMC proliferation [37, 46, 54].

eNOS function is regulated via phosphorylation of the enzyme. Activation of the inactive eNOS dimer occurs via Ca^{2+} mediated protein modification through myristolyation, phosphorylation and palmitoylation resulting in a conformational change of the enzyme. Although phosphorylation of eNOS can occur at its multiple Serine (Ser) or Threonine (Thr) sites, phosphorylation at Ser1177 and Thr495 sites are more commonly studied and observed to be involved in its regulation [20, 21, 55, 56]. In cardiomyocytes, phosphorylation of Ser1177 is observed to activate enzyme activity, whereas phosphorylation of Thr495 has inhibitory effect [20, 21]. Under non-stimulated condition Thr495 tends to be phosphorylated by Protein kinase C (PKC), which results in interference for binding of calmodulin to its binding site on the enzyme resulting in inactivation of eNOS [56, 57]. In contrast, phosphorylation at Ser1177 stimulates the flux of electrons within the reductase domain thereby activating the enzyme [49, 56]. Although Ser/Thr kinase (Akt) and adenosine monophosphate activated protein kinase (AMPK) are involved in the phosphorylation of Ser1177, Akt is the major regulator of phosphorylation in response to various triggers such as estrogen, vascular endothelial growth factor (VEGF) and insulin [49, 58].

eNOS derived NO exerts its biological effects by targeting various Ca^{2+} channels such as L-type calcium channels (LTCC), ryanodine receptor (RyR), sarcoplasmic Calcium ATPase (SERCA) via c-GMP dependent and independent signaling pathways [46, 59, 60], which is discussed later in this chapter. Nonetheless, for effective and targeted signaling, eNOS is primarily localized in plasmalemma caveolae in the

spatial vicinity of its target proteins [46, 61, 62]. Myristolyation and palmitoylation of eNOS on glycine and cysteine target eNOS to plasmalemma caveolae. Alternatively, it can translocate to other subcellular compartments including Golgi apparatus, cytosol and endothelial cell junctions [63]. Dissociation of eNOS from caveolin by interaction of proteins such as heat shock protein 90 promotes phosphorylation of eNOS by recruitment of Akt [38, 46, 61, 64]. Heat shock protein 90 also plays an important role in maintaining dimeric form of eNOS [65].

At low level of eNOS, derived NO is involved in maintaining various cardiovascular functions. Loss of bioactive NO as a result of eNOS uncoupling is implicated in the progression of many cardiovascular dysfunctions [53, 66]. Loss of cofactor BH4 leads to uncoupling of NOS changing it from NO producing enzyme to superoxide producing enzyme [67, 68].

Neuronal Nitric Oxide Synthase (nNOS)

nNOS, a 161 kDa NOS isoform, although first characterized in neuronal cells, is also expressed in several other cell types such as cardiomyocytes, smooth muscle and skeletal muscle. Similar to eNOS, nNOS is constitutively expressed where enzyme activity is regulated by Ca^{2+}/calmodulin to produce low (pM-nM) amount of NO [46, 47]. In cardiomyocytes, nNOS is localized in the sarcoplasmic reticulum (SR) membrane and nNOS mediated NO is involved in the regulation of Ca^{2+} handling proteins such as SERCA, LTCC and phospholamban [47, 60, 69]. In cooperation with eNOS, nNOS mediated NO plays an important role in β-AR mediated excitation–contraction coupling in cardiomyocytes [69, 70]. Although the exact mechanism for the cardioprotective role of nNOS is unclear, genetic manipulations resulting in loss of nNOS manifested in blunting of βAR response [70]. However, nNOS mediated NO plays a major role in the regulation of blood pressure in the central nervous system [49].

Inducible Nitric Oxide Synthase (iNOS)

This NOS isoform is not constitutively expressed, rather its expression is induced particularly in the presence of pathological stimuli such as cytokines, bacterial LPS or stress [38, 44]. Though initially recognized to be restricted to inflammatory cells, recent evidence confirms that the expression of iNOS can be induced in several cell types including cardiomyocytes [20, 21, 46, 49, 69]. Unlike eNOS and nNOS, once expressed iNOS is constantly active and produces μM concentration of NO. iNOS is active even in the absence of changes in Ca^{2+} and does not depend on post translational modifications or Ca^{2+} for the regulation of its activity [43, 44]. Although NO produced by iNOS has a crucial role in defense against pathogens, parasites,

tumor cells and microbes; it also exerts deleterious effects on neighbouring healthy cells [42, 43].

The production of NO by iNOS is controlled at the level of transcription via nuclear factor κB (NFκB). Activation of pro-inflammatory cytokines such as TNFα and IL-1 also facilitates activation of iNOS via translocation of NFκB from the cytosol to nucleus or upregulation of IFNγ mediated Jak-STAT signaling [44, 71, 72]. The promoter region of iNOS gene has binding regions for several transcriptional factors like NFκB, AP1, Jun/Fos, CREB and STAT family of transcription factors. The binding of NFκB and AP1 transcription factors to the promoter region mediate expression of various inducible genes such as iNOS, COX-2, ICAM-1 and VCAM-1 [43, 73].

iNOS mediated NO exerts deleterious effects in healthy cells via multiple mechanisms. NO can bind to iron and inhibit the activity of key iron containing enzymes such as in the mitochondrial electron transport chain, cis-aconitase, enzymes of complex I and II as well as ribonucleotide reductase [30, 74, 75]. NO at high concentration can also directly interfere with DNA resulting in strand break and fragmentation [49, 74]. It can also form peroxynitrite resulting in apoptosis through the activation of various caspases and PARP [17]. iNOS upregulation can exacerbate the pathophysiological conditions of myocardium and can modulate cardiac contractility by targeting multiple Ca^{2+} handling proteins involved in EC coupling [38, 47]. Increased myocardial iNOS can also initiate various cardiac remodeling events such as ventricular hypertrophy and dilatation [42, 60].

Nitric Oxide Signaling

Nitric oxide (NO) mediated intracellular signaling is mainly via two distinct pathways: cGMP dependent and cGMP independent. The initial discoveries identified increased cGMP as a critical mediator to carry out biological action of NO. NO induced increase in sGC was observed to be crucial to mediate vaso-relaxation in response to an increase in NO [37, 76]. Constitutively active NOS mediated generation of NO interacts with heme moiety of sGC. The latter further causes activation of sGC leading to conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) [77]. cGMP activates cGMP dependent kinases, protein kinase G (PKG) resulting in phosphorylation of a number of proteins involved in Ca^{2+} regulation, reducing the levels of $[Ca^{2+}]_i$ [22, 47]. This stimulates muscle relaxation, increase in vascular permeability, anti-platelet and anti-oxidant effects through targeting protein kinases and ion channels [48]. The stimulatory effect of NO is abrogated by enzyme phosphodiesterase-5 which converts cGMP to GMP [76].

Another alternative mechanism for NO signaling was discovered to be independent of an activation of sGC. NO was observed to directly alter proteins by the formation of NO-protein adducts resulting in more stable complex [76, 78]. NO triggers nitrosylation of proteins by targeting the sulfhydryl groups of the proteins

resulting in reversible nitrosothiol compounds or nitration of proteins at tyrosine residue [48]. These reactions are highly specific and require low concentration of NO compared to cGMP mediated alterations [63]. However, the propensity of nitrosylation depends on many factors including the redox microenvironment [48]. Upon nitrosylation, proteins can change their properties similar to the effect of protein phosphorylation. Low level of nitrosylation is critical for the activation of a number of proteins [38, 77, 78]. In fact, s-nitrosylation in coordination with phosphorylation regulates the function of many Ca^{2+} handling proteins such as LTCC, SERCA, RyR and phospholamban involved in β-AR mediated cardiac contractility [79]. The formation of S-nitrosothiols is maintained and cleared by enzyme S-nitrosothione reductase degrading the former into glutathione disulphide (GSSG) and ammonia (NH_3) [22, 79]. Hence, deficiency of s-nitrosothione reductase results in enhanced levels of s-nitrosothiols in tissue and contributes to pathological signaling [60]. To achieve specificity, NO is produced in close vicinity of the target molecule allowing direct interaction between NO and its target. In fact NOS is believed to be part of protein complex in which s-nitrosothiol signaling occurs [63, 78]. Extensive protein nitration is involved in disease initiation and progression as a result of gain or loss of function by stimulation or inhibition of protein phosphorylation respectively [28, 36].

Nitrosative Stress

Reactive nitrogen species (RNS) such as peroxynitrite are involved in the pathogenesis of a variety of cardiovascular pathologies such as myocardial infarction, chronic heart failure, diabetes, neurological diseases such as Alzheimer's and Parkinson's disease [26, 80–82]. Increased plasma levels of NO and peroxynitrite are reported in patients with cardiovascular complications including Dox-induced cardiotoxicity [80, 83]. Dox treatment also lead to increased NO, peroxynitrite and protein nitrosylation levels in isolated adult cardiomyocytes [20]. Peroxynitrite plays a critical role in exacerbating Dox mediated cardiotoxicity [21]. These changes not only exacerbates the toxicity by direct oxidation of DNA, proteins and lipids [29], but they also inhibit the activity of antioxidant enzymes [32] and accelerate the production of ROS such as superoxide [35]. As peroxynitrite is formed by near diffusion reaction of superoxide with NO [23, 29, 82], it has been speculated that an increase in the generation of superoxide through the upregulation of NADPH oxidase, xanthine oxidoreductase would act as an initial trigger for the generation of peroxynitrite [84]. Further stimulation for the production of higher concentration of peroxynitrite is through increased generation of NO by upregulation of iNOS [84].

Although low level of nitrosylation has an important role in cardiovascular signaling and regulation of angiogenesis, cardiac contractility, vascular relaxation, apoptosis and inflammation [78, 79, 85, 86], high levels of nitrosylation have inhibitory effects [85]. Peroxynitrite at higher levels induce nitration and nitrosylation of various proteins and thereby alter their function and downstream signal transduction (Fig. 7.1). Extensive protein nitration and nitrosylation is observed in

both in vitro and in vivo models of Dox- induced cardiotoxicity [20, 21]. Increased peroxynitrite leads to nitration and nitrosylation of cardiac myofibrils, apoptosis, inflammation as well as calcium handling proteins resulting in ventricular dysfunction (Fig. 7.1) [25, 33, 86]. Under the conditions of increased oxidative stress, high levels of NO had an inhibitory effect on mitochondrial respiratory chain enzymes by increased nitrosylation [85]. S-nitrosylation of proteins is dependent on the redox state of the cell and can be reversed in the presence of antioxidants such as glutathione, thioredoxin and ascorbic acid [85, 87]. We demonstrated that the treatment with Vit C significantly reduced Dox mediated nitration and nitrosylation of proteins [20, 21] (Fig. 7.2).

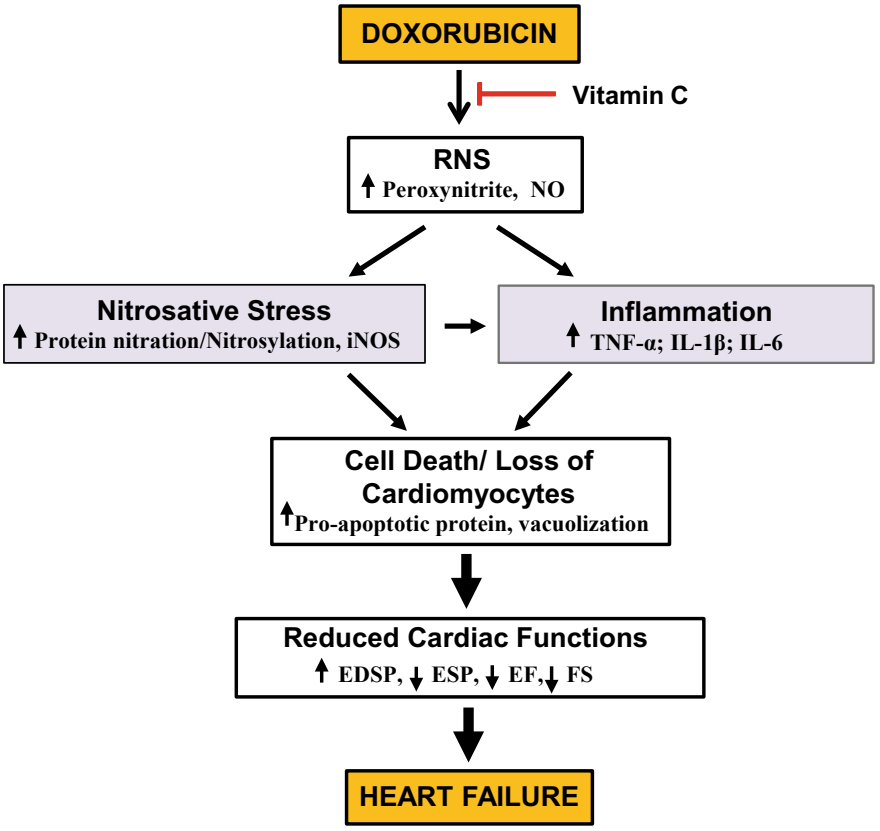

Fig. 7.2 *Doxorubicin induced nitrosative stress is mitigated by Vitamin C.* Doxorubicin induced nitrosative stress involves production of peroxynitrite leading to an increase in proinflammatory cytokines which collectively cause cell death and contribute to depressed cardiac function eventually leading to heart failure. These cardiotoxic effects of doxorubicin are mitigated by Vitamin C. RNS, Reactive nitrogen species; NO, Nitric oxide; iNOS, inducible nitric oxide synthase; TNF-α, Tumor necrosis factor alpha; IL-1β, Interleukin 1 beta; IL-6, Interleukin-6; EDSP, End diastolic pressure; ESP, End systolic pressure; EF, Ejection fraction; FS, Fractional shortening

Various experimental models demonstrated upregulation of iNOS expression by Dox administration [20, 21, 23, 83, 88–91]. Additionally, an imbalance in the expression of iNOS and eNOS is observed after administration of Dox [90]. Reduction in peroxynitrite using peroxynitrite scavenger abolished Dox-induced apoptosis and NT formation [23]. Peroxynitrite leads to increased NT formation after administration of Dox in isolated cardiomyocytes and cardiac tissue of Dox treated animals [33, 89]. Dox causes extensive protein nitration and nitrosylation of cardiac myofibrils and contractile proteins and affects cardiac contractility resulting in LV dysfunction [25]. Thus reduction of Dox induced nitrosative stress is critical for attenuation of its cardiotoxicity. We have recently demonstrated a reduction in nitrosative stress by Vitamin C in isolated cardiomyocytes as well as animals exposed to Dox [20, 21].

Modulation of NOS

Physiologically NO produced by different NOS isoforms has differential function as well as regulation. Since eNOS is compartmentalized in plasmalemma caveoli [46] which is in close proximity to the sarcoplasmic reticulum, eNOS derived NO in physiological levels (low concentration) plays an important role in cardiomyocyte contraction [49, 69]. As all forms of NOS are stable in their dimeric state, maintaining the dimeric form of eNOS is crucial for its catalytic activity [49, 92, 93]. Nevertheless, dissociation of the dimeric form into monomeric form under the conditions of nitrosative stress or cofactor oxidation can lead to uncoupling of enzyme resulting in the production of superoxide instead of NO [65]. Despite iNOS being a more potent source of generation of NO and superoxide than eNOS, uncoupling of eNOS is implicated in many pathological conditions. This is because of its inability to produce physiological levels of eNOS derived NO crucial for normal cardiovascular function.

Uncoupling of eNOS is associated with the progression of many pathological conditions such as endothelial dysfunction, diabetes, obesity and aging [67, 94, 95]. Although the mechanism for uncoupling of eNOS is not clearly understood, it can be a consequence of monomerization of eNOS as a result of oxidation of the cofactor BH4, involved in binding of two monomers. However, conditions involving increased production of ROS can also trigger uncoupling of eNOS. The disruption of dimeric form of eNOS into monomeric subunits is enhanced in Dox treated cardiomyocytes [20, 51, 67]. Enhanced nitrosylation of eNOS also results in the disruption of dimer stability [87]. Monomerization and uncoupling of NOS results in synthesis of superoxide anion instead of NO [51]. On the other hand, maintaining redox potential through an upregulation of thioredoxin reductase resulted in the protection of eNOS dimer and restoration of enzyme activity [87]. Furthermore, loss of eNOS in the eNOS knockout mice provides cardioprotection against Dox-induced cardiotoxicity [96, 97].

eNOS being constitutively expressed, phosphorylation is the key mechanism for regulation of its activity. eNOS enzyme activity is regulated by differential phosphorylation of the enzyme at its activating site Ser1177 or inhibitory site Thr495. Phosphorylation at Ser 1177 enhances enzyme activity, whereas, phosphorylation at Thr495 reduces enzyme activity [49, 58]. Phosphorylation of eNOS at its activating site leads to conformational change in eNOS resulting in its activation [49]. Under non-stimulated conditions, eNOS is phosphorylated at Thr495. Phosphorylation of eNOS is regulated by various factors such as estrogen, VGEF, bradykinin which activates various kinases such as Akt, AMP activated protein kinase (AMPK) and protein kinase C (PKC) as well as protein phosphatase 2A (PP2A) [49, 58]. Akt dependent phosphorylation is required for the activation of NOS in endothelial cells [98]. We reported a downregulation in the protein expression of phosphorylated eNOS at Ser1177, while an upregulation of phosphorylated eNOS at Thr495 in Dox treated cardiomyocytes. This may be possibly due to Dox mediated downregulation in Ser1177 activating kinases Akt and AMPK [16]. In our studies on the rat animal model we found a downregulation of Akt by Dox which was prevented by Vit C [21].

Inflammatory Responses

An increased protein expression of iNOS in Dox-treated cardiomyocytes as well as in Dox treated animals has been reported by us [20, 21]. Under physiological conditions, iNOS is usually not expressed in cells, but pathological stimuli such as lipopolysaccharide and Dox exposure can induce its expression [21, 63]. Upregulation of iNOS is implicated in various pathological conditions including heart failure [99]. Increased iNOS activity and expression were demonstrated in the myocardium and vasculature of both animals and patients with heart failure [34, 83]. iNOS mediated enhanced NO levels in high μM range for prolonged period of time are responsible for enhanced peroxynitrite formation as well as protein modification by s-nitrosylation and nitration. Studies using genetic and pharmacological modulation of iNOS have highlighted the role of iNOS in the pathogenesis of heart failure [38]. Disruption of iNOS has been shown to reduce protein nitrosylation [100] as well as improve cardiac function [101] via reduction in cardiac NO. Thus an increase in NO levels and total NOS activity by Dox treatment in isolated cardiomyocytes as well as in adult rat cardiac tissue observed in this laboratory can be attributed to upregulation of iNOS protein expression and not via eNOS which is rendered inactive by the interplay of phosphorylation at the Thr495 and Ser1177 sites [20, 21].

An increase in pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in isolated cardiomyocytes as well as in the hearts has also been observed following treatment with Dox in dose-dependent manner [20, 21, 89, 102]. These cytokines are involved in nuclear translocation of NF-ƘB, which then activates the iNOS and generates more NO [103]. Dox possibly recruits these cytokines and induce apoptosis through activation of iNOS-mediated nitrosative stress. In this regard, Vit C has been

shown to be beneficial in reducing the translocation of NF-ƘB via inhibiting TNF-α and IL-1β and subsequently resulting in attenuation of iNOS activation [20, 21, 104].

Conclusions

A production of RNS through elevated cardiac NO levels under Dox treatment is well documented. In our lab, we demonstrated change in different NOS isoforms in in-vitro and in-vivo models of Dox-induced nitrosative stress. Dox was involved in the production of NO and peroxynitrite. The latter is able to activate different stress signaling molecules to cause protein nitrosylation and cell death. In the in-vivo rat model, Dox was able to significantly decrease systolic function and increase in cardiac levels of inflammatory cytokines like TNFα, IL-1β and Il-6. Vitamin C (Vit C), which is a potent water soluble antioxidant crucial for maintaining redox state and scavenging reactive free radicals, was able to mitigate dox-induced cardiomyopathy. Vit C reduced activation of iNOS by Dox and thus, reduced protein nitrosylation and overall NO production. The reduced action of iNOS by Vit C decreased the load of pro-inflammatory cytokines in dox-treated animals as well as in isolated cardiomyocytes. These inhibitory effects of Vit C were enough to promote overall survival of cardiomyocytes and improve cardiac function in Dox-treated animals. In any adjuvant therapy, it is important to maintain anti-tumor activity of Dox without causing damage to the heart. Thus it is important to understand the molecular basis of cellular actions of Dox separately not only in cardiomyocytes but also in cancer cells.

Acknowledgements Dr. P. K. Singal is the holder of the Dr. Naranjan S. Dhalla Chair in Cardiovascular Research supported by the St. Boniface Hospital Foundation.

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Chapter 8
Role of Nitric Oxide Synthase and Nitric Oxide Signaling in the Neutrophil Ontogeny and Functions

Sachin Kumar, Samreen Sadaf, and Madhu Dikshit

Abstract Neutrophils are the essential guards of the immune system that inactivate different pathogens as well as instruct specific immune responses. Nitric oxide (NO), a pleiotropic signaling molecule produced from nitric oxide synthases, regulates neutrophils at diverse levels. This includes the development of neutrophils from hematopoietic stem cells through granulopoiesis processes; furthermore, nitric oxide regulates neutrophil maturation from committed progenitor cells. In addition, nitric oxide regulates most of the neutrophil functions, such as adhesion, chemotaxis, respiratory burst, apoptosis, NETosis, intruder killing, and tissue damage. Intriguingly, neutrophils provide substantial amount of NO with the presence of nitric oxide synthases and their regulation in inflammatory conditions. Overall, this work discusses the role of nitric oxide signaling in neutrophil ontogeny and functions.

Keywords Neutrophil functions · Ontogeny · Inflammation · Nitric oxide · NO synthase · NADPH-Oxidase

Neutrophil Ontogeny

Neutrophils (PMNs), the most abundant yet short-lived leukocytes in the blood, are produced in the bone marrow by the hematopoiesis process in an adult subject. They are the prominent players of innate immunity and provide the first line of defense by directly killing the intruders through phagocytosis and/or NETosis. The indiscriminate killing is due to the high amount of reactive oxygen species (ROS), nitric oxide (NO), the release of myeloperoxidase, proteases, and cytotoxic peptides in the

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_8

phagolysosomes. Methodological advancements in cell biology research unfolded several vital aspects of neutrophil biology and helped identify their crucial role in immunity.

Neutrophils have a high turnover rate i.e., 10^{11} cells per day in a healthy human and that is under circadian rhythmic regulation. PMNs are derived from hematopoietic stem cells (HSCs) by the processes of proliferation, differentiation, and maturation [1, 2]. HSCs reside in a specific local microenvironment in the bone marrow, known as the stem cell niche that modulates survival, self-renewal, and cell fate decision [3]. HSC niche was initially divided into two main zones i.e., endosteal niche and vascular niche, however, currently, it is believed that almost every hematopoietic or non-hematopoietic cell in the bone marrow regulates HSC function [4]. Stromal or non-hematopoietic cells in the BM include majorly osteoblast cells, mesenchymal stromal/stem cells (MSCs), and CXCL12-abundant reticular (CAR) cells adjacent to sinusoids colocalize with HSCs and are also required for their maintenance. HSCs derived short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) that give rise to common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs) [5, 6]. CMPs form the megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte–macrophage progenitors (GMPs). Granulocytes and monocytes differentiate from GMPs [7, 8]. The earliest recognizable neutrophil precursors are myeloblasts (MBs), which differentiate into promyelocytes (PMs), myelocytes (MCs), metamyelocytes (MMs), band cells (BCs) and segmented neutrophils [9]. The high demand for neutrophil generation is met through a highly controlled granulopoiesis process in the bone marrow.

Granulopoiesis engages the orchestration of different transcription factors, growth factors, cytokines, and cell cycle regulators (Zhu and Emerson 2002). Thrombopoietin (TPO), stem cell factor (SCF), FMS-like tyrosine kinase-3 (FLT-3) ligand, and cytokines (IL3, IL6) released from stromal cells promote HSCs self-renewal and number in vitro as well as in vivo [10–14]. G-CSF and its receptor (G-CSFR) are critical for PMNs differentiation [15]. Homologues deletion of G-CSF and G-CSFR in mice reduces neutrophil maturation (about 20% of normal levels) and induces their mobilization [16, 17].

G-CSF released from the stromal cells enhances the release of matrix metalloproteinases (MMPs), elastase, and cathepsin G from neutrophils. These disrupt the interaction of stromal-derived factor-1 (SDF1) or CXCL12 with receptor CXCR4, as well as vascular cell adhesion molecule 1 (VCAM-1) and very late antigen-*4* (VLA-4, CD49d/CD29, alpha4 beta1) interaction or N-cadherin dimerization between HSCs and osteoblasts, to promote mobilization of HSCs and neutrophils into circulation [18–22]. CXCR4 is expressed on the neutrophil surface, and its level is altered during different stages of maturation and activation. The interaction of SDF1 with the chemokine receptor CXCR4 is essential for neutrophil retention in the bone marrow. Cultured peripheral neutrophils or aged neutrophils increased CXCR4 expression over 48 h [23]. Moreover, CXCR4 and CXCL12 are down-regulated by G-CSF [24, 25]. Chemokine receptors CXCR2 and CXCR4 are thus indispensable to sustain neutrophils in the bone marrow pool, and their expression augments the number of aged neutrophils [26]. CXCR4 mutation or deletion of CXCR2 also induces

neutrophil retention in the bone marrow [27, 28]. Moreover, CXCR4 deficient mice die perinatally due to excessive release of PMNs in the circulation [28]. CXCR4 modulates neutrophil extravasation [26], while its inhibition impedes neutrophil homing [25]. CXCR4 deficiency results in the decrease of bone marrow neutrophils but increased peripheral neutrophils in mice [29, 30].

NOS/NO Signaling

Nitric oxide (NO) was initially identified as the regulator of vasodilation and neurotransmission, however, it was subsequently recognized as a critical regulator of immune responses and immune functions [31–33].

NO synthesis is catalyzed by enzyme nitric oxide synthase (NOS) from substrate L-arginine in the presence of oxygen and several cofactors. Three isoforms of NOS include neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), designated based on their initial identification in a particular cell type. Interestingly, nNOS and eNOS are constitutive and depend on calcium-calmodulin for their enzymatic activity, while iNOS is inducible in nature, and its activity is independent of calcium. Constitutive NOSs produce a low level of NO, in contrast to the inducible iNOS isoform that produces higher NO for a prolonged duration [34]. Initially, iNOS was found to be present in the immune cells like macrophages and neutrophils, however, subsequent studies demonstrated the presence of almost all three isoforms in the bone marrow cells and neutrophils both at mRNA and protein levels [35]. Importantly, circulating neutrophils help to maintain blood pressure by suppressing bacterial and IFNγ-dependent iNOS expression in the vasculature of healthy mice [36] as neutrophil depletion led to a decrease in blood pressure, suggesting the requirement of PMNs in maintaining the optimal vascular tone.

NOS/NO and Granulopoiesis

All three NOS isoforms, are expressed in hematopoietic stem cells as well as in the circulating blood cells [37–42]. In hematopoietic system, NO by modulating the action of several cytokines and growth factors, regulate HSC self-renewal, proliferation, and differentiation in human [43–46] and rodents [47–51]. Studies conducted using NO donors, or NOS inhibitors suggested that NO mediates TNFα, IFNγ, and GM-CSF induced hematopoiesis and hematopoietic maturation [47, 52, 53]. Punjabi et al. [52] observed that inflammatory stimuli, IFN-gamma, and LPS caused NO production in bone marrow cells [52]. Likewise, granulocyte–macrophage (GM)-CSF, IL-3, and TNF-alpha act synergistically with IFN-gamma and LPS to produce NO. Among the hematopoietic BM cells, granulocytes are the primary cells to generate NO. High NO generation by the combined action of GM-CSF and LPS or IFN-gamma markedly suppresses cellular proliferation in the BM, which was

reversed by NOS inhibitor, NG-monomethyl-L-arginine [52]. Moreover, inhibition of NOS by L-NAME in LPS-treated rats increased neutrophil infiltration by enhancing the expression of ICAM-1. At the same time, NO donors prevented neutrophil migration [54], suggesting the role of NO in bone marrow cell growth and development. Exposure of NO to BM or CD34+ cells inhibited colony formation in a dose-dependent manner [53]. iNOS mRNA was present in the highly purified CD34+ cells. It was further induced by IFN-gamma or TNF-alpha, leading to the apoptosis of progenitor cells, which was reversed by NG-Monomethyl-L-arginine [53]. NO thus seems to be an essential mediator of cytokine-induced hematopoietic suppression [53]. Another study identified that NO donors sodium nitroprusside (SNP) and S-nitroso-acetyl penicillamine (SNAP) in vitro differentially regulate growth and differentiation of normal human bone marrow cells and CD34+ cells as inhibition of colony-forming unit-erythroid (CFU-E), while increase in the colony-forming unit-granulocyte macrophages (CFU-GM) [47].

On the other hand, stromal cells derived NO from nNOS, iNOS, or eNOS modulated actin conformation and cell–cell adhesion to regulate HSCs proliferation, differentiation, and mobilization [55–58]. Moreover, induction of iNOS in the bone marrow and CD34+ cells following stimulation with IFNγ or TNFα adversely affected the hematopoiesis [53]. North et al. demonstrated induction of HSCs number by NO donor, SNAP, while nNOS/eNOS knockdown blocked HSCs development [44]. Moreover, a previous study on murine bone marrow HSCs exhibited preferential myeloid commitment of HSCs following an in vitro exposure to NO donor [59]. In addition, NO, through its antioxidant and anti-proliferative effects, maintains HSCs quiescence [60]. In hematopoietic cells, neutrophils are known to generate a large amount of NO at a rate of 10–100 nmoles/5 min/10^6 cells [61]. Furthermore, neutrophils and their precursors constitute the significant fraction of the BM niche, and NO produced from these cells might acts as a paracrine effector to regulate hematopoiesis in the BM. NOS inhibition was found to augment the number of neutrophils in the mouse bone marrow and blood; this study however did not investigate neutrophil apoptosis [62] which is also regulated by NO [63]. NO thus regulates hematopoiesis [62], and also erythropoiesis [64], while monocytic differentiation from non-lymphocytic leukemia cells [65], HL60 cells [66] and U-937 (Yamazaki et al. 1995) has also been demonstrated using NO donors.

Further studies suggested the role of NO in neutrophil generation by the application of NO donors and NOS inhibitors in vivo, interestingly NOS inhibitors treatment in mice led to an increase in the number of stem and progenitor cells in the bone marrow without any significant change in the peripheral neutrophils [62]. While in the irradiation-transplantation model, this HSPC increase was followed by a transient increase in the number of circulating neutrophils [62]. Another study identified the role of nNOS in the regulation of hematopoiesis, which is mainly expressed in the stromal cells [55]. Levels of nNOS expression and the ability of different stromal cell lines to support hematopoietic stem cells were strongly correlated [55]. The additive effect of NO donors was also observed by stromal cell lines to support hematopoietic stem cells, suggesting that NO produced by stromal cells regulate hematopoietic cells in a paracrine manner [55]. The role of NO is also being investigated in the

development of HSC during embryogenesis [44], and NO plays a critical role in the shear stress-induced hematopoiesis from mouse embryonic stem cells [67].

Further, Nasrallah et al. have suggested the role of NO in hematopoiesis by monitoring hemogenic endothelium (HE) transition to hematopoietic cells. Endoglin (ENG) is an accessory TGF-β receptor required for the normal development of hemogenic precursors [68]. A high ENG expression in ES cell line, mesodermal, or HE cells accelerated the emergence of $CD45^+$ definitive hematopoietic cells and thus hematopoiesis [68]. The increased pSMAD2/eNOS expression and NO synthesis in hemogenic precursors were observed with high ENG expression. Blocking of eNOS activity mitigated the ENG-induced increase in hematopoiesis [68]. NO is also known to regulate ESC differentiation by arresting the loss of self-renewal markers and promoting cell survival by inhibiting apoptosis [69], and a high amount of NO increases ESC differentiation towards definitive endoderm, cardiomyocytes, and neurons [70]. While another study suggested that bone marrow mesenchymal stromal cells exhibited rapid differentiation of $CD11b^+$ myeloid cells from progenitors in the presence of NO [71]. Interestingly, more neutrophils ($CD11b^+Gr1^{hi}$) cells were formed from NOS2 deficient MSCs [71]. This study thus suggests the fundamental role of stromal NOS2 in hematopoietic homeostasis.

Treatment of murine Lin^-/LSK-$CD34^-$ cells, the hematopoietic stem cells, with SNP, a NO donor, enhanced the numbers of LSK-$CD34^+$ cells [43]. Furthermore, it was observed that the acquisition of CD34 expression by LSK-$CD34^-$ cells was not due to the proliferation of LSK-$CD34^+$ cells [43]. During the development, mouse hematopoietic stem cells switch their phenotype from CD34+ to CD34– cells in between 6–8 weeks of life span. This contrasting effect of NO observed in 6–8 weeks of juvenile mice than 10–12 weeks young mice was due to the up-regulation of self-renewal and differentiation genes by differentially affecting their reconstitution potential [43]. Tiribuzi et al. observed that depletion of paracrine or autocrine NO using oxy-hemoglobin and NOS inhibitor during the commitment stage blocks CD34+ HSCs differentiation towards dendritic cells, and sustains undifferentiated highly proliferating cell population [72]. Moreover, hematopoietic stem cell development has shown to be dependent on blood flow, NO donors regulated HSC numbers even when treatment occurred before the initiation of circulation and also rescued HSCs. Knockdown of nNOS/eNOS blocked HSC development [44]. Endogenous NO causes vasodilation in rat bone marrow, bone, and spleen during accelerated hematopoiesis [73]. A recent study from our group demonstrated the role of NO generated from iNOS in neutrophil differentiation by using diverse approaches [74]. iNOS-overexpressing K562 cells and iNOS KO murine progenitor cells shown to increase and decrease in neutrophilic differentiation respectively [74]. An enhanced neutrophil differentiation with NO donor was also observed in these models. Furthermore, a significant upregulation in NO levels was observed during neutrophil differentiation and apoptosis [45, 46], together establishing the role of iNOS-NO in neutrophil proliferation and differentiation.

Molecular Mechanisms in NOS/NO Driven Granulopoiesis

NO stimulation results in the dynamic cooperation of multiple signaling pathways to promote the expansion and differentiation of HSCs [59]. Overall hematopoietic niche in BM via angiopoietin 1 and its receptor Tie2 maintains the quiescence of hematopoietic stem cells [60, 75]. Interestingly, NO has been shown to induce the expression of angiopoietin 1 as well as Tie2 [76]. Cell surface expression and mRNA of CXCR4 on CD34+ cells were reported to be increased in a dose- and time-dependent manner in response to NO donors [77]. SDF1 and its receptor CXCR4, along with matrix metalloproteinases, regulate stem cell migration and mobilization into circulation. Stem cell mobilization is mediated by SDF1 and its receptor CXCR4 and proteinases such as elastase, cathepsin G, and MMPs [78]. Similarly, expression of PU.1, elastase, cathepsin G, and MMPs were found to be increased in iNOS-derived NO-mediated neutrophil differentiation [45, 46]. Furthermore, Hall et al., using a zebrafish model, showed that C/EBPβ-dependent iNOS activity was necessary for the enhanced shift toward neutrophil lineage in response to infection [79]. They also observed that macrophages produce elevated serum GCSF during inflammation resulting in the increased expression of C/EBPβ by GCSF-responsive HSPCs. This consequently elevated iNOS expression and maintenance of HSPC proliferation/expansion dispensable for neutrophil commitment [62]. Moreover, MSC, the myeloid DC precursors when treated with GM-CSF suppressed allogeneic and OVA-specific CD4 + and CD8 + T cell responses via cell contact and NO production [80]. In yet another study, bone marrow stromal-cell- eNOS was identified as an important component of the stem cell niche and is essential for the mobilization of stem and progenitor cells [58]. Studies in mice lacking eNOS, which showed a reduction in hematopoietic recovery and dysfunctional endothelial cell mobilization, further deciphered the significance of eNOS in hematopoiesis in adult animals [58, 81]. IL-17 upregulates the expression of mRNA for both iNOS and eNOS isoforms in murine bone marrow cells, as well as enhances the phosphorylation of p38 MAPK [82]. Asthma exacerbates the number of CD34+ circulating progenitors expressing high levels of iNOS, implicating the role of NO in preventing cell growth and colony formation in a paracrine and autocrine manner but it was not sufficient to prevent their proliferation in the circulation [83]. Blocking of endogenous NO increases white blood cell accumulation in rat lung [84], suggesting cell specific paracrine and autocrine effect of NO.

Studies from our lab characterized the presence of NOS isoforms in rat neutrophils and their precursors by using biochemical and molecular techniques [49]. Subsequently, NOS as well as NO were demonstrated to contribute to the generation of reactive oxygen species (ROS) in human PMNs [85]. Furthermore, we have reported a proliferative effect of nitrite on HL-60 cells, which was NO-mediated and Cdk2 activation-dependent [86]. Moreover, the DETA-NO-mediated biphasic effect on HL60 cells depended on the Cdk2 nitrosylation/activation and the loss of mitochondrial potential to mediate proliferation and cell death, respectively [51].

NO/NOSs in Neutrophils

Neutrophils, representing 50–60% of the total circulating leukocytes, could add a substantial amount (10–100 nmole/5 min/10^6 cells) of NO in circulation, impacting circulating cells and vascular homeostasis [87, 88]. Neutrophil's nitric oxide synthase activity was first established by its ability to relax aortic rings [89] or platelet aggregation [90]. NOS activity in rat and human PMNs was demonstrated by the conversion of radiolabeled L-Arginine to radiolabeled L-citrulline in the presence of active NOS enzymes [91]. The inhibitory activity of neutrophils was found to be prevented by the pre-incubation of cells with N^G-monomethyl L-Arginine [90, 92]. An increase in the release of NO from PMNs after thrombosis [90] and hypoxia-reoxygenation [93] indicate an essential role of PMNs in the regulation of homeostasis.

Reports depicting the characteristics of NOS present in neutrophils were initially limited compared to the investigations in other cells/cell lines, possibly due to high proteolytic activity in neutrophils. Expression of iNOS mRNA and protein, as well as iNOS enzymatic activity, was first observed in cultured neutrophils [94] in cytokine-stimulated [95] or bacteria-infected human neutrophils and in the primary granules [96]. Failure in the detection of constitutive expression of iNOS in human PMNs was explained based on the incomplete release of membrane-bound enzyme and inadequate proteinase inhibition in the resting PMNs [97], as more than 90% of iNOS is tightly bound to membrane in human PMNs [96]. Wheeler et al. (1997) identified neutrophils as the primary source of iNOS in leukocyte-enriched pellets isolated from the urine of patients with bacterial infection [96]. iNOS mRNA, protein and enzymatic activity was also reported in circulating rat PMNs after culture Miles et al. [94] and in human PMNs after cytokine-treatment [95] and bacterial infection [96]. Human and rat neutrophils have been shown to also express neural nitric oxide synthase mRNA constitutively [98, 99]. However, Greenberg et al. [98] failed to detect the presence of NOS protein, while the presence of nNOS mRNA and 150 kDa protein in circulating human PMNs was found by Wallerath et al. [100]. Later constitutive expression of iNOS in human neutrophils was also observed by flow cytometry, Western blotting and enzymatic activity [97]. Western blotting revealed that iNOS protein was highly dependent on di-isopropylfluorophosphate mediated potent protease inhibition. Immunofluorescent staining further documented the presence of nNOS and iNOS in human PMNs, while eNOS was not detected [100]. Similarly, RT-PCR transcripts for nNOS and iNOS but not of eNOS were detected in human PMNs [100]. The presence of eNOS in neutrophils is still controversial as there is only one report on the presence of endothelial nitric oxide synthase isoform in human neutrophils [101]. Furthermore, decreased eNOS expression has been suggested during acute myocardial infarction or TNFα treatment. This study also demonstrated that the 3'-untranslated region of eNOS mRNA binds with cytosolic proteins of human neutrophils [101]. The presence of both nNOS and iNOS in human and rodent neutrophils has now been accepted unequivocally [38, 97, 99, 102, 103].

Studies conducted in our lab using RT-PCR, Western blotting, and immuno-electron microscopy demonstrated the presence and localization of nNOS/iNOS

in nucleus, granules, phagosome, and mitochondria and in the membrane of rat and human neutrophils [38]. NOS activity and expression was found to be regulated by ascorbate in guinea pig, rodent, and human PMNs [103]. Availability of reduced tetrahydrobiopterin, L-arginine, and oxygen is crucial for the NOS activity regulation in resting and activated human, guinea pig, and rat neutrophils [38, 40, 41, 93, 103–112]. Moreover, NO-mediated free radical generation was noted in PMNs under reoxygenation after hypoxic conditions [93]. Neutrophil iNOS was augmented in LPS treated [105], spontaneously hypertensive rat neutrophils [104]. Moreover, neutrophil nitrite content and NO generation as well as circulating/CSF nitrite content, were altered during various CNS and other diseases, indicating their role in pathology and possible marker of some of the pathological indications.

Further studies revealed that the expression of iNOS was constitutively augmented following the maturation of rat neutrophils, while nNOS expression was almost comparable during the various stages of neutrophil development [49]. In contrast, maximum eNOS expression in the immature rat neutrophils was attenuated with neutrophil maturation [49]. Detailed and multipronged studies conducted in iNOS and nNOS overexpressed K562 cells, mice Lin^{-ve} cells, and CD34+ cells from human marrow have further helped in establishing the role of NOS in human neutrophil differentiation and survival [45, 46].

Pathological Conditions

Leukocyte-enriched pellets from the urine of urinary tract infections patients and bacterial-infected leukocytes exhibited induction in iNOS levels [96]. Plasma nitrate concentration has been reported to be significantly higher in patients with septicemia who have a normal or elevated number of neutrophils in peripheral blood than in those with neutropenia [113]. Similar results were obtained by us, with a significant increase in plasma nitrate, nitrite, and TNF alpha levels in patients with sepsis [114]. Likewise, we also observed an increase in the plasma MPO levels in sepsis correlating with increased neutrophil proliferation, a marker of the severity of inflammation [115]. Enhanced ROS and RNS generation as well as secretion of inflammatory mediators, led to apoptosis in the dropsy patients [116]. Moreover, neutrophil ROS and RNS have been associated with tissue damage in myocarditis, myocardial infarction, and ischemia–reperfusion injury, thus, inhibition of neutrophil recruitment and ROS generation improve cardiac function [117]. Furthermore, nitrite levels in neutrophil precursors as well as in blood plasma and BM fluid, were significantly less in CML patients compared to the values in controls [118].

Additionally, high oxidative stress led to reduced iNOS expression in PMNs of CML patients [119]. It has been demonstrated that neutrophil-derived NO is responsible for the augmented free radical generation following hypoxia-reoxygenation [93]. On the contrary, induction of thrombosis in rats was associated with a reduction in the free radical generation and augmentation in NO release [107]. However, an increased release of NO by PMNs was found to be associated with the inhibition

of platelet aggregation by the neutrophils obtained after pulmonary thromboembolism [90]. An increase in the neutrophil nitrite content and its role in Parkinson's disease has also been suggested [108]. On the contrary, unaltered nitrite level is also observed in CSF of Parkinson's disease patients as compared to the controls [120]. A significant decrease in NO synthesis while an unaltered antioxidant enzyme activity was observed in PMN of schizophrenia patients suggesting that the decrease in NO synthesis by PMN might lead to oxidative stress in such patients [121]. Similarly, PMNs of patients with affective disorders displayed altered NOS activity [122]. In addition, during the head-ache-free period of patients with migraine, the blood nitrite levels were not significantly altered [123]. Similar results were obtained from the patients suffering from motor neuron disease [124]. Moreover, no significant change was found in neutrophil ROS levels in migraine patients compared to controls suggesting that neutrophils are not the cause of oxidative stress observed in migraine patients [125]. Moreover, in diseases like sepsis, diabetes, glucose-6-phosphate dehydrogenase deficiency, glycogen storage diseases (GSDs), systemic lupus erythematosus (SLE), rheumatoid arthritis, and cancer the metabolic reprogramming of neutrophils by inflammatory mediators or during pathologies are desired to be undertaken [126]. Circulating neutrophils from hypertensive patients release more ROS than their normotensive counterparts [127]. In the hypertensive rat model, the induction of NOS in neutrophils along with augmented oxidative stress was observed, suggesting the association of oxidative stress with endothelium might be leading to inflammatory changes in hypertensive conditions [40, 104]. Circulating neutrophils have also been shown to maintain the physiological level of blood pressure by suppressing bacterial, and IFNy-dependent iNOS expression in the vasculature of healthy mice [36] as neutrophil depletion led to low blood pressure and suggested the requirement to maintain optimal vascular tone. NO generation by neutrophils is also involved in their antimicrobial function [128]. During inflammation, the rate of synthesis of NO and superoxide is increased leading to the generation of peroxynitrite mainly by mitochondria and immune cells including macrophages and granulocytes [129].

Nitric Oxide-Mediated Modulation of PMNs Functions

Nitric oxide has been shown to regulate most of the neutrophil functions, such as chemotaxis, respiratory burst, adhesion, apoptosis, NETosis, and bacterial killing or tissue damage [42, 51, 61, 130–134]. Here we discuss the key functions of neutrophils and their modulation by NOS/NO.

(a) ***Rolling & adhesion***

NO is an important homeostatic regulator of leukocyte rolling and adhesion [135–137]. Extravasation of neutrophils is a complex and highly coordinated phenomenon that involves initial low-affinity rolling of neutrophils mediated by L, P, and E

selectins followed by high-affinity interactions with integrins on vascular endothelium facilitating the process of transmigration [138]. It has been demonstrated that inhibition of NO synthesis promotes P-selectin-dependent leukocyte rolling [139]. Surprisingly exogenous NO also decreases leukocyte rolling [140, 141]. In the iNOS deficient mice increase in the leukocyte-endothelium interaction and neutrophil transendothelial migration following LPS-induced endotoxemia has been reported [142]. A similar observation was also observed in NOS inhibitors; L-NAME, amino guanidine, 1400 W or with guanylate cyclase inhibitor, ODQ treated mice, or endothelial cells [142–144].

L-Arginine supplementation enhanced and prolonged fMLP triggered neutrophil aggregation in a NO-dependent manner involving ADP ribosylation and rearrangement of the actin cytoskeleton [145]. NO prevents neutrophil-endothelium interaction by reducing CD11b/CD18b expression and inhibiting β_2 integrins by interfering with the cell surface transduction of signals linked to particulate guanylate cyclase activity [135, 146]. LPS treatment induced NOS and upregulated expression of E-selectin and ICAM-1, thus influencing intercellular adhesion [147, 148], a phenomenon opposed by NO donors. NO donors generating NO in higher than physiological levels inhibited LPS or TNF-α induced neutrophil adhesion to endothelial cells. Furthermore, endogenous NO or supplementation with L-arginine was effective in preventing reperfusion injury and target organ infiltration and damage attributed to neutrophils, as in sepsis [149–151]. NO prevents the leukocyte-endothelial cell adhesion by reducing the CD11/CD18 expression [135, 137], and also by inhibiting the β_2 integrins in a concentration-dependent fashion by dampening the transduction of signals linked to the activity of membrane-bound guanylate cyclase [146]. Cell permeable analogs of cGMP also inhibit leukocyte-endothelial cell adherence [152], suggesting involvement of NO/cGMP signaling in leukocyte-endothelial cell adherence.

(b) ***Chemotaxis***

Chemotaxis is the directed movement of cells in response to a concentration gradient of a chemo-attractant stimulation inducing a cascade of events, which include actin reorganization, shape changes, development of polarity, and reversible adhesion, culminating in directed migration. NO from both exogenous and endogenous sources limit leukocyte recruitment into normal and inflamed vessels [141, 153, 154]. While NO also enhanced neutrophil adhesion to endothelial cells [155, 156]. Moreover, exogenous NO enhanced the random migration of rabbit peritoneal neutrophils in a concentration-dependent manner, which was associated with a rapid and transient increase in cGMP levels [157]. The role of endogenous NO in migration has also been assessed by using NOS inhibitors and L-arginine [152, 153].

Neutrophil chemotaxis in response to invading pathogens and chemokines upregulated iNOS. Intra-peritoneal inoculation of a lethal dose of *Staphylococcus aureus* in a sepsis model prevented neutrophil migration to the site of infection, which was prevented by aminoguanidine pretreatment [158]. In a similar study, Benjamin et al.

[159] observed that iNOS (-/-) mice subjected to lethal sepsis induced by cecal ligation and sublethal sepsis by cecal ligation and puncture (SL-CLP) suffered high mortality due to a lack of microbicidal activity in neutrophils of iNOS(-/-) mice. Zymosan injection into the peritoneal cavity in both wild-type and iNOS knockout mice elicited a similar chemotactic response of neutrophils yet a subtle difference in the kinetics pointing towards fortifying effects of NO on neutrophil chemotaxis [160].

Higher concentrations of NO donors inhibit chemotaxis in a cGMP-dependent manner, whereas lower concentrations promote this response, suggesting a biphasic regulation of chemotaxis by NO [157, 161]; [147, 162–164]. An increase in chemotaxis at lower concentrations of NO was cGMP-independent [157, 161]. A subsequent study on NO-induced cGMP-independent neutrophil chemotaxis was found to be mediated by IL-8 production [165].

(c) ***Phagocytosis***

Neutrophils eliminate microbes and segregate them intracellularly into the phagocytic vacuole by phagocytosis. NO production in the human PMNs, along with ROS and myeloperoxidase (MPO), is important to execute the antimicrobial activity. Human neutrophils require the cytokine trigger in the form of interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFNγ) to induce iNOS and subsequently nitration or nitro-tyrosine modification of the bacterial target proteins following the formation of peroxynitrite [95].

Endogenous enzymatic generation of NO has been implicated in bacterial endocytosis and subsequent killing by neutrophils. These observations can be further categorized as a response of peripheral and peritoneal neutrophils. Rat peritoneal neutrophils constitutively generate NO and exhibit pronounced fungal killing in vitro, in comparison to the peripheral neutrophils, which produce less amount of NO. The phagocytic activity of human neutrophils was augmented by supplementation with L-arginine, but was antagonized by N^G nitro-L-arginine (L-NNA), L-canavenine (L-CAN), or aminoguanidine [166]. Reduction in phagocytosis and pathogen killing was also observed in L-NG-mono-methyl arginine (L-NMMA) pre-treated anucleate granule-poor neutrophil, which was neutralized in the presence of L-arginine [128]. Following phagocytosis, sustained NO production in the PMNs was well-maintained by redox-sensitive cofactor, BH_4, and substrate, L-arginine, to enable microbial killing [167].

Moreover, phagocytosis-induced NO production in CGD patients was nearly half compared of healthy individuals and is associated with poor survival of the patients. Phagocytosis-induced NO generation persuades nitro-tyrosine formation through intermediate production of peroxynitrite [95]. NO donors at high concentrations, however, inhibited phagocytosis [168]. Furthermore, the immune functions of neutrophils were found to be neutralized by microbial siderophore's iron-scavenging property by depleting ROS [169]. Enhanced nitrosative stress resulted in S-glutathionylation of L-plastin, leading to impaired chemotaxis, polarization, and bactericidal activity of human PMNs, providing a mechanistic basis for their impaired functions in diabetes mellitus [133].

(d) ***Degranulation***

Neutrophil activation involves the degranulation of its enzymes in the phagocytic vacuoles or the extracellular milieu. Enzyme release is a complex multi-step process, which is influenced by migration, membrane recognition, adherence of particle and ingestion, as well as granule exocytosis. Among the various granules present in the neutrophils, azurophilic and specific granules show different degranulation dynamics, specific granules being mobilized selectively, albeit to a varying extent, in response to most soluble stimuli. This is primarily due to different Ca^{2+} requirements for exocytosis, specific granules being more sensitive to a rise in intracellular Ca^{2+} and, consequently, released before the azurophil granules [170]. Moreover, Moilanen et al. found that NO donors inhibited degranulation in PMNs [171], supporting the idea that PMNs-derived NO could act as a negative feedback signal to restrict the inflammatory processes. Exogenous NO enhances fMLP induced exocytosis in rabbit peritoneal neutrophils. Higher concentrations, however, strongly inhibited exocytosis [172]. Cyclic GMP and its analogs or agents, which increase intracellular cyclic GMP, enhance degranulation [173]. NO donors impede the release of β glucuronidase from human PMNs.

(e) ***Respiratory burst/Free radical generation***

A respiratory burst was first reported by Baldridge and Gerald [174] observed during the process of phagocytosis in neutrophils due to the activation of NADPH oxidase, a multi-subunit enzymatic complex**.** Respiratory burst is responsible for more than 90% of the total oxygen consumption by these leukocytes [175]. This leads to the generation of O2- into the phagosomes or to the exterior milieu. Superoxide anions are relatively noxious, but form additional, toxic oxygen species, in particular H_2O_2, by spontaneous dismutation, which may then oxidize halides, in particular, Cl^-, to hypohalous acid, e.g., HOCl, catalyzed by myeloperoxidase, released from the azurophil granules following degranulation. After the encounter the invading organisms, the neutrophils sequester the organism into an enclosed vacuole, known as a phagosome. Upon stimulation, cytoplasmic proteins $p47^{Phox}$, $p67^{Phox}$, and a Rac-related GTP protein translocate to the plasma membrane, binding to the sites located on a unique b-type hemoprotein, Cytochrome b_{558}. This hemoprotein, a dimer consisting of $gp91^{Phox}$ and $p22^{Phox}$, binds FAD and NADPH which results in a flow of electrons to the terminal acceptor Cytochrome b_{558} [176, 177]. Transfer of an electron from the Cytochrome to oxygen yields superoxide. The production of superoxide initiates a series of oxidative events, which result in the microbial killing. Patients with the chronic granulomatous disease face life-threatening infections primarily because their phagocytic cells are unable to generate superoxide [176], highlighting the importance of phagocyte-derived superoxide in host defense.

NO and oxidative burst in neutrophils have been extensively investigated in our lab [38, 40, 41, 93, 103–112]. The observations convincingly indicate NO-mediated augmentation of free radical generation from PMNs [93, 105, 106]. Moreover, PMNs have preferential oxygen utilization for ROS generation over NO synthesis [178]. Furthermore, sustained ROS generation by PMNs is associated with S-Glutathionylation of NADPH subunit p47phox [179]. Intracellular and extracellular

calcium levels also have a modulatory impact on NOS activity and free radical generation [109]. Ascorbate enhances RNS generation in the PMNs by maintaining the levels of redox-sensitive tetrahydrobiopterin [110]. Ascorbate also enhanced NOS expression and activity in vivo and reinforced the anti-microbial activity of neutrophils by augmenting oxidative mechanisms [111]. Further, the release of NO from the PMNs was observed to be enhanced by intracellular ascorbate that prevents the activation of platelets by subsequently activating guanylyl cyclase in the platelets [180]. $ONOO^-$ exhibited a biphasic effect like NO, being stimulatory at lower concentrations through the MEK/ERK/MAPK pathway but inhibitory at higher concentrations due to the direct inhibition of NADPH oxidase [93]. NO donor-augmented respiratory burst also involved the activation of K^+ channels and various kinases [181]**.** Clancy et al. [182] showed direct interaction of NO with the membrane subunit of the NADPH oxidase complex, while Fuji et al. [183] demonstrated an inhibitory association of NO with both membranous and cytosolic subunits. Lee et al. [184] also reported an inhibitory effect at a higher NO concentration. Recently, NO donors were found to decrease PMA- and/or fMLP-induced phosphorylation of p47 on tyrosine and serine/threonine residues and PKC on serine residues and ROS production with MAPK phosphorylation [185]. Recent studies from our lab also demonstrated that the sustained release of ROS was due to S-glutathionylation of p47phox cysteine residues in the activated PMNs (Nagarkoti et al., 2018). Moreover, the use of different probes like DCF and DHE to assess the superoxide scavenging ability of NO requires confirmation by other methodologies [85, 179].

The antioxidant defense mechanisms are on constant vigil to maintain the redox balance of the neutrophils. Neutrophils are protected against self-destruction by the intracellular superoxide dismutase, ascorbate, GSH, and catalase [186]. Factors instigating oxidative burst may simultaneously trigger NOS in neutrophils. Lipopolysaccharide (LPS), a membrane component of gram-positive bacteria, a potent inducer of iNOS lead to a significant increase in L-arginine uptake and free radical generation from peripheral and peritoneal neutrophils [105]. NOS inhibitors, aminoguanidine, and 7-nitroindazole, inhibited arachidonic acid-induced free radical generation from LPS-treated neutrophils. Moreover, pre-incubation with nitrite also elevated the free radical generation and myeloperoxidase (MPO) activity [105]**.** Moreover, hypoxic neutrophils following oxygenation exhibited a significant increase in the respiratory burst in a NO-dependent manner [93]. Thus NO seems to mediate the damaging effects of neutrophils in the hypoxic environment at the inflammatory loci.

(f) ***Apoptosis***

NO and apoptotic regulation of neutrophils is indicated, but a decisive and distinctive picture is still awaited. The role of NO in modulating gene expression and cell survival has been extensively elaborated [130, 131, 187–189]. The role of endogenous NO is controversial, showing both pro and anti-apoptotic outcomes. Levels of nitrite increase in spontaneously aging neutrophils, and the anti-apoptotic effect of GM-CSF in prolonging neutrophil survival is associated with decrease of nitrite content in these cells [190]. On the contrary apoptotic trigger from anti Fas ligand or TNF-α relates to a reduction in the nitrite content suggesting a survival signal

from NO [190]. Exogenous NO or $ONOO^-$ delivered by the NO donors at much higher than average physiological concentrations clearly showed enhancement in the rate of spontaneous apoptosis [191–193]. We have also demonstrated a crucial role of NO/iNOS in neutrophil apoptosis via enhanced ROS generation and caspase-8 mediated activation of the mitochondrial death pathway [63]. During inflammation, neutrophil survival is prolonged, and it is interesting to see that both NO donors and NOS inhibitors provide protection. It thus highlights the complexity in the action of such a simple molecule like NO on neutrophils. Prolonged treatment of human PMNs or mice neutrophils with NO led to enhanced ROS generation, caspase-8/caspase-3 cleavage, reduced mitochondrial membrane potential, and finally apoptosis [63]. Upon induction of apoptosis in the thymus by x-ray, iNOS KO mice exhibited higher levels of neutrophil infiltration and production of MIP-2 and keratinocyte-derived chemokine (KC) [194]. Mechanistically we have shown that extenuation of catalase activity by S-glutathionylation by NOX, and mitochondrial ROS compromised neutrophil survival [195]. Recently we have reported that cell death in the human leukemic cell line, K562 of myeloid origin upon overexpression of iNOS and nNOS by activating distinct mechanisms leads to pyroptosis and apoptosis of K562 cells, respectively [45]. Furthermore, NO generated from iNOS induced neutrophil differentiation by using a multipronged approach of inducing NOS in K562 cells, mice, or human HSCs [45, 46].

(g) ***NETosis***

Following the sensing of pathogens, neutrophils also release web-like structures or amalgams of nuclear DNA, histones, and granular proteases as neutrophil extracellular traps (NETs) to extracellularly eliminate pathogens [196]. This process is also termed as NETosis and is distinct from apoptosis of PMNs. NETosis release decondensed euchromatin and heterochromatin along with granular protein in the extracellular space. NETosis is regulated by ROS, myeloperoxidase, azurophilic granulated proteins, and neutrophil elastase (NE) [197, 198]. NE released from azurophilic granules degrades the linker histone H1 and the core histone proteins following chromatin decondensation [199]. Moreover, NE release and chromatin decondensation are significantly augmented in the presence of MPO [199]. In addition, hypercitrullination of histone H3 following conversions of histone arginine to citrulline by peptidyl arginine deiminase 4 (PAD4) is critical for chromatin decondensation [200, 201], as it reported that PAD4 knockout mice decreased histone hypercitrullination and NETs release [202]. NETosis can be grouped into two main categories based on the dependency on ROS production. The molecular basis of NETosis is still the least defined/poorly understood. However, accumulated pieces of evidence on the type of inducers have grouped it into two types: The inducers which involve activation of NOX2-mediated oxidative burst are termed NOX-dependent NETs inducers like pro-inflammatory cytokines [203], chemokines [204], PMA [196, 197], NO [205] and oxLDL [206]. An essential role is attributed to NOX2 in regulating NETosis as PMA-induced NETs are prevented either by using diphenylene iodonium, DPI, inhibitor of NOX activity or in patients with chronic granulomatous disease (CGD) which have congenital defect in NOX2 subunits [207]. Moreover, the NOX-dependent pathway

occurs slowly over 2–4 h. In contrast, the other types of inducers do not require the formation of ROS and NOX activation. These include calcium ionophore [208], uric acid [209], soluble immune complexes [210], and a few microorganisms [211] and are termed as NOX- independent NETs inducers. NOX-independent NETosis is known to occur rapidly, and NETs release is observed at an early time point i.e. within 1 h. Moreover, recent evidence suggests that neutrophils can catch and kill pathogens extracellularly using the similar bactericidal approach as NETs. Studies have shown that cells with very long and very thin filopodia can directly communicate with cells across a distance of many cell diameters. These filopodia, also called cytonemes were first observed in a variety of embryonic cells [212–214]. Live neutrophils are also reported to create a cytoneme network using filamentous tubulovesicular secretory protrusions. Granular bactericides are localized in membrane vesicles and tubules of which cytonemes are composed. The cytonemes are comparatively short-lived structures. Unlike NETs these are formed within 10–20 min and can begin to break down almost immediately as a result of the splitting and lysis of the vesicles at the end of the cytoneme or because of the detachment of cytonemes from the neutrophil surface. By proteomic analysis it was discovered that cytonemes contain the primary (myeloperoxidase, cathepsin G, and defensins) and secondary (lactoferrin, lipocalin) secretory granules of neutrophils and numerous cytosolic proteins. Cytosolic proteins include: (i) energy metabolism enzymes such as a number of glycolytic enzymes and glucose-6-phosphate dehydrogenase; (ii) cytoskeletal proteins like beta and/or gamma actin, L-plastin etc. [215–219]. In addition to antibacterial activity, cytonemes are also involved in the cell adhesion and communications [218].

Studies from this lab demonstrated that treatment of human neutrophils with NO donors induced NETs formation in a concentration and time-dependent manner, which was attenuated in the presence of NADPH oxidase inhibitor, N-acetyl cysteine, DPI and MPO inhibitor, ABAH [205]. In addition, NO-induced NETs contained both nuclear and mitochondrial DNA as well as proteolytic enzymes [203, 220]. Furthermore, NADPH oxidase and MPO-mediated enhanced NETs release was observed in human PMNs following in vitro stimulation with inflammatory cytokines (TNFα, IL-1β, and IL-8) or treatment with the plasma of SIRS patients showing high pro-inflammatory cytokines induced NETosis [203, 220]. Inhibition of inflammatory cytokines (TNFα, IL-1β, and IL-8) or pre-incubation of SIRS patient plasma with inflammatory cytokines antibodies attenuated the NETs formation [203]. Furthermore, we also demonstrated ROS-dependent activation of ERK and p38 MAPK, leading to PMA-induced NETs release from human neutrophils [221]. Further studies unraveled the significance of glycolysis in both NOX-dependent and independent NETosis and revealed the importance of lactate in NETs formation [134]. Moreover, ROS depletion led to attenuating neutrophil functions like NETosis using iron-scavenging bacterial siderophores [169]. In addition, inhibition of NOS by L-NAME or 7-NI inhibited PMA-induced NETs formation in mice and human neutrophils [36, 222]. Participation of Rac2 in PMA or LPS-induced NETs formation in mice neutrophils, has also been shown [223]. Reportedly, NETs formation is completely abrogated in Rac2 null mice or Rac2 mutants as compared to Rac1 or wild-type control mice, as they are unable to produce ROS, suggesting that Rac2 isoform is

crucial for NETosis [223]. However, Rac2 mutants can be rescued by the exposure of H_2O_2 to neutrophils. Thus, the involvement of iNOS in NETs formation can be speculated as Rac2 has been observed to interact with iNOS [42]. Furthermore, phosphorylation of eNOS was found to be increased upon treatment of diabetic mice with ruboxistaurin, which accelerated wound healing, thereby suggesting inhibiting NETs formation in these mice by PKCb inhibition [224]. Furthermore, a study of PMA or bacterial infection-induced NETs formation in teleost (*Cynoglossus semilaevis)* demonstrated enhanced production of factors like ROS, NO, and MPO in NETs in this fish which was inhibited upon blocking these factors indicating the formation of NETs in teleost with antibacterial effects in a ROS-, NO-, and MPO-dependent manner [225]. Furthermore, enhanced NETs release was observed in our lab in iNOS overexpressed-neutrophil differentiated K562 cells, further suggesting a direct role iNOS derived NO in NETs formation [46]. Similarly, a recent report demonstrated RNS, ROS, and PI3K-dependent NETs formation when stimulated with PMA and calcium ionophore (A23187) in PMNs and human promyelocytic leukemic cells (HL60) [226]. NADPH oxidase activity was required to release NETs upon stimulation with NO, as shown in NADPH-deficient neutrophils isolated from patients with the chronic granulomatous disease [226]. Additionally, the role of NO is reported in cytoneme formation, from live neutrophils which possess bactericidal activity like NETs. Cytonemes can develop within minutes of infection through the action of NO or actin-depolymerizing alkaloids of invading pathogens [218]. It is speculated that the formation of cytonemes in neutrophils is initiated by intercellular NO. In a study by Galkina et al., NO-induced binding and aggregation of *Salmonella enteric serovar* Typhimurium bacteria extracellularly by *tubulovesicular extensions* was observed. This was not observed using NOS inhibitor L-NAME; rather, a phagocytic response was generated [219]. In yet another study, by the same group it was observed that extensions were formed on the neutrophil cell bodies and in the presence of NOS inhibitor, neutrophils were well spread and had no micro extensions. These tubulovesicular extensions were observed under scanning microscopy *as* extensions of neutrophils having the capacity of distant adhesion and binding substances for phagocytosis, such as serum-opsonized zymosan particles and erythrocytes [227]. However, in our lab, it has been demonstrated that the addition of NO donors (SNAP and SNP) to adhered PMNs led to a time and concentration-dependent NETs release as observed under scanning electron microscopy/Confocal microscopy. The NO-mediated NETs formation was further confirmed by extracellular DNA release, and NET-bound elastase activity. The NETs formation was abrogated upon inhibition of NOX using NOX inhibitor NAC, suggesting the role of free radicals in NO-induced NETs generation [205]. Host NO often inhibits microbial cell-to-cell contact and eliminates staphylococcal virulence by attacking the Agr quorum sensing mechanism and destroys zinc homeostasis in Salmonella *enterica* Serovar *typhimurium* [228]. The scope of antimicrobial activity by NO involves the ability of this natural agent to cause cytonemes formation and change the interaction of bacterial and fungal pathogens with neutrophils from phagocytosis to binding of microbes extracellularly by cytonemes. NETs made of DNA and protein expulsion trap bacterial pathogen and kill them [219, 227, 229]. Unlike NETs, the activation of neutrophils and the

formation of reactive oxygen species (ROS) are not necessary but hinder the formation of cytonemes. Commonly used ROS activators, such as LPS, fMLP and PMA, did not initiate cytonemes formation in neutrophils [230]. Activation of neutrophils with PMA or other stimuli results in the formation of superoxide anion radicals (O2) produced by NOX. The NO radical interacts rapidly with the O2 radical, producing ONOO (peroxynitrite) anions [231]. This reaction lowers the NO bioavailability which plays a crucial role in the formation of cytonemes. In addition, peroxynitrite formed in the form of reactive oxygen can initiate oxidative processes responsible for the destruction of neighboring cells and tissues [232], including oxidative destruction of cytonemes. Nitric oxide may initiate cytoneme formation through glycolysis inhibition and/or V-type ATPase [233]. A recent study demonstrated that pediatric patients with childhood asthma showing all the symptoms of asthma showed more NETs formation when induced with SNP (NO donor) than the conventional inducers like PMA and LPS [234]. Furthermore, the NETs forming ability in these patients was blocked by inhibiting NOS using NOS inhibitors and L-NAME [234]. Thus, NOS inhibitors could serve as a therapeutic target for childhood asthma. Interestingly, iNOS was found to be upregulated in ICAM-1^+ neutrophils present in the lungs of sepsis patients [235]. Together, these reports suggest a positive regulation of iNOS/NO in NETs for the destruction of invading microbes. This information can be exploited for the therapeutic intervention of NETs in diseases like sepsis, cystic fibrosis, SLE etc. where the prevalence of NETs is significantly exaggerated.

Conclusion and Future Perspective

Neutrophils produce a high amount of NO via constitutive iNOS and nNOS. Neutrophils abundance in the blood and BM seem to impact hematopoiesis via NO signaling. In the BM, diverse hematopoietic and non-hematopoietic stromal cells also express NOS isoforms and produce NO in the hematopoietic niche. We have discussed the role of NOS/NO in granulopoiesis and also described the molecular mechanisms involved in NOS/NO-mediated modulation of granulopoiesis by multiple mechanisms under steady state and various disease conditions. Furthermore, NO in BM niche has been suggested to function in both autocrine and paracrine manner. In addition, recent research have identified novel pathways and molecular mechanisms regulating NOS/NO-mediated neutrophil functions that might have future translational applications.

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Chapter 9
Cell Death-NO-Today: Effect of NO and RNS on Non-apoptotic Regulated Cell Death

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Abstract Nitric oxide (NO), generated either enzymatically or non-enzymatically plays a significant role in cellular physiology. At low concentration, NO acts as a signalling molecule in cell, however, deregulated production of NO generates several reactive nitrogen species (RNS). Through diffusion controlled reaction NO and RNS can exert detrimental effects on cellular lipids, proteins and DNA. NO and RNS influence glycolysis, mitochondrial respiration, permeability of different ion channels and even release of proinflammatory cytokines. NO and RNS are known to regulate positively or negatively many essential enzymes, transcription factors and proteins through post translational modifications which can also stimulate cell death. Apoptosis is one of the major forms of programmed/regulated (PCD/RCD) cell death and NO mediated induction or suppression of apoptosis is well comprehended. NO and RNS have been found to be associated with an increasing number of non-apoptotic RCD in tumor etiology and various neurodegenerative, autoimmune, inflammatory, infectious diseases in recent times. Hence use of NO and its derivatives might prove beneficial to manipulate different non apoptotic RCD pathways to develop novel pharmacological strategies. Here we summarize the cutting edge evidences supporting pleiotropic nature of NO and reactive nitrogen species influencing nonapoptotic cell death.

Keywords Nitric oxide · Reactive nitrogen species · Nitrosative stress · Cell death · Autophagy · Apoptosis · Pyroptosis · Ferroptosis · Parthanatos · Necroptosis

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*, Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_9

Introduction

Nitric oxide is a colourless, odourless ubiquitous signalling molecule that is produced enzymatically or non-enzymatically in almost every biological system. Being a pleotropic regulator, it is involved in neuronal regulation, vasodilation, redox homeostasis, regulation of iron uptake, tumorigenesis, pathogen killing etc. [1, 2]. NO interacts with the heme moiety of soluble guanyl cyclase and couples with cGMP dependent protein kinase G and other metal containing enzymes. Enzymatic synthesis of NO can be catalysed by three nitric oxide synthase (NOS) isoforms namely eNOS, nNOS and iNOS [3]. Under acidic condition NO can be generated from nitrite also [4]. NO shows a Janus faced function, at low concentration it plays an important role in signalling whereas at high concentration it causes ER stress [5], mitochondrial dysfunctionality [6], ATP depletion, DNA damage etc. which can stimulate cell death. Furthermore, it regulates activity of cell signalling proteins and transcription factors by post translational modifications. Regulated cell death (RCD) pathways have specific effects on disease pathology. Recently, Nomenclature Committee on Cell Death (NCCD) has categorized almost 12 types of RCD [7]. Remarkable conflicting finding regarding NO and $ONOO^-$ mediated initiation or prevention of apoptosis, necroptosis and other cell death modalities are present in the literature.

In this review we tried to emphasize on the effect of nitric oxide and reactive nitrogen species (RNS) on major non apoptotic RCDs, including—autophagy dependent pathway, pyroptosis, ferroptosis, parthanatos, necroptosis and netotic cell death.

Sources of Nitric Oxide

Endogenous NO is synthesized by Nitric Oxide synthase (NOS) enzyme that catalyses the conversion of L-Arginine into L-Citrulline and Nitric oxide (NO). Activity of NOS is dependent on NADPH, FAD, FMN and tetrahydrobiopterin (BH_4) that serve as coenzymes [3, 8]. NOS are classified in to three groups- 1. Neuronal NOS (nNOS/NOSI) expressed in central and peripheral nervous system and other cells. 2. Inducible NOS (iNOS/NOSII) expressed in many cells (macrophage, leukocytes) in response to proinflammatory cytokines, lipopolysaccharides (LPS), damage associated molecular patterns (DAMP) etc. and finally 3. Endothelial NOS (eNOS/NOSIII) expressed mainly in vascular endothelial cells [8]. In addition, α-isoform of nNOS has been reported to be constitutively expressed in mitochondria hence named mitochondrial NOS [9]. All three NOSs have a calmodulin binding site. Calmodulin binding brings conformational change in structure, which is necessary for proper transfer of electron between N terminal oxygenase and C terminal reductase domain of NOS enzyme [8]. Interestingly, while CAM binding of nNOS and eNOS has been discovered to be highly dependent on cellular Ca^{2+} status, iNOS was found to be independent of Ca^{2+} level [10]. NOS enzymes can exist in two forms. In its

homodimeric form, it mainly produces NO in the presence of O_2. However, release of tetrahydrobiopterin (BH_4) leads to uncoupling of NOS, i.e., depletion of BH_4 uncouples oxygen reduction and oxidation of L-Arginine and keeps NOS in monomeric form, which results in oxygen becoming the terminal electron acceptor instead of L-Arginine. This results in the formation of superoxide and thereby increases generation of reactive nitrogen species (RNS) [8, 11]. Many physiological conditions can trigger superoxide generation from NOS such as-i. inhibition of hsp90 and nNOS coupling or ii. low level of L-Arginine or increase in arginase activity [12, 13]. Interestingly, peroxynitrite has been demonstrated to oxidize both Zinc-thiolate cluster of eNOS and BH_4 and prevents binding of BH_4 to eNOS. Moreover, activity of eNOS has been demonstrated to be regulated by S-glutathionylation [8]. Clinically NO and RNS can be generated in cells using a group of chemicals known as NO Donors. Some most common NO donors used in experimental setups are- sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), nitrates, nitrites, NONOates (SperminNONOate, DETA NONOate), Sydnonimines (SIN1) etc. [14–17]. NO donors are capable of producing NO, nitrosonium ion (NO^+) or nitroxyl anion (NO^-) independent of endogenous NOS. Interestingly, exogenous GSNO, which can act as an endogenous NO reservoir, has been identified to modulate iNOS activity by S-glutathionylation [18]. All these events indicate the presence of a complex feedback mechanism by which NO and RNS can regulate NOS activity.

Reactive Nitrogen Species (RNS) and Nitrosative Stress

Superoxide radical (O_2^-) either generated in mitochondria or by NADPH oxidase or Xanthine Oxidase [8] readily reacts with excess NO and produces deleterious peroxynitrite ($ONOO^-$) and other primary reactive nitrogen species. In acidic pH, peroxynitrite transforms into peroxynitrous acid (ONOOH) that can either 1. Dissociate into $\cdot NO_2$ and $\cdot OH$ radical or, 2. Isomerize into NO_3^- [8, 19]. All these are strong oxidizing agent and can modify nucleic acids, proteins, lipids etc. Furthermore, $\cdot NO$ can interact with $\cdot NO_2$ to produce N_2O_3 and all together they take part in protein nitration [20].

$$ONOOH \rightarrow NO_3 + H^+$$

$$ONOOH + ONOO^- \rightarrow NO_2^{\cdot} + {}^{\cdot}OH$$

$$2NO_2 + 2NO \rightarrow 2N_2O_3$$

Thus excess amount of NO and other reactive nitrogen species give rise to a condition termed as Nitrosative stress, a phenomenon associated with many inflammatory, autoimmune disease, cancer etc.

Protein Modification in NO Signalling

NO and other RNS are capable of modifying protein activity through post translational modifications. The major post translational modifications (PTMs) involved in NO signalling are S-nitrosylation, S-glutathionylation, nitration and oxidation.

The mechanism of S-nitrosylation involves transformation of cysteine thiol firstly into thiolate ($R\text{-}S^-$) followed by oxidation into thiyl radical ($RS\cdot$) that interacts with NO to form SNO-protein [8]. This particular reversible modification is highly dependent on overall NADPH/$NADP^+$, GSH/GSSG ratio [21]. Furthermore, NO can be transferred from one nitrosylated protein (-SNO protein) to thiol group of another protein by a process known as transnitrosylation [22]. Activity of many crucial proteins like RIPK3, TSC2, ERK1/2, transcription factors like, NF-κB, enzymes like GSNOR, Catalase, COX2 etc. have been reported to be modulated by S-nitrosylation [17, 23, 24]. As Cysteine is abundant in the catalytic and active sites of many enzymes as well as in their co-enzymes, it is presumable that dysregulated S-nitrosylation is bound to cause trouble.

S-glutathionylation is the formation of di-sulfide bond between protein and GSH and it is catalysed by Glutathione-S-transferase [8]. S-glutathionylation not only regulates functionality of different proteins but also helps to maintain GSH-GSSG pool [25].

Finally, covalent attachment of NO_2 to the phenolic group of tyrosine leads to the formation of 3-Nitro-Tyrosine, which is considered as footprint of nitrosative stress [8]. Like S-nitrosylation, tyrosine nitration also depends on bio-availability of oxidants, RNS generation, and subcellular compartmentalization, which facilitates nitration of certain proteins [26–28]. Because of mitochondrial genesis of superoxide and abundance of metal centre rich proteins, mitochondrial proteins have been found to be enriched in nitration. Tyrosine nitration may impart both loss of function or gain of function outcome on the target proteins [16, 24, 29].

S-nitrosylation has been shown to regulate ubiquitination of many proteins. S-Nitrosylation prevents proteosomal degradation of BCL2 and stabilizes HIF1α [30]. Both positive and negative correlations have been established between S-glutathionylation and S-nitrosylation [31]. Jin et al. showed that S-nitrosylation of ERK1/2 prevents its phosphorylation and subsequent activation [32]. All these examples give us a mere idea of why nitrosative stress mediated PTMs are so much important and how it can affect different crucial protein through molecular crosstalk and influence the cellular outcome under stress.

Defence Against Nitrosative Stress

The antioxidant system plays pivotal role in maintaining cellular homeostasis by fighting against Oxidative and Nitrosative stress and involves both enzymatic and non enzymatic components. Examples of non enzymatic ROS/RNS scavenger include

vitamin C, α- tocopherol, retinol, GSH and other metal ions like Fe^{+2}, Cu^{+2}, Mn^{+2}, Zn^{+2} etc. On the other hand, the key enzymes that play important functions in maintaining cellular homeostasis are Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), GSNO Reductase (GSNOR), Thioredoxin Reductase (TR_X) and Peroxiredoxin. Apart from the conventional role of SOD in conversion of O_2^- into H_2O_2 and O_2, it limits formation of peroxynitrite from NO thus confer protection against mitochondrial dysfunction [8]. Catalase eliminates H_2O_2 from the system by converting it to H_2O and O_2. Furthermore, it has been reported that catalase can slowly consume NO to protect against nitrosative stress and on the other hand, NO can also inhibit hydrogen peroxide scavenging activity of catalase [33, 34].

Nitrosative stress is also kept in check by GSNO that serves as a reservoir of endogenous NO. GSNO takes part in trans-nitrosylation and gets converted to GSSG by GSNOR. GPx contributes to the GSSG pool as well. As the ratio of reduced Glutathione to oxidized form (GSH/GSSG) is a well-known marker of cellular oxidative stress, it is strictly maintained by GR that catalyses reduction of GSSG to GSH using $NADPH/H^+$ [35]. Thioredoxin and its associated TrxR enzyme are one of the major system that take part in protein denitrosylation the other being GSNOR system. In addition, peroxiredoxin (a Trx dependent peroxidase) is able to reduce peroxynitrite. Now all these may seem enough to combat nitrosative and oxidative stress, the fact is all these enzymes has been demonstrated to be regulated by NO and other RNS. Peroxynitrite has also been documented to modify catalytic activity of Catalase [36, 37]. In plants also S-nitrosylation of GSNOR has been observed to induce its autophagic degradation indicating the crucial role of GSNOR in maintaining cellular homeostasis [38]. Mitochondrial SOD, GSNOR, Trx, all are negatively regulated by peroxynitrite mediated nitration or S-nitrosylation [39]. Endogenous NO has been found to induce peroxiredoxin I and VI synthesis in murine macrophage [40] and this phenomenon adds up another dimension to the redox control machinery.

Programmed Cell Death

The balance between cell survival and death is the foundation of tissue homeostasis in any living organism. In 1973, Schweichel and Merker classified cell death into 3 forms. Type I referred to as apoptosis that exhibits distinguishable features like pyknosis, karyorrhexis, chromatin condensation, membrane blebbing. Type II-autophagy dependent cell death which involves formation of autophagic vacuoles and subsequent degradation of cytosolic components and type III- Necrosis, a rather unregulated form characterized by oncosis and disintegration of plasma membrane [41]. However, many regulated forms of necrosis have gained recognition later including necroptosis, pyroptosis. Regulated cell death (RCD), also known as programmed cell death [42] that involves different subtle molecular machineries during normal physiological modulation. These molecular machineries can be regulated by different physical, chemical or mechanical stimuli and protein modification

which distinguishes RCD from Accidental Cell Death (ACD). Specific cell death pathways are involved in pathology of autoimmune disease, senescence, aging, tumour growth, infection, inflammation and enormous knowledge regarding new pathways have surfaced over time. At present about 12 types of distinct cell death forms are recognized by NCCD based on genetic, biochemical, and pharmacological characteristics [43]. One of the most studied forms of programmed cell is apoptosis which is also referred to as the PCD I. This form of cell death is characterized by distinctive morphological features like rounding-up of the cell, membrane blebbing, shrinkage of cytoplasm, chromatin condensation and nuclear fragmentation [44, 45]. The role of NO in inducing apoptotic response has been extensively investigated and been linked with regulation of molecules that are part of apoptosis, like p53, Bcl-2, caspases and heat shock proteins [46]. NO has been reported of being capable of regulating both intrinsic and extrinsic modes of apoptotic pathways [47, 48]. But we in this current issue will be focusing mainly on non-apoptotic programmed cell deaths.

NO in Autophagy Induction

Numerous studies in the past implied a complex association between nitrosative stress and autophagic machinery (Fig. 9.1) [49]. NO reacts with cGMP to produce 8-nitro-cGMP in the presence of ROS and it is a known regulator of autophagy. It has been reported that 8-nitro-cGMP triggers S-guanylation of cell surface proteins of Group A Streptococcus (GAS) bacteria. This S-guanylation induces Lys63-linked ubiquitination of the bacteria and facilitates its recognition by the autophagic machinery. Exogenous 8-Nitro-cGMP even induces non cytotoxic autophagy without involving mammalian target of rapamycin (mTOR) [50, 51]. NO and peroxynitrite mediated PTM is also vital for induction of mitophagy and mitochondrial dysregulation related cell demise. Recruitment of Dynamin-related protein 1 (Drp1) to damaged mitochondria is one of the crucial steps in regulating induction of mitophagy as Drp1 regulates the mitochondrial fission. Peroxynitrite ($ONOO^-$) and exogenous NO donor have been reported to induce post translational modification of Drp1, which then translocate and get recruited in mitochondria. This translocation of Drp1 results in increased mitophagy which also involves PTEN-induced kinase 1 (PINK1) and Parkin RBR E3 ubiquitin-protein ligase (PARKIN) [52]. Another key factor in this cascade is GSNOR. Impairment of GSNOR activity is linked with mitophagy and autophagy as it regulates denitrosylation of many proteins and its inactivity triggers protein misfolding. Again, protein misfolding cause ER stress and upregulation of Ca^{2+} in cytoplasm which further activate NO production. In pericyte cells, treatment with ZnO nanoparticle resulted in generation of $ONOO^-$ which increased S-nitrosylation of transient receptor potential melastatin-related 2 (TRPM2). This turnover of TRPM2 resulted in ER stress induced autophagy. This study also identified calmodulin to be one of the TRPM2-interacting proteins. Thus it not only expresses the complex relation between NO mediated PTM but also reported the

involvement of calmodulin in ER stress induced autophagy facilitated cell death [53]. One of the major pathological outcomes of dysregulated mitochondrial activity has been observed in Parkinson disease. PINK1 and Parkin were found to play a regulatory role in mitophagy. Upon dissipation of mitochondrial membrane potential PINK1 phosphorylates Parkin and ubiquitin. Phosphorylation of ubiquitin facilitates translocation of phosphorylated Parkin to mitochondria and this activated parkin then triggers mitophagy by polyubiquitinating mitochondrial outer membrane proteins. Impairment of mitochondria leads to S-nitrosylation of PINK1 and thereby inhibits its kinase activity which in turn inhibits Parkin phosphorylation and thereby blocking mitophagy [54]. On the other hand, reports show that S-nitrosylation in parkin at Cys323 residue activates its E3 ligase activity to induce mitophagy [55]. However, in PINK1 null dopaminergic neurone, nNOS derived NO was found to compensate for PINK1. Mitochondrial accumulation of p-nNOS (ser1414) can facilitate NO generation at optimal concentration. Moreover, nitrosative stress caused by SNP and other NO donor failed to translocate Parkin [56]. ATG4 and LC3II oxidation affect their activity. In endothelial cells, knockdown of ATG3 was found to inhibit autophagy which resulted in impairment of NO production in response to shear stress due to reduced phosphorylation of eNOS [57]. Continuous laminar stress has shown to increase NO/RNS induced autophagy, though the molecular mechanism remained unclear [58]. RNS induced autophagy has been found to play pro-survival as well as cytotoxic role. SNP has been found to regulate autophagy by increasing expression of BCL2 related Ovarian Killer (BOK) and reducing expression of Bcl2 family protein Myeloid Cell Leukemia Sequence 1 (MCL1). Administration of SNP reduced MCL1 level thus disrupting MCL1-Beclin1 interaction. Upregulated BOK was also suspected to disrupt MCL1-Beclin1 association by interaction with MCL1. Beclin1 thus released from inhibitory effect of MCL1, upregulates Autophagy [59]. In osteoblast cell, SNP activate Autophagic cell death under GSH depleted condition [60]. In many cells, NO can induce both autophagy and apoptosis where apoptosis was the cause of cell death, autophagic upregulation was found to be protective or assisting apoptosis [61]. In human umbilical vein endothelial cells, IR injury was observed to induce migration and apoptosis in an iNOS, autophagy dependent manner [62]. Furthermore, in these cases it was found that NO induced autophagy through upregulation of AMPK that inhibits mTORC1 and its downstream target proteins, as well as activates the Unc-51 like autophagy activating kinase 1 (ULK1) via phosphorylation [63–65]. NO donor, S-nitrosocysteine (SNOC) has been found to induce cell death and mitophagy in mixed cortical neuronal cultures by inducing mitochondrial fission [66]. However, the concept of NO being a stimulus of autophagy mediated or dependent cell death has been established quite recently. MCF7 breast cancer cells showed increased mortality when subjected to steady state NO. SperminNONOate and NO were found to induce p-ATM, ACC and p-AMPK expression level which correlated with upregulated TSC2 level and inactivation of mTORC1 pathway. Indeed, reduction in pp70S6K, p-S6K and 4EBP1 and raptor (inhibitory phosphorylation by p-AMPK) expression was found by western blot analysis [67]. It is worthy of mentioning that AMPK mediated phosphorylation of raptor aids its association with 14–3-3 and that inhibits mTOR activity [68]. Upregulation of

p-AMPK is also important because it phosphorylates Beclin 1 present in Vps34-Atg14L-Beclin1 proautophagic complex thus induce autophagy, while upon stress condition, inhibit nonautophagic Vps34-beclin1 complex by phosphorylating Vps34 [69]. While inhibitor studies and flow cytometry analysis proved that the cell death was not related to apoptosis or necrosis; upregulation of p-ULK1 (phosphorylation at S317 site was increased by AMPK and phosphorylation at S757 was decreased), ATG5, LC3II puncta proved this to be autophagic cell death [67]. In accordance with this data our lab [70] reported that treatment with DETA NONOate for 8 h caused reduced viability in MCF7 cell line expressing features of autophagy dependent cell death but not of apoptosis. Increased cell morbidity was associated with increased expression of SIRT1, pAMPK and p53 and its target DRAM1. Although Mitochondrial Membrane Potential (MMP) and cellular redox status remain unaltered, treated cell expressed 2 folds increase in NAD^+/NADH ratio which correlate with SIRT1 induction. In depth analysis revealed that SIRT1 stability was dependent on phosphorylation status of AMPK. SIRT1 knockdown by si-RNA treatment showed that the cell death process was indeed dependent on it and SIRT1 maintained p53 in its de-acetylated state. It has previously been reported that p53 can induce autophagy through DRAM1 [71, 72] and while deacetylated p53 induce autophagy its acetylation leads to apoptosis [73–74]. Interestingly, in caloric restricted condition SIRT1 is capable of inducing eNOS activity without involving AMPK [75, 76] and can trigger autophagy by regulating FOXO1, or TSC2 [77]. NO upregulates ULK1 expression and that stabilizes SIRT1, independent of autophagy. eNOS knock down in rat abrogates SIRT1 stabilization. ULK1 was found to negatively regulate 26S proteasomal function which was partly mediated by O-linked GLCNAC transferase activity [78]. Previously our lab reported distinct apoptotic or autophagy associated cell death in CML K562 cell line based on RNS species. Exposure to DETA-NONOate for 8 h resulted in increased acidic vacuole formation (AVO), autophagic flux, Beclin1, ATG5, LC3II expression which indicated autophagy induction. Whereas, absence of PARP or Caspase3 cleavage, no loss of membrane integrity and unaltered MMP showed no involvement of apoptosis; indicating autophagy was responsible for lowered cell viability. While peroxynitrite and SIN1 induced cell death was found to be apoptotic and partly necrotic. Moreover, it was found that the autophagy dependent cell death occurred only in the presence of pure NO, not RNS. NO induced autophagy was mediated by phosphorylation and subsequent activation of AMPK. Inhibition of autophagy or p-AMPK shifted the mode of cell death towards apoptosis which clearly indicate crosstalk between these two RCDs under NO stress [79]. We also found that TAP73α was related to this autophagy dependent cell death. Tebbi et al., previously reported upregulation of p73 by Chk1/2 phosphorylation upon administration NO donor [80]. It is noteworthy to mention that p73 can initiate autophagy by modulating ATG5 and DRAM1 [81, 82]. S-nitrosylation of ERK1/2 prevents its phosphorylation [32]. Phospho-ERK1/2 blocks TSC2 activity and thus inhibits autophagy [83]. Another NO donor JS-K has been found to induce apoptotic and autophagy dependent cell death in many cancer cell lines such as (MDA-MB-435, SK-BR-3 MDA MB 231) however, cytotoxic effect was more severe in malignant cell lines compared to premalignant, non-malignant and non-malignant

immortalized cells [84]. JS-K induced autophagic and apoptotic cell death in mouse tumor xenograft and ovarian cancer cells in vitro [85]. A variety of drugs have been found to affect autophagy and autophagy dependent cell death through modulation of either NOS or NO [86]. In cardiac HL1 cells, LPS induced autophagy was found to be dependent on NO [87]. Administration of Cocaine triggers autophagic cell death via NO-GAPDH axis. S-Nitrosylation of GADPH was found to be essential in this event [88]. Oxygen–glucose deprivation (OGD) induced autophagy was found to be attenuated by eNOS knockdown in endothelial cells [89]. Cotreatment with Vitamin D and menadione prevent MCF7 proliferation through oxidative/nitrosative stress and induced autophagy [90]. Caloric restriction protects kidney cells from ischaemia/reperfusion (I/R) injury by upregulation of autophagy via eNOS-PGC-1α axis [91]. Platinum coated gold nanoparticles were found to induce non-apoptotic cell death in A549 cells with features like NO mediated mitochondrial dysfunction and increased autophagy [92]. In breast cancer, quinacrine initiate autophagic and apoptotic cell death. Quinacrine (QC) has been reported to upregulate p21 and DR5 by inhibiting mTOR-PI3K-Akt pathway which leads to excessive generation of ROS and RNS. This induced both autophagy and apoptosis through the increased interaction between DR5 and p21 in the death-inducing signalling complex (DISC) [93]. Nitro polycyclic aromatic hydrocarbons are known substrates of NOS, Nitro reductase and Xanthine Oxidase. One such metabolite has shown to elicit caspase independent RCD (having autophagic characters) in Hepa-1c1c7 cell [94]. In Oxaliplatin resistant cells, cotreatment with Oxaliplatin and Cannabidiol have been reported to cause autophagic cell death by changing expression of p-NOS3 and NO, RNS generation [95]. HeLa cells subjected to Silibinin (extract from Silybummarianum) showed increased ROS/RNS generation accompanied with p53 mediated GSH depletion that results into cell mortality [96, 97]. From the same group, it was reported that Silibinin mediated cell death regulated by ROS-JNK-p53 positive feedback loop. Silibinin upregulation increased ROS-p-JNK mediated upregulation of p53 which in turn increased ROS generation and mitochondrial dysregulation by modulating MMP and PUMA [98]. Similar effect was observed in A431 cells [99]. In *Clamydomonas reinhardtii*, high intensity light increases NO that interacts with H_2O_2 and generates ROS/RNS stress which in turn induces upregulation of autophagic genes and cause autophagic cell death [100]. Gangliosides activate NF-κB that a) Induces autophagic cell death and b) Increase inflammatory response through iNOS-NO generation in astrocyte [101]. Erythrocytic stage of *Plasmodium falciparum* shows autophagy like cell death characterized by cytoplasmic vacuolization under SNP treatment [102]. Air pollutants with aerodynamic diameter 2.5 μM upregulates inflammatory cytokines, NOS2 and cause autophagy dependent cell death of bronchial epithelial cells [103]. In human bronchial cells single wall carbon nanotubes elicit autophagic cell death by causing mitochondrial dysregulation and NO synthesis [104].

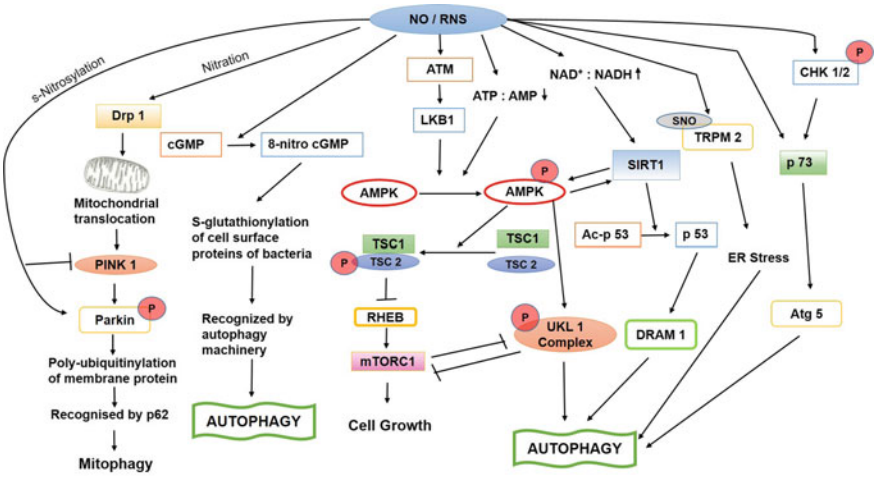

Fig. 9.1 Nitric oxide can induce autophagy by various mechanisms. Nitrosative stress activates AMPK either by inducing ATM pathway or by decreasing ATP/AMP ratio. Activated AMPK phosphorylates TSC2 and enhances TSC1/TSC2 interaction which in turn blocks mTORC1 by inactivating RHEB. p-AMPK activates ULK1 complex to induce autophagy. Nitrosative stress can also increase NAD^+/NADH to induce SIRT1. SIRT1 and AMPK can be in a feedback activating network. SIRT1 de-acetylates p53 to induce autophagy by DRAM1 upregulation. Upregulation of p73 by NO/RNS directly or by CHK1 can induce autophagy by activating ATG 5. NO induced TRPM2 S-nitrosylation can also induce autophagy by causing ER stress. Formation of 8-nitro-cGMP can also activate autophagy by S-glutathionylation of bacterial surface proteins. NO mediated nitration of DRP1 enhances its translocation to mitochondria leading to Parkin phosphorylation by PINK1 which finally activates mitophagy. NO mediated S-nitrosylation of PINK1 or Parkin can inactivate or activate respective proteins to regulate autophagy

NO Mediated Inhibition of Autophagy

On the contrary to the reports indicating positive regulatory role of NO in inducing autophagy, inhibitory effects of NO on autophagy has also been well documented in previous studies (Fig. 9.2). The most extensive evidence of the negative impact of Nitric Oxide on Autophagy was reported by Sarkar et al. [105]. They showed that treatment with different NO donors such as DEANONOate, DETANONOate or SIN-1 were able to inhibit autophagy in primary cortical neurones, HeLa cells and in Huntington disease models in vivo. Inhibition of NOS had the same effect on HEK293 cells. NO was found to inhibit autophagy by two separate pathways independent of cGMP. S-Nitrosylated JNK1 (p-JNK1 level was reduced) was found to be unable to phosphorylated Bcl2 which hindered Beclin1-hVps34 complex formation leading to inhibition of autophagosome formation (inhibition of early autophagosome formation was also prominent by reduced expression of Atg16). In fact, there have been previous reports suggesting JNK1 S-nitrosylation could reduce its phosphorylation modification [106, 107]. In meniscal cells NO donor inhibits autophagy by JNK1 suppression [108]. It is important to mention that JNK1 can transactivate autophagic

machinery through DRAM1 and Sestrin 2 [109–111]. The second pathway involved inhibition of IKK - AMPK - TSC2 mediated inactivation of mTORC1. IKKβ has been found to be nitrosylated in presence of NO. S-nitrosylation of IKKβ leads to its inactivation by reduced phosphorylation was previously reported [112–113]. Lee et al. stated that IKKβ is also able to activate m-TOR and increase tumor angiogenesis by interacting with TSC1 following its inactivation by phosphorylation [114]. BCL2 protein, though majorly related to its role in apoptosis it also plays a crucial role in autophagy. Under starvation JNK1 mediated phosphorylation of BCL 2 or DAPK mediated Beclin 1 phosphorylation releases Beclin 1 from BCL-Beclin 1 complex to induce autophagy [115–117]. Interestingly, BCL2 has also been demonstrated to be S-nitrosylated at Cys158 and Cys229 [118, 119]. Furthermore, NO mediated oxidation, S-nitrosylation of Bcl2 was found to inhibit its ubiquitination degradation [118]. Interestingly, the contradiction between activation and inhibition of AMPK by NO can be explained by the fact that AMPK phosphorylation is also regulated by lowered ATP/AMP ratio or by its upstream liver kinase B1 (LKB1) or through sGC-Ca^{2+}-CaMKKβ, eNOS and inactivation of these components due to post translational modifications or mutation might have a severe effect on the outcome [120, 121]. Thus the effect of NO might be cell specific or depend upon crosstalk between these kinases in this case. In human melanoma cells A375, inhibition of iNOS by pharmacological inhibitor or by the use of si-RNA resulted in suppression of p-mTOR and its downstream targets - p70S6K, pS6RP, p-4EBP1 are responsible for protein translation and cell proliferation. Exogenous NO was found to reversibly nitrosylate TSC2 (along with Bcl2) and that affected its interaction with TSC1 and GTPase activity. Moreover, NO has been reported to reverse the inhibitory effect of BRAF inhibitor vemurafenib on mTOR [122]. Intriguingly previous reports suggest a positive correlation between high levels of BRAF and autophagy as BRAF has been known to activate ERK and inhibit mTORC signalling [123]. Autophagy inhibitor bafilomycin (BAF) was found to inhibit insulin induced eNOS and this suggests NO bioavailability is also autophagy dependent [124]. In LPS induce macrophages; IRF-1 induces mTOR by eliciting NO production by iNOS [125]. In Ovine Trophectoderm cell, administration of arginine which is the substrate for endogenous NO production, elicits PI3K-Akt pathway and subsequent mTOR activation [126]. Previously Rapamycin was found to inhibit eNOS activity through PKB/Akt pathway causing reduction in NO synthesis thus the idea of positive correlation among eNOS and mTOR cannot be neglected [127, 128]. NO nitrosylates PTEN (a known suppressor of PI3K I/AKT/mTOR pathway) at Cys 83 site causing its subsequent degradation by NEDD4-1 and activation of Akt-mTOR pathway which results in inhibition of autophagy [129–133]. Many drugs have been shown to selectively upregulate particular cell death pathway via modifying expression of NOS enzymes. Very recently 2 enantiomers of Goniothalamin (Goniothalamus derived styryl lactone) have been shown to anti proliferative effect on renal cancer cells by reducing nNOS and eNOS expression which was further increased by co administration of L-NAME. Interestingly, while the natural R enantiomer was found to induce apoptosis, the synthetic one (ENT1) induced autophagic cell death [134]. In hepatic stellate cells NO was demonstrated to inhibit autophagy while inducing

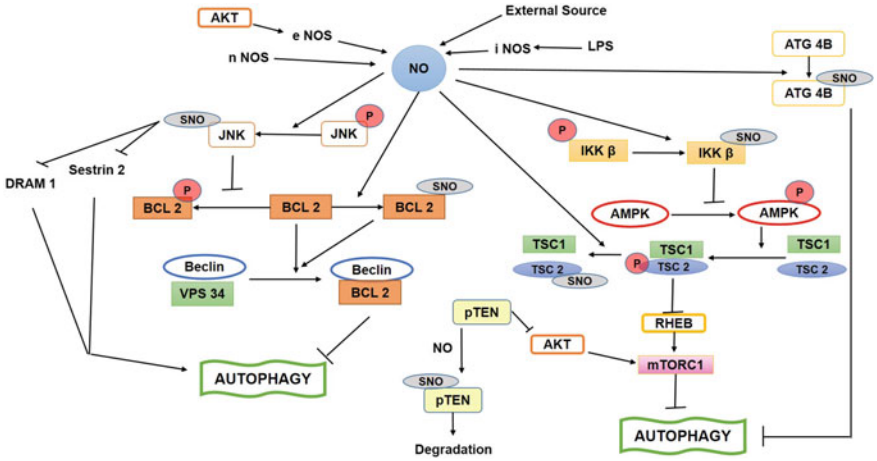

Fig. 9.2 Different pathways are involved to inhibit autophagy by nitric oxide. S-Nitrosylation of JNK and BCL2 proteins enhance BCL2-Beclin interaction and thereby inhibit autophagy induction. JNK S-nitrosylation can also inhibit autophagy by blocking DRAM 1 and Sestrin 2. IKKβ S-nitrosylation by NO/RNS blocks AMPK activation to inhibit autophagy. NO can also S-nitrosylate TSC2 protein to block autophagy or can S-nitrosylate PTEN to induce its degradation culminating in inhibition of autophagy. NO mediated S-nitrosylation of Atg 4B can also inhibit autophagy by blocking LC3B formation

apoptosis [135]. Another way by which NO inhibits autophagy is by activating Akt-mTORC1. Akt phosphorylates TSC2 [136], thus activates mTOR. NO mediated PTM of PI3K and JAK-STAT pathway leads to upregulation of Akt [137, 138]. In glioma cell, hypothermic shock and administration of NO donor has been reported to inhibit autophagy [139]. In neural culture cell and hippocampus of GK (Goto–Kakizaki) rat high glucose stress cause S-nitrosylation of ATG4B by NO and impair autophagy [140].

Positive Interaction Between Nitric Oxide and Inflammasome

Several studies indicate a positive correlation between pyroptosis and NO (Fig. 9.3). Cytoplasmic flagellin triggered NLRC4 induces NOS2 activation through Caspase1 and NF-κB and PARP1 activation [141]. NLRC4 and Naip5 inflammasome can also upregulate iNOS expression in macrophages treated with extra or intracellular flagellin in Caspase1 independent or dependent manner respectively [142]. On the other hand, inhibition of iNOS or nNOS has been found to reduce neural inflammation by abrogating NLR1, NLR3, Caspase1 and IL-β1 [143]. Overexpression of iNOS and nNOS cause cells to accumulate in G_0/G_1 phase of cell cycle, alter mitochondrial membrane potential, reduced PI3K/Akt/mTOR signalling, increased

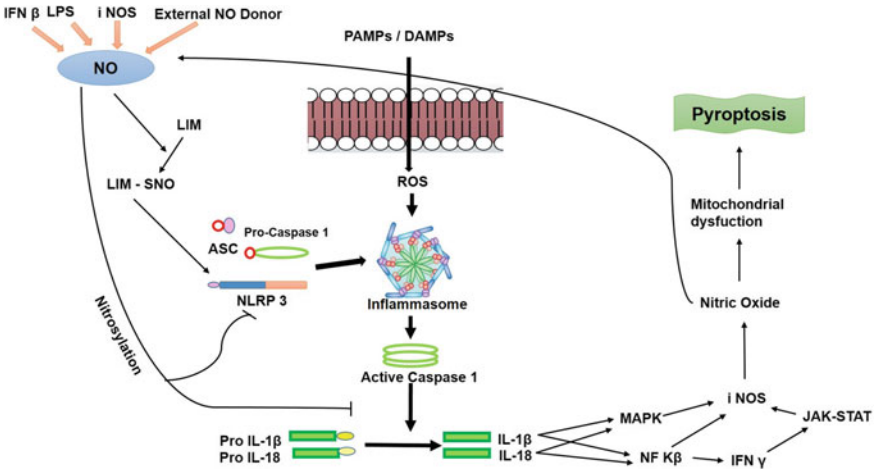

Fig. 9.3 Nitrosative stress can regulate pyroptotic cell death. Inflammasome is formed in response to PAMPs and DAMPs. Active caspase activates IL-18 and IL-1β to induce NO generation either in MAPK dependent pathway or in NF-κβ–INFγ–JAK-STAT mediated pathway. NO generation from iNOS leads to mitochondrial dysfunction and pyroptosis. NO can also activate NLRP3, a key component of inflammasome by nitrosylating LIM protein which aids in pyroptosis. On the other hand, direct S-nitrosylation of NLRP3 and Caspase1 by NO results in inhibition of pyroptosis

generation of ROS and NOS and upregulation of JNK-p38 MAPK-ERK1/2 pathways in K562 leukemic cells. Apoptotic gene microarray, western blot analysis and gene microarray showed upregulation of extrinsic and intrinsic apoptotic pathway and induction of caspase-1/4 and caspase-3 in iNOS and nNOS expressing cells respectively. Treatment with LPS in iNOS expressing cell showed an increase in release of IL-1β indicating overexpression of iNOS primes the cells towards pyroptotic cell death whereas nNOS overexpressing K562 stimulated apoptotic cell death [144]. Pyroptosis is often associated with mitochondrial dysfunctionality, disruption of mitochondrial membrane, electron transport chain, excessive ROS generation and a shift from oxidative respiration to glycolysis to produce more ATP. Fleetwood et al. reported that macrophage cells when treated with OMV (outer membrane vesicle) of *P.gingivalis*, showed increased iNOS/NO expression, secretion of pro-inflammatory cytokines and increased glycolysis. They suggested that NO modulate mitochondrial activity by itaconate generation that might have a role in induction of pyroptosis [145].

NO as Inflammasome Inhibitor

Though the previously mentioned studies show the role of NO in inflammasome inductions, some reports have also been documented about the inhibitory role of NO in inflammasome activation. Nitric Oxide can exhibit cytoprotective activity

and inhibit inflammasome activation. In fact, nitric oxide acts as a vital regulator of inflammation and host- pathogen interactions. It is well known that host cell produces NO and RNS as a defence mechanism, interestingly there are few denitrifying bacteria that can produce NO to inactivate inflammatory response of the host cell. *Brucella abortus* is such bacteria which have been found to produce NO with its nitrate reductase enzyme within the host cell and the NO thus produced reduces secretion of proinflammatory cytokines via suppression of inflammasome [146]. In mice, LPS induces hyperalgesia (an inflammatory condition characterized with enhanced sensation of pain and reduced pain threshold), which was found to be associated with rise in expression of NF-κB, Caspase 1 p20, Caspase 11, p20, NLRP3, ASC, NOX2, $gp91^{phox}$, $gp47^{phox}$, IL-1β and protein nitration. LPS has also been found to reduce expression of iNOS, eNOS and overall NO bioavailability. MCC950 (3 mg/kg dose), a NLRP3 inhibitor prevented LPS mediated activation of NF-κB, inflammatory complex proteins and increased expression of NOS and NO level indicating antagonistic relationship between NO and NLRP3 [147]. NO prevents mitochondrial dysfunctionality and inhibits NLRP3 mediated activation of Apoptosis associated spec like protein containing a CARD (ASC) inflammasome, ASC oligomerization, ASC caspase1 colocalization, caspase1 activation and lL-1β secretion in LPS primed human and in murine cells treated with pyroptotic inducers such as ATP, nigericinetc (Fig. 9.3) [148].

Inflammasome and S-nitrosylation

NO can also directly modulate the key proteins present in inflammasome positively or negatively via S-nitrosylation. Endogenous NO can inhibit NLRP3 activation in response to IFN-β or long time exposure to LPS and SNAP, a NO donor has been found to directly inhibit NLRP3 by S- nitrosylation. However, NLRC4 and AIM2 seemed to have more resistance against NO mediated inactivation [149]. LPS induced NO prevents IL-1β secretion from macrophages by S-nitrosylating NLRP3, Caspase1 and pro-apoptotic Akt [150]. IFN-γ or LPS primed macrophage and RAW264.7 cells showed reduced Caspase-1 activity and IL-1β level in response to NO donor SNAP. While NO inhibitor NG-Monomethyl-L-Arginine reversed the inhibitory effect on caspase1. Treatment with DTT reversed caspase1 inhibition by NO suggesting involvement of S- nitrosylation [151].

On the other hand, NO can help in NLRP3 activation through S-nitrosylation of NLRP3 associated proteins. In Cardiac hypertrophy, a condition characterized by increase in cardiac muscle fibre or cell mass due to prolonged stress or injury, S-nitrosylation of Muscle Lim Protein at Cys79 site aided NLRP3 activation. Thoracic Aortic Constriction or ANG II induced hypertrophy showed an alleviated GSNOR level which was associated with increased SNO-MLP. Furthermore, SNO-MLP interacted with TLR3 and RIPK3 without affecting cellular mortality and these complexes activated NLRP3 and induced IL-1β secretion [152]. Taken together the knowledge of relationship between NO, inflammasome and pyroptosis still remains

fragmentary; however, it is clear that they are critically regulated by cellular redox system, cytokines and other signalling molecules.

Ferroptosis and Nitric Oxide

In recent reports NO has been documented to play a regulatory role in ferroptosis induction.

Intriguingly, a recent study demonstrated in M1 macrophage that does not show sensitivity to RSL3 (GPx4 inhibitor) induced ferroptosis, knockdown of iNOS in M1 type RAW 264.7, EOC 20 or microglial cells induced cell mortality. Moreover, RAW 264.7 and MLE cells when primed with LPS and IFN-γ showed increased level of iNOS/NO and provided protection against ferroptosis. On the other hand, M2 cells which do not express iNOS/NO undergo cell death under different ferroptotic stimuli such as RSL3, ML162 or IKE which was abrogated by overexpression iNOS or use of NO donors like DPTA NONOate or DETANONOate [153]. Furthermore, using in vivo and in vitro study they found out that the activity of iNOS can compensate antiferroptotic functions of GPx4. Employing different experiments such as genetic knockdown, fluorescence microscopy, MS etc. it was found that RSL3 mediated ferroptosis was associated with accumulation of lipid hydroperoxides, oxidised PE, 15-HP ETE-PE and 15 LOXA enzyme which can be abolished by endogenous or exogenous NO. In light of this data it was evident that iNOS can bestow resistance against ferroptosis by two distinct mechanisms i.e.

(a) NO can inhibit catalytic activity of LOXA by reducing its non-haem iron and thereby inhibits lipid peroxidation which in turn blocks ferroptosis. Indeed, dioxygen molecule mediated inhibition of LOX family protein has been reported previously [154], and (b) NO interacts with lipid radical intermediates to produce oxidatively truncated derivatives which are not capable of inducing ferroptosis [155]. Now it is worthy of mentioning that the balance between NO and RNS is critically important for regulation of lipid peroxidation. In fact, NOS uncoupling in absence of BH_4 has been reported to induce superoxide generation instead of NO as mentioned earlier [155]. Interestingly, a study showed that upregulation of BH_4 via overexpression of GTP Cyclohydrolase-1 can inhibit ferroptosis independent of iNOS expression [156]. Various reports have showed a connection between upregulated RNS and induction of ferroptosis. Fer-1, a ferroptotic inhibitor has been found to inhibit ROS-RNS production in SH-5Y5y cells administered with rotenone [157]. Deng et al., revealed that in Concanavalin A (ConA) induced liver hepatitis and Erastin (a common ferroptotic inducer) treated LO2 cells, ferroptosis was associated with increased expression of redox activated iron, malondialdehyde, iNOS, $ONOO^-$ and 3-NitroTyrosine. However, treatment with 1400 W (an iNOS inhibitor) and PDC (peroxynitrite scavenger) could revert these effect. Using GdCl3 (another iNOS inhibitor) they found that reduction in the number of Kupffer cell can help to subdue ferroptosis. In the light of these data they suggested that as Kupffer cells are known source of NO [158],

the overall production of RNS accompanied with redox iron and other inflammatory condition regulate AIH associated ferroptosis. Moreover, using in vivo and in vitro model they found that the induction of ferroptosis in hepatocytes was associated with reduced expression of Caveolin-1 [159]. Indeed, Cav-1 has been found to protect against binge drinking-related liver injury and hepatic ischemia–reperfusion injury by regulating nitrosative stress [160, 161]. In a parallel study it was found that indoleamine 2, 3-dioxygenase 1 (IDO1) works upstream of antiport system Xc^-in ConA aggravated ferroptosis in AIH (Autoimmunity mediated hepatitis). IDO1 negatively regulates Xc^- and GPx4, helps in accumulation of RNS thus provide sensitivity to ferroptosis [162].

Lipid Oxidation, Iron and Nitrosative Stress

As mentioned earlier, the two metabolic keystones of ferroptosis are lipid peroxidation and iron metabolism. Peroxynitrite can modify biomolecules such as unsaturated fatty acids, thiols and protein tyrosine residues, arachidonic acid, low density lipoprotein by nitration or oxidation and produce nitrito, nitro, nitroso, peroxo, nitrated lipid monoaldehyde conjugates, lipid hydroperoxide. Both peroxynitrite anion and peroxynitrous acid (ONOOH) can participate in these reaction in a pH dependent manner. A recent study demonstrated that a group of drug consists of a Phenothiazine core can inhibit NO consumption in brain and inhibit lipid peroxidation [163]. However, antioxidant properties of Nitroxides are not yet clear [164]. $ONOO^-$ has been found to negatively regulate COX1, COX2 through nitration at Tyr430, 385 [19]. The primary substrates of lipid oxidation are phosphatidyl ethanolamine (PE) phospholipids and esterified polyunsaturated fatty acids (PUFA) which are catalysed to produce lipid peroxides and aldehydes [165]. Lipoxygenase family (ALOX5, ALOX12, ALOX15) catalyse PUFA while free fatty acids can be oxidized by cyclooxygenase and cytochrome 450 [166–168]. Acetyl CoA Synthetase Long chain family member 4 (ACSL4) catalysed Arachidonoyl (AA) or Adrenoyl (AdA) acetyl CoA are esterified by Lyso-phosphatidylcholine Acetyltransferase 3 (LPCTA3) into Phosphatidyl ethanolamines (AA PE, AdA PE) which are finally oxidised by 15-lipoxygenase to generate lipid hydroperoxides [169, 170]. Intracellular Iron pool is maintained in four forms- vesicular iron, labile iron pool (LIP) in Fe^{+2} form, functional iron and stored in proteins. Fe facilities ROS generation trough Fenton reaction, where Fe^{2+}catalyzes H_2O_2, to produce hydroxyl radicals. Fe also assists ALOXs to produce PUFA-PEOH and NOXs to produce NO. A complex interaction is present between NO, $ONOO^-$ and iron. Having an affinity for heme and nonheme iron [171], NO is also capable of influencing the binding of iron responsive elements to iron regulatory proteins [172]. NO reacts with ribonucleotide reductase, ferritin and heme containing proteins [171]. S- nitrosylation of iron regulatory protein 2 (IRP2) results in its degradation followed by accumulation of iron and ferritin in cell [173]. HNO nitroxyl ion can react with thiols and heme proteins [171]. NO

regulate ferritin, HO-1 (catalyzes Fe^{+2} from heme protein) and ferroprotein transcription via Nrf2/ARE axis [174]. Nrf2 Is a transcription factor that under oxidative stress translocate to nucleus and activates GCLC (glutamate cysteine ligase), GPx4, Transferrin receptor (TfR1), Ferritin, Ferroprotein (FPn), HO-1 (extracts Fe from heme) and thus provides cytoprotectivity in cancer cells [175, 176]. Intriguingly, NO has been found to inhibit Fenton reaction [177]. The bidirectional relationship has been supported by more observations. In mice lacking Nrf2, upregulation of eNOS provide protection against myocardial ischemia reperfusion (I/R) injury [178]. ERK and p38 pathways are involved in NO mediated translocation of Nrf2 and antioxidant response in vascular endothelium [179]. While NO has been found to activate IRP1, NO^+, $ONOO^-$ are capable of inhibiting IRP1 and IRP2 [180, 181] and this helps to understand how macrophage polarity can influence activities of IRP1 and IRP2 [182].

Peroxynitrite Mediated DNA Modifications

N_2O_3 and ONOOH (peroxynitrous acid) catalyse nitrosation of primary and secondary amines and nucleic acid [183]. Peroxynitrite is able to damage both nitrogenous base and sugar phosphate backbone [184]. Peroxynitrite ($ONOO^-$) removes hydrogen atom from deoxyribose forming single strand breaks (SSBs) that can be recognized by PARP1 [185, 186]. SSB forming interaction between $ONOO^-$ and 2'deoxyriobose or deoxynucleotides yields malondialdehyde [187].

Peroxynitrite ($ONOO^-$) affects cytosine methylation, and convert guanine into 8-oxoguanine, 8-nitoguanine. Nitration of 3-alkyladenine DNA glycosylase (AAG) hinders DNA repair [188, 189]. 5-hydroxy hydantoin, 5-hydroxy methyl uracil, thymine glycol,4–6-diamino-5- formamide pyramide (FAPy adenine), 2,6-diamino-5-formamide pyrimidine (FAPy Guanine), 8 oxoadenine, hypoxanthine are notable among other base modification that are caused by nitrosative stress [184]. Moreover, exo- and endogenous NO inhibits binding of DNA repairing proteins like ligase, alkyl transferase, SP1 etc. at the repair site [190]. Peroxynitrite can also hinder DNA repair by inactivating PARP by S-nitrosylation in its zinc motif [191]. In fact, NO can S-nitrosylate PARP and regulate its binding to iNOS promoter which creates a negative feedback loop [192]. In macrophage induced tumor cell, iNOS was found to disrupt dNTP supply by ribonucleotide reductase (RNR) thus hamper DNA synthesis and impart cytotoxicity [193].

NO Mediated Parthanatos

The pathophysiology of COPD includes remodelling of bronchial tissue due to death of human bronchial epithelial cells. This event is often found associated with cigarette smoking which imparts oxidative stress in human bronchial epithelial cells (HBE

cells) and ultimately develops ROS, $ONOO^-$ mediated DNA stand breaks [194, 195]. Kunzi et al. observed that fully differentiated HBE cells exposed at air–liquid interface of smoke resulted in PARP1 activation and subsequent nuclear translocation of AIF (apoptosis inducing factor) and Endonuclease G leading to parthanatos (Fig. 9.4) [196]. LPS mediated drastic production of superoxide and reduced iNOS activity in murine peritoneal as well as J774.2 macrophages orchestrate the production of $ONOO^-$ that causes $NAD^+$and ATP depletion and PARP activation following DNA damage [197]. Abrogation of nicotinamide phosphoribosyl transferase (NAMPT) involved in NAD biosynthesis increases ROS, RNS, CO_2, H_2O_2 expression that induce in Jurkat an ML2 cells upon APO866 treatment [198]. Parthanatos is also documented to be involved in neural degradation. Death of basal ganglia and accumulation of α-syn fibrils (termed as Lewy body) are observed in Parkinson's disease. In primary cultured neuron or mice brain, administration of α-syn PFF induces PARP activation by inducing NO expression and DNA damage. PARP1 increases PAR accumulation which accelerates α-syn fibrillization and conversion to more toxic form and activates parthanatos. Indeed, PAR- α-syn was found to be more neurotoxic [199]. In human cortical neuron culture system, excitotoxicity or ischemia developed due to O_2 and glucose deprivation triggers parthanatos which has been found to be regulated by NMDA (N-methyl D-asparate) receptors, NO and PARP. Using nNOS inhibitor (NPLA) and PARP inhibitors and knockdown of PARP it was discovered that NO acts upstream of PARP as PARP inhibition was only able to abolish cell death but could not alter NMDA or NO expression. In essence, glutamate (can be upregulated by nNOS) triggers NMDA receptor and increases Ca^{2+} influx and nNOS expression that causes $ONOO^-$ mediated DNA damage [200]. Administration of exogenous NO has been found to induce PAR activation in RAT brain [201]. The positive association between nNOS and excitotoxicity was further justified by observation of inhibition of neuronal death upon NPLA administration and occurrence of less cell mortality in retinoic acid deprived neuron culture where nNOS expression remains marginal [200]. In contradiction, nNOS derived NO can inactivate NMDA receptor by S-nitrosylation and confer protection against excitotoxicity [202, 203].

NO and RNS as Necroptosis Inducers

The association between Nitric Oxide and necroptosis is quite complicated. NO mediated PTMs have been found to exert profound effect on major effector proteins involved in necroptosis (Fig. 9.5). RNS trigger nitration of proteins and impaired mitochondrial respiration by modulating respiratory chain complex 1 leading to apoptosis and RIPK1, RIPK3 dependent necrosis [204]. In line with this data another publication showed that NO mediated nitration of mitochondrial complex I subunit NDUFB8 alters mitochondrial homeostasis and triggers RIPK1/3 dependent necrosis [205]. These discoveries prove peroxynitrite and RNS to be potent inducer of necroptosis. Vast number of drugs has been found to upregulate proinflammatory NO which

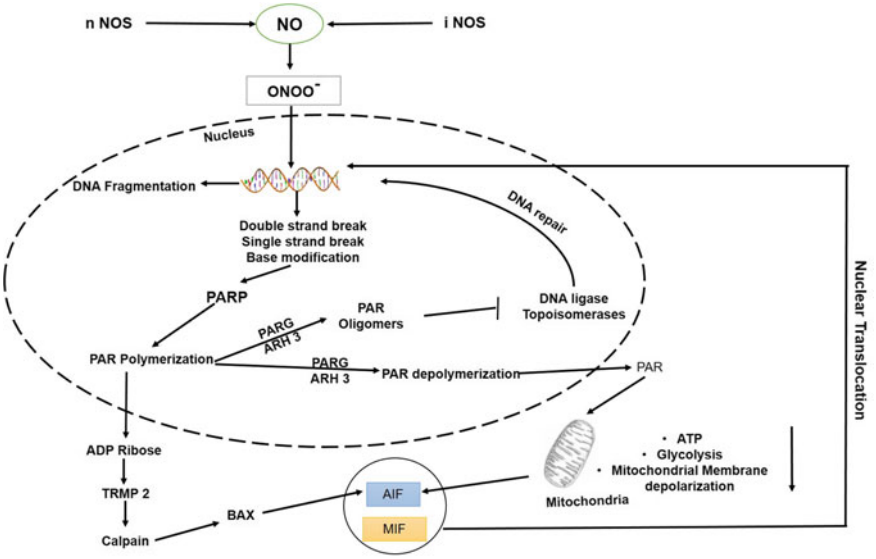

Fig. 9.4 Nitric Oxide mediated Parthanatos. Formation of peroxynitrite from NO can lead to DNA damage. Excessive DNA damage leads to PARP hyper-activation resulting in PAR polymerization. PARG and ARH3 mediated PAR depolymerization results in the formation of PAR oligomers. PAR oligomers block the DNA repair enzymes like topoisomerase and DNA ligase. PAR can also translocate to mitochondria from nucleus leading to mitochondrial membrane depolarization drop in glycolysis rate and ATP level. BAX activation in an TRMP2-calpain mediated pathway or PAR translocation to mitochondria leads to release of AIF from mitochondria. Released AIF along with MIF further translocate to nucleus to aggravate DNA damage and parthanatos

then elicit TNF α, IL-6 expression etc. and thus triggers necroptosis or RIPK dependent apoptosis in many scenarios. Recent study projected NO to be a regulator of BPA (BisphenolA) mediated necroptosis in SH-SYY cells [206]. In A549 lung cancer cells, mediated necroptosis which involves H_2O_2 dependent JNK-iNOS upregulation [207]. Gallic Acid has been identified to confer protection against neuroinflammation caused by increased apoptosis and necroptosis by lowering proinflammatory IL-β and NO [208]. eNOS plays a protective role in myocardial IR injury, recently a drug named Baicalin has been shown to reduce this damage by upregulation of PI3k-ATK-eNOS mediated NO production and down regulation of necroptosis which accounts for about 50% cell death during IR injuries in heart [209]. Nonetheless, S-nitrosylation has been proven to be a great modulator of this pathway. Glyceryl-trinitrate (GTN) promotes S-nitrosylation of cIAP1, thus treatment with GTN inhibits ubiquitination and by stabilizing RIPK1 it induces RIPK1 dependent apoptosis [210]. Interestingly, NO has been found to inhibit many Caspases [3, 8, 9] through S-nitrosylation and their inactivity might evoke the cells to undergo necroptosis [211–214]. Moreover, exogenous and endogenous NO can directly facilitate necroptosis by nitrosylating RIPK3. In HEK293 cells, treated with GSNO or NMDAR-nNOS mediated cerebral ischemia, S-nitrosylation of RIPK3 at cys119

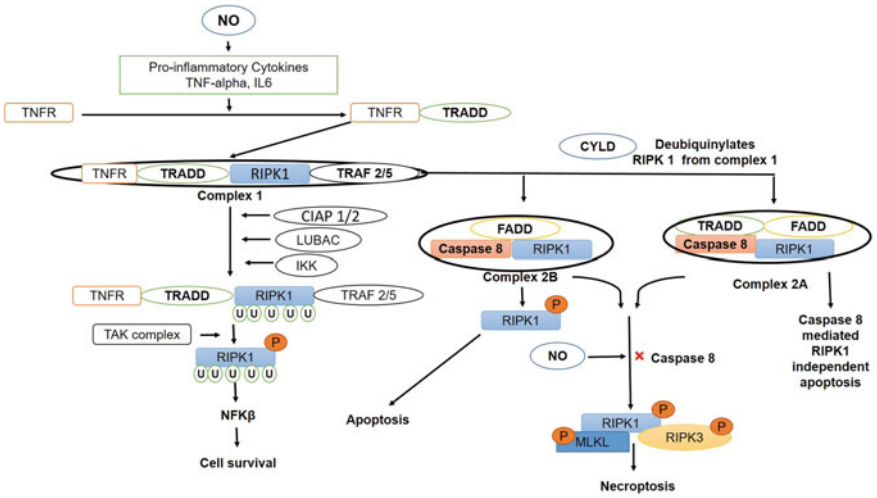

Fig. 9.5 Necroptosis can be regulated by nitric oxide. Release of pro-inflammatory cytokines like TNF-α, IL-6 induce association of TNFR and TRADD which in turn leads to formation of Complex-I. Further association of CIAP1/2, LUBAC and IKK induces RIPK1 ubiquitinylation in the Complex-I. RIPK 1 phosphorylation by TAK complex culminates in NF-κB activation and cell survival. CYLD can de-ubiquinylate RIPK 1 to form either Complex 2B or Complex-2A. Complex 2B formation results in RIPK1 phosphorylation and apoptosis; whereas Complex 2A formation leads to RIPK1 independent caspase 8 mediated apoptosis. NO enhances the phosphorylation of RIPK1 and RIPK3 by S-nitrosylation and also inactivates Caspase 8 which leads to increased interaction between RIPK1 and RIPK3 resulting in phosphorylation and activation of MLKL. Activation of MLKL leads to induction of necroptosis

facilitates its activation by phosphorylation. This phosphorylation further facilitates interaction between RIPK3 and RIPK1 [215].

Role of RNS in NETs Formation

nNOS and iNOS produce considerable amount of NO and it regulates free radical generation in neutrophils [216–218] in a NOX dependent (ERK mediated activation of NADPH oxidase) and independent manner [219]. NETs are involved in pathology of many NO stress related disease such as sepsis, Alzheimer's etc. [2, 220–222]. Patel et al. provided the first evidence of involvement of NO or RNS in NETosis in human Neutrophil. Addition of NO donor SNAP or SNP to adherent PMN induced NETs formation in a time and concentration dependent manner. Furthermore, inhibitor study suggested the involvement of NOX, MPO and free radicals in NET generation by NO [223]. Later on, treatment with pure NO donor DetaNONOate (100–500 μM) was found to significantly increase NOX and myeloperoxidase (MPO) mediated superoxide and free radical production that in turn facilitated NETs release from

human neutrophil. Using confocal microscopy and PCR (for nuclear and mitochondrial DNA specific genes) they concluded that the NET contains both nuclear and mitochondrial DNA. Elastase was found to be abundant in these NETs. Platelets and THP-1 cells when incubated with NETs released IL-1β, IL-8 TNFα which further supports proinflammatory and pathogen killing properties of NETs [224]. Interestingly, these proinflammatory cytokines are capable of enhancing ROS/RNS generation which further attribute to NETs generation in SIRS (Systemic Inflammatory Response Syndrome) suggesting presence of a positive feedback mechanism [225]. PMA is a common inducer of NETosis. In murine bone marrow derived neutrophils, NOS inhibition by L-NAME attenuated effect of PMA. RAC 1 and 2, two Rho-GTPases are involved in ROS formation by NOX and they also regulate NOS activity [226]. Interestingly, RAC2 has been found to be involved in NO mediated NETosis [226, 227]. The role of NO derived RNS/ROS was further explored by Manda et al. It was found that exogenous NO and peroxynitrite mainly exert its function by production of RNS. Apart from NOX and MPO, the process is strictly dependent on PI3K and ROS generation and is autophagy independent. Astonishingly they found that RNS mediated NETs formation did not require histone citrullination for chromatin relaxation rather occurred due to H2A and H2B degradation. Most importantly they found that in human PMN, PMA or calcium ionophore induced NETosis can be diminished by inhibition of NOS or NO and $ONOO^-$ scavenging further confirming positive role of nNOS and eNOS in NETs formation. Moreover, in chronic granulomatous disease granulocyte, SNAP reduced NET formation which is plausible because of NOX deficiency. In this condition, NO produced by SNAP might not be able to produce RNS, however, administration of $ONOO^-$ successfully produced NETS suggesting they can act without involving NOX [228].

Conclusion and Further Perspective

In this review we tried to put together the pieces of information on the impact of NO and other RNS on various cellular moieties engaged in non-apoptotic RCDs. In spite of the complexity and diversity of NO and NO derived RNS, it is evident that the balance between NO and other radicals and their interaction with other proteins, lipids, metal ions tune the fate of cell survival. A wealth of data convicted nitrosative stress to be the accelerator of inflammation, tumour metastasis, autoimmunity and regulated or unregulated cell death. While excess NO, $ONOO^-$ have been proved to be detrimental for nucleic acid structure or integrity of mitochondria, ER or plasma membrane; contrarily NO at lower concentrations could act as cytoprotectant. Thus it seems that the effect of NO is indeed dependent on the redox status, pH, concentration, proximity and bioavailability of interacting molecules and other biochemical aspects. However, the effect of NO and its derivatives are not at all limited only by dysregulation of these cellular modalities. Over the past few decades, NO mediated S-nitrosylation, nitration, oxidation, S-glutathionylation have gained concern and tons of research are going on to dismantle their effect and their

implication to modulate cell signalling nexus. It is now clear that regulated cell death does not only include apoptosis but other highly complex non apoptotic pathways such as autophagy dependant pathway, necroptosis, pyroptosis, ferroptosis, parthanatos, etc are also involved. Moreover, all of them were found to be somewhat interconnected and to have specific impact on cellular pathology or physiology. While apoptosis is considered to suppress inflammation, other regulatory pathways such as necroptosis or parthanatos have been identified to trigger neuroinflammation. In fact, there are pathways designated to specific cells such as netosis that can be regulated by RNS. NO interacts with the key regulatory proteins of cell death such as Caspase, RIPK1/3, MLKL, NLRP3, GPx, PARP etc., modulates transcription factors such as p53, KEAP1, NF-κB etc. and triggers cytokine release or can act as secondary messenger which could initiate or inhibit the specific pathway. These vast activities make it understandable why the effect of nitric oxide depends on NO donor, the genetic background of the cell and RNS that are generated. In addition, it has been found that NO mediated inhibition of one pathway can induce another form of RCD. Although large numbers of studies have unveiled how ROS and thiol system control specific RCDs, they are mostly related to apoptosis and how exogenous and endogenous RNS are involved in regulation of non-apoptotic RCD is still quite not clear. From the early discrimination of three types of cell death, today RCD includes more than 12 forms and new pathways such as oxeiptosis, autosis [229, 230] are still getting unravelled. It is intriguing that NO has been found to interact with some of the modulator of these pathways such as KEAP1 (involved in oxieptosis) [231] which needs to be investigated further. NO mediated depletion of ATP has been found to upregulate AMPK which in turn upregulate autophagy by activating ULK1 or TSC2, on the other hand NO has been shown to activate MLKL [232]. Many tumor cells have been found to become resistant against a specific type of cell death pathway. So it would be beneficial if it is possible to decipher the alternative cell death pathway elicited by NO and RNS. Thus further elucidation is required to solve the puzzle so that NO or RNS can be used as novel therapeutic strategies.

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Chapter 10
Involvement of Nitric Oxide in Insulin Secretion to Carbohydrate Metabolism

Somanjana Khatua, Sarbashri Bank, and Krishnendu Acharya

Abstract Since the discovery of nitric oxide (NO) as an important mediator of vasoregulation, the molecule has been found to be involved in several physiological and pathological processes in human. One area of recent interest is the potential role of NO in modulation of insulin secretion. Emerging data suggest that NO augments insulin release from pancreatic beta cells through increasing intracellular Ca^{2+} level or via S-nitrosylation of glucokinase, as well as vasodilation of islet vasculature. Besides, synthesis of NO is also a prerequisite for effective insulin sensitivity in targeted tissues. Thus, NO is involved in glucose uptake and disrupted NO pathways play a role in pathogenesis of insulin resistance in hypertension, obesity and type 2 diabetes mellitus. In this review, we summarize the updated paradigms on the involvement of NO in insulin secretion from islets of Langerhans and glucose uptake by various tissue system. Hence, a better understanding of nitric oxide synthase (NOS)–NO system in regulation of glucose homeostasis can hopefully facilitate the development of new treatments.

Keywords Glucose uptake · Insulin sensing · Nitric oxide synthase · Pancreatic β cells · Skeletal muscle cell

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_10

Introduction

Nitric oxide (NO) is one of the smallest molecules in chemistry and its structural and functional properties have been studied by the chemists for years. NO was primarily considered as an environmental pollutant until the late 1980s, when the three-pioneer worker Furchgott, Ignarro and Moncada recognized NO as an endothelial-derived relaxing factor (EDRF). Their research showed that NO can work as a signalling factor for regulation of blood pressure and vasodilation, thereby triggering research worldwide [1–3]. In the year 1992, Science journal attributed NO as "Molecule of the Year" because hundreds of research articles unveiled the importance of this simple molecule from digestion to blood pressure regulation to antimicrobial defense [4]. In 1998, three pioneer workers of this field got Nobel prize for discovering the signaling role of NO in cardiovascular function. Since the discovery of its role in biological system, it received special attention from most of the branches of biological sciences like physiology, medicine, genetics and biochemistry. NO is produced by the enzyme nitric oxide synthase (NOS) which catalyzes a five electron oxidation of amidine nitrogen of amino acid L-arginine to an intermediate L-hydroxyarginine that is tightly bound to the enzyme to generate NO and L-citrulline (Fig. 10.1) [5]. The enzyme comprises three main isoforms: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) isoforms [6, 7]. Due to its ubiquity and versatile properties it has been recognized as a key molecule in different patho-physiological conditions as a regulator of blood pressure [8], signal molecule in smooth muscle and nerve [5, 9], inhibitor of platelet aggregation and addition [10], antioxidant [11], neuromodulator of central nervus system, inhibitor of neutrophil adhesion [5], modulator cytokine production [12], antithrombotic [13], wound healer [14], antineoplastic [15] and immune generator [16].

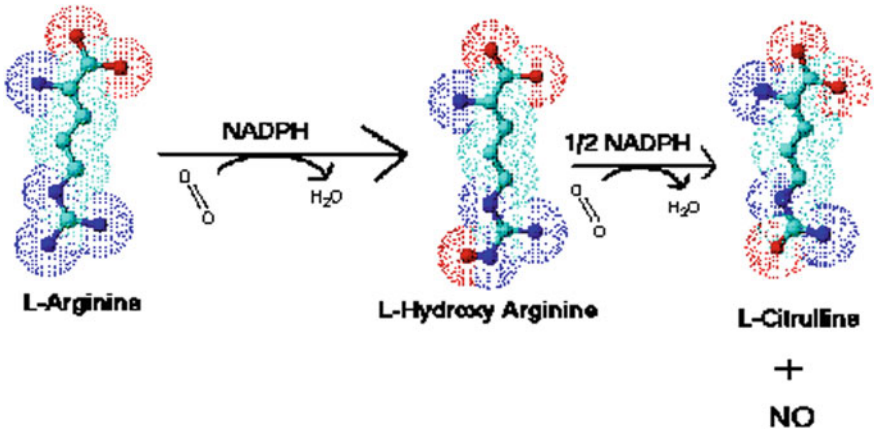

Fig. 10.1 Biosynthesis of NO: Nitric oxide synthase (NOS) catalyses a five electron oxidation of amidine nitrogen of L-arginine to generate NO and L-citrulline. L-hydroxyarginine is formed as an intermediate that is tightly bound to the enzyme

Recent data suggest that NO has an important role in glucose metabolism which is the primary source of metabolic energy for most cells of the body and is of critical importance for the brain and red blood cells. Rising of blood sugar level leads to secretion of insulin from pancreatic beta cells. Insulin, an endocrine dipeptide hormone composed of an A chain of 21 amino acids and a B chain of 30 amino acids connected together by disulphide bond with a molecular weight of ~6000 Da. It is primarily responsible for carbohydrate metabolism [17]. The key function of insulin is to help the entry of glucose and amino acids in three most responsible energy storage tissues i.e. muscle, liver and adipose tissue. After release to the circulatory system, insulin travels through the blood and binds to insulin receptor of the target cell. Binding of insulin with receptor initiates signal transduction and activates glucose transporter properties. Glucose transport protein binds to cell membrane to uptakes glucose leading to fall blood glucose level. Lowering of sugar level in the blood inhibits insulin secretion by β-cells through a negative feedback mechanism [17]. In resting state of the body, insulin acts as a primary hormonal regulator of metabolism [18, 19]. In the absence of insulin, glucose uptake by the tissue decreases and metabolism of lipids in adipocytes increases [20]. Major functions of insulin are (a) facilitating the transport of glucose, (b) decelerating the gluconeogenesis and glycogenesis, (c) stimulating enzyme system responsible for conversion of glucose to glycogen and (d) regulating lipogenesis, promoting protein synthesis and growth [17].

In this review, we have tried to draw a systematic correlation between NO and its involvement in insulin secretion to action on carbohydrate metabolism.

NO in Insulin Secretion

The pancreas is an organ of the digestive system which serves two major functions (a) exocrine function (helps in digestion) and (b) endocrine function (regulation of blood sugar level). Millions of islet of Langerhans are scattered within the pancreas and are responsible for the endocrine function. About three hundred β cells are present in each islets of Langerhans that secrete insulin, which is responsible for the regulation of glucose metabolism. Each β cell contains about one hundred insulin secretary granules. The release of insulin is stimulated by glucose and other secretagogues oscillate with a reproducible frequency [17].

Glucose enters β cells through glucose transporter protein (GLUT) [21]. The GLUT 2, glucose transporter isoform, is expressed in hepatocytes, pancreatic islet beta cells, intestinal mucosa, kidney and in the central nervous system. GLUT-2 is a facilitated diffusion glucose transporter with a high capacity of glucose transport and low affinity for glucose (Km 17 mM) [22, 23]. Glucose is then phosphorylated by glucokinase and pyruvate is generated through glycolysis in the cytoplasm of β cells [24]. Pyruvate metabolism by pyruvate dehydrogenase leads to increase adenosine triphosphate (ATP) in cytoplasm thereby changes ATP/ADP (adenosine diphosphate) ratio in the cytoplasm. Elevation of cytoplasmic ATP/ADP ratio blocks ATP-sensitive K^+ channels (K_{ATP}) in the β cells [25, 26]. As K_{ATP} channel is the primary determinant

of the membrane potential, closing of the channels depolarizes the membrane. The membrane depolarization opens Ca^{2+} channels (voltage-dependent Ca^{2+} channels) and subsequently elevates cytosolic Ca^{2+} concentration due to Ca^{2+} influx, which rapidly increases the release of insulin from storage granules via exocytosis [24].

In pancreatic β-cell, glucokinase is associated with insulin secretary granules which is controlled by interaction with NOS, reversed by S-nitrosylation of glucokinase [27–29]. Insulin secretion is regulated by NO and NO donor, sodium nitroprusside (SNP), that stimulates insulin secretion in rat islets. At the same time, increase of NO production and insulin secretion was also reported in HIT-T15 (clonal pancreatic β cell line) and in isolated mouse islets [30, 31]. Inhibition of NO production by N^G-Monomethyl-L-arginine monoacetate (L-NMMA) in rat islet or by scavenging of NO by carboxy-2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (CPTIO) in glucose-responsive INS-1 cells reduces insulin secretion [32, 33]. Treatment of Mim6 β-cells with NO resulted activation of pancreatic and duodenal homeobox factor-1, the insulin gene promoter thereby increases insulin mRNA level within the cells [34]. Similar observation was also recorded in isolated islet cells in rat [35, 36]. Henningsson et al. [37] reported that cNOS and iNOS of islet of Langerhans cell are activated differently by glucose concentration. Among three NOS isoforms (nNOS, eNOS and iNOS), nNOS is mainly found in insulin secretary granules (ISG) [38]. Exposure of β-cells with higher cytoplasmic glucose concentration (>10 mM) increases the level of expression of iNOS whereas it is not detectable at the basal glucose concentration (7 mM) [37, 39]. Increase of NO via nNOS activation stimulates glucokinase activity that mediates glucokinase dissociation from ISG and helps in insulin secretion [29]. cNOS derived NO is found to cause intracellular Ca^{2+} mobilization from mitochondria and endoplasmic reticulum or through the blocking of K_{ATP} channels (Fig. 10.2) [33, 40]. NO also induces cGMP (Cyclic GMP) elevation, sequestration of Ca^{2+} into ER, thereby protecting β-cells from increased Ca^{2+} concentration [41]. Furthermore, secreted insulin pool is also maintained by the inhibition of a cytosolic protease, an insulin degrading enzyme by NO via S-nitrosylation [42]. Decrease in insulin secretion is also observed in case of β-cells dysfunction by interleukin 1β [43].

NO in Insulin Sensing

Insulin binds to plasma membrane receptor to trigger its known physiological effects. Insulin stimulates the rate of entry of glucose into the cells in a selective fashion. The insulin receptor consists of two identical α chains protruding from the outer face of the plasma membrane and two transmembrane subunits with their carboxyl termini protruding into the cytosol [45, 46]. Alpha subunits of insulin receptor bind to insulin resulting a conformational change in the intracellular domains of the β-chains that contain the protein kinase activity which transfers a phosphoryl group from ATP to hydroxyl group of Tyr residues i.e. cis-auto inhibition to trans-auto phosphorylation of tyrosine kinase domain [44]. The binding of insulin to the receptors is the initial

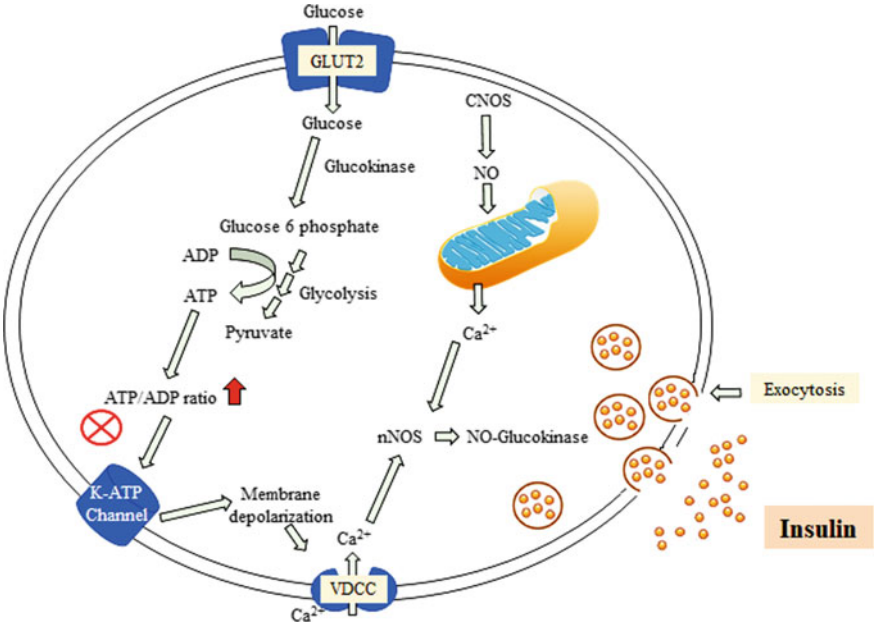

Fig. 10.2 Insulin secretion from pancreatic β cells

step in the signal transduction process and triggers the consumption and metabolism of glucose [45]. Similarly, membrane bound phosphatidylinositol 3-kinase is also activated by insulin which is involved in hormone signaling pathway. Administration of physiological concentration of insulin in mice model resulted in the reduction of plasma glucose concentration and formation of methaemoglobin signifies the production of NO in the system [13]. Kahn et al. [46] reported that incubation of different types of whole tissue from mice and human red blood cells with insulin showed NO production by activating a membrane bound NOS. Insulin does not show any effects on cytosolic NOS and the activity of insulin-activable membrane bound NOS (IANOS) is not dependent on ATP and NADPH, but the presence of Ca^{2+} is essential for the production of NO. The effect of NO on IANOS is biphasic in nature. At low concentrations of NO, it is activated, whereas at higher concentration of NO, the degree of stimulation decreases gradually. The purified IANOS showed some similarities to the insulin receptor itself [47]. Insulin activated NOS has a very important role in the pathophysiological condition where there is severe impairment of NO production [15]. Enzyme kinetics demonstrated that the insulin mediated activation of NOS activity is directly correlated with the increase in V_{max} and simultaneous decrease in K_m. The blocking of insulin receptor by anti-insulin receptor antibody also inhibits NO production. Use of L-NAME (N^G-Nitro-L-arginine Methyl Ester), a potent inhibitor of NOS, in the reaction mixture totally inhibits the insulin-inducible NOS activity and insulin activated carbohydrate metabolism both in vitro and in vivo. This inhibition by L-NAME has no effect on tyrosine and PI3-kinase activity which

signifies that activation of tyrosine kinase and PI3-kinase is not obligatory in the transduction of insulin effects for the carbohydrate metabolism [46]. In contrast, genestein mediated inhibition of tyrosine kinase or wortmannin mediated inhibition of PI3-kinase inhibits insulin mediated NO production [49]. Young et al. [48] also reported that NO can stimulate glucose transport and metabolism in vitro in the skeletal muscle of rat. Insulin increases eNOS activity by phosphorylation at serine 1177 in the endothelium cells through PI3-AKT-eNOS pathway, one of the major pathways for insulin mediated insulin action [50–53]. Kahn et al. [46] proposed NO as the "second messenger" of insulin for its diverse effects in various physiologic and pathologic events.

NO in Glucose Metabolism

NO has a significant role in glucose uptake and glucose transport [54, 55]. In glucose uptake, the role of NO was first understood when inhibition of NOS in rat limb muscle attenuated 2-deoxyglucose uptake [55]. This observation was further supported by the application of exogenous NO that increased the uptake of 2-deoxyglucose. Local infusion of L-NMMA, a well-known NOS inhibitor, inhibits glucose uptake in skeletal muscle [56]. Furthermore, Young et al. [48] reported that NO stimulates glucose transport and oxidation in vitro in rat skeletal muscle. Skeletal muscle is the primary target for glucose transport for glucose homeostasis during insulin stimulation. The occluded intracellular tubulo-vesicular reservoir of the cells stores glucose transporter protein 4 (GLUT 4). Insulin activates translocation of GLUT 4 from the reservoir to cell surface [57].

Insulin increases blood flow in skeletal muscle and glucose uptake in a NO-dependent mechanism [58]. Blocking of NOS activity in skeletal muscle results in the impairment of insulin-mediated glucose uptake, hyperglycemia and insulin resistance [59]. Furthermore, treatment of isolated muscle tissue with NO donor, SNP or S-nitrosoglutathione increases insulin-stimulated glucose transport, uptake and oxidation [55, 60, 61]. Change in the energy level in cells, the AMP-activated protein kinase (AMPK) plays the key role in the regulation of energy metabolism [62]. Activation of AMPK by 5-amino-4-imidazolecarboxamide riboside, an indicator of AMPK in muscle increases glucose transport through the translocation of GLUT 4 to the plasma membrane and inhibition of NO production by NOS inhibitor nullifies the AMPK mediated glucose uptake [63–65].

In hepatocytes, glucose uptake is physiologically controlled by the balance between intercellular glucose phosphorylation and dephosphorylation. Glucokinase activity stimulated by insulin also helps in glucose uptake across plasma membrane indirectly [66]. iNOS expression is found in periportal hepatocytes, while eNOS expression is found in hepatocytes and endothelium of hepatic arteries [67]. Increase in NO level by NO donor or treatment with L-arginine or tetrahydrobiopterin plays an important role in carbohydrate metabolism by decreasing PEP-Carboxylase activity

and by suppressing hepatic gluconeogenesis [68]. NO also modulates activities of different enzymes involved in glycogenesis and glycogenolysis in the hepatic tissue.

Cardiac muscles are highly dependent on glucose as a rich energy source. So, the expression of GLUT 4 is subjected to change the metabolic activity and endocrine regulation. During ischemia, GLUT 4 translocation, stimulated by AMPK cascade, leads to the activation of glucose uptake and glycolysis [48, 69]. Besides AMPK mediated glucose uptake, NO/cGMP pathway also plays an important role [70]. Jensen and his colleagues [71] reported that treatment of cardiomocytes with SNP activates nitrogen activated protein kinase which stimulates glucose uptake. It has also been reported that lipid has a role on the downregulation of GLUT4 gene and reduces insulin-stimulated glucose uptake in the heart [72].

Insulin induced NOS activity helps in glucose uptake in adipocytes [73]. In human adipose tissue, eNOS expression was first reported by Ribiere and collegue [74]. Later on, the expression of eNOS and iNOS was reported in murine white adipocytes (3T3-L1) [75]. Blocking of NOS activity in brown and white adipocytes inhibits glucose uptake by insulin action [73]. In adipose tissue mainly eNOS is membrane bound and iNOS is cystolic [76]. Treatment of 3T3-L1 adipocytes with SNP increases glucose uptake via GLUT 4 translocation. Glucose uptake due to SNP mediated stimulation is inhibited by cPTIO signifies the role of NO in glucose uptake in adipose tissue [75].

Conclusion

Endogenous NO is involved in insulin secretion, insulin sensing and glucose uptake and thus play a key role in carbohydrate homeostasis. Thus NO-releasing drugs can restore disrupted NO signalling and improve carbohydrate metabolism. Designing such rational therapies to improve insulin action in vascular endothelium might have beneficial effects on disorders with metabolic syndrome, such as in type 2 diabetes.

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Chapter 11
Nitric Oxide as a Diagnostic and Therapeutic Tool in Respiratory Diseases

Kavita Gulati, Suresh Kumar Thokchom, and Arunabha Ray

Abstract Nitric oxide (NO) is a gasotransmitter that plays a vital role in diverse biological processes. NO is a fundamental component in regulating cardiovascular functions, smooth muscle tone, and neurotransmission. It acts as a critical signaling molecule in the body that widens blood vessels in the lungs when inhaled. Several lines of evidence indicate that endogenous NO is responsible for the physiological regulation of airways and is involved in various respiratory diseases. The primary sources of NO in the respiratory tract are epithelial cells, inflammatory cells (macrophages, neutrophils, mast cells), and endothelial. The highest output of NO is from epithelial cells and macrophages. The concentrations of NO are different for each airway inflammatory disease, and these changes in the level help in the evaluation and management of respiratory disorders. The amount of NO found in expired air is detectable by a non-invasive method in animals and humans. Several research findings have pointed out the role of NO in the pathogenesis of various diseases affecting airways, and this can be translated to future application in clinical practice. This review summarizes the basic understanding of NO in various respiratory disorders, and the fractional exhaled levels of NO can be an important non-invasive economical diagnostic marker. Further, the current version of the role of endogenous NO may provide new insight into the regulation of the airways, and inhaled NO may potentially contribute as treatment strategies to various respiratory diseases in clinical practice.

Keywords Nitric oxide · Respiratory diseases · FeNO · COPD · Inhaled NO · Exhaled NO

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*, Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_11

Introduction

Nitric oxide (NO) is a colorless, tasteless, and short-lived endogenously produced gas. It acts as a signaling molecule in nearly almost every organ. It acts as a ubiquitous intercellular messenger in all vertebrates, regulating blood circulation, blood clots, and neuronal networks. Interestingly, neurons in the human brain can produce NO for 80 years without causing toxicity [1]. However, during pathological conditions such as cerebral ischemia, the formation of NO may increase a great danger to the neurons in only a very few minutes. Such damaging action results from the reaction of NO with superoxide anion ($O2\cdot-$), forming a strong intoxicant oxidant, i.e., peroxynitrite ($ONOO-$), which results in the production of contradictory characteristics of NO in the body, mediating tissue destruction, inflammation, and vasoconstriction, especially in various lung diseases [2]. Several studies have shown the role of endogenous NO in regulating airways physiology and its involvement in respiratory diseases. The primary purpose of this review is to summarize the basic understanding of endogenous NO in the physiological regulation of airways and its application as a diagnostic and therapeutic tool in patients with respiratory diseases.

Source and Biosynthesis of NO

NO is endogenously generated in mammalian cells by the oxidation of L-arginine to NO and L-Citrulline, catalyzed by nitric oxide synthase (NOS) enzyme. NO is produced via two successive mono-oxygenation reactions in the presence of at least five cofactors. The cofactors involved in this process include oxygen, nicotinamide-adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) [3]. The physiological effects of NO are mediated through to produce per mole of NO, two moles of O_2 and 1.5 mol of NADPH are utilized.

Nitric oxide synthase enzymes exist in three closely related isoforms. They are mainly categorized into either calcium- and calmodulin-dependent (cNOS) or calcium- and calmodulin-independent (iNOS). The isoforms of NOS are neuronal (nNOS), macrophase (iNOS), and endothelial cells (eNOS). Both endothelial (eNOS) and neuronal (nNOS) isoforms of NOS are chiefly expressed in mammalian cells (e.g., cardiovascular system and nervous system, respectively) and inducible (iNOS) isoforms of NOS are expressed only when activated by an immune response [4]. Thus, the expression of iNOS is triggered by exposure to bacterial endotoxin and pro-inflammatory cytokines such as TNF-α, INF-γ, and IL1-β. The activity of iNOS is regulated at the transcriptional level and is not affected by the changes in intracellular calcium concentrations or by the cofactors, i.e., NADPH and BH_4 [5]. High NO levels indicate increased iNOS expression, which is raised in circumstances involving inflamed airways, such as asthma, pollen inhalation, and airway infections. iNOS can

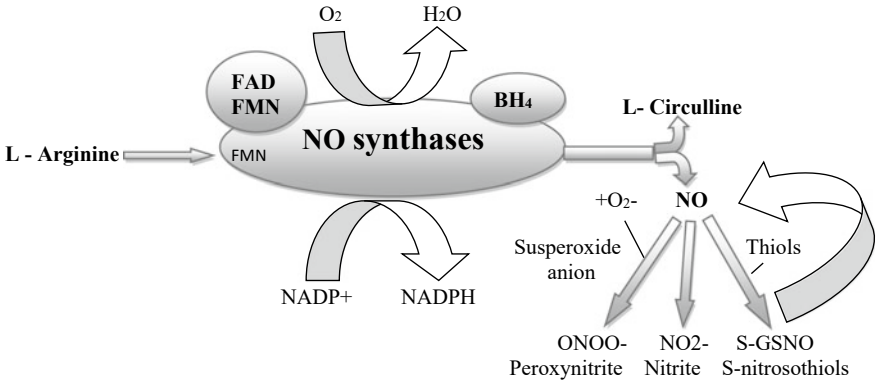

Fig. 11.1 Schematic representation of nitric oxide synthesis and its major effector targets

produce a higher NO concentration than cNOS, and it may last for several hours or days [6].

The physiological effects of NO are mediated by forming cyclic guanosine monophosphate (cGMP), a signal transduction molecule. NO diffuses easily into surrounding cells, where it activates soluble guanylate cyclase, resulting in the formation of cGMP. Heme protoporphyrin IX is present as hemoglobin in soluble guanylate cyclase. This hemoglobin presence is associated with iron in the form of ferrous, which has a great affinity in binding with NO. In the targeted site, cGMP activates cGMP-dependent kinases, which leads to the modulation of intracellular calcium levels, thereby regulating varieties of functions in the target tissues. Thus, the main three effector sites of NO include (i) Metalloproteins, in which NO interacts with metals, especially iron in the heme group; (ii) Thiols, where NO interacts with thiols compounds having the -SH group (thiol) and forms nitrosothiols; (iii) Tyrosine nitration, where peroxynitrite (ONOO-) is formed when NO combines with superoxide [7]. Figure 11.1 shows the simplified representation of nitric oxide synthesis and its primary effector targets.

Therefore, the fast reactivity of NO with metals, oxygen, and reactive oxygen species (ROS) determines its labile factor or inactivation in vivo. Proteins haviing iron-containing prosthetic groups, such as heme and hemoproteins, react with NO the fastest. Thus, the reaction of NO with hemoglobin or S-nitrosylation leads to its inactivation and its transport throughout the vasculature. Inactivation of NO is also achieved by reacting NO with O_2 to form nitrogen dioxide. Further, it reacts with superoxide to form peroxynitrite, an endogenously produced and highly reactive oxidizing species for the NO-associated pathogens [7].

Nitric Oxide—Cytotoxicity Versus Cytoprotection: A Question of Balance

NO acts as a signaling molecule in many physiological functions at optimal concentrations. At high concentrations, it is a cytotoxic mediating defensive mechanism against pathogens. Thus, it's perplexing that NO can serve as a physiological intercellular messenger molecule while potentially cytotoxic in vivo. The factors that decide whether NO is beneficial or harmful include (i) the cellular environment where NO is released; (ii) the influx rate of NO depends on the activation of the NOS enzyme, and (iii) the variety of second messenger cascades that might be used to signal beneficial or harmful cells [8]. NO may serve as an antioxidant at low concentrations and as a pro-oxidant at higher concentrations. In the cells, the antioxidant effects of NO are acquired by the activation of signaling transduction pathways. This increases the synthesis of endogenous antioxidants and thus downregulates the pro-inflammatory stimuli [9]. NO shows cytoprotective interaction with lipid radicals and iron. The cytotoxic effects of pro-oxidants, H_2O_2, or t-Butyl hydroperoxide (tBH) are decreased by NO-releasing diazen-1-ium-1,2-diaolates [10]. NO can inhibit the neutrophils' superoxide anion formation via inhibition of NADPH oxidase [10].

Further, NO has also been reported to reduce the involvement of neutrophils by lowering neutrophil deformity. That results in decreased interleukin-8 (IL-8) formation and endothelial adhesion molecules by lung epithelial cells [11]. NO has also been reported to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation triggered by inflammatory stimuli (e.g., lipopolysaccharide) [12]. The mechanism responsible for this inhibition of NF-kB binding to DNA induced by NO may be due to inhibition of IkBa degradation and nitrosation of cysteine residues on the p50 subunit of NF-kB bringing NF-kB to maintain in its inactive form [13, 14]. NF-kB is a transcription factor responsible for many inflammatory chemokines, cytokines, and growth factors; therefore, this character of NO may be responsible for protecting against inflammation by pathogens.

Generally, NO exhibits low reactivity as a free radical. In high doses, however, the interaction of NO with superoxide (O2•) produces peroxynitrite (ONOO), a potent neurotoxic oxidant that causes tissue destruction, inflammation, and vasoconstriction. It is responsible for mediating the cytotoxic effect, especially in various lung diseases [2]. NO's reaction with O_2•− takes place at a close diffusion-limited rate and is irreversible. Peroxynitrite is a powerful oxidant, and its reaction with tyrosine residues gives nitrotyrosine. This nitrosation leads to changes such as interruption of the actin filament, mitochondrial enzyme inhibition, oxidation of surfactant protein A, depletion of plasma sulfhydryls and antioxidants [15]. Peroxynitrite-induced oxidative damage has been linked to enhanced lipid peroxidation and DNA damage. Further, this results in the inactivation of enzymes and proteins, thereby enhancing tumor formation and proliferation. Besides, NO plays a pro-inflammatory role by activating NF-kB in response to inflammatory agents [2, 16]. This activation of NF-kB is associated with the presentation of tumor necrosis factor-alpha (TNF-α) as well as interferon-γ (IFN-γ) [17, 18]. Further, NO generators have been reported to enhance

the formation of prostaglandin E_2 (PGE_2) by lung fibroblasts in vitro and alveolar macrophages [19].

Role of NO in the Physiology of the Lungs

Nitric oxide (NO) is involved in various functions in the airways, including immunomodulation, bronchodilation, secretion control, and cell signaling. Several reports have highlighted the potential role of NO in the airways. The following is a summary of the physiological significance of NO in the airways:

NO and Bronchodilation

Since its discovery, the ability of inhaled NO to produce a bronchodilator response in humans and animals has been explored. NO is the neurotransmitter inducing the dilatation of the human airways by activating guanylate cyclase and raising cGMP. Dupuy et al. [20] found that breathing NO in a dose-related way reduces bronchoconstriction caused by methacholine inhalation in sedated guinea pigs. They discovered that inhaling a large concentration of NO resulted in a small amount of baseline bronchodilation. Further, adding 80 ppm of NO to inspiration gas prevented enhanced resistance to nebulized methacholine [21]. But, a NO concentration of 80 ppm had no impact in healthy people or individuals having chronic obstructive pulmonary disease (COPD), however, it did have a minor bronchodilator response in those with bronchial asthma. NO is also implicated in the dilation of airways through a different mechanism than guanylyl cyclase activation. NO is engaged in thiol metabolism to generate nitrosothiols (RS-NO), which are found in healthy people's airways. RS-NO has substantial bronchodilator properties, and they are independent of the cGMP cascade. They generally present in healthy humans in adequate amounts to modulate bronchial tone. In severe asthma, the concentration of RS-NO is reduced in the airways. This suggests that the lack of such endogenous bronchodilator substances may be responsible for inflammation and the degradation process in the airways of severe asthmatic patients. This contributes to the severity and refractory bronchospasm in bronchial asthma. There is a nonadrenergic noncholinergic (NANC) neural system that directs bronchomotor tone in addition to the classical adrenergic and cholinergic network. This system regulates whether airways constrict (excitatory NANC) or relax (inhibitory NANC) in both animals and humans [22]. NOS immunoreactive nerves in parasympathetic and sympathetic and sensory ganglia support providing bronchial smooth muscles, vessels, and the lamina propria. When comparing the proximal and distal airways, NOS immunoreactive neurons are more prevalent in the proximal airways. nNOS mediates NO's release from peripheral nerves, and calcium entry in a depolarized state activates it. Approximately half of the inhibitory nonadrenergic noncholinergic (iNANC) response is mediated by

NO. In the second step of the iNANC relaxant response, the vasoactive intestinal peptide is implicated. In humans, however, NO is entirely responsible for the iNAMC response in both the central and peripheral airways [23]. Besides, cholinergic neural bronchoconstriction is potentiated by NOS inhibitors without any impact on neural acetylcholine release. Thus, it demonstrates that NO generated by nNOS serves as a functioning antagonist to the excitatory cholinergic network in the postjunctional state but not in the prejunctional state. Although there is no change in nNOS expression, allergic airways inflammation causes a failure in neural NO-induced relaxation. This suggests that the activity of nNOS is altered in the presence of airway allergic inflammation, which leads to exacerbation in asthmatic patients [24].

NO and Bronchoprotection

Increased bronchoconstriction in response to several direct and indirect stimuli is a fundamental hallmark of obstructive airway disorders (OAD) such as asthma and COPD. Endogenous NO plays a vital role in airway hyperresponsiveness in animal models, suggesting that NO is bronchoprotective in OAD [25, 26]. Histamine causes bronchoconstriction (in vivo and in vitro), potentiated by NOS inhibitors, implying that endogenous NO plays a modulator function in airway hyperresponsiveness. Within the airways, NO triggers soluble guanylyl cyclase in target cells, thereby increasing cGMP and relaxation of the smooth muscle. In asthmatic patients, inhalation of a high amount of NO causes bronchodilatation. The inhibition of NO production with NOS inhibitors accentuates airway responsiveness to various stimuli in experimental and clinical asthma studies [27]. Apart from its direct action on respiratory smooth muscle, NO plays an essential role in stabilizing mast cell activity, a necessary step as these cells are involved in the pathogenesis of asthma and airway responsiveness [28]. Based on these findings, several clinical studies have been undertaken to corroborate the potential of endogenous NO to protect against excitatory airway responses during allergen exposure. The effect of the inhaled NOS inhibitor N (G)-monomethyl-L-arginine (L-NMMA) on the airway hyperresponsiveness to bradykinin before and after the allergen challenge was studied in asthmatic patients. According to the findings, allergen exposure in asthma increased airway hyperresponsiveness to bradykinin by impairing the generation of bronchoprotective nitric oxide, which was linked to the downregulation of NOS isoforms (ecNOS) [29].

In another study, the bronchoprotective role of NO in hyperpnea-induced bronchoconstriction and airway microvascular permeability was studied in animals. The results suggested that constitutive (but not inducible) NO seems to have a bronchoprotective effect on hyperpnea-induced bronchoconstriction [30]. Further, researchers have investigated the bronchoprotective mechanism of a potent peroxynitrite-releasing compound named S-morpholinosydnonimine (SIN-1). They have a considerable bronchoprotective effect against acetylcholine. Bronchoprotection by SIN1 appears to be mediated in part by peroxynitrite and NO regeneration, which may involve glutathione (GSH) and airway thiols due to peroxynitrite exposure [31].

Lee et al. evaluated the possible involvement of the eNOS gene and its association with patients with bronchial asthma [32]. When comparing the asthma group to the control group, the presence of one genotype (bb) of eNOS was striking. However, there was no significant difference within the group of subjects with varying degrees of asthma. These data suggest that variations in the eNOS gene may be linked to asthma development; however, the severity of the disease is not to be affected by polymorphisms of the eNOS gene.

NO and Pulmonary Vasculature

The NO is a key signaling molecule that regulates blood flow and tissue oxygenation. NO plays an essential function in controlling O2 supply through paracrine modulation of vasomotor tone locally and respiratory responses centrally. It can account for the biological activity of endothelium-derived relaxing factor (EDRF), an endogenous vasodilator that causes vascular smooth muscle to relax. The importance of pulmonary NO generation in the management and mechanism of the systemic vasculature is still being explored through extensive research. NO regulates vascular tone and blood flow in the vascular smooth muscle by activating soluble guanylate cyclase (sGC). In the classical NO signaling pathway, activation of sGC emphasizes cGMP formation, which ultimately brings vasodilatation. Further, mitochondrial O_2 consumption is controlled by inhibiting cytochrome c oxidase. NO is involved in regulating pulmonary circulation that prevents the vascular basal tone and counteracts hypoxic vasoconstriction. Lung diseases with chronic hypoxia have decreased NO release [33, 34]. The oxidation of NO to nitrite or the interactions of NO with protein thiols to create S-nitrosothiols (SNOs) are two alternate NO signaling pathways. These NO derivatives can serve as vasodilators or post-translational protein function modifiers [35, 36]. New theories about the relationship between nitrite and SNOs with hemoglobin have developed. The allosteric characteristic of hemoglobin inside red blood cells is exploited, resulting in NO signal transmission towards the periphery. Its vasodilator capabilities allow it to deliver oxygenated blood to hypoxic tissue selectively.

The results of genetically modified experimental studies in each NOS isoform have greatly simplified our understanding of the importance of individual NOS isoforms in pulmonary vascular biology. The endothelium of pulmonary arteries contains isoforms of eNOS in healthy persons, however, its activity is diminished in individuals with a diagnosed pulmonary hypertension [37, 38]. Reduced eNOS expression causes respiratory vasoconstriction and a rise in the smooth muscle layer in the airway arterial system, which are common signs of the condition. A loss of NO bioavailability causes endothelial dysfunction and vascular pathology in pulmonary hypertension. COPD patients' pulmonary arteries have been found to have reduced endothelium-derived NO release [39]. Moreover, the basal bronchial vascular tone is

regulated by endogenous NO, whereas pulmonary vasodilatation following inhalation of cigarette smoke results from exogenous NO. In experimental studies, the dilatation of the bronchial vasculature is greatly influenced by endothelial NO [38].

NO and Airway Secretions

The respiratory system possesses a complex muociliary protective network to maintain the delicate mucosal system's balance. The respiratory mucosa is made up of pseudostratified and ciliated epithelium interspersed with mucus-secreting submucosal glands and goblet cells. Mucociliary transport helps remove inhaled particles from the respiratory mucosal surface and is a crucial defense system for the airway mucosa, and it increases the mucus secretion in the airways. A large quantity of NO produced by iNOS during pathological conditions triggers the chemotaxis of inflammatory cells, mainly due to the recruitment of eosinophils and T-lymphocytes to the lung. This results in vasodilatation, plasma extravasations, and mucus secretion in the airways [40]. On the other hand, the reaction of NO with anion superoxide enhances the oxidative stress pathway [41]. It thus causes cellular injury by dysfunction of the protein or DNA injury and airway hyperresponsiveness. NO can regulate the arginase pathway by substrate competition and cause bronchial remodeling, smooth muscle contraction, and mucus production [40].

In healthy individuals, NO is produced by persistent expression of iNOS in epithelial cells; thus, it plays a vital role in protecting the airways from several respiratory infections. NG-nitro-L-arginine-methyl ester (L-NAME) is a cNOS inhibitor, and its topical application reduces nasal NO concentrations. The NO donor sodium nitroprusside increases nasal NO while lowering nasal saccharine transit time, indicating mucociliary activity. Furthermore, L-NAME is shown to prolong the transport time. Such findings show that altering nasal NO generation artificially could change mucociliary activity in the airways [42]. NO concentrations are reduced by prolonged cough, rhinosinusitis (acute and chronic), primary ciliary dysfunction, cystic fibrosis, etc. Exposure to cigarettes and alcohol produces the same result. Changes in upper airway ciliary mucosal histology are linked to these disorders [43]. Patients with rhinosinusitis and septicemia have lowered NO levels due to decreased iNOS expression in the maxillary sinuses, which is linked to a lower defensive capacity and a higher risk of secondary infections [44]. Blanco et al. [44] investigated NO's effect in controlling ciliary mobility under normal conditions. They looked at how NO synthase enzyme inhibitors (L-NAME and aminoguanidine) affected mucociliary transportability. The data suggest that NO can promote or reduce mucociliary transport depending on the pathway, demonstrating that NO has a dual role in mucociliary transport. Thus, on one hand, NO can act as an essential physiological regulator and on other hand, it involves the pathogenesis of airways, leading to hypersecretion of surface liquid.

Exhaled NO as a Non-invasive Biomarker for a Variety of Respiratory Conditions

Analysis of exhaled human breath constituents has recently become a fast-expanding subject of study, particularly for analyzing chest diseases and oxidative stress of the lungs. Nowadays, it has given a great impetus to medical diagnosis and monitoring of diseases in a non-invasive way, particularly respiratory diseases [45]. Volatile organic compounds/gas present in expelled air becomes the fingerprints of biophysical processes in the human body and thus helps in the early detection of diseases. The presence of an excess amount of organic compounds/gas in exhaled breath is an indicator of disease state, and thus it is used as a biomarker of different diseases. NO is one of the most extensively studied markers among various exhaled markers. It is now recognized as a biological mediator of many physiological functions in animals and humans. Several airway diseases have reported irregularities in exhaled NO levels [46, 47]. Under physiological conditions, fractions of NO are found in the exhaled breath. It is derived endogenously from pro-inflammatory cells of airway epithelium by the action of the inducible isoform of NOS (iNOS). The fractions of gaseous mediators, i.e., NO present in the expelled air, can be measured quantitatively by a non-invasive method and used as a clinical biomarker to evaluate and manage various respiratory disorders. Many study reports have emphasized the role of NO as a non-invasive biomarker for assessing airway inflammation, particularly in chronic respiratory diseases.

The NO fractions in exhaled breath are measured directly by using NO analyzers and expressed in parts per billion. Fractional exhaled Nitric Oxide (FeNO) is an excellent marker that a high value (>50 parts per billion) strongly suggests airway eosinophilic airway inflammation and steroid responsiveness [48]. Lung diseases, including asthma, COPD, bronchiectasis, cystic fibrosis, interstitial lung disease, etc., are characterized by chronic airways inflammation. However, because of the challenges in assessing inflammation, they are not directly quantified in ordinary clinical practice. The significance of FeNO monitoring in various respiratory disorders, as well as its future potential, are highlighted here.

FeNO and Asthma

Asthma is a disease of airway inflammation accompanied by altered exhaled breath composition. Measurement of FeNO level in asthmatic patients is a valid and reproducible non-invasive marker with a high discriminatory capacity. It can be used with more than 90% specificity for diagnosing asthma in both adults and children. In asthmatic patients, the level of FeNO is found to be increased. These increased NO levels in asthmatic patients indicate inflammation in the lower respiratory tract [47]. Exhaled NO levels rise in untreated asthma or during acute asthma exacerbations and decrease with appropriate anti-inflammatory corticosteroid treatment. Hence,

the determination of NO fractions in exhaled air may be considered an ideal tool for monitoring the corticosteroid response in patients with bronchial asthma [49]. In the area of therapeutic monitoring of asthma, it has been observed that FeNO correlated with the frequency of respiratory symptoms. Thus, clinicians can recommend a specific bronchodilator or dosage based on FeNO level but not FEV_1 [50, 51]. The level of FeNO rapidly decreases after inhaled corticosteroids and anti-leukotriene treatment. However, such a response is not observed in the case of theophylline or nedocromil therapy. Early anti-inflammatory medication can prevent subsequent airway remodeling and worsen asthma symptoms. Thus, the change in FeNO level with corticosteroid therapy helps identify the disease pattern, whether poor disease control or uncontrolled airway inflammation, making it easy for the clinicians to decide on further dose regimens [52]. Therefore, the monitoring of FeNO has great potential in the diagnosis and monitoring of asthma.

The high levels of FeNO in asthma predominantly reflect the lower airway origin. They are most likely because of the activation of iNOS isoform of NOS in airways inflammatory and epithelial cells [53]. Yet little involvement from nNOS may be involved as the expression of nNOS gene connects with FeNO [54]. The level of FeNO may further increase by increased L-arginine (substrate for NO), not specific for asthma; however, an elevated level of it may pose a significant indication in distinguishing asthma from other reasons behind chronic cough [55]. In a study by Dupont and colleagues [56], the FeNO value was used to distinguish between healthy participants with or without airway symptoms and asthmatic patients. In another study, the intra-individual coefficient of variation was used as a variation index to measure the change in the level within a defined interval. Results showed that the FeNO in normal subjects within seven days was 15.8%, increasing to 16.8% within 23 days. The study's finding suggests that the percentage change in FeNO level by 30−35% or even more within the time interval (1–3 weeks) was considered abnormal [57]. In another study on mild to moderate asthmatic patients, the increased FeNO level was reduced significantly after three months of yogic intervention, thereby reducing the inflammation in the airways [58]. Several studies have investigated the performance of FeNO as an indicator for measuring the severity of asthma, but the findings are equivocal. Some studies reported increased levels of FeNO in asthmatic patients [59–61]; however, many failed to prove such correlations [62, 63]. The estimation of FeNO as a novel biomarker for the control of future asthma outcomes is more accurate when combined with spirometric parameters [64, 65]. Therefore, an integrated FeNO and pulmonary function test assessment are suggested in clinical care. This could aid clinicians in predicting the response to pharmacological treatment in terms of asthma control.

FeNO and Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a common preventable and treatable disease characterized by progressive airflow limitation that is not fully reversible.

The incorporation of FeNO into clinical practice is currently undergoing critical assessment. Measurement of FeNO level has been considered a surrogate marker for eosinophilic airway inflammation, especially in asthma. There have been few studies to characterize the clinical values of FeNO in individuals with established COPD, and the results are conflicting. Smoking is considered to be one of the important factors that influence the level of exhaled NO in these patients. Recent findings show a mild elevation in FeNO levels in stable patients with COPD than in healthy volunteers; however, much higher FeNO levels were found in ex-smokers than current smokers. The reduction in FeNO in current cigarette smokers maybe because of tobacco, which down-regulates the expression of eNOS, indicating a high risk of developing the pulmonary disease. Besides, the relatively low value of FeNO in current smoking COPD patients is maybe because of the presence of more peripheral inflammation when compared to asthma patients. The down-regulation of eNOS and increased oxidative stress consumes NO to form peroxynitrite [66]. However, high levels of FeNO are documented in patients with unstable COPD when compared to the stable COPD patients with present or former smokers [67]. Thus, there is a chance that cigarette smoke may dramatically conceal any likelihood of a disease-associated rise in exhaled NO levels [67, 68]. A significant number of patients with COPD comprise some of the features of asthma conditions such as infiltration of airway inflammatory cells, eosinophilic inflammation, etc. The illness is defined as asthma–COPD overlap syndrome (ACOS). It is characterized by chronic airway obstruction and various symptoms common in asthma and COPD [69]. The assessment of both FeNO level and blood eosinophils count helps differentiate ACOS from COPD [70].

As per a recent finding, patients with corticosteroid-naive having FeNO level > 25 ppb associated with a blood eosinophil count >250 cells/μl is indicative of high specificity (96.1%) for differentiating ACOS from COPD [71]. However, in the case of stable COPD, a FeNO level > 50 ppb is considered eosinophilic inflammation [72]. Further, some studies reported that patients with stable COPD with elevated FeNO levels are likely to respond better to corticosteroids [73] and may predict better FEV_1 response to inhaled corticosteroids [74].

COPD exacerbations are linked to a significant increase in airway eosinophilia capable of expressing eNOS and generating NO [75]. Respiratory acidosis is usually seen during COPD exacerbation, leading to a further rise in FeNO level. Patients with mild/moderate COPD, particularly in combination with cor pulmonale, have a higher concentration of FeNO than severe patients [76]. FeNO levels have been shown to increase during acute exacerbations and return to normal levels after a few months of appropriate steroid treatment. Therefore, pharmacotherapy with anti-inflammatory agents may be beneficial in preventing the progression of the disease and the subsequent damage to the lungs [68]. Thus, the change of FeNO level after pharmacotherapy with corticosteroids may help the clinicians identify the disease pattern, whether poor disease control due to uncontrolled airway inflammation and help decide on a different dose regimen [74]. Exhaled NO monitoring may thus be a valuable biomarker for early identification and illness management in inflammatory diseases like COPD. A significant reduction in the FeNO level

following three months of the yogic intervention was observed in the case of asthmatics [59] and COPD patients [77]. Further, it was accompanied by a reduction in the specific markers of inflammation, thus correlating the levels of FeNO with airway inflammation.

FeNO and Cystic Fibrosis

In contrast with asthma and COPD, the expression of NOS and its function in the lungs of cystic fibrosis (CF) is little known. FeNO measurements at various flow rates assist distinguish between alveolar and bronchial NO emissions in CF patients. Therefore, the measure of FeNO concentration may be beneficial to estimating NO deficit in the airways of patients with CF. Lower FeNO value with moderate to severe grade of CF is probably due to lower bronchial NO output. FeNO levels in CF patients are lower than in healthy people. Strong neutrophilic inflammation in the airways produces superoxide anions, converting NO to nitrate and potentially producing peroxynitrite [78]. According to Thomas et al. [79], FeNO levels are lowered in patients with CF despite the airway inflammation. However, it does not correlate with cystic fibrosis genotype. The mechanism for the decreased level of FeNO in CF patients is incompletely understood. However, the possible reasons may be reduced expression of eNOS or deficiency of the enzymes in airway epithelial cells, or reduced availability of L-arginine in the airways [80]. Further, the enhancement of eNOS expression by neutrophils is seen in the epithelial cells of normal human airways, but it was not observed in patients with CF [81]. Sexual hormones may also play a role in cystic fibrosis transmembrane mRNA expression. Worsening of lung symptoms before menstruation in female patients with CF has been reported. Further, the lowest value of FeNO levels with a significant lowering of FEV_1 values has been observed during the menstrual cycle. However, the physiologic significance of this finding is far from clear [82].

Further, the FeNO levels assessment may help differentiate the underlying diseases having common symptoms of inflammation. As a result, measuring FeNO levels can assist in distinguishing cystic fibrosis patients from those with atopic bronchial asthma. FeNO levels in adults having atopic bronchial asthma are substantially greater than those in individuals with extensive cystic fibrosis [83]. Eshghi et al. [84] investigated the proportion of expelled nitric oxide in CF children and its relationship with sputum culture to monitor infections. After weeks of antibiotic treatment, sputum culture was negative, and FeNO levels in these patients were substantially lower than FeNO before starting treatment. Participants having pseudomonas sputum culture did not differ considerably from others with non-pseudomonas sputum culture in terms of original FeNO levels and changes following treatment.

FeNO and Bronchiectasis

Bronchiectasis is a lung disease marked by chronic airway inflammation and infection, as well as abnormal airway dilation. The clinical applications of exhaled nitric oxide in bronchiectasis are less evident. An increase in FeNO level is found in bronchiectasis but lower than in other respiratory diseases. The rise in FeNO levels in patients with bronchiectasis may correlate with the severity of the disease. Shoemark et al. [85] investigated the use of FeNO levels to determine airway inflammation in bronchiectasis patients. Results showed that bronchiectasis patients had higher FeNO levels than healthy controls. Thus, its level reflected the severity of bronchiectasis, but it didn't provide enough information to guide treatment options.

The elevated FeNO level in bronchiectasis may suggest the presence of active inflammation in the airways. Therefore, it has the potential to anticipate the severity of the disease. It is further strengthened by observing the increased expression of eNOS in the airways of bronchiectasis patients [86]. But contradictory results were reported by Foley et al. [87], who showed no elevation in FeNO level in clinically stable patients with bronchiectasis compared to normal subjects. NO was thought to be removed or trapped in viscous airway secretion due to its interaction with ROS. Therefore, to clarify these contradictory results, further research is highly needed to fully explain the mechanism involved and the role of NO in the induction and progression of the disease.

FeNO and Rhinitis

FeNO is rarely measured in allergic rhinitis patients. However, measuring FeNO may be considered a valid, promising, non-invasive inflammatory marker adjunct to clinical assessment, used in other airway diseases (such as asthma, COPD, etc.) and in people who have persistent rhinitis. It may help diagnose and manage patients with rhinitis and can be useful to rule out the difficulties in the clinical interpretation of the disease [88]. The amount of NO emitted by the upper airways is 100 times more than that released by the lower airways. Thus, it causes a large release of NO in human paranasal sinuses [89]. In normal subjects, nasal NO may remarkably decrease by the action of L-NAME. Thus, inhibition of NOS by activation of eNOS can cause airway hyperresponsiveness in the nasal mucosa [90]. High eNOS and low iNOS immunoreactivity has been suggested in the submucosal glands and nasal epithelium in healthy participants. However, iNOS expression was found to be increased in rhinitis patients. Further, in patients with permanent perennial rhinitis, the immunoreactivity of nitrotyrosine was found to be increased in the nasal mucosa, which can be correlated with the severity of the sinonasal symptom. However, the increase in the expression of iNOS may not be associated with the increased intensity of nitrotyrosine labeling. This reflects that NO derived from iNOS can have the power to take part in the pathophysiology of rhinitis [91]. However, peroxynitrite

generation in rhinitis sufferers is dependent on iNOS levels. Eotaxin is an eosinophil-specific chemoattractant that raises the immunoreactivity of nitrotyrosine in the nasal mucosa, resulting in increased nasal NO availability in clinically severe allergic rhinitis patients [92]. Patients with allergic and persistent rhinitis have been also found to have higher levels of nasal NO [89].

FeNO and Interstitial Lung Diseases

The application of exhaled NO has become a potential marker of many interstitial lung diseases (ILD), including systemic sclerosis, idiopathic pulmonary fibrosis (fibrosing alveolitis), and sarcoidosis. Many ILDs have unclear etiologies, which necessitates ongoing monitoring due to the disease's creeping, unpredictable, and irreversible character. Many studies have been conducted to explore the utility of FeNO in monitoring disease progression and response to therapy in patients with ILD.

Systemic sclerosis: FeNO levels in patients with systemic sclerosis, whether they have pulmonary hypertension or not, are lower than in healthy people. The possible reason behind this is a decrease in the pulmonary vascular endothelial surface or a reduction in the expression of eNOS in pulmonary vessels. But studies on the expression of eNOS in airway vessels in patients with systemic sclerosis are limited, and the results are equivocal. Recently, increased FeNO levels have been reported in systemic sclerosis patients with fibrosing lung disease, whereas those with pulmonary hypertension have relatively low FeNO levels [93, 94].

Idiopathic pulmonary fibrosis (fibrosing alveolitis): ILD remains a life-threatening, heterogeneous group of disorders. When diagnosed at the stage of idiopathic pulmonary fibrosis (fibrosing alveolitis), the underlying lung disease can sometimes be difficult to identify. Fractions of exhaled NO are modulated during the pathogenesis of ILD and can be used to determine the differences in subtypes of fibrotic ILD [95, 96]. The expression of iNOS in macrophages, nitrotyrosine, neutrophils, and alveolar epithelium was observed in the airways of ILD patients with active inflammation [97]. This was found to be consistent with increased levels of FeNO in patients with idiopathic pulmonary fibrosis. The level of FeNO is directly associated with disease severity. A high FeNO value may indicate the need for systemic treatment, and thus FeNO can be a useful biomarker for ILD management [98].

Sarcoidosis: As sarcoidosis is characterized by granulomatous airway inflammation, studies have been conducted to evaluate whether exhaled NO levels change in sarcoidosis and correlate with the morphological extent and functional severity of the disease. However, conflicting FeNO levels have been reported in clinical practices. Exhaled NO levels in pulmonary sarcoidosis are not elevated, according to several investigations [99, 100], but in contrast, opposite results are reported summarizing increased expression of eNOS in airway epithelium and granulomata in sarcoidosis patients [101]. The rise in the level of FeNO in patients with sarcoidosis may reflect

the severity and progression of the disease. The raised level of FeNO in sarcoidosis is lowered by pharmacotherapy with steroids, and this may be the reason behind conflicting results reported in patients with pulmonary sarcoidosis.

FeNO and Pulmonary Hypertension

The pathogenesis of pulmonary hypertension (PH) or pulmonary arterial hypertension (PAH) is complex and not well understood. Reduced NO availability is one of the characteristic features of the pathogenesis of PAH. Vasoconstriction of arteries of the lungs causes increased blood pressure, and a decrease in endogenous NO is directly correlated to the development of PAH. In contrast to asthma or COPD, earlier investigations have found low exhaled NO levels in PAH patients, indicating decreased endothelial NO release. This may be due to reduced endothelial expression of eNOS, which reduces NO concentration in the airway wall [102, 103]. The use of FeNO as a precise measure of NO generation may not be suitable in the case of PAH. Further, there are previous studies that have reported contradictory results. Malekmohammad et al. [104] investigated the association between FeNO levels and disease severity in PAH patients and therapy outcomes. The data showed no differences in FeNO between healthy controls and PAH patients. Further, continued treatment for three months had no significant effect on FeNO levels. Thus, the use of FeNO as a marker for monitoring severity or therapy was not recommended in patients with PAH. Inhaled prostacyclins, such as epoprostenol, have been developed to treat PAH. In one study, nebulized epoprostenol increased FeNO levels in PAH patients but not in healthy people, suggesting that PAH is regulated by a NO-related mechanism [105]. However, the opposite effect was observed by treatment with enalapril (inhibitor of angiotensin-converting enzyme) used to treat PAH, which elevates the level of FeNO in subjects with normal arterial pressure, but not in subjects with systemic arterial hypertension [106].

FeNO and Infections

NO is thought to play a critical role in diverse functions, including microbial defense against various bacteria and virus infections. One possible host defense mechanism involving NO is S-nitrosylation of cysteine proteases by NO that creates unfavorable for replication or virulence of several bacteria, viruses, and parasites. The decreased production of endogenous NO causes a declining level of FeNO. This may be one of the contributing factors for recurrent airway infection, particularly in patients with CF, as discussed earlier.

The level of endogenous NO was found to be higher during experimental human influenza and thus demonstrated that NO contributes to the pathogenic outcome of viral infections [107, 108]. The increased respiratory NO levels were also reported

in experimental studies [109]. The high generation of NO during viral infection appears helpful as it blocks the synthesis of viral RNA and S-nitrosylation of cysteine proteases [110]. NO has been reported for its antimicrobial activity against various viruses, including the influenza virus. However, in a study, inhaled NO therapy failed to improve outcomes in severe experimental influenza, and no difference in viral lung load was observed between experimental groups [111]. In another experiment, exhaled NO was evaluated in babies with acute respiratory syncytial virus (RSV) bronchiolitis [112]. It was observed that there was a temporary decrease in FeNO level during acute RSV, which was elevated during recovery to normal levels and higher. In a recent study, inhaled NO therapy was given in acute bronchiolitis and showed rapid oxygen saturation improvement and a reduced length of stay in the hospital [113].

The therapeutic potential of NO in coronavirus disease 2019 (COVID-19) infection is currently being explored. COVID-19 is associated with respiratory illness caused by a beta-corona virus closely linked to the severe acute respiratory syndrome (SARS) coronavirus. Recent evidence suggests that NO has a potentially significant role in suppressing the replication of a respiratory coronavirus and thus may help in the clinical management of patients with COVID-19 by restoring pulmonary physiology [114]. Alvarez et al. [115] recently evaluated the major therapeutic benefits of inhaled NO in COVID-19 and summarized multiple properties, including selective pulmonary vasodilatation, improved ventilation, and oxygenation restoration. Moreover, early clinical investigations suggested that NO could act as an anti-inflammatory, antibacterial, antiviral, and antithrombotic properties in *vitro* and in vivo.

NO Inhalation: A Therapeutic Tool in Respiratory Diseases

NO is a gas that regulates smooth muscle cell relaxation in the vascular system. Considering the understanding that NO relaxes endothelial smooth muscles, several studies were conducted to analyze NO's effects on airway vasculature [116, 117]. NO has a strong affinity for reacting with oxyhemoglobin. The gas is quickly scavenged by oxyhemoglobin in red blood cells. The vasodilating actions of inhaled NO are restricted to ventilate lung regions. NO has the unique ability to cause pulmonary vasodilation exclusively in areas of the lungs with excellent ventilation, improving blood oxygenation and decreasing intrapulmonary right to left shunting. Clinically, NO is presently used as a selective pulmonary vasodilator to treat a variety of respiratory disorders in children and adults, including pulmonary hypertension, hypoxemia, airway inflammation, and pulmonary edema. The US Food and Drug Administration (FDA) permitted the use of inhaled NO for the management of hypoxic respiratory failure in term and near-term (>34 weeks) newborns with clinical or echocardiographic indications of pulmonary hypertension in December 1999. Low-concentration inhalation of gaseous NO enhances oxygenation and reduces the extracorporeal membrane oxygenation required [118, 119]. Conditions like persistent

pulmonary hypertension of the neonate (PPHN) and COPD can benefit from inhaled NO. Although the widespread use of inhaled NO is unappealing for logistical and budgetary reasons, it is the only allowed indication for the treatment of PPHN.

The mechanism of vasodilatation involves eNOS-induced NO that acts on endothelial smooth muscle cells. NO interacts with the soluble guanylyl cyclase and converts GMP to cGMP by entering the cellular membrane. Further, cGMP binds to cGMP-dependent protein kinase, triggering a cascade of actions that lower smooth muscle tone. In simple words, the active protein kinase binds to ionic channels in the cellular membrane and the sarcoplasmic reticulum, reducing calcium influx, increasing calcium ejection, sequestering calcium inside the sarcoplasmic reticulum, and lowering calcium mobilization. These processes have the net effect of reducing the amount of calcium supplied for depolarization and contraction, resulting in smooth muscle relaxation. NO then diffuses into the bloodstream and is subsequently inactivated. The concentration of NO reported to relax vascular smooth muscle is 10^{-10} M [120].

Inhaled NO in Lung Transplantation

The development of Lung Ischemia/Reperfusion Injury (LIRI) is commonly seen in patients after lung transplantation. It is the main reason for the incidence of early postoperative mortality and acute primary graft dysfunction. The donor's alveolar macrophage appears to be important in the induction of lung damage following reperfusion. Currently, it's unclear what role NO has in LIRI management; however, several animal studies have conducted the positive effects of inhaling NO in LIRI therapy. In an experimental study, pretreatment with inhaled NO preceding lung harvest or lung ischemia in the donating animal was found to dramatically minimize reperfusion of pulmonary injury. The mechanism involved may be due to a decrease in IL-8 production, neutrophil infiltration, and free radicals [121, 122]. Further, intravenous administration of nitroglycerin was found to improve lung damage in models of LIRI [123, 124].

Inhaled NO In Respiratory Distress Syndrome

Adult respiratory distress syndrome (ARDS) has a morbidity and mortality rate in the United States and Europe that ranges from 10 to 90%, depending on how the disorder is defined. ARDS was classically described as an acute-onset inflammatory disease with increased vascular permeability linked with clinical, radiologic, and physiologic abnormalities but may coexist with left atrial or pulmonary capillary hypertension [125]. The alveoli are inflamed and filled with an exudate containing blood protein, water, and electrolytes that reduce the surface area available for gas exchange. This results in more volume of blood passing through the lungs without

participating in gas exchange. This possibly leads to a reduction in PaO_2, despite breathing 100% oxygen [126].

Inhaled NO therapy may help in treating ARDS partially due to its vasodilator properties. It causes vasodilation of the blood vessels that supply the ventilated regions [127]. This reduces the pulmonary artery pressure and thus shunt fraction and causes a concomitant increase in PaO_2. Although inhaled NO therapy takes care of the pulmonary component of ARDS, this disease is a condition with a systemic inflammatory component complicated by multiple organ failures, and the treatment is still being researched. Various investigations have already shown the impacts of inhaled NO on gas exchange and hemodynamics. Rossaint et al. [128] conducted a study to evaluate the effects of inhaled NO in individuals with severe ARDS. They have suggested that inhaling NO reduced mean pulmonary pressure and enhanced arterial oxygen pressure (PaO2). In another study, Young et al. [129] found that the magnitude of improvement in PaO2 following NO inhalation therapy is proportional to the severity of pulmonary hypertension before treatment.

Inhaled NO in Chronic Obstructive Pulmonary Disease (COPD)

COPD is one of the leading causes of illness and mortality worldwide. There is epidemiological evidence of an increase in cases of COPD, especially in elderly subjects. Cigarette smoking is the leading cause of developing COPD. The treatment of COPD includes pharmacotherapy, cessation of smoking, controlling exacerbations, etc. Inhaled NO therapy in COPD helps to improve pulmonary function by maintaining sufficient gas exchange, but continuous inhalation of NO (at concentration 10–40 ppm) may worsen gas exchange in the lungs [130]. This seems paradoxical as NO inhalation is beneficial in ARDS and improves the situation, but it may exacerbate COPD. This can be explained on the basis that in ARDS, NO cannot reach many alveoli as they are filled with fluid and unventilated. NO predominantly functions as a vasodilator in well-ventilated lung areas. Shunting, however, is not the only factor that contributes to the pattern of COPD ventilation/perfusion mismatch. There are several partially ventilated zones in the lungs of COPD patients, as opposed to completely ventilated or unventilated areas. Thus, if inhaled NO produces vasodilation in certain sections of the lungs, it will reduce the amount of blood moving through better-ventilated regions, resulting in a lower PaO2 level.

Therefore, one of the other ways to approach this problem is to deliver a combined supplementation of oxygen and NO. However, the results of investigations conducted utilizing this strategy are mixed. Studies show increasing PaO2 levels, and others that show the opposite by supplementing oxygen and NO [130, 131]. Alternatively, NO can also be administered to well-ventilated parts of the lungs alone or with oxygen. Pulsed inhaled NO administration at predetermined intervals at the start or throughout inhalation may help control COPD.

Inhaled NO in Severe Acute Respiratory Syndrome in Response to COVID-19

Currently, infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) is associated with a number of health issues. Inhaled nitric oxide possesses antiviral properties, improves oxygenation, and is safe in infants with respiratory conditions. In research by Goldbart et al. [113], inhaled nitric oxide was utilized as a therapy in hospitalized babies with acute bronchiolitis. They were given a high dosage of inhaled nitric oxide (160 ppm) with oxygen/air for 30 min or oxygen/air alone (control) five times per day for up to five days. In comparison to standard therapy, they found that high dosage inhaled nitric oxide (160 ppm) was safe, well-tolerated, decreased hospital stay, and improved oxygen saturation quickly.

Several potential roles of inhaled nitric oxide in managing COVID-19 associated lung complications have been reported by recent studies. Fakhr et al. [132] used high concentrations of inhaled nitric oxide as a rescue therapy to manage and stop further progression of COVID-19 infection in patients with hypoxic respiratory failure. They found that breathed nitric oxide at a concentration of 160–200 ppm is simple to utilize, well responded, and is beneficial in patients with COVID-19 with hypoxic respiratory failure. In another research, inhaled NO was administered in COVID-19 patients spontaneously breathing [133]. For all patients, the beginning dose of inhaled nitric oxide was 30 parts per million, and the average treatment time was 2.1 days. They discovered that more than half of spontaneously breathing COVID-19 patients who received inhaled nitric oxide treatment won't require respiratory support (mechanical ventilation). This evidence proves that inhaled nitric oxide therapy could help COVID-19 patients avoid future hypoxic respiratory failure.

Conclusions

The recent discovery of multiple roles of endogenous NO in the balance of several physiological processes, particularly in cellular functions in the airways, has brought a new, rapidly growing field in the science of the respiratory system. It has enhanced our understanding of the potential role as a diagnostic and therapeutic tool in respiratory diseases. NO, along with its metabolites, serves as a non-invasive biomarker that helps evaluate the severity of various chronic inflammatory airway disorders. The potential bronchoprotective effects of NO include relaxation of the smooth muscle, and reducing airway hyperresponsiveness to bronchoconstrictor stimuli. As NO has already made it from the bench to the bedside, it is not really strange to think that this potent molecule will become a therapeutic target in the coming years. Pharmaceutical companies may develop techniques to alter the expression of specific NOS enzymes, which could keep the equilibrium between the beneficial and harmful actions of NO and could be a viable therapy option for a variety of respiratory disorders. However,

some studies revealed various contradictory findings in many aspects; therefore, further detailed investigations are required to use NO as a therapeutic tool to validate its efficacy in various respiratory disorders.

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Chapter 12
Therapeutic Potential of Nitric Oxide in the Management of COVID-19 Induced Acute Respiratory Distress Syndrome (ARDS)

Jagdish Chandra Joshi and Bhagwati Joshi

Abstract The severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), transmitted by human to human, is a causative pathogen of the fatal Corona virus disease 2019 (COVID-19) pandemic. The SARS-CoV-2 virus has caused massive health as well as the socioeconomic crisis globally. The virus induces an airway infection, affecting gas exchange by primarily targeting alveolarepithelial and endothelialvessels,resulting in acute respiratory distress syndrome (ARDS) followed byacute lung injury (ALI)and leads to multiple organ failure and death. There is no specific pharmacological agent for the treatment of COVID 19, only symptomatic and supportive therapy are used to treat patients. Various endogenous agents generated during the pathological insult of COVID-19 are critical to regulatethe homeostasis and repairing lung injury. Nitric oxide (NO) is one of the essential gaseous substances synthesized via various respiratory cells. It is well-known for its bronchodilator and pulmonary vasodilator function to ease the breath. It also plays a significant role as viricidal and microbicidal. These potential activities of NO in the pulmonary systemmake ita likely candidate to treat the COVID-19 induced ARDS. In this chapter, we addressed the possible mechanism and targets of NO to suppress the SARS-CoV-2 induced ALI/ARDS and its complications.

Keywords Acute lung injury · ARDS · SARS-CoV-2 · COVID-19 · Nitric oxide · iNOS

Introduction

There have been various global epidemics caused by viral pathogens over the past 240 years, such as influenza A (H1N1), Zika virus, SARS-CoV, Ebola virus, and, recently, SARS-CoV-2. COVID-19 pandemic caused by a novel SARS-CoV-2 is the biggest disaster of 2020. It has resulted in more than 63 million infections and

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_12

1.46 million deaths globally which is less than a year of its appearance, [1–3]. The lack of effective drugs or vaccines was the major hindrance for adequate protection against these emerging viral threats [4]. Thus, it remains a significant challenge and is of supreme importance to develop therapeutic strategies for the emerging COVID-19 pandemic. COVID 19 outbreak, a zoonotic transmitted viral infection, was first observed in a patient of Wuhan city of China on December 12, 2019. The patient was showing the presence of unknown etiology of pneumonia in bronchoalveolar lavage (BAL) [3, 5–7]. It drew global attention quickly due to its rapid spread all over the world [5, 8, 9]. The rate of COVID-19 transmission is very high through air droplets and direct contact of human to human [2]. SARS-CoV-2 primarily attack respiratory cells in the same way as other SARS coronaviruses [3, 5]. Newborn babies, geriatric, pregnant women, immunocompromised patients, or patients having comorbidities like diabetes mellitus and cardiovascular disease are more prone to COVID-19 infection [10–12].

According to the WHO database, approximately 3000 studies are being conducted by variousresearch institutes, pharmaceutical and biotech companies. However, to date, there is nospecific pharmacological tool to combat the deadly virus so far, only maintaining social distance and quarantine is the most effective way to stop its transmission. As the scientists are in the arduous search for its proper cure, this chapter focuseson how NO can act as a promising compoundfor the prevention of ALI/ARDS in COVID-19 patients (https://clinicaltrials.gov/ct2/who_table).

SARS-CoV2 or COVID-19 Induced Pathological Changes

SARS-CoV-2 infection shows a broad spectrumof symptoms ranging from asymptomatic/mild symptomatic to severe life-threatening conditions. The primary clinical sign includes cough, fever, expectoration, throat pain, Chest tightness, chill, fatigue, and myalgia. The atypical manifestations are productive cough, dyspnea, pleuritic chest pain, lymphopenia, hemoptysis, diarrhea, GI distress, nausea, and vomiting [12–18]. In most cases, it is associated with severe pneumonia and ARDS, leading to respiratory failure and multiple organ dysfunction. The primary cause of morbidity and mortality in SARS-CoV-2 infected patients is ARDS, which isaccomplished with alveolar microvascular disruption, infiltration of polymorphonuclear cells, and activation of platelet-activating factors [2, 19]. The virulence of SARS-CoV-2 starts with the endocytosis or internalization of its positive single-stranded RNA genome into the host cell cytoplasm. SARS-CoV-2 virus RNA is enclosed in a phospholipid bilayer envelope that contains various types of structural proteins, viz. the spike trimeric glycoprotein (S), membrane (M) glycoprotein, envelop (E), and nucleocapsid (N) [4, 20]. The densely present trimeric S glycoprotein, critical in the pathogenesis, has two subunits (S1 and S2).The glycoprotein spike (S) of SARS-CoV-2 must be cleaved at two different sites by host cell proteases, furin, and TMPRSS2, to enter the cell. The furin enzyme that is highly expressed in human lungs cleaves the trimeric S protein of the virus at a specific furin-like protease recognition pattern,

making it ten-times more efficacious for host cells [21, 22]. After cleavage of trimeric spike protein S, it divided into an N-terminal S1-ectodomain and a C-terminal S2 membrane-anchored protein [20, 21]. The S1 subunit of the glycoprotein recognizes the ACE2 in the surface of the hostcell [4, 22–24], while the S2 subunit helps in the fusion of the virus membrane to the host cell membrane. In the airway, the cellular proteasetransmembrane protease serine 2 (TMPRSS2) is the primary protease to cleave COVID-19 S protein [22]. TMPRSSs proteases co-expressed with ACE-2 in upper respiratory epithelial cells and detach the ACE2 receptor along with the viral glycoprotein. This further leads to the internalization of viral RNA into the cytoplasm of various cell types viz., lymphocytes, alveolar macrophages, epithelial and dendritic cells [5, 25]. Incorporated and internalized viral RNA reaches the nucleus of the host cells, where it is transcribed and translated, thereby multiply its genome number [21]. The viral RNA also activates various signaling pathways through the membrane receptors. Several membrane receptors, prominently Toll-like receptor4 (TLR4) or cytosolic TLR7, and retinoic acid-inducible gene 1-like receptors (RLRs) referred to as pattern recognition receptors (PRRs) recognize the ssRNA of SARS-CoV-2 virus. Recognition of viral ssRNA by PRRs is essential to initiate an innate immune response and synthesize various inflammatory cytokines including type 1 interferon (Fig. 12.1). The cytokines storm induced by COVID-19 leads to severe pneumonia, ARDS, septic shock, resultant multiple organ failure, and death [21, 25–29]. Patients infected with SARS-CoV-2 showed a promisingly higher level of TNF-α, IFN-γ, IL2, IL7, IL8, IL9, IL10, IL15, IL17, GCSF, GMCSF, MCP1, MIP1α, and increased various growth factor viz. PDF, FGF2, VEGFA [18], and markedly decrease the number of natural killers (NK), $CD4^+$, and $CD8^+$ T cells, which are critical for the control of viral infection and immune response [25, 30].

The radiological studies from multicentric hospitalized patients showed abnormal features, such as RNAaemia, acute cardiac injury, patchy lesion, and incidence of grand-glass opacities in subpleural regions [31, 32]. X-ray radiography at nine to ten days of COVID-19 infection showed increased left basilar opacity, atypical pneumonia, which was rising over time [13, 33]. Histological examination of biopsy studies showed bilateral diffuse alveolar damage with cellular fibromyxoid exudates, desquamation of pneumocytes, and formation of the hyaline membrane [15]. Pathological changes are associated with a localized and systemic immune response that results in the influx of neutrophils, leukocytes, and recruitment of proinflammatory cytokines. Blood analysis showed increased leukocytes, D-dimer, and ESR, lymphocytopenia, and thrombocytopenia [13, 15, 34]. Biochemistry of blood samples shows increase lactate dehydrogenase, creatinine level, elevated creatinine kinase, liver enzymes, (alanine aminotransferase, aspartate aminotransferase), increased C-reactive protein, decrease in total protein level [13, 15, 17, 32, 34, 35]. Sputum of COVID-19 patientsshows a positive real-time polymerase chain reaction [15, 32]. The pathological study confirms the influx of proteinaceous fluid, multinuclear cells, edema formation, accumulation of fibrinoid cells, vascular congestion, the proliferation of pneumocytes [25, 34].

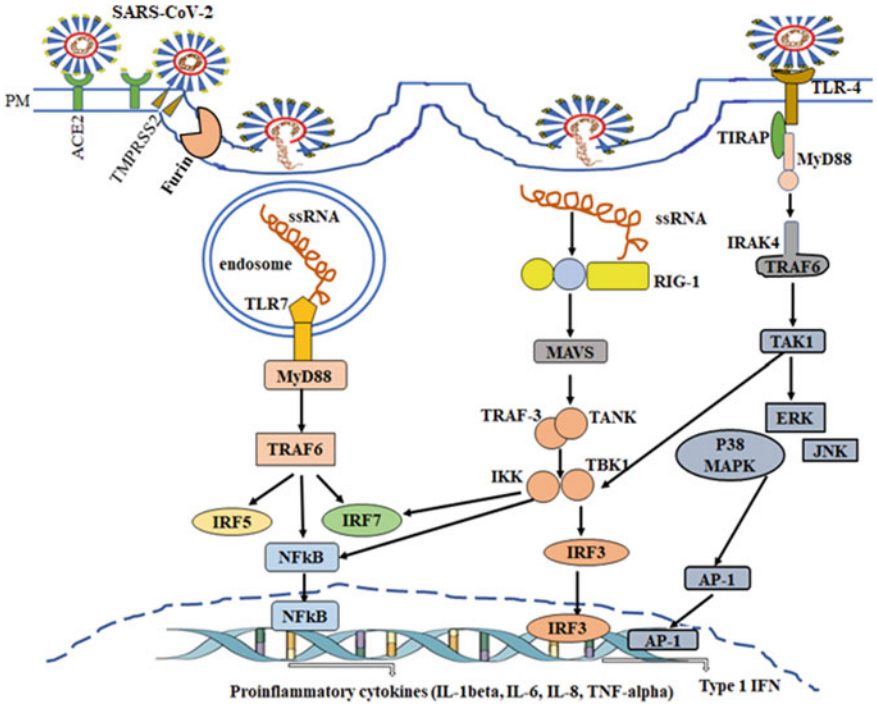

Fig. 12.1 Pathogenesis of COVID-19 and immune response, ACE2 receptor on the cell membrane recognizes the COVID-19 virus. The cellular protease transmembrane protease serine 2 (TMPRSS2) detaches the ACE2 receptor along with the viral glycoprotein and leads to the internalization of the viral RNA into the human cell. TLR4 is activated by virus glycoprotein, while internalized ssRNA is recognized by cytosolic TLR7 and retinoic acid-inducible gene 1-like receptors. Binding of viral ssRNA to 4 and TLR7 leads to the recruitment of the adaptor protein MyD88 complex with TRAF6. Adaptor protein MyD88 directly or indirectly recruits and activates the 5, IRF7, and NFkBsignaling and promotes transcription of proinflammatory cytokines. The activated MyD88 recruit TRAF6 and IRAs, which, in turn, results in the activation of TAK1. TAK1 phosphorylates IKK, leading to activation of NF-kB. NF-kB translocates to the nucleus to promote the transcription of proinflammatory genes (such as IL-1α/β, IL-18, IL-6, and TNFα). In other cytosolic pathways, the ssRNA genome of the virus binds with RIG-1 and gets oligomerize. Following oligomerization, on the mitochondrial membrane, RIG-I activates and dimerizes MAVS. After that, dimerized MAVS associate with TRAF-3 and TRAF family member-associated NF-kappa B activator (TANK) leads to the activation of Tank binding kinase-1 (TBK1) and I kappa B kinase (IKK). TBK1 and IKK phosphorylate IRF3 and IRF7, which then homodimerize and translocate to the nucleus to promote the expression of type I IFNs

Classification of Drugs for COVID-19 Treatment

To date, there are no specific drugs developed against COVID-19. Clinicians have the only choice to provide symptomatic relief and reduce the respiratory discomfort associated with COVID-19 induce infection.

Following drugs are currently under use for symptomatic reliefin COVID-19 patients.

a. **Antivirals:** Remdesivir, Lopinavir, Ritonavir, Favipiravir, Umifenovir
b. **Corticosteroids:** Methylprednisolone, Dexamethasone
c. **Antibodies to neutralize the virus:** Immunoglobulins or convalescent plasma
d. **Nitric Oxide:** Inhaled nitric oxide (iNO) and oral nitric oxide (NOViricid)
e. **Monoclonal antibodies and other:** Tocilizumab, Siltuximab, Sarilumab, bevacizumab, eculizumab, Bamlanivimab, REGN10933 and REGN10987, GSK4182136, Anakinara
f. **Antibiotics:** Azithromycin
g. **Immunosuppressant:** Sirolimus
h. **Anti-coagulant**
i. **Anti-inflammatory**
j. **Miscellaneous:** ACE-2 inhibitors, Vasoactive intestinal peptide, Neurokinin 1 antagonist, Baricitinib, Nitazoxanide, Niclosamide and Ivermectin, Colchicin, QingfeiPaidu, Lianhuaqingwen, Yinqiao San, and Vitamin C

Apart from the medication, patients with severe respiratory infections, hypoxemia, or shock need oxygen therapy with an initial flow rate of 5 L/min to reach the required oxygen saturation level.

Nitric Oxide and its Impact on the Pulmonary System

Nitric oxide (NO) is an essential gaseous signaling molecule that plays a broad role in the various physiological and pathological processes [36]. In the airway, different cell types, viz., alveolar macrophages, alveolar type II cells, and pulmonary endothelial cells synthesizeNOconstitutivelyusing nitric oxide synthase3 (NOS3),precursor L-arginine,and oxygen in the presence of NADPH [37–39]. Another NOSenzyme,NOS2, also called iNOS, is activated by cytokines released during infectious and inflammatory injury such as IFN-γand chemotactic peptideproduced inducible NO [37, 38]. However, NO has a very short half-life (0.1–2 s), but because of its high diffusion capability and reactivity to the cellular and free radical, it reached almost all the cells. Diffused NO activates soluble guanylate cyclase (sGC) and generate cyclic-guanosine 3', 5'-monophosphate (cGMP). cGMPplays a vital role in physiological tasksand regulates various ion channels, oxygen consumption, and cellular contraction [39, 40]. NO also increasesmucus secretion and ciliary movement, which helps remove viral particles from the airways. It is well known that NO generated by iNOS modifying proteins and nucleic acids and have viricidal activity [41].

NO and it's Potential in COVID-19 Induced Lung Injury

In vitro studies suggested that treatment with organic NO precursor following post-infection and induction of iNOS in cells reduced the growth of SARS-CoV significantly and inhibited its replication during early steps [36, 42–44]. Apart from this, comorbidities associated with the low NO production in the pulmonary system, such as cystic fibrosis or Kartagener'ssyndrome, are more prone to infect SARS-CoV-2/COVID-19. Studies in mice model found thatinhibition of NO, using its inhibitor, have more susceptible to COVID-19 infection [44]. A clinical study suggested that following inhalation NO (iNO) therapy 83% of patients were negative fornasopharyngeal swab test within twenty-two days of SARS-CoV-2 infection (https://www.massgeneral.org/news/press-release/nitric-oxide-benefit-pregnant-covid-patients).

ACE-2 is a carboxypeptidase enzyme responsible for the synthesis of Ang-1–7 bybreaking Ang-II, a vasoconstrictor, andconstitutively produced NO by binding to its receptors (Fig. 12.2). ACE-2 is also the cell surface receptor for SARS-CoV-2. The binding of SARS-CoV-2 to ACE-2 reduces its availability to Ang-II and thus reduces the generation of NO. This results in pulmonary vasoconstriction, increase platelet aggregation, thrombosis, and reduced viral clearance, which leads to the precipitation of ALI/ARDS and its complication in COVID-19 patients (Fig. 12.2) [45–47].

Infiltration of neutrophils,which leads to neutrophilic lung injury,is common in SARS-CoV-2 virus infection. Evidencedemonstrated that iNO reduced the neutrophil infiltration and their activation, suppresses the release of inflammatory cytokines, regulate the cell-mediatedlung injury,and inhibits the lung parenchymal damage [48, 49].

(https://www.massgeneral.org/news/press-release/nitric-oxide-benefit-pregnant-covid-patients).

Pulmonary macrophages play a significant role in recognition of pathogenic infection and initiation of innate immunity. They are very plastic and play a significant role in regulating ALI/ARDS induced by pathogenic insultand secreting various inflammatory and anti-inflammatory cytokines. However, persistent activation and their proliferation at the M1 stage aggravate the ALI/ARDS and responsible for unresolved lung inflammation [50]. Evidence suggested that NO orchestratesmetabolic reprogramming in macrophages and suppresses the secretion of inflammatory cytokines viz. TNF-α, IL-1β, etc. and promote the conversion of inflammatory macrophage to anti-inflammatory, thus helping in the resolution oflung inflammation in ALI/ARDS patients [49, 51].

Inflammatory cytokines storm due to SARS-CoV-2 infection disturbs the pulmonary endothelial barrier, thereby reducing endothelial release relaxing factor, nitric oxide, and increased vasoconstrictor endothelin level results in endothelial dysfunction and thrombus formation which precipitate the ARDS [42, 48]. Studies suggested that iNO and dietary organic nitrate use in COVID-19 patients reduce vasoconstriction, thrombus formation, leukocyte adhesion, thus improve endothelial function and pulmonary performance [42, 48]. SARS-CoV-2virus infection cause early

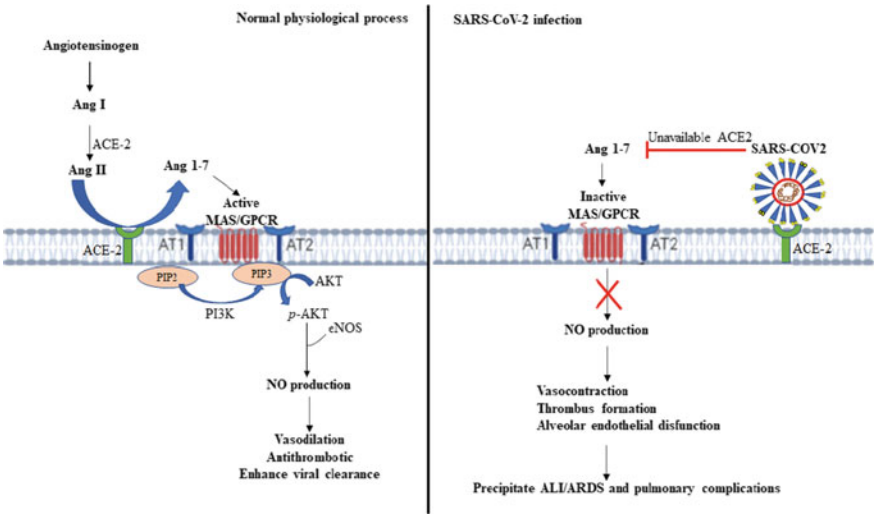

Fig. 12.2 Effect of SARS-CoV-2 in the constitutive production of NO. ACE-2 is a crucialvascular system enzyme, and in normal physiological processes, it generates Ang I, Ang II, and Ang-1–7 sequentially from plasma globulin angiotensinogen. Later product of angiotensinogen, i.e., Ang-1–7 binds with MAS-GPCR. MAS-GPCR activates PI3K-dependent Akt phosphorylation, results in the phosphorylation and activation of endothelial NO synthase, finally leads to the generation of constitutive NO.In the case of SARS-CoV-2 infection, the virus binds to ACE-2 to get entry into the host cells. The binding of SARS-CoV-2 leads to the shedding of ACE2 on cell surface receptors and loss of its protective function. The MAS-GPCR get inactivated and thus pause NO production. Thus, loss of ACE-2 leads to an imbalance of Ang-II and An-1–7, results in vasoconstriction, thrombosis, endothelial dysfunction

arterial hypoxia by Ventilation/Perfusion [V/Q) mismatch, thereby increase P(A-a) O_2gradient [48, 52]. Inhaled-NO has been used to improve the arterial oxygenation and PaO2/FIO2 in COVID-19 patients. Clinical evidence suggested that inhaled nitric oxide provides rapid relief from shortness of breathing and decreases respiratory rate [43, 44].

Phosphodiesterase are the group ofenzymes that degrades the cGMP, an important secondary messenger produced by NO, into its inactive component. Several phosphodiesterase inhibitors are being used to treat pulmonary hypertension and respiratory disorder, for instant, COPD and asthmatic inflammation. These phosphodiesterase inhibitors are FDA-approved and mimic the NO signaling by protecting cGMP degradation, a secondary messenger produced by NO [53–55]. There are extensive experimental studies that suggest the beneficial effect of phosphodiesterase inhibitors in protecting pathogen induced ALI/ARDS, suppressing neutrophil accumulation, and inflammatory cytokine release [56].

Various clinical trials are being conducted for the iNO, and few are in the phase-II study in mechanically ventilated COVID-19 ARDS patients [48]. A clinical Phase, IIb/IIIa outpatient study for the safety and efficacy of the 30 mg sodium nitrite oral lozenge (NO viricid], has been approved by the US Food and Drug

Administration(FDA]for treating African Americans patient who is diagnosed with SARS-CoV-2 infection.

https://www.europeanpharmaceuticalreview.com/news/131413/trial-to-evaluate-oral-nitric-oxide-therapy-in-covid-19-patients/.

Conclusion

In summary, preclinical and clinical evidence suggests that NO is an excellent anti-inflammatory compound to reduce the infiltration of neutrophils, adhesion of leukocytes, enhance pulmonary circulation, and ease breathing. NO is a pharmacoeconomic and safe compound and has been used to treat various respiratory disorders, including pulmonary hypertension.Thus, it may be a promising therapeutic compound for COVID-19 induced ALI/ARDS treatment and to reduce its complications.

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Chapter 13
Emerging Novel Therapies for COVID-19: Implications for the Use of Nitric Oxide as an Anti-COVID-19 Therapy

Ramesh K. Goyal, Chandragouda R. Patil, and Kalpesh R. Patil

Abstract The pandemic of COVID-19 is an unprecedented calamity that has seriously affected entire human race. The treatment guidelines for COVID-19 were obviously unavailable to the lack of prior data on the mode of transmission of this viral infection and due to the unknown pathogenesis of symptoms associated with it. Due to an obvious lack of guidelines for selecting proper therapeutic modalities, led to a chaotic situation and numerous clinical trials were simultaneously undertaken to evaluate the clinical efficacy of antivirals, steroids, hydroxychloroquine and so on. These trials were based on the prior evidences of antiviral and cytokine suppressing effects of such drugs. Nitric oxide (NO) is known to exert antiviral effect, induces vasorelaxation and suppresses the cytokine storm. On the basis of this, NO has been extensively studied in preclinical and clinical trials for its therapeutic utility in the COVID-19 infection and its consequences. Present chapter highlights the rationale for therapeutic use of NO in COVID-19 infections and provides an update on its status as a treatment modality for COVID-19.

Keywords Covid-19 · Nitric oxide · Cytokine storm · Vasorelaxation

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_13

Introduction

Corona viruses are RNA viruses affecting livestock and birds. These viruses may sometimes induce cross-species infections including humans. The earlier pandemics of coronavirus like SARS (severe acute respiratory syndrome) and MERS (Middle East Respiratory syndrome) induced sporadic infections and deaths during the last couple of decades. However, the recently discovered novel coronavirus, which is also named as SARS-2 and COVID-19 is the most contagious strain of the coronavirus [1]. COVID-19 has affected almost 48.5 million and killed more than 1.23 million people worldwide. With the increasing number of infected individuals, more clinical data on the pathogenesis is emerging. This accumulation of the clinical data has provided insights into the therapeutics regimens which may prevent, cure, and salvage the critically affected patients of COVID-19 infection. Multiple drugs have been clinically tested for the prevention and treatment of the COVID-19 associated morbidity and mortality.

COVID-19 virus can stay active in the atmosphere and on the surfaces for a quite long time (3 days) [2]. The incubation period for common flu is 1−4 days whereas for the COIVID-19 it is 1−14 days. During incubation and even after infection, a large proportion of patients may remain completely asymptomatic. Unfortunately, these asymptomatic carriers can spread the infection, and hence, the spread of COVID-19 may occur more rapidly. The case fatality rate for the COVID-19 infections ranges from 2.2 to 3.9% which is comparatively higher than the common flu infection (<0.1%) [3] (Johns Hopkins University. Coronavirus (COVID-2019) Global Cases). In certain countries, the average mortality rates have been reported as high as 4.34 to 28.37% in countries like Yemen [4]. Even after reduction of the viral load, the patient may still suffer from the consequences of the COVID-19 infection like neurological ailments (headache, dizziness, encephalopathy, demyelination, seizures, CNS vasculitis, skeletal muscle damage, optic nerve neuritis, and generalized myoclonus) along with the cardiovascular problems (myocardial injury, myocarditis, acute myocardial infarction, arrhythmias, shock and cardiac arrest, venous thromboembolic events [5, 6]. The typical clinical biochemistry findings in the COVID-19 patients include increased serum levels of C-reactive protein, glutamate pyruvate transaminase, creatine kinase, and creatinine [7, 8]. The clinical symptoms of COVID-19 infection include sore throat, cough, weakness, headache, fatigue, shortness/difficulty in breathing, and pneumonia (Public Health Agency of Canada).

During the initial stages of this pandemic, there was a scarcity of clinical data on the mechanisms of morbidity and mortalities dues to COVID-19 infection. Rapid spread of the infection and considerably higher incidences of mortality necessitated rapid development of the therapeutic regimens and multiple drugs and their combinations were evaluated as a treatment for COVID-19. These approaches included use of antiviral agents to reduce the viral load, use of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers to prevent the interaction of virus with the lung cells, antibiotics to prevent secondary infections, drugs and biologicals

to suppress the cytokine storm, convalescent plasma therapy, and, therapy to prevent intravascular blood clotting.

COVID-19 Problems and Challenges in the Treatment

The COVID-19 infection is a terrible, rapidly spreading infection with higher morbidity and mortality rates. Unfortunately, there is no vaccine available for this viral infection during the emergence of pandemic. Though multiple COVID-19 vaccines are available or in the pipeline, their safety and efficacy is still being investigated. The logistics and costs involved in the vaccination may keep awaiting a large portion of the human population. The availability of the vaccine may provide protection to the frontline health workers. However, the duration of protection after each cycle of vaccination may still remain a major concern for the usefulness of the vaccine. Along with the systemic vaccines, nasal spray vaccines targeting the viral load in nasal and respiratory tract are also being clinically tested for their efficacy. Further, questions are being raised regarding the significance of vaccinating the individuals who are naturally immune to the COVID-19 infection.

Presently, there are no specific anti-viral agents to be used in the treatment of this infection. The antivirals like remdesivir, favipiravir, ribavirin, lopinavir, and ritonavir have been proved to either provide a marginal protection or no benefits in terms of survival of the severely affected COVID-19 patients [9]. Recently, US-FDA approved the use of remdesivir in the treatment of adults and children above 12 years age with corona infection. The controlled clinical trials have concluded that remdesivir reduces the hospital stay of the patients, however it does not alter the outcome in term of death indicating that it may not prove effective as a life-saving drug in the severe infections. None of the other antivirals have shown any significant clinical benefits above the remdesivir. Even in case of remdesivir, certain adverse effects like hepatotoxicity, rectal haemorrhage, vomiting, and nausea [10]. Apart from the antiviral agents, the repurposed drugs to inhibit the binding of virus to target host cells has also been extensively evaluated in the treatment of COVID-19 infection.

ACE2 (Angiotensin converting enzyme II) is a widely expressed in multiple organ systems and receptors for ACE-2 (ACR-2) are present on the cell surface of various organs. ACE-2 functions as a carboxiypeptidase enzyme and plays important role in cardiovascular physiology. The COVID-19 virus enters the human cells through the receptor binding domain of the S protein which has a strong affinity for the ACR-2 [11]. Thus, the drugs that bind to ACR-2 receptors have been proposed to inhibit the binding of this virus to the human cells and this blocking the entry of the virus into the cells and making them more vulnerable to the antiviral therapy. Both chloroquine (CQ) and hydroxylchloroquine (HCQ) are reported to interfere with the binding of S protein domain of COVID-19 to the ACR-2 on the host cells. These drugs also increased the endosomal pH and thereby affecting the viral replication. These drugs also alter the MAPK pathway and affect the process of viral integration

and replication [12]. Initial clinical trials projected both these drugs as effective in treating the COVID-19 infection and reducing the associated complications like pneumonia [13]. However, the severe cardiovascular adverse effects of these drugs led to their reduced clinical use. Ultimately, the WHO suggested discontinuation of these drugs in the treatment of COVID-19 patients (Solidarity clinical trial for COVID-19 treatments].

Thus even today, the main focus of the treatment of COVID-19 infections remains the supportive care in which severely affected patients are provided with oxygen, ventilation, and symptomatic treatment with steroids, immunosuppressant and antibiotics [14]. In summary, we are facing a terrible virus with greater infectivity than the SARS-CoV pandemic of 2003. There is presently no specific anti-SARSCoV-2 drug regimen to treat critically ill patients. Most of the potential drugs for treatment of COVID-19 are being investigated for safety and efficacy against SARS-CoV-2. Remdesivir is the most promising agent. However, the emerging data from the recent clinical trials has revealed only marginal or no benefit of remdesivir treatment in the COVID-19 patients. In addition, favipiravir and combination therapy with hydroxychloroquine plus azithromycin appear to be acceptable alternatives for treatment of COVID-19 patients. For patients with SARSCoV-2 infection. Finally, low-dose steroid (hydrocortisone) might be prescribed for treatment of refractory shock in patients with COVID-19.

Failures of Repurposed Drugs in Treatment of COVID-19

Chloroquin (C) and hydroxychloroquin (HC) are decades-old drugs being used in the treatment of malaria. Mutliple mechanisms were proposed for their anti-COVID-19 actions. The C and HC inhibit the endocytosis of COVID-19 virus and release of its genetic material. The C exerts alkalinisation of the cellular pH, inhibits the proteases that cleave the viral S protein and thus inhibit the interaction of the virus particles with the lung epithelium [15]. The C also inhibits the fusion of the lysosomes with autophagosomes which is necessary for the release of the viral genome into host cells. Thus, it prevents both endocytosis of virus and release of viral genome. The HCQ inhibits the transfer of virus particles from endosome to lysosome and thus prevents the release of the viral genome into cytoplasm of the host cells [16]. Considering these mechanisms of anti-COVID-19 effects, both C and HC were clinically evaluated for their efficacy and safety in the COVID-19 patients. Unfortunately, the meta-analysis of the clinical studies recently concluded that both these drugs do not reduce the death associated with COVID-19 and also do not provide any clinical benefits either as prophylaxis or as treatment of COVID-19 infection [17]. Though C and HC are used in treatment of malaria, their use in absence of medical supervision can lead to severe adverse effects including arrhythmia, changes in vision, headache, dizziness, vertigo, and skin reactions. Thus, the adverse effects of C and HC overwhelm the proposed meagre clinical benefit and hence their use in the COVID-19 infections is not recommended [18].

Another repurposed drug extensively tested through clinical trials in azithromycin, an antibacterial antibiotic of macrolide class. This class of antibiotics have immunomodulatory potentials and have been tested in the treatment of viral infections. Azithromycin has ability to reduce the pro-inflammatory cytokines like TNF-α, IL-1, IL-6 along with oxidative stress and alteration of the helper T-cell functions [19]. However, azithromycin is devoid of any antiviral properties [20]. Despite of its claimed efficacy in viral infections, the clinical trials on combination of HC and azithromycin revealed no advantage of this combination in reducing the death rate in the COVID-19 patients [21]. However, certain recent clinical trials on the combination of HC and azithromycin or these drugs administered alone have reported clinical benefits of azithromycin in reducing the COVID-19 associated deaths [22]. The oxford University scientists conducted a clinical trial named Randomised Evaluation of Covid-19 Therapy (RECOVEY Trial) indicated that low dose dexamethasone reduces the COVID-19 associated death by one third of the patients on ventilation. The low dose dexamethasone (approximately half of the active corticosteroid dose) therapy also increased the survival of patients who developed acute respiratory distress syndrome.

Similar to these repurposed drugs, an approach of using the convalescent plasma from the recovered COVID-19 patients has also been sufficiently tested through the clinical trials. The basis for the use of convalescent plasma is the presence of antibodies against the COVID-19 in the plasma of recovered patients which can provide passive immunity against the virus. A propensity score–matched control study on the use of convalescent plasma in the treatment of COVID-19 patient indicated that this transfusion therapy provides clinical benefits to the patients and reduces the oxygen requirements in the hospitalized COVID-19 patients. This study involved only 39 patients and raised hopes for the efficacy of the convalescent plasma therapy [23]. However, an open-labled, phase–II, multicentric clinical trial conducted in Indian adult patients (PLACID trial) has concluded that the convalescent plasma therapy did not provide any clinical benefits either in terms of reduction in the progression of severe COVID-19 or in terms of reducing death rate [24]. This trial included 464 Indian patients and has concluded about the lack of clinical benefits of the plasma therapy. Thus, the repurposed drugs which had appeared promising in the treatment of the COVID-19 proved to exert only marginal clinical benefits and these clinical failures of the repurposed drugs necessitated further exploration of novel approaches.

Emerging Anti-COVID-19 Therapies

Antivirals

Immediately after the onset of COVID-19 pandemic, antivirals including ritonavir, lopinavir, remdesivir and hydroxychloroquine possessing in vitro activity

against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replication were emerged as the treatment for COVID-19 [25]. Monotherapy with antiviral drugs might not be sufficient to defend against COVID-19 in moderate to severe cases of infection. However, combination of antivirals and immunomodulators is promising approach to treat the more severe cases of COVID-19 [26]. Fusion inhibitors including umifenovir and camostat mesylate demonstrated antiviral activity against SARS-CoV-2. It was noted from the in silico computational studies that protease inhibitors like lopinavir, elbasvir, carfilzomib, eravacycline and valrubicin were found to inhibit the main protease in SARS-CoV-2. Reverse transcription inhibitors including remdesivir and ribavirin showed promising effects against COVID-19 infection. Currently, there are no FDA approved antiviral drugs as an anti-COVID therapies. However, the fusion, protease and transcription inhibitors targeting at various steps of SARS-CoV-2 life cycle could be promising therapies [27].

Immunosuppressants

The immunosuppression is recommended for patients of COVID-19 disease. However, the corticosteroid therapy at higher dosage and presence of risk factors for severe COVID-19 disease are contraindications for the use of immunosupressants [28]. Few immunosuppressive drugs including IL-6 inhibitors (tocilizumab) and corticosteroids have ability to decrease the mortality and mechanical ventilation in COVID-19 patients. Based on the results of in-vitro studies conducted against SARS-CoV and MERS-CoV, thiopurine analogues and mTOR (mammalian target of rapamycin) inhibitors could be effective against SARS-CoV-2 [29]. Immunosupressants like rituximab having long term effects need cautious use in COVID-19 disease. However, systemic immunoglobulins & doxycycline that do not alter the antiviral immunity as well as calcineurin inhibitors, chloroquine, and hydroxychloroquine can be the suggested alternatives [30].

ACE Inhibitors

SARS-CoV-2 and angiotensin-converting enzyme 2 (ACE2) are linked with each other. ACE2 is a co-receptor for SARS-CoV-2 viral entry and possess role in COVID-19 pathology. It is suggested that angiotensin-converting enzyme inhibitors (ACEIs) could inhibit ACE2. However, the clinically prescribed ACE inhibitors are ineffective in direct inhibition of ACE2 [31]. Although theoretical benefit of ACEIs are demonstrated in the physiological SARS-CoV infection model, the same outcomes are not inferred to SARS-CoV-2 responsible for the development of COVID-19 [32]. Evidence based literature search suggested that patients at the risk of COVID-19

including hypertensive patients should be continued with the ACE inhibitors and angiotensin receptor blockers therapy [33]. Some reports signifies the lack of sufficient evidence regarding effectiveness of ACE inhibitors or angiotensin receptor blockers (ARBs) in COVID-19 [34]. However, other studies suggested the use of ACE inhibitors and ARBs in hypertensive COVID-19 patients [35].

Vitamins

Dysregulated vitamin D metabolism is linked with the respiratory diseases which implies the raised vitamin D deficiency during pulmonary inflammation [36]. Vitamin D is recommended for healthy and COVID-19 susceptible population for the prevention and protection against COVID-19 [37]. Risk of severe COVID-19 event has been linked with the vitamin D deficiency. Covid-19 pandemic resulted into decreased opportunities to sun exposure and vitamin D synthesis. Fortified foods, dietary advice and vitamin supplementation are suggested to prevent this deficiency states, especially in the COVID-19 pandemic [38]. Risk of COVID-19 infection and mortality is proposed to be decreased with the Vitamin D supplementation. Higher vitamin D_3 doses are recommended for the treatment of COVID-19 patients [39]. However, further evidences like cohort studies and clinical trials are warranted to establish the link between severity of COVID-19 and vitamin D levels [40].

Add on Therapies

Bromhexine in combination with HC or quercetin is proposed as an effective add on therapy for the prophylaxis and treatment of COVID-19 infection [41]. Add on use of ivermectin to hydroxychloroquine (HC) and azithromycin (AZT) in COVID-19 treatment suggested that ivermectin is effective, safe and reduces the hospital stay. However, it is recommended to validate such effects through further extensive studies [42]. Use of azithromycin in severe COVID-19 patients receiving standard of care treatment including hydroxychloroquine showed no improvement in clinical outcomes. Therefore, the use of azithromycin along with hydroxychloroquine is discouraged in treating severe cases of COVID-19 [43]. COVID-19 infection is associated with the risk of thromboembolism. Low molecular weight heparin such as enoxaparin is projected for prophylactic anticoagulation in moderate to severe cases [44].

Nitric Oxide

Nitric oxide (NO) has pivotal role in the vascular function maintenance and inflammatory cascades regulation. No therapy targeted at optimal infection stage could be convincing and approachable option for COVID-19 treatment [45]. Nitric oxide (NO) has been effective in reducing hypoxia and SARS-CoV replication in severe acute respiratory syndrome patients. On the basis of in vitro studies against SARS-CoV and clinical trials, NO is suggested for the treatment of COVID-19 [46].

Respiratory Tract Pathology During COVID-19 Infection

SARS-CoV-2 is single-stranded RNA virus that possess a spike (S)-protein at the envelope. Host proteases mediated activation leads to its binding with human ACE2 receptor and subsequent viral fusion and endocytosis. Arrival of virus in the cell causes transcription and translation of viral genome through viral RNA-dependent RNA polymerase and host ribosomes, respectively. Synthesised viral proteins in the form of virions are assembled for exocytosis [47]. Angiotensin-converting enzyme 2 (ACE2) is established receptor for SARS-CoV-2 [48]. Normally, ACE2 is expressed on both types of alveolar epithelial cells. However, the type-II alveolar epithelial cells have maximum ACE2 expression. Increased ACE2 expression occurs following the SARS-CoV-2 binding with ACE2 that leads to alveolar cell damage. Furthermore, it promotes several systemic reaction and even the death [49].

Multistep pathology of COVID-19 infection involves asymptomatic phase, invasion & infection of upper and lower respiratory passage followed by development of acute respiratory distress syndrome. Asymptomatic phase involves entry of SARS-CoV-2 through highly expressed ACE-2 in the nasal epithelial cells. Virus propagates through replication and ciliated cell infection. This phase is short lasting having limited immune response. Upper respiratory tract is infected through viral migration from the nasal epithelium. The involvement of immune response is manifested as release of interferons and C-X-C motif chemokine ligand 10 (CXCL-10) from infected cells [50]. Severe symptoms of COVID-19 develop at the stage which involves the lower respiratory passage and progress to the acute respiratory distress syndrome. Viral nucleocapsids are developed following the viral invasion of alveolar epithelial cells through ACE-2 receptor. Virus infected pneumocytes releases various inflammatory markers and cytokines including tumour necrosis factor-α (TNF-α), interleukins (ILs), interferons (IFs), CXCL-10, macrophage inflammatory protein-1α (MIP-1α), and monocyte chemoattractant protein-1 (MCP-1) [51]. Association of these inflammatory mediators termed as cytokine storm, chemoattract the several cells including neutrophils, CD8 and CD4 T cells. While, fighting against the virus these cells develop an inflammation and lung injury. Host cell apoptosis followed by release of viral particles and infection of alveolar (type-2) epithelial cells leads to severe alveolar damage and acute respiratory distress syndrome [44].

Rationale for Nitric Oxide Use in COVID-19

The treatment of COVID-19 is based on the knowledge of acute respiratory distress syndrome (ARDS). The contemporary supportive ARDS therapies are limited to mechanical ventilation and endotracheal intubation. As the NO has potent vasodilator effect primarily on the pulmonary vasculature and circulation, the inhaled NO can be considered as rescue therapy for hypoxemia associated with ARDS and COVID-19 [52]. NO is produced by several types of cells including endothelium and unique signaling molecule. NO is reported to improve survival rate of SARS-CoV infected mammalian cells by inhibiting the viral replication. SARS-CoV-2 the causative agent of COVID-19 infection shares most of the SARS-CoV genome. This implies the potential of inhaled NO therapy as a promising anti-COVID-19 therapy [53]. Most frequent complication and cause of death in critically ill COVID-19 patients is acute respiratory distress syndrome (ARDS) [54]. It is characterized as raised intrapulmonary blood shunting and pulmonary hypertension. NO has a unique ability to cause vascular smooth muscle relaxation and vasodilatation. Therefore, NO induced pulmonary vasodilation leads to improved oxygenation of blood and declined intrapulmonary shunting. Accumulating evidences suggests the efficacy of inhaled NO therapy in decreasing the severity of ARDS [55].

Preclinical Evidences on Role of NO/iNOS Inhibitors in Viral Infections

Respiratory mucosal epithelium is common site of viral contact and inflammation and infection. Nitric oxide (NO) derived from the respiratory epithelium is important with respect to antiviral defence in the airway. Impaired NO synthesis is associated with declined antiviral defence and NO therapeutics shown promises in patients with weakened antiviral defence [56]. NO is vital molecule in the infectious disease pathology [57].

The inducible nitric oxide (iNOS) is produced in response to viral infection and NO cause inhibition of viral replication. In-vivo evidence showed that viral infection induces iNOS expression in the heart. NO demonstrated protective effect against virus mediated injury in murine model of CVB3 myocarditis. NO is proposed as in vivo nonspecific immune defence against viruses [58].

L-N^G-monomethyl-arginine (L-NMMA) supress NO production in experimental animals. L-NMMA is studied along with oseltamivir against influenza A/California/04/2009 (H1N1) virus infection in BALB/c mice. Results demonstrated that L-NMMA combined with oseltamivir is beneficial against influenza virus infections [59].

Arenaviruses cause haemorrhagic fever characterized by edematous skin swelling, pleural effusions, cytokine storm and hypovolemic shock. Earlier study conducted using HLA-A2-expressing mice infected with a monkey-pathogenic strain of

lymphocytic choriomeningitis virus showed that the edema formation and hypovolemic shock is abolished in mice lacking iNOS. Thus iNOS was identified as a mediator of arenavirus haemorrhagic fever and prevention of iNOS induction by Interferon-γ blockade decreases vascular leakage and terminal shock [60].

NO production in response to Rabies virus (RABV) causes dose dependent increase in T cell differentiation or T cell function suppression. Earlier study evaluated in vivo effect of NO in the immune response regulation during Rabies virus infection in mice. Intracerebral challenge of mice with RABV resulted in altered population of NK, CD4+, CD8+ cells in blood and raised NO levels. However, the subsequent treatment of mice with iNOS inhibitor, aminoguanidine showed NK, CD4+, CD8+ cells and decreased NO level. This in vivo study suggests role of NO and iNOS in immune response against RABV infection [61]. The antiviral effect of NO was studied in suckling C57BL/6 and C57BL/6 iNOS–/–mice. NO exerted potent in vivo inhibition of hantavirus replication in infected mice [62]. Infection of animals with varity of viruses and treatment with NO inhibitors showed raised viral replication [63]. Mouse model was used to study the role of NO in HSV-2 vaginal infection. NO demonstrated in vitro and in vivo antiviral activity against HSV-2. NO could be the antiviral effector mechanism against viruses. However, NO may contribute to pathology during immune response and can be damaging [64]. To understand the role of NO in viral infection it is suggested to examine the stage of viral diseases at which NO exerts prominent antiviral effects [65].

NO in Physiology and Pathology of Viral Infection

Nitric oxide (NO) plays a variety of roles in physiological and pathophysiological processes. During viral infections and also may exert direct antiviral effects on certain type of virus [62]. NO exerts effects like the generation of the peroxynitrite radicals and modulation of the innate as well as acquired immune responses. During microbial and viral infections there is activation of the iNOS which exerts increased production of NO which further participates in generation of oxidative radicals and modulation of T-cell response from Th1 type to Th2-biased response. It is also proposed that the viral mutations are accelerated by the NO. It has been observed in the experimental studies involving various types of viral infections (herpes simplex virus, rabies virus, influenza virus, and coxsackievirus) in mice trigger the iNOS which leads to a consistent increase in the generation of the NO in the localised tissues [66]. It is proposed that the morbidity associated viral pneumonia arises as a consequence of the overproduction of NO. The production of the NO in respiratory system epithelium is also triggered during the viral infection of respiratory tract. This increased level of NO is also proposed to exert antiviral effects and contribute to the exacerbation of the asthma [56, 67]. The in vitro experimental study has clearly indicated that the NO inhibits the replication of cycle of the SARS CoV during the initial phase of infection. This indicates that the iNOS induced NO has direct antiviral activity [68]. The age-related decline in the immune response is also correlated with the decreasing

levels of NO and bradykinin. Such a reduction in the NO is further correlated to the severity of the COVID-19 infection [69].

Clinical Studies on NO in Treatment of Viral Infections

The benefit of inhaled NO therapy was reported in spontaneously breathing patients of Covid-19. Among the 39 spontaneously breathing patients of Covid-19 who received the inhaled NO therapy, fifty percent patients didn't required mechanical ventilation. These results implies the potential role of inhaled NO therapy in preventing the respiratory failure in COVID-19 patients [70]. The potential of inhaled NO for the treatment of COVID-19 patients with pulmonary hypertension was studied retrospectively in critically ill COVID-19 patients. Inhaled NO therapy was found to beneficial through the reduction and stabilization of raised pulmonary artery systolic pressure and reduced risk of COVID-19 associated heart failure [55]. The summary of clinical trials related with use of NO as anti-COVID-19 therapy is summarized as Table 13.1. However, majority of these trials are still recruiting the patients and volunteers and none of them have yielded any outcomes to support the clinical use of NO in the health workers or COVID-19 patients. The case reports however, support its use in prevention and treatment of the COVID-19.

NO in COVID-19/Viral Infections

NO is recognised therapy for the treatment of viral diseases like influenza and coronaviruses [71]. Recently, NO treatment was employed for the treatment of immunocompromised SARS-CoV-2 positive patient. It was observed that NO has important role in the treatment of impaired alveolar perfusion and hypoxic pulmonary vasoconstriction [72]. Viral infection is manifested as "cytokine storm" having elevated levels of inflammatory mediators that leads to pulmonary hypertension and pneumonia [73]. Monocyte activation induces iNOS which generates the NO that is reported to inhibit SARS CoV replication cycle. Inflammation signaling including cytokine storm stimulates iNOS and generate NO against viral infection [71]. NO is potent vasodilator and antimicrobial agent effective against SARS-CoV replication and hypoxia in SARS patients. Recent study evaluated the in vitro effect of NO on SARS-CoV-2 replication. NO inhibited the viral replication and proposed as therapy for the treatment of other human coronavirus infections including COVID-19 [46]. The role of NO in COVID-19 is depicted as Fig. 13.1.

Table 13.1 Summary of clinical trials on use of nitric oxide (NO) in COVID-19 patients

Study design and title	Dose and route of administration	Subjects (*n*)	Primary endpoint	Secondary endpoint	NCT number
RCT, multicentre, open-label; pulsed, inhaled nitric oxide (iNO) for the treatment of patients with mild or moderate COVID-19; **Time Frame:** 14 days	iNO 160 ppm 15 min b.i.d	n = 470 Health workers attending COVID-19 patients for ≥ 3 days per week Age > 18 Yrs	Incidence of COVID-19 infection on 14th day of enrolment	(1) RT-PCR positivity for COVID-19 (2) Proportion of patients needing quarantine	NCT04312243
Phase-II, RCT; NO therapy: COVID-19 infection in the emergency department (ED) **Time Frame:** 28 days	iNO 130−300 ppm 20−30 min/day	n = 260; patients presented to ED due to COVID-19 related respiratory problems	Likelihood to return to ED during 28 day timeframe due to worsening of symptoms	Likelihood of: (1) inpatient hospitalization (2) Rates of intubation (3) Death	NCT04338828
Parallel assignment, open-lable, phase-II RCT; NO therapy: spontaneous breathing COVID-19 infection **Time Frame:** 28 days	Inhalation; 140−180 ppm, for 20−30 min, b.i.d	n-70, Spontaneously breathing mild/moderate COVID-19 patients	Reduction in intubation and mechanical ventilation	(I) Mortality (II) Clinical recovery	NCT04305457

(continued)

Table 13.1 (continued)

Study design and title	Dose and route of administration	Subjects (*n*)	Primary endpoint	Secondary endpoint	NCT number
Multicentre, open-label, single blinded, phase II RCT; nitric oxide gas inhalation therapy for mechanically ventilated patients with severe acute respiratory syndrome caused by SARS-CoV2: **Time frame**: 28 days	Continuous inhalation, initial 80 ppm (48 h) followed by 40 ppm	n = 200 Age > 18 Yrs COVID-19 infected patients in ICU who are on mechanical ventilation	Improvement in oxygenation	(1) Time to reach normoxia (2) Covid-19 negativity (3) Survival	NCT04306393
Multicentre, open-label RCT; high-dose NO for COVID-19 (ICU Patients) **Time frame**: 3 days	iNO, 160 ppm 6 h/day for 2 days	n = 20 age > 18 Yrs COVID-19 patients, < 14 days diagnosis and < 7 days from intubaton	Rate of PCR positivity	Not available	NCT04383002
Open-label RCT; iNO for preventing progression in COVID-19 (NO-COVID-19) **Time frame:** 28 days	20 ppm	n = 42 Age- 18–85 Yrs, Hospitalize patients of COVID-19 requiring O2 supplement, with comorbidities	Prevention of progressive de-oxygenation	(I) Prevention of progression (II) Clinical improvement	NCT04388683
Open label, RCT; Pulsed NO in Mild or Moderate COVID-19, **Time frame:** 28 days	20 ppm	Not available	Prevention of progression of the disease	Not available	NCT04358588

(continued)

Table 13.1 (continued)

Study design and title	Dose and route of administration	Subjects (*n*)	Primary endpoint	Secondary endpoint	NCT number
Randomized, placebo controlled RCT; iNO pulse for COVID-19 **Time frame:** 14 to 28 days	iNO, 40 ppm as an acute treatment of hypoxemia	n = 30	Incidence of ADR	(I) Prevention of progression (II) Clinical improvement	NCT04398290
Multicentre, phase-2, RCT; NO releasing solutions to prevent and treat COVID-19, **Time frame:** 14–21 days	Gargle, nasopharyngeal irrigation, Nasal spray 5 times a day	n = 200 age > 19 yrs **Prevention study:** Healthy volunteers and health workers who are in contact with the COVID-19 patients **Treatment study:** COVID-19 positive patients	Prevention of COVID-19	(1) Prevention of progression (2) antiviral effect (3) negative test for COVID-19	NCT04337918
Phase-2, Open label safety study, Sequential assignment; Inhaled Gaseous Nitric Oxide (gNO) Antimicrobial Treatment of Difficult Bacterial and Viral Lung (COVID-19) Infections	iNO 0.5% / Nitrogen 99.5%, up to 160 ppm	n = 20 Probable or known COVID-19 patients	Safety and efficacy	(I) reduction in mortality (II) antiviral effect (III) reduced incidence of mechanical ventilation	NCT03331445

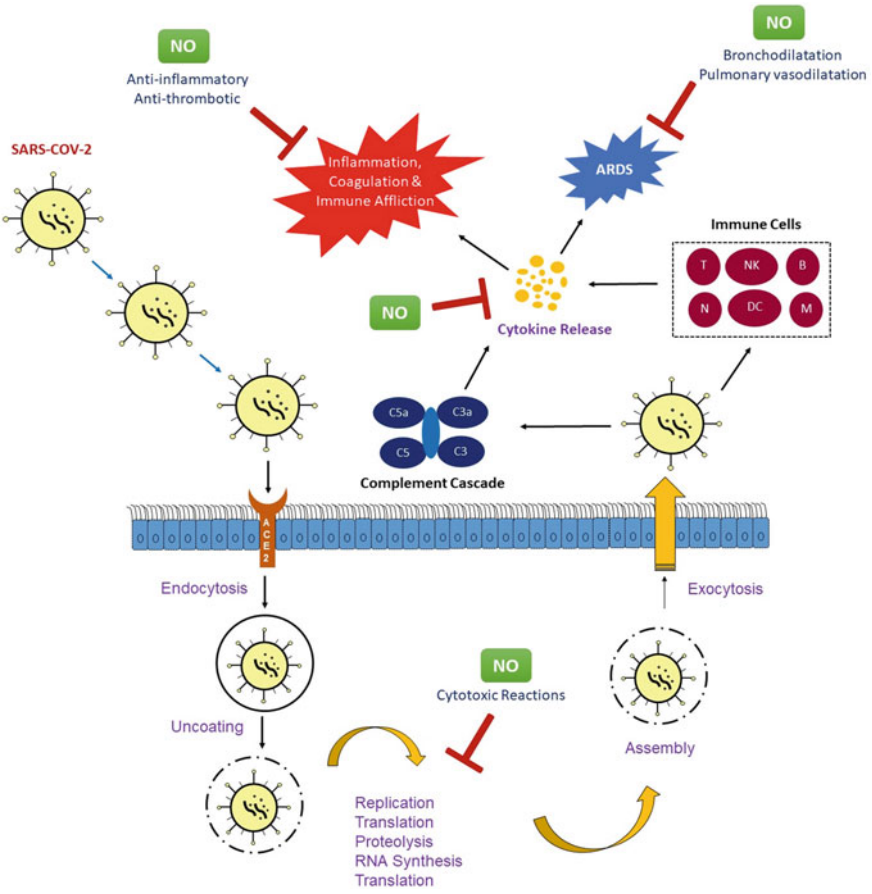

Fig. 13.1 Role of NO in COVID-19

Conclusion

The efforts to repurpose antiviral agents like remdesivir, antibiotics like azithromycin, steroids like dexamethasone and others have yielded marginal benefits in the prevention and treatment of COVID-19 infection and its consequences. The inflammatory and fibrotic consequences affecting lungs and thromboembolic events post-COVID-19 infection appear to be the major contributors to morbidity and mortality during this pandemic. There is continued search for the novel therapies and also repurposed drugs in preventing the spread of infection and also to treatment consequences of COVID-19. Recent evidences on the antiviral, anti-inflammatory, immunomodulatory and cardiovascular effects of NO have raised hope for its use as a therapy at different stages of COVID-infection and its consequences. However, these claims will be reinforced only through systematic clinical studies and in near future there

is possibility of addition of NO in the therapeutic arsenal against the COVID-19 infections.

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Chapter 14
Sex Differences in Stress and Stress Related Neuropsychiatric Disorders: Focus on Nitric Oxide

Ayanabha Chakraborti, Kavita Gulati, and Arunabha Ray

Abstract Sex differences in neuropsychiatric disorders are well reported although the mechanisms remain poorly understood. The prevalence of major depressive disorders and anxiety disorders are substantially higher in women as compared to men. Moreover, sex differences also exist in terms of symptom severity as well as comorbidities of such ailments with other neurological disorders. Nitric oxide containing neurons are widely distributed within the brain and nitric oxide synthase may co-localize with gonadal hormones' receptors. Estrogen as well as other gonadal hormones may influence nitric oxide synthase expression. Neuronal nitric oxide synthase (nNOS), is abundant in multiple regions of the brain closely associated with the pathophysiology of affective disorders including the prefrontal cortex, hippocampus, amygdala, and the hypothalamus. This review article critically examines the clinical and basic research findings on sex differences in stress-related neuropsychiatric diseases with a particular focus on the role of nitric oxide action in the pathophysiology of such disorders. The interplay between gonadal hormones and NO signaling in the brain, as well as how such interactions affect mood disorders, are also discussed. Recent advances in therapeutic approaches for developing appropriate NO modulators and targeting NO signaling pathways for stress related disorders have been briefly covered, as well as priorities for future research.

Keywords Stress · Nitric oxide · Sex differences · Estrogen · Anxiety

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_14

Introduction

Stress has an important role in the pathophysiology of a wide range of neuropsychiatric disorders, and the incidence and prevalence of psychiatric ailments including anxiety, depression, schizophrenia, and bipolar disorder differs between men and women [1]. Anxiety and depressive disorders are most common in adolescence and early adulthood, with females having a higher risk than males [2]. Females have greater lifetime incidence for depression and most anxiety disorders [3]. Gender variations in the monoamine transmitter system and the HPA axis have been discovered, implying that these could be the underlying causes of depression susceptibility. Serotonin, norepinephrine, and dopamine are monoaminergic neurotransmitters that have sex-related differences in their receptor expression and binding, according to rodent research [4]. Pronounced sex differences in baseline HPA axis activity have been reported, *i.e.*, females have been shown to have a stronger HPA response to stressors [5]. Multiple brain areas have shown sexual dimorphism in neuronal gene expression [6]. However, the molecular mechanisms that mediate sex differences in stress-related neuropsychiatric illnesses, remain poorly understood.

Nitric oxide (NO) is an important signaling molecule that regulates a wide range of brain functions. Overproduction of NO, on the other hand, stimulates the generation of peroxynitrite, protein SNO and tyrosine nitration, all of which have been linked to the pathophysiology of a variety of neuropsychiatric and neurodegenerative brain disorders [7, 8]. NO has the ability to regulate neurotransmitter production [9]. NO also influences hippocampus neurogenesis, brain plasticity, nerve growth factor synthesis, hypothalamic-pituitary-adrenal (HPA) axis activity, and other depression-related targets [10]. As a result, NO dysfunction in the brain has been related to the pathogenesis of anxiety disorders, major depressive disorder, bipolar disorder, and schizophrenia [11].

Gender differences in NO production and oxidative/nitrosative stress in a variety of brain regions have been reported and such changes have been associated to both gonadal hormones and genetic variables [12]. Estradiol, positively modulate hippocampal NO generation in nNOS-deficient mice via receptor mediated mechanism [13]. Stress was observed to boost males' glucocorticoid-dependent NO production while reducing females' NO production in the hippocampus. In the anterior hypothalamus and preoptic area, sex-dependent differences in nNOS mRNA expression have been described [14]. Females have been shown to be significantly more resilient to oxidative/nitrosative stress than men in both *in vitro* and *in vivo* investigations [15]. Synaptic processes related with NO and SNO differ between male and female mice. In fact, in contrast to males, the female cortex was shown to be considerably enriched in these activities. The neurochemical mechanisms that drive sexually dimorphic synaptic patterning, on the other hand, are largely unknown.

The findings of clinical and basic research on sex differences in stress-related neuropsychiatric diseases are critically examined in this review, with a particular focus on the role of nitric oxide action in the pathophysiology of such disorders. The connection of gonadal hormones and NO signaling in the brain, as well as

how such interactions affect mood disorders, are also discussed. Current therapeutic approaches for developing appropriate NO modulators and targeting NO signaling pathways for affective disorders have been briefly covered, as well as priorities for future research.

NO Signaling in the CNS: An Overview

Garthwaite and colleagues' seminal study [16] demonstrated that stimulation of NMDA receptors boosted the release of a diffusible messenger in a calcium dependent way, which was eventually identified as NO. NO is produced from L-arginine via enzymatic conversion by NO synthase enzymes [17]. NO synthase comprises of three isoforms-neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS and eNOS enzymatic activity are regulated by calcium-calmodulin and more prominently active in neurons and endothelial cells, respectively. Normally there is modest iNOS level expression under basal conditions, but under inflammatory stimuli the expression is increased resulting in significant NO generation [18]. The nNOS that is connected to the NMDA receptors, serves as the primary source of NO generation in neuronal cells [16], although all the NOS isoforms can affect CNS signaling. Other stimuli including muscarinic activation, changes in serotonergic neurotransmission relevant receptor/transport proteins that alters intracellular calcium concentration can modulate nNOS in the brain [19]. cGMP is activated via protein–kinase G (PKG) dependent signaling, and NO-cGMP pathway also plays key role in CNS physiology and pathophysiology [20]. Furthermore nitrosylation of neuronal proteins can lead to change in their activity and have important implications for neuronal signaling and plasticity [21].

NO Signaling in the Brain and Gonadal Hormones

Gonadal hormones can affect NO production and NO-mediated signaling in both peripheral and CNS tissues. Endothelial nitric oxide synthase (eNOS) and neuronal and nitric oxide synthase (nNOS) in neural tissues increase the activity, expression, and synthesis of NO [22]. In mouse brains, both testosterone and estrogens affect the expression of neuronal nitric oxide synthase [23]. Furthermore, nitric oxide synthase and gonadal hormone receptors may co-localize. Endocrine hormones can change the expression of nitric oxide synthase during maturity and development [24]. Deletion of ER alpha in rodents have been shown to substantially reduce NO synthase expressing neurons in specific areas of the brain. Although they are crucial in physiological conditions like the estrous cycle, the impacts of sex steroid hormones have primarily been studied after long-term therapy [25].

In mammalian brains, the distribution of nNOS overlaps that of gonadal hormone receptors in numerous brain locations. In the bed nucleus of the stria terminalis, the

amygdala, the preoptic area, and the mediobasal hypothalamus, estrogen receptors (ER-alpha and ER-beta), androgen receptors (AR), and progesterone receptors (PR) are abundant [26]. Sex differences in colocalization of ER-alpha with nNOS cells in different brain areas have been reported. For example, in males ER-alpha colocalizes with 90% nNOS cells in medial amygdala and 50% nNOS cells in the BNST while a relatively small number of nNOS cells are colocalized with the receptor in these brain areas in females [27].

Studies have shown that nNOS expression in different areas of the brain are controlled by steroid hormones and immunoreactivity of nNOS in preoptic hypothalamic area have been shown to be reduced by castration in rodent studies [28]. The effect of hormonal therapies on nNOS expression in brain areas have been inconclusive. E2 treatment has been shown to increase nNOS-positive neurons in the PVN [29]. On the other hand, nNOS expression in hypothalamus have been shown to have minimal effect by hormonal treatment [30]. Using Aromatase knockout mice it has been demonstrated that the immunoreactivity of nNOS is reduced in PVN and VMH of the brain [27]. As compared to wild type mice, substantial alterations in hypothalamic and limbic areas of the brain are found in ER alpha knockout mice [31]. suggesting a key role of estrogens in modulation NO signaling in the limbic-hypothalamic areas of the brain. Studies using a double mutant mouse deficient in both functional ER and AR, has shown that ER and AR interact in a site-specific manner to modulate NOS in males and females. E2 treatment increases the number of nNOS-IR cells in the posterior ventral area of MeA and the PVN while testosterone treatment results in more nNOS-IR cells and immunoreactive area staining in the MPA. Overall, these studies highlight a critical role of gonadal hormones in the regulation of nitrergic signaling in the brain.

NO and Sex Differences in Brain and Behavioral Responses to Stress

In animal and human studies, gender disparities in specific cognitive capacities have been established, particularly under stressful situations [32, 33]. Sex dependent changes in neuronal function and oxidative stress markers in the hippocampus due to prenatal stress has been reported. Stressful experiences facilitate associative learning and memory consolidation in males, but when females are exposed to the same stress, their cognitive abilities are severely affected. An expanding amount of research suggests that structural differences in the brain play a role in how stress affects memory consolidation in men and women. Functional dimorphisms are known to exist between male and female brains in the realm of emotional control and other higher order brain functions. There have been reports of significant sex differences in the function of the amygdala during processing of emotional stimuli [34]. Differences in emotional state perception and experience between the sexes have been associated to subtle structural differences brain areas. Limbic system activation due to negative

emotions occurs significantly more in women than in men, who rely more on cortical components. Female rats, on the other hand, do not demonstrate the spatial memory loss seen in male rats after chronic restraint stress. Acute stress also changes males' anxiety behaviors and impeded escape learning, but not females' [2].

Corticotropin-releasing factor (CRF), a stress neuropeptide has been shown to contribute to the pathophysiology of stress related disorders. Sex related variations in CRF receptor density, expression, distribution, trafficking, and signaling are well known and differences in CRF responses between males and females may play a prominent role in the sex specific vulnerability in neuropsychiatric disorders [35]. Important studies on sex differences in CRF receptors in the brain has been summarized in Table 14.1.

Both human and preclinical studies show that exposure to stress induces neurobehavioral alterations and elevated corticosterone levels [45]. Stress effects on the brain is dependent on the intensity, type, and duration of stressors. While exposure to chronic unpredictable stress leads to behavioral dysfunction, homotypic stressors

Table 14.1 Summary of key studies on sex differences in corticotropin releasing factor (CRF) factor in the brain

CRF receptor	Reported sex difference	References
Receptor binding	CRF1 receptor binding is higher in amygdala and cortex in adult female rats as compared to male rats. CRF2 receptor binding is higher in regions of the amygdala and hypothalamus in male rats and as compared to females.	[35–37]
Receptor number	CRF1 receptor expression is elevated in the dorsal and ventromedial portion of dorsal raphe in female as compared to male rats. CRF2 receptor expression is also higher in females than males in ventrolateral dorsal raphe.	[35, 38]
Receptor distribution	CRF1 receptor co-localizes with dorsal raphe parvalbumin neurons more in male than in female mice. In hippocampal CA1 area female rats have more CRF receptors in delta opioid receptor-containing dendrites than males.	[35, 39, 40]
Receptor trafficking	In male but not female rats' exposure to acute stress causes β-arrestin2 to bind to the CRF1 receptor and CRF1 receptor internalization in locus coeruleus (LC) dendrites. LC neurons in CRF-OE female mice fire three times faster than those of males leading to increased arousal in the females but not in males	[35, 41, 42]
Receptor signaling	CRF1 receptor signals more through β-arrestin2-mediated pathways in males and more through Gs-mediated pathways in females Overexpression of CRF increased the phosphorylation of proteins in Alzheimer's disease pathways more in female than male mice	[35, 43, 44]

often result in diminished responses suggesting stress adaptation, an inbuilt protective mechanism to counter the effects of chronic stress [46, 47]. Exposure to acute or chronic stress may have varied biological responses and the processes underlying these reactions are poorly understood and need to be clarified [48].

Studies from our lab has demonstrated that acute restraint stress induces anxiogenesis which ameliorated by pretreatment with L-arginine, an NO precursor suggestive protective role of NO under stressful conditions. In a subsequent study we investigated the role of NO following acute and recurring restraint stress using behavioral and biochemical approaches [49]. The interplay between reactive oxygen and nitrogen species, was also investigated under such situations. Both 1 hr. and 6 hrs. of RS evoked anxiogenic responses which were ameliorated by prior administration of L-arginine. The levels of nitric oxide metabolites, lipid peroxidation marker and antioxidant GSH levels were altered in brain homogenates and were associated with the behavioral changes. Interestingly there was increased lipid peroxidation in the brain while the levels of NOx and GSH were lower indicating that RS contributes to prooxidant-antioxidant imbalance in the brain.

Although men and women differ in their sensitivity to stress and how they react to it, the mechanism underlying these variances is unknown. Using a rodent model of stress, we therefore investigated the role of nitric oxide in stress induced anxiogenesis and how such responses vary as a function of sex. Exposure to RS caused anxiogenesis in the elevated plus maze (EPM), and these changes were more pronounced t in males than females. Behavioral changes were associated with higher asymmetric dimethylarginine (ADMA) and lower levels of NOx in rat brain homogenates, and effects being of higher magnitude in males than females. This was the first demonstration of that ADMA, an inhibitor of endogenous nitric oxide synthase, plays a crucial role in stress-induced neurobehavioral changes [50]. RS induced behavioral and neurochemical changes were reversed by L-Arginine in a dose dependent manner while NO synthase inhibitor exhibited opposite effects suggesting that both re-active oxygen and nitrogen species have a substantial modulatory role in the differential anxiogenic stress response between males and females [50].

In addition to neurobehavioral responses, stress also induces gastric ulcerogenesis via complex brain-gut axis interaction. The role of NO during gender-specific stomach ulcerogenesis during cold constraint stress has been explored (CRS). CRS exposure caused gastric ulcers in both male and female rats, although the rate of ulceration was substantially higher in male rats than female rats, L-arginine administered prior to CRS reduced the number and severity of ulcers in both male and female rats in a dose-dependent manner, but the effect was substantially more evident in males. Inhibition of NO production by L-NAME, on the other hand, consistently increased stress ulcerogenesis in males. CRS-induced stomach ulcerogenesis was linked to lower NOx and GSH levels and elevated MDA levels in male and female brain homogenates, and the responses were more pronounced in males than females, which is intriguing. Pre-treatment with formestane (an aromatase inhibitor) but not tamoxifen (an estrogen receptor blocker) exacerbated the development of stress ulcers in female rats when compared to vehicle-treated exposed CRS rats. Formestane administration resulted in larger reductions of brain NOx and GSH, as well as higher brain

MDA levels, when compared to vehicle-treated CRS rats. These findings suggested that estrogen, and its interactions with oxidative stress markers and NO, play a key role in sex related differences in stress-induced stomach ulcerogenesis, CRS may lead males to have lower brain NO levels and more oxidative injury, which may contribute to the severity of stomach ulcers. Estrogen's protection, on the other hand, may be due to females' greater tolerance of CRS' ulcerogenic effects, which appear to be linked to interactions with brain NO [51].

NO and Sex Differences in Neuropsychiatric Disorders

(i) ***Anxiety Disorders***

Anxiety disorders are a type of neuropsychiatric ailment that is characterized by persistent anxiety with a wide range of associated physiological and behavioral responses [52]. Anxiety disorders include generalized anxiety disorder, phobia, panic disorder, post-traumatic stress disorder. A variety of neurotransmitters and transcription factors have been implicated in the etiology of anxiety [52]. Benzodiazepines (BZPs) increase the activity of GABA on the GABAA receptor, causing calming effects [53]. Anxiety has been linked to changes in the serotonergic and glutamatergic systems. Anxiogenesis is aided by inhibiting the 5-HT1A receptor, and selective serotonin reuptake inhibitors (SSRIs) are extensively used as anxiolytics [54]. Both neuropeptide Y and CREB signaling has been linked to pathophysiology of anxiety [55].

According to epidemiological studies, the incidence and prevalence of anxiety disorders are higher (~2-fold) in women as compared to men [56]. Such higher prevalence among females is true for all the disorders under anxiety disorders spectrum including social anxiety, generalized anxiety and panic disorder. Anxiety symptoms in women may exacerbate during different stages of the reproductive cycle like adolescence, pregnancy, post-partum when hormonal fluctuations are prominent [57]. These times of increased risk that correlate with hormonal changes suggest a key influence of that gonadal hormones in the precipitation and exacerbations of anxiety disorders in women. The sex specific vulnerability of developing anxiety disorders may also be linked to variations in the controlling negative emotional responses to stressors between the two sexes.

Several studies have linked NO to anxiety [58–60] and NO synthase expressing neurons are found in amygdala, hypothalamus, hippocampus etc.–the brain regions that play a key role in regulating anxiety [61, 62]. 7-NI, a common nNOS inhibitor, exhibits anxiolytic effects similar to that of diazepam. Rodent studies have shown that 7-NI lowers anxiety induced by social stressors [63, 64]. NO decrease has an anxiolytic impact, as evidenced by the relationship between NO and typical anxiolytic drugs. Fluoxetine has been suggested to work as an anti-anxiety medication by reducing nNOS activity and CREB expression. Zhang et al. [65] discovered that the fluoxetine 5-HT1A receptor's regulatory role is mediated by suppression

of the nNOS-NO pathway in the hippocampus, resulting in anxiolytic effects [65]. Anxiolytic effects were seen in the actions of neurosteroid dehydroepiandrosterone sulphate (DHEAS), which was potentiated by the NO precursor L-arginine [66]. On the other hand, pretreatment with L- NAME [66] a kind of NOS inhibitor, entirely reduced DHEAS' anxiolytic effects. Furthermore, the anxiolytic effects of morphine have been found to be partially regulated by NO. However, whether and how increased nNOS-NO signaling contributes to anxiety is still poorly understood. Anxiety-inducing stress has been associated to increased nNOS expression in the PFC and hippocampus [67]. An increase in 5-HT in the hippocampus following a stressful situation contributes to the development of anxiety. 5-HTR1 is deactivated. A signaling mechanism that control anxiety-related behaviors regulate the expression of nNOS in the hippocampus [65]. ERK phosphorylation is significantly inhibited by the nNOS-NO pathway [68]. The nNOS-CAPON-Dexras 1 complex is activated in response to mild stress, inhibiting ERK phosphorylation. The anxiolytic impact of disrupting the interaction between nNOS and CAPON reverses this strategy.

There are few studies that have investigated the role of NO in sex differences in anxiety. Using ovariectomized (OVX) rats, [69], the effects of the NOS inhibitor L-NAME and the NO precursor L-arginine on the anxiety modulatory capabilities of exogenous ovarian hormones have been investigated. Cycling rats spent more time in open arms and had lower serum NOx levels during metestrus than other cycle phases and OVX rats, while they spent less time in open arms and had lower serum NOx levels during proestrus. L-NAME had an anxiolytic effect in OVX rats, whereas L-arginine had no impact. Estradiol benzoate significantly elevated serum NOx levels and had an anxiogenic impact when compared to controls, which was dose-dependently decreased by L-NAME but not by L-arginine. Progesterone, on the other hand, dramatically reduced serum NOx levels and had an anxiolytic effect, which was eliminated by L-arginine but not by L-NAME. These findings suggested that the NO system may be involved in variations in anxiety levels associated with the estrous cycle, most likely via regulating the influence of ovarian sex hormones. Munoz-Castaneda et al. [70] examined sex differences in motor coordination and anxiety-related responses in response to nicotine therapy and genetic NOS1 activity depletion. The open-field and rotarod tests were used as behavioral assays in both male and female mice. NOS1 knockout mice were studied to better understand the role of NO. Nicotine was delivered continuously via osmotic mini pumps over a 2-week period. Control NOS1 KO males exhibited an enhanced anxiogenesis as compared to control NOS1KO females and control wild-type (WT) males. However, these differences were not apparent in the nicotine administered NOS1 KO males. NOS1 deletion also differentially affected motor function in the males and females. Overall, these findings suggested that NO affects motor and anxiety behaviors in a sex-dependent manner.

(ii) ***Depression***

Major Depressive Disorder (MDD) is a neuropsychiatric condition characterized by a persistent feeling of sadness, reduced motivation and sometimes may also be life threating [71]. Several neurotransmitters including serotonin, norepinephrine,

and dopamine have been linked to pathogenesis of depression Postmortem brain studies have suggested that patients with depression have a deficiency of serotonin and several antidepressants increase the concentration of serotonin in multiple brain regions [72]. Sex differences exist not only in the in the prevalence and symptoms of MDD [73] but also the determinants of functional outcomes differ between males and females [74].

nNOS has been shown to play an important role in the regulation, synthesis, release, and absorption of 5-HT. nNOS knockout mice has elevated 5-HT levels in various brain areas including hypothalamus, hippocampus, cerebral cortex, and amygdala. NO donors have been found to differentially regulate 5-HT release in raphe nucleus and frontal cortex [75]. Intracranial as well as systemic infusion of 7-NI elevates 5-HT levels in the hippocampus. Both glucocorticoids and chronic stress have been demonstrated to cause a decrease in 5-HT1A receptor density and messenger RNA (mRNA) content in the hippocampus Endogenous NO produced from nNOS may thus have a role in the pathophysiology of depression by modulating the 5-HT pathway in the hippocampus in response to chronic stressors [76]. Treatment with the nNOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole (TRIM) increased the behavioral effects of imipramine, citalopram selective serotonin reuptake inhibitor (SSRI), and fluoxetine or tianeptine [77]. The antidepressant-like action of bupropion, a dopamine reuptake inhibitor, was inhibited when it was pretreated with L-arginine, a nitric oxide synthase substrate. Furthermore, pretreatment of mice with 7-nitroindazole enhanced the impact of bupropion [78]. The behavioral effects of imipramine and fluoxetine were improved when they were given the nNOS inhibitor 7-nitroindazole [79].

Studies investigating the role of nitric oxide in the context of sex differences in depressive behavior is limited. Hu et al. [13] showed that hippocampal NO contributes to the sex difference of depression-like behavior in mice. Chronic mild stress promotes nNOS expression and elevates NO expression in the male hippocampus, while it inhibits nNOS expression and causes NO shortage in the female hippocampus and NO donor infusion in the female hippocampus mended the sex gap of affective behaviors. Heydarpour et al. [80] conducted a study to investigate the antidepressant-like effects of acute estradiol administration in female ovariectomized (OVX) mice and the possible role of nitric oxide (NO)/cyclic GMP (cGMP) pathway. OVX mice showed significantly prolonged immobility time in comparison with the sham group. Estradiol administration 1 h prior to FST, exerted antidepressant-like effects in OVX mice. Both L-NAME and 7-NI significantly reduced the immobility times of OVX mice. Administration of a sub-effective dose of L-NAME 15 min after a sub-effective dose of estradiol had a robust antidepressant-like effect in OVX mice. Both L-arginine and sildenafil prior to estradiol treatment prevented the antidepressant like effect of a potent dose of estradiol suggesting that suppression of the NO synthase/NO/cGMP pathway may be involved in the antidepressant-like effects of estradiol in OVX mice.

(iii) ***Bipolar Disorder***

Bipolar disorder (BD) is a serious psychiatric condition characterized by recurrent bouts of mania and depression [80, 81]. About 1% of the population globally is

affected with this disorder and is an important cause of morbidity [82]. Sex differences in the clinical symptoms and course of this disorder has been reported. The general course of BD varies between men and women with marked differences between episodes of mania or depression, length of individual depressive episodes as well as the age of onset of the disease [83].

Alterations in NO signaling have been linked to the pathophysiology of BPD [84]. Lower levels of systemic nitric oxide levels have been reported in BD patients [85]. Lithium- a drug commonly used in the treatment of BD has been reported to elevate NO levels especially during depressive episodes [86]. On the other hand, increased NO and total nitrite levels in BD patients have also been reported with serum NO levels have been found to be higher in patients with euthymic-phase BD [87]. Reports on nNOS mediated nitrergic dysfunction in the locus coeruleus has been linked to BD pathogenesis. The levels of nNOS protein in the LC of suicidal people is considerably lower than in controls, according to a postmortem investigation [88]. However, more research into the relationship between NO signaling and BD pathogenesis especially with reference to sex specific variations is warranted.

(iv) ***Schizophrenia***

Schizophrenia is a neuropsychiatric condition with clinical symptoms and cognitive changes differing by gender. Both neurodevelopmental and social factors contribute to the sex disparities in this disorder. Sexual dimorphism in brain areas that regulate mood and emotions has also been associated to the differences in prevalence and symptoms severity between males and females. Sex differences between cognitive deficits and white matter abnormalities in first episode and drug-naive schizophrenia has been reported [89]. Levels of Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, was found to be higher in patients with schizophrenia, suggesting that ADMA may play a role in the pathophysiology of schizophrenia-related cognitive impairments and that plasma ADMA could be used as a peripheral biomarker for assessing cognitive function in schizophrenia [90]. Patients with schizophrenia had significantly higher plasma nitrate and nitrite concentrations, with female patients having significantly higher quantities than male patients [91]. Because of its role in glutamate neurotransmission, the nitric oxide synthase 1 adapter protein gene (NOS1AP) had previously been identified as a schizophrenia susceptibility gene. Cheah et al. [92] looked at the link between NOS1AP polymorphisms and schizophrenia depressive characteristics. A cohort of 235 schizophrenia patients were genotyped for nine SNP variants and one NOS1AP SNP was linked to the general diagnosis of schizophrenia, while eight others were linked to depression-related phenotypes in schizophrenia. Overall, these studies implicate a critical regulatory role of NO in the pathogenesis of schizophrenia and sex related factors may modulate such changes.

Targeting NO and NO-Mediated Signaling as a Treatment for Stress and Mood Disorders

The role of the NO cascade on depressive pathophysiology is becoming increasingly recognized in clinical and preclinical research [93, 94]. Several studies have implicated that NO synthase inhibitors exert antidepressant effects. L-NAME, a non-specific NO synthase inhibitor has been shown to have antidepressant like response in forced swim test and those effects were reversed by L-Arginine treatment. Another non preferential NO synthase inhibitor L-NNA was found to potentiate the behavioral effects of antidepressants. Co-treatment with the certain 5-HT receptor antagonists have also been found to attenuate the L-NA-induced reduction of immobility in forced swim test [95]. NOS inhibitors, including as L-NAME, aminoguanidine, and sildenafil, were found to reduce LPS-induced depression-like behavioral and neurochemical alterations [96, 97].

Selective nNOS inhibitors like 7-NI have been shown to produce antidepressant behavioral effects and improve imipramine and fluoxetine behavioral effects in the FST. Stress induced cFos expression and immunoreactivity levels are also reduced by 7-NI and TRIM in a similar manner like classical antidepressants indicating that these drugs have similar neurobiological substrates [97, 98]. 7-NI also modulates CREB signaling pathway suggesting a possible involvement of this signaling mechanism in 7-NI induced antidepressant effects [99]. It has been demonstrated that mice in which the nNOS gene has been knocked out, display an antidepressant phenotype. Chronic stress increased nNOS expression in the hippocampus and inhibiting nNOS function ameliorates stress-induced depressive like behaviors [76]. Chronic treatment with 7-NI via increasing BDNF protein levels in the hippocampus exerts protective effect in the learned helplessness model of depression [97, 100]. Role of nitric oxide in the antidepressant-like effects of ketamine has also been suggested [101]. Taken together, extensive data suggest that the nNOS-NO-sGC pathway has a critical role in mood and depression and NO signaling pathway can serve as a therapeutic target in such disorders.

Conclusion and Perspectives

Male and female pathophysiology of stress-related neuropsychiatric diseases differs significantly, according to compelling data from both clinical and pre-clinical investigations. The mechanisms that cause sex variations in stress responses and create sex biases in disease risk or resilience are complex, but they appear to include an interaction of sex chromosome genes with periods of dynamic hormonal changes that may compound over time. Women's dynamic hormonal variations and other aging-related cellular processes in limbic brain regions contribute to sex-specific variations in stress reactivity in the aging brain. For homeostasis and survival, the brain's ability

to sense and respond effectively to stress must be maintained throughout one's lifetime. As a result, it is critical to understand how gender differences in stress reactions can predict disease risk and resiliency when creating prevention and treatment strategies. As part of our best effort in mental health, studies that incorporate sex as a factor remain a vital necessity across the lifespan. Many stress effectors are known to be regulated by NO. Under both basal and stressful situations, there is a preponderance of evidence that NO mediated signaling in the brain differs between females and males. There is still a need for a more comprehensive study of sex related variations in brain and behavioral responses to stress to better understand the mechanisms that mediate differential vulnerability between males and females. Studies focusing on time-specific measures in relation to the period of stress are crucial for establishing a temporal relationship between stress and NO in females, as well as the influence of the estrous/menstrual cycle. More research into the interaction of stress and NO in specific brain regions could aid in the identification of NO's role in stress-related diseases in both men and women. Male and female monoaminergic systems are structurally and functionally distinct, which is thought to underpin sex-bias in mood disorders. An important translational goal would be to identify disease pathways associated with the sex bias in NO signaling. One possible strategy for achieving this goal is to compare phosphoproteomes under optimal NO overexpression conditions. Another method for determining causality is to manipulate NO-expressing neurons to have a specific signaling bias and then observe the effects. Another crucial topic is how sex differences in NO coupling to interacting proteins emerge. Recent advancements in transgenics, neuroimaging, and *in vivo* optogenetics are opening the way for a deeper understanding of gonadal hormone-NO interactions at the molecular, cellular, and circuitry level. Although NO is unlikely to be the only sex-specific component underlying brain sex differences, a greater understanding of its role in the regulation and function of the brain will likely lead to better prevention and treatment options for devastating neurological illnesses. Further research into the sex differences in the NO system could provide new vistas on the mechanisms underlying females' increased sensitivity to stress-related neuropsychiatric illnesses, as well as in developing better therapeutic strategies. Inhibiting NO production has shown promise to have antidepressant-like effects in preclinical studies. Direct inhibition of nNOS and/or iNOS, are some of the pharmacological strategies that can be used to produce these results. As a result, lower NO levels may allow for proper monoaminergic signaling during stressful situations, which could aid behavioral adaptability. Continuous reduction of NO generation, in this circumstance, may enhance neuroplastic pathways connected to the antidepressant effect, such as increased BDNF-TrkB signaling and neurogenesis, following chronic stress exposure. Despite substantial progress, developing drugs that differentially inhibit the 'correct' NOS isoform at the appropriate brain region remains a challenge. However, in the future development of antidepressants and antipsychotics, NO signaling pathway definitely can serve as a viable therapeutic target.

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Chapter 15
Nitric Oxide in Major Depressive Disorder

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Abstract The pathogenesis of mood disorders remains elusive, but it is evident that multiple factors, genetic and environmental, play a crucial role in adult psychopathology and neurobiology. Concerning therapy, a significant proportion of affective disorder patients are partial or non-responders. There has been no breakthrough in finding novel, valuable drug targets since introducing the current marketed antidepressant drugs in the 1950s to the 1980s, which all are based on monoaminergic pharmacological effects. Consequently, there is a pressing need to develop novel treatment strategies—and ultimately understand the aetiology and pathophysiology of affective disorders. Nitric Oxide serves an essential role in the nervous system. It acts as a messenger molecule in several physiological processes, including processes linked to major psychiatric diseases. The present chapter will review the general aspects of the NO system in Major depressive disorder (MDD) and focus on reducing NO production as putative therapeutic agents towards depression.

Keywords Major depressive disorder · Mental health disorders · Stress · Inflammation · Serotonin · Neuroplasticity · Nitric oxide

Introduction

Data from Europe [1–3] indicate that brain disorders account for 12% of all direct costs in the health system, and 9% of the total drug consumption was used to treat brain diseases. Expenses for brain diseases constituted 3–5% of the gross national products, and the whole European expenditures for all investigated brain diseases reaching almost 800 billion EURO in 2010 [4, 5]. Among brain disorders, affective

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_15

disorders were among the costliest diseases (110 billion Euro), and anxiety disorders were among the most prevalent.

The pathogenesis of mood disorders remains elusive, but it is evident that multiple factors, genetic and environmental, play a crucial role in adult psychopathology and neurobiology [6]. Concerning therapy, a significant proportion of affective disorder patients are partial or non-responders. Consequently, there exists a pressing need to develop novel treatment strategies—and ultimately understand the aetiology and pathophysiology of affective disorders.

Nitric Oxide (NO), described initially as the Endothelial-derived relaxing factor (EDRF) with essential roles in the cardiovascular system and macrophages [7, 8], has also been shown to have a crucial role in the nervous system [9, 10]. It serves as a messenger molecule in several physiological and pathological processes, including processes linked to major psychiatric diseases [11–14]. The present chapter will review the evidence for the involvement of the NO signalling in Major Depressive Disorder (MDD), the role of NO in antidepressant action, and the role of NO synthesis inhibitors as putative therapeutic agents.

General Aspects of Nitric Oxide in the Brain

The initial evidence indicating NO as a possible signalling molecule in the brain came from the seminal work by Garthwaite and colleagues [15, 16], showing that the activation of N-methyl-D-aspartate (NMDA) increased intracellular Ca^{2+} and cyclic GMP (cGMP) levels in cerebellar cells. Since both the NMDA antagonist and haemoglobin (which traps EDRF/NO) blocked the increase in cGMP after NMDA stimulation, it was suggested that EDRF was released in brain cells in response to NMDA receptor activation by glutamate. Later on, it was identified that increased intracellular Ca^{2+} could activate the NO synthase, which then converts L-arginine to NO and L-citrulline, as reviewed by Guix and colleagues [17]. L-citrulline has no signalling function, but NO can influence several targets, as described below.

Three major isoforms of NOS have been identified: neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), and inducible NOS (iNOS or NOS2). NOS1 and NOS3 are Ca^2 calmodulin-dependent enzymes constitutively expressed primarily in neurons and endothelial cells, respectively. The constitutive isoforms produce low NO concentrations, usually associated with soluble guanylyl cyclase (sGC) activation. NOS1 is widely expressed throughout the brain, especially in the cerebellum, the basal ganglia, hippocampus, hypothalamus, frontal cortex, raphe nuclei, amygdala, and other regions [18]. NOS2, on the other hand, is not expressed or is expressed in very low levels under basal conditions, requiring de novo synthesis triggered by immunological or inflammatory stimulation in macrophages, astrocytes, microglia, and other cells, to produce NO [19]. Because NOS2 has a high affinity for Ca^{2+}-calmodulin, it is usually active when expressed in the cell, thus producing large amounts of NO for longer periods, thereby causing several cytotoxic and immunotoxic effects [19].

The enzyme sGC remains the primary target for NO in the brain. The binding of NO to this enzyme increases its activity more than 200 times and catalyzes GTP conversion into cGMP. cGMP activates protein kinase G (PKG)-dependent signalling [20, 21]. Both NOS1 and sGC are co-localized in several limbic brain regions, supporting the idea of an integrated NO-cGMP signalling system in the brain [22]. In addition to that, NO can also interfere with different signalling mechanisms by inducing "S-nitrosylation" of many other proteins, thereby affecting their activity. S-nitrosylation is characterized by adding a NO group to a cysteine thiol/sulfhydryl (RSH) with significant consequences for neuronal signalling and neuroplasticity [23].

Various upstream signalling cascades regulate NOS1 activation. Specifically, NOS1 is physically attached to the NMDA receptor complex via the Postsynaptic density protein 95 (PSD-95) [24] in glutamatergic neurons [25], as NOS1 has a PDZ-domain interacting with the PDZ2 domain belonging to PSD-95, thereby anchoring NOS1 at the post-synaptic density [26]. Moreover, the GluN2 subunits of the NMDA complex attach to the PDZ domains of PSD-95, bringing the NMDA receptor complex close to NOS1. The physical proximity allows NMDA receptor-mediated Ca2+ influx to interact with CaM, activating NOS1 by phosphorylation, increasing NO synthesis. NO interacts not only with the NMDA receptor via the PDZ domains but also with other proteins. For example, the interaction between NOS1 and CaM is blocked by the Calmodulin protein kinase (CaMKII) via phosphorylation of nNOS [27, 28]. Another important example is the NOS1 adapter protein (NOS1AP - previously known as CAPON). As NOS1AP binds directly to NOS1, it competes with the interaction between NOS1 and PSD-95. It may thereby alter the subcellular localization of NOS1 [29], making it less likely to be activated following NMDA mediated Ca2 + influx or facilitating interaction of NOS1 with other proteins [30, 31]. The binding of NOS1 to NOS1AP has also been shown to shift the signalling towards the activation of a downstream MAP kinase (MAPK) cascade and, thus, modulate nuclear transcription of cAMP-response element-binding protein (CREB), as well as other transcription factors such as N-myc proto-oncogene protein (N-Myc), nuclear factor kappa B (NF-κB) [28, 32–35].

The amount of NO produced can vary significantly between tissues and even within cells. The levels and bioavailability of NO will be dependent on variations of NOS enzyme levels, glutamate receptor coupling/expression, and inactivation/scavenging mechanisms in a given tissue/cell, therefore determining the dominant role of NO (signalling, neuroprotection, vs neurotoxicity). An excessive amount of NO and neurotoxicity are often associated with increased iNOS expression rather than nNOS activation. The promoter region of the iNOS gene contains binding sites for transcription factors such as NF-κβ, and high levels of proinflammatory mediators can thus promote iNOS expression in resting cells, including macrophages, astrocytes, and microglia, which can therefore synthesize NO in high amounts lasting hours or days independent of intracellular calcium [36]. The combination of NO and free radicals like the superoxide anion will form peroxynitrite, which is highly reactive and can then nitrate tyrosine residues on proteins to 3-nitrotyrosine, induce

lipid peroxidation, and cause DNA damage. Therefore, under pathological conditions involving high levels of reactive oxidative or nitrosative species (RONS), NO can severely damage and facilitate neurotoxicity [37].

Therefore, by distinct mechanisms, NO can modulate several neuronal physiological functions ranging from cell excitability and synaptic plasticity to learning and memory. On the other hand, due to its multifaceted nature, NO can also trigger important mechanisms associated with cell death and neurodegeneration, as well as behavioural abnormalities [28, 33–35], described further in the text below in Fig. 15.1.

No and Mental Health Disorders

The NO signalling pathway has been established in several different psychiatric disease entities, including Schizophrenia, Bipolar Disorder, and Major Depressive Disorder. A detailed overview of all these disorders is beyond the scope of this text. Therefore, the present summary will primarily relate to the diagnostic construct 'Major Depressive Disorder,' where several lines of evidence support an association between abnormalities in NO and mood disorders. However, it should be noted that since diagnosis in psychiatry is phenomenological, significant overlaps between the diagnostic entities exist.

Clinical Evidence

Postmortem Studies

Although not with unequivocal results, postmortem material from patients with major depression has reduced NOS1 activity and protein content in various brain regions. In a study of 8 patients (including two with schizoaffective diagnosis and two with bipolar depression) diagnosed according to DSM-IIIR, a reduced number of NOS1 containing neurons in the paraventricular hypothalamic nucleus was observed [38]. This finding was later expanded and confirmed in 11 patients and 11 matched controls [39]. In another study, a strong trend ($p < 0.06$) in decreased activity of the constitutive NOS in the prefrontal cortex of 15 patients with unipolar depression diagnosed versus 15 non-psychiatric controls from the Stanley Consortium was observed [40]. A study examining 12 depressed subjects and 12 psychiatrically normal control subjects, obtained at autopsy at the Coroner's Office of Cuyahoga County, Cleveland, OH, USA, found a significantly lower amount of NOS1 in locus coeruleus of depressed subjects [41]. However, no changes were observed in the cerebellum.

Since the hippocampus is a crucial region in affective disorders' pathophysiology, the possible hippocampal involvement is of great interest. Findings from the CA1 hippocampal area in brains from the Stanley Consortium have reported an increase in

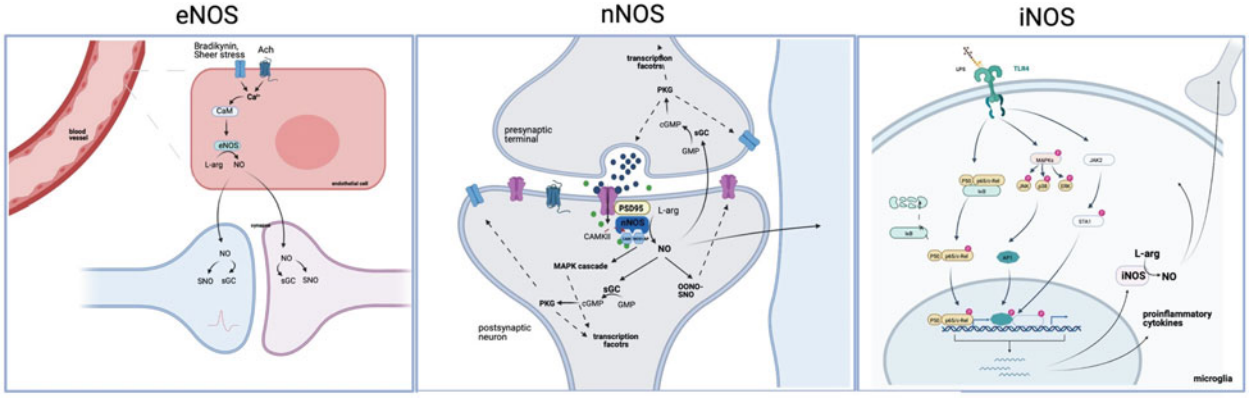

Fig. 15.1 Major pathways involved in NO synthesis in the brain from NOS1, NOS2, and NOS3. NOS3 (eNOS) is mainly expressed in blood vessels in the brain. Its activation by sheers stress, for example, can lead to NO synthesis, which can diffuse and interfere with the excitability of surrounding neuronal cells. NOS1 (nNOS) is the most expressed isoform in the brain, and its activation occurs in response to increased intracellular calcium levels, mainly due to NMDA receptor activation. NO can target sGC and other molecules (enzymes, ion channels, transcription factors) in the cell where it has been synthesized or diffuse to other cells and interfere with the synaptic transmission in a group of neurons. NOS2 (iNOS) has low expression in the healthy brain, and its synthesis is triggered by immune stimuli, such as LPS (lipopolysaccharide). Once synthesized by activated microglia or astrocytes, iNOS can produce more significant NO and prolonged periods than other isoforms, thus increasing oxidative/nitrosative stress neurotoxicity. The activity of NOS2 is tightly associated with its expression levels and is induced by inflammatory stimuli, while NOS1 and NOS3 activities depend on increased intracellular calcium

NOS1 immunoreactivity in depression and bipolar disorder [42]. In the same study, no changes were observed in the brains of schizophrenic patients.

More recently, alterations in NOS1AP were examined in a sample from the Netherlands Brain. It was found that NOS1AP-immunoreactivity was significantly increased in the dorsolateral PFC and anterior cingulate cortex in major depressive disorder, accompanied by an upregulation of spinophilin and downregulation of synapsin [43]. Given the potential role of NOS1AP competing with NOS proximity to the NMDA receptor, this may indirectly decrease NO production in the postmortem brains. Whether this finding is relevant in the living brain remains to be established.

Peripheral Markers

Several studies have examined peripheral NO metabolism in major depression, however, with somewhat mixed results. In a study of suicide attempters, increased NO metabolites (NO_2 and NO_3) have been observed [44, 45], indicating a nitrergic system's hyperfunction. The same finding was reported a few years earlier, where it was found that 17 drug-naïve patients suffering from depression, diagnosed according to DSM-IV, had elevated nitrite levels [46]. In the same study, treatment with antidepressants normalized the nitrite levels, correlating with clinical response [46]. Finally, in a study including 36 depressed patients, diagnosed according to DSM-IV, and 20 healthy subjects, there was no correlation between depressive symptoms and nitrate levels, but a significant effect of antidepressant treatment, lowering the nitrate levels [47]. Besides, some studies demonstrate NO's involvement in some, but not all, forms of IFN alpha-induced depression [48]. However, measurement of nitrate in serum will only detect the overall nitrate pool. Indeed, a study by Srivastava and co-workers examining 66 cases of depression and 114 controls revealed a 73% decrease in nitrite content in the polymorphonuclear leukocytes [49]. Since human polymorphonuclear leukocytes express NOS1 like neurons [50], this measure may be hypothesized to be more relevant than serum values. This assumption is also reflected in a study where decreased platelet NOS activity and plasma NO metabolites in depressed patients were found [51, 52].

Interestingly, in a recent extensive study, 460 patients with a current episode of depression were compared to 895 healthy controls for NOS activity (L-Cit/L-Arg plasma ratio); depressed patients had a lower NOS activity than healthy controls at baseline, which increased significantly after antidepressant treatment [53]. The study is in disagreement with the other studies previously discussed but also uses another methodology. However, as recently reported, this finding may reflect the lower bioavailability of arginine in depressed subjects, reflected in lower NO production [54].

Finally, several human association studies have been published linking endogenous inhibitors of NOS with the disease. The levels of the endogenous inhibitors, NG-monomethyl-L-arginine (SDMA) and NG-dimethyl-L-arginine (ADMA) [55–58], are changed in Depression, Schizophrenia, and Alzheimer's disease [59–62]. However, it is not clear whether these associations are clinically meaningful.

Taken together, although human studies have been predominantly carried out on peripheral tissue samples (e.g., plasma or serum), support for the role of the NO system in psychiatric disease exists. Findings from the peripheral tissue are conflicting and suggest both a decreased and an elevated NO metabolism in depressive states. However, it is worth mentioning that very different methodologies were used. Several of the measures discussed here were carried out on specific sub-components in the blood compartment, and the generalization may be limited.

Genetic Studies

Several association studies have been performed with the emergence of genetic techniques, linking abnormalities in the NO system's genetic architecture with depression. A recent review examining the whole mental health spectrum can be found elsewhere [63].

A population-based association study investigating NOS1 in unipolar depression tested whether the NOS1 C276T polymorphism confers susceptibility to unipolar depression and treatment response to fluoxetine. No association with disease or SSRI treatment response was found in Chinese patients [64], but due to the study's restricted design, it was concluded that other variants of the NOS1 gene might play a role. Similarly, in a study from Denmark and Britain, no significant differences in the frequencies of SNP rs2682826 were observed among the study subjects. However, a difference in genotypes between the Danish and control groups was observed. However, no overall allelic association was reported due to the smaller Danish sample size versus the British [65]. In another Japanese genetic association analysis of case–control samples, using single nucleotide polymorphism, no associations between one marker in NOS1 and mood disorder patients were detected [66]. However, the paper did not perform an association analysis based on linkage disequilibrium and a mutation scan of NOS1 [66].

Similarly, in a more recent study of the association between polymorphisms of the genes related to oxidative and nitrosative stresses (including NOS1 and NOS2 genes) in a Polish population and a risk of depression, the frequency of NOS1 failed to reveal significant differences between samples [67]. In a large genome-wide association study, an association of NOS1 with the disease was present. However, the size of the NOS1 gene makes the authors cautious about the finding [68]. Similarly, it was shown that polymorphisms associated with the NOS2A and NOS1 genes might confer an increased risk of recurrent depressive disorder [69]. Again, NOS1 was shown to influence the human life span and the quality of life in old age [70]. In another study, the effect between the most common NOS1 SNP, stress, and depressive disorders was examined, with significant associations between 8 out of 20 different NOS1 polymorphisms and human liability to depression during conditions of financial and psychosocial stress factors [71]. NOS2 transcription is increased in the peripheral blood of patients with recurrent MDD, and a polymorphism in the NOS2 promoter associates with a higher risk of recurrent depressive disorder [72, 73].

While the following studies are not directly related to NOS1, it is (due to the regulatory function on NO production) relevant also to highlight importing findings examining NOS1AP in a cohort of Vietnamese combat veterans with PTSD and a group of healthy control individuals, suggesting that the NOS1AP gene is associated with PTSD and that a genetic variant in NOS1AP may increase the susceptibility to severe depression in patients with PTSD, and increased risk for suicide in untreated combat veterans [74]. Similarly, another study reported that the NOS1AP) was associated with a broad diagnosis of schizophrenia, and eight NOS1AP SNPs were associated with depression-related phenotypes within schizophrenia rs1415259 SNP showed a strong association with sleep dysregulation phenotypes of depression [75].

Limited information regarding NOS3 is available, and in a Japanese study, no associations were observed between any of the polymorphisms of the eNOS gene and the Hamilton Rating Scale for Depression. However, plasma NOx level was significantly associated with a polymorphism of the eNOS gene [76].

In conclusion, a diverse spectrum of findings related to the NOS1 and NOS2 gene exists. Several more extensive genetic replication studies are needed before a firm conclusion upon NO's role in depressive disorders, based on genetic evidence, can be made.

Preclinical Evidence

Stress Effects on NO Signalling in the Brain

Stress is a major environmental factor contributing to depression development. A large number of evidence indicates that both acute and chronic exposure to stress can enhance the expression and activity of NOS1 in brain regions related to the pathophysiology. For example, the dorsal hippocampus's nitrite and nitrate level was increased following acute restraint stress [77]. Another study elevations in NADPH- or NOS1-positive neurons in the entorhinal cortex and hippocampal CA1/CA3 subregions following acute and chronic restraint stress [78]. Similarly, a 21 established mild stress paradigm elevated NOS1 expression in hippocampal CA1, CA3, DG, and subiculum area [79]. A 5 day escapable/inescapable water stress paradigm increased NOS1 gene expression, NOS1 protein levels, and NOS1 activity in the whole hippocampus in the FSL rats, a genetic animal model depression [80].

Increased iNOS levels have also been detected in the hippocampus and prefrontal cortex of animals exposed to acute and chronic stress, contributing to the delayed and sustained NO levels observed after stress exposure [81]. It has been identified that the increased expression of iNOS in microglia is one crucial mechanism mediating neuronal death under chronic stressful situations and neuroinflammatory stimuli [82, 83]. Interestingly, increased neuroinflammation is observed in chronically stressed rodents and depressed patients, thus posing an essential role for iNOS

as a central mediator of the stress-induced neuroinflammation associated with depression [84]. Accordingly, the immunological challenge with LPS promotes increased iNOS expression and neuroinflammation in the hippocampus and the prefrontal cortex and precipitates depressive-like behaviour in animals [85]. Excessive NO levels can further aggravate neuroinflammation induced by stress and mediate neuroplasticity impairments in brain regions relevant to mood, emotion, and cognition, such as the prefrontal cortex and the hippocampus [86].

Increased expression of both nNOS and iNOS has also been described in hypothalamic regions. The prefrontal cortex and hippocampus play an essential modulatory role in the HPA axis activation during stress [87]. Although increased NO levels can promote activation of the HPA axis and glucocorticoid release, the role played by NO in the regulation of HPA activation during stress is rather complex [88, 89]. Of particular importance, hippocampal NOS1-derived NO significantly downregulated local glucocorticoid receptor expression in chronically stressed mice and elevated HPA axis activation, thus implicating NO in the HPA dysregulation observed in depressed patients [79].

Behavioural Changes in Mice with Impaired NO Synthesis

The synthesizing enzymes and the main targets for NO are expressed in different brain regions, including many areas involved in controlling emotional, behavioural, and endocrine responses to stress [18, 90–92]. Consistent with that idea, mice with a targeted disruption of the NOS1, NOS2, or NOS3 show behavioural changes that reflect an important role for NO in controlling behaviours associated with stress consequences and depression development. For example, NOS1 knockout mice exhibit increased locomotor activity in a novel environment, increased social interaction, decreased depression-related behaviour, impaired spatial memory retention, and impaired social recognition memory [93–98]. Therefore, the lack of NOS1-derived NO synthesis indicates decreased anxiety and depressive-like behaviours, but this can also result from impaired cognitive skills due to neurodevelopment changes in the absence of NO. Nevertheless, NOS1 knockout mice present significant neurochemical changes in different brain regions and increase resistance to stress-induced behavioural consequences, consistent with a large body of evidence showing that NO can control the release of various neurotransmitters that affect behavioural regulation. Furthermore, deficient NOS1 mice present fewer impairments in neurogenesis and anhedonia induced by chronic stress exposure [98]. This indicates that NOS1-derived NO contributes to stress-induced depression by suppressing neurogenesis.

Consistent with the primary role of NOS2-derived NO in mediating inflammatory responses, NOS knockout animals are more resistant to developing behavioural and endocrine changes induced by neuroinflammatory and neurotoxic stimuli [99–101]. The absence of iNOS could protect from excessive NO and peroxynitrite levels that would be formed under such circumstances [102]. NOS2 deficient mice have also shown increased struggling in the forced swimming test, similar to the treatment with conventional antidepressant drugs in this model [103]. These mice, however,

present increased NOS1-derived NO levels in the prefrontal cortex, which renders them more anxious and makes it more challenging to understand the role of NO in regulating depressive-like behaviours [104].

Surprisingly, mice lacking NOS3 also show impaired exploratory behaviour in the open-field test, increased anxiety in the elevated plus-maze, and facilitated learning in the water-maze test, along with imbalanced levels of acetylcholine noradrenaline and serotonin in the striatum [105, 106].

Another study indicated that NOS3 provides tonic levels of NO in the vessels that could affect neuronal signalling, whereas NOS1 provides phasic changes in neuronal NO levels with both being able to control LTP in the hippocampus, thus influencing learning and memory [107]. This could explain, for example, cognitive changes observed in knockout animals or individuals with depression.

Altogether, the information from animals deficient in specific NOS indicates that NO derived from the three isoforms may play essential roles in controlling neurotransmitter release, neuronal signalling, and behaviour. It is, however, necessary to have in mind that neurodevelopmental changes due to the absence of the specific gene might have contributed to the differences observed later in life. In this context, pharmacological tools have been crucial in understanding the NO involvement in stress and depression neurobiology.

Behavioural Effects of NO Synthesis Inhibition

In 1996, Jefferys and co-workers demonstrated that the unspecific NOS inhibitors L-N-arginine methyl ester or NG-nitro-L-arginine methyl ester (L-NAME) decreased the immobility of rats in the FST, an effect counteracted by pretreatment with the NOS substrate L-arginine [108]. Similarly, not only acute, but also chronic L-NAME and NG-monomethyl-L-arginine (L-NMMA) displayed antidepressive-like response in the FST [109], and later work extended these findings, since NG-nitro-L-arginine (L-NA or L-NNA) also resulted in antidepressant-like effects in the FST, together with augmentation of behavioural effect of conventional SSRI antidepressants [110–112]. More selective NOS1 inhibitors such as 7-nitroindazole (7-NI), Nω-propyl-L-arginine (NPA), and 1-(2-trifluoromethylphenyl)imidazole (TRIM) were subsequently tested and shown to produce antidepressant-like effects in the FST [113–116] 0.7-NI was also shown to augment the behavioural outcome in the FST of a TCA and an SSRI [110]. Selective inhibition of iNOS can also promote an antidepressant-like effect in the FST, thus indicating that NO derived from both nNOS and iNOS can modulate depressive-like behaviours [103].

Since the FST has a questionable face and construct validity, the effect of NOS inhibitors has been tested in more valid animal models. Selective inhibition of nNOS induced antidepressant effects in animals submitted to the learned helplessness [117] and the chronic mild stress [98, 118]. However, contradictory results have been described with nNOS inhibitors inducing similar chronic stress exposure changes [119].

Both inhibiting the downstream NO signalling by inhibiting the sGC using [1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one] (ODQ) and scavenging NO by Carboxy-PTIO (c-PTIO) induced an antidepressant-like effect [118, 120, 121].

The inhibition of NO synthesis in specific brain regions has been proven sufficient to elicit antidepressant effects since central administration of 7NI into the hippocampus [114] or the prefrontal cortex [122] produced antidepressant-like effects in the FST. These brain regions seem to play a central role in stress adaptation and depression neurobiology, thus indicating that increased NO in response to stress in both regions can predispose to depressive behaviours.

Interestingly, a class of antagonists may be considered endogenous inhibitors. These include L-Citrulline, Agmatine, NG, ADMA, SDMA, and arginNOS2uccinic acid. While L-citrulline is a very weak inhibitor, a derivate L-Thiocitrulline is much more potent [123]. Agmatine, de-carboxylated arginine [124], is potentially significant, as there is evidence of antidepressant effects in animal models of depression [125–128], as well as in humans [129–132]. No reliable preclinical data exist for the other endogenous inhibitors, although there are reports of their presence in animals [133].

It was also demonstrated that the antidepressant action of ketamine, which has proven to be efficacious for treating a patient with severe treatment-resistant depression, could be attenuated with L-Arginine, supporting the hypothesis that NO may play an essential role in the mechanism of action of ketamine [134]. Curiously, the inhibition of NO synthesis does not elicit acute antidepressant effects, and repeated treatment is required to promote behavioural effects in the learned helplessness model [117].

As mentioned above, NOS1AP has been implicated in depressive psychopathology. Preclinically, NOS1AP was increased in the PFC of mice exposed to the Chronic Unpredictable Mild Stress procedure [43]. Interestingly, the indirect disruption of the NOS1/PSD-95/NMDA receptor complex has been proposed to control NO synthesis. It has been shown that both lentiviral disruption and the use of small-molecule inhibitors of the PSD-95/nNOS interaction may produce antidepressant-like effects in preclinical assays. For example, IC87201 and ZL006 produce antidepressant-like responses in the forced swimming test (FST) and tail suspension test (TST) following a single administration in mice [26, 135]. Viral-mediated NOS1AP downregulation in the medial PFC reversed the depression-like behaviours in mice exposed to the Chronic Unpredictable Mild Stress procedure [43], although not all studies could detect an effect [136]. In later studies, it was observed that the small molecule inhibitors protected against glutamate-induced neuronal atrophy in primary cortical neurons [137], a finding relevant for the potential pathophysiology of depression.

NO, and Classical Antidepressants

Direct interaction between clinically used antidepressants and nitrergic signalling has been shown in a few studies. In a study with patients with ischemic heart disease and depression, 17 received paroxetine and 14 patients nortriptyline. It was observed that serum nitrite and nitrate levels were significantly decreased following paroxetine treatment but not nortriptyline [138]. Besides, paroxetine was also a considerably more potent inhibitor of the NOS enzyme activity than nortriptyline [138]. Similarly, several established antidepressants of distinct chemical classes, including imipramine, paroxetine, citalopram, and tianeptine, have been shown to inhibit hippocampal NOS activity in vivo applied locally in the brain in therapeutic relevant concentrations [139]. This can result from antidepressant effects on glutamatergic signalling and NMDA receptor activation and directly impact nNOS activity [140, 141].

It has also been reported that the precursor of NO, L-Arg, antagonizes the effects of the classic tricyclic antidepressant, imipramine [109]. This observation has led to hypotheses regarding the potential contribution of serotonergic/noradrenergic mechanisms in the observed antidepressant-like effects of the NOS inhibitors (see below). Corroborating this hypothesis, a recent study reported that treatment with 7-NI, venlafaxine, and fluoxetine attenuate stress-induced neuronal activation in overlapping brain regions, thus suggesting that NOS1 inhibitors and monoamine antidepressants may share common neurobiological substrates [115].

Subsequently, it has been demonstrated that low and ineffective L-NAME doses could potentiate the behavioural effects of imipramine and fluoxetine but not reboxetine, a noradrenaline reuptake inhibitor, in the FST [110, 142]. It was also shown that a serotonergic mediation of the antidepressant-like effects of L-NA, 7-NI was present since serotonergic depletion abolished the antidepressant-like effect of the inhibitors [142]. In the hippocampus, the antidepressant-like effect induced by local administration of a selective NOS1 inhibitor (N-propyl-L-arginine) could be prevented by co-administering a 5-HT1a antagonist, implicating endogenous serotonin in such impact [143]. However, not all inhibitors seem to display this profile, as it was also demonstrated that agmatine's effect was independent of the 5-HT depletion [128]. However, as already discussed, agmatine likely has multiple impacts on several receptor systems.

Finally, NO has also been implicated in the antidepressant role of several other substances, like tramadol [144], bupropion [145], and lithium [146].

Although these studies further corroborate the idea that 7-NI shares common molecular mechanisms. Monoaminergic antidepressants, a recent study that compared the gene expression pattern in rats treated with imipramine or 7-NI by serial analysis of gene expression (SAGE), found that there are also significant differences in the genes regulated by such treatments [147]. Nevertheless, this study conformed to the overlapping regulation of genes involved in oxidative stress and neuroplastic responses. However, further studies are still necessary to evaluate the contribution of such molecular changes for the antidepressant-like effects induced by NOS1 inhibition.

NO and Serotonin

A substantial number of studies show close interaction between NOergic and 5HTergic signalling, and NOS1 transgenic mice have been shown to have elevated levels of 5-HT in several brain regions involved in emotion, such as the cerebral cortex, hypothalamus, hippocampus, and amygdala [148]. It has been suggested that NOS1 contributes to regulating the synthesis, release, and reuptake of 5-HT. First, the rate-limiting enzyme in the biosynthesis of 5-HT, Tryptophan Hydroxylase, is inactivated by peroxynitrite in a concentration-dependent manner [149]. Second, modulating NO in distinct brain areas is followed by alterations in 5HT in a brain region-dependent manner. For example, systemic administration of the NO donor S-nitroso-*N*-penicillamine (SNAP) decreased 5 HT release in the raphe nucleus but increased release in the frontal cortex [150].

In contrast, local administration of SNAP induced NO increases in the striatum [151]. Both local and systemic administration of the NOS1 inhibitor 7-NI into the hippocampus significantly increased the extracellular level of 5-HT [152, 153]. In contrast, administration of the NO precursor L-arginine decreased hippocampal 5HT [154] but increased 5HT in the striatum [155]. Third, using rat brain synaptosomes, some NO donors inhibited the reuptake of 5-HT without affecting the serotonin transporter [156]. Still, distinct NO donors were shown to inhibit 5HT uptake through human SERT expressed in COS cells [157]. Finally, a substantial number of NOS1 immunoreactive cells co-labelled with 5HT or SERT can be found in the DRN [141, 158]. It was also observed that NOS1 had a physical association with the SERT through a PDZ domain, attenuating SERT activity in DRN [141].

NO and Neuroplasticity

Neuroplasticity is an essential property of neuronal adaptation, and it is compromised in depression [159, 160]. It is believed that various neurotrophins, such as Brain-derived neurotrophic factor (BDNF), regulate neuroplasticity, which includes proliferation, differentiation, survival, and death of neuronal cells and supporting tissue [161]. BDNF binds to the Tropomyosin kinase B (TrkB) receptor and subsequently activates intracellular signalling pathways governing transcription and dendritic translation of proteins necessary for cellular survival, differentiation, and learning/memory formation in the hippocampus [162]. Interestingly, NO seems to modulate BDNF levels since it was demonstrated that NO donors (SNP, NOR3] decrease BDNF release in hippocampal cell culture, whereas the inhibition of NO production increase these levels [163]. Accordingly, in vivo experiments showed that chronic treatment with L-NAME increased BDNF mRNA and protein levels in the hippocampus and rats' prefrontal cortex [164, 165]. In line with that observation, the antidepressant-like effect induced by chronic treatment with the selective NOS1 inhibitor 7-NI was associated with increased expression of hippocampal BDNF protein levels [117].

Similarly, increased levels of BDNF have also been observed after treatment with other NOS inhibitors, either in cultured or in vivo neocortex [166]. However, in another study, the antidepressant effect induced by aminoguanidine, a preferential NOS2 inhibitor, was not correlated with increased BDNF signalling in the prefrontal cortex of FSL rats [167]. On the other hand, mice with deficient NOS2 expression presented increased BDNF levels in the PFC and hippocampus associated with the antidepressant-like phenotype [168]. Therefore, it is likely that both NOS2 and NOS1-derived NO can modulate BDNF signalling in stress adaptation. Although NO usually has been shown to downregulate BDNF levels, peroxynitrite formation derived from NO and O^{2-} was observed to trigger trkB signalling [169], suggesting BDNF signalling to be affected. Evidence from cultured hippocampal neurons indicates that BDNF secretions' inhibition is more pronounced in response to exogenous NO levels or under exacerbated NO concentrations.

In contrast, low endogenous levels of NO would facilitate BDNF-TrkB signalling [170]. Bioinformatic analysis has predicted a direct action of NO on the amino acid residues of BDNF or TrkB, suggesting protein s-nitrosylation or tyrosine nitration in both rodents and humans quoted molecules [171]. These direct actions of NO on BDNF or TrkB proteins could trigger useful negative feedback to control protein function or drive a reinforcement of downstream BDNF/TrkB signalling.

Conversely, neurotrophins can modulate NO or NOS levels since BDNF has been found to upregulate NO signals in either hippocampal or neocortical neurons [166, 170]. Similarly, the ratio of NOS1-positive neural progenitor cells (NPCs) is increased following treatment with BDNF [172]. On the other hand, BDNF can suppress NO production in microglia, thus counteracting the brain's inflammatory processes [173].

More recent evidence indicated that the interplay between NO and BDNF-TrkB signalling is more complex and involves more signalling cascades. Both NMDA and TrkB can be associated with PSD-95 and induce downstream signalling mechanisms that regulate synaptic plasticity [174]. In this scenario, PSD-95-NOS1 interaction may down-regulate BDNF expression via inhibiting ERK activation. On the other hand, NMDA-PSD-95 uncoupling would increase BDNF levels and facilitate BDNF-TrkB-PSD-95 signalling mechanisms related to the contribution of neuroplasticity to the behavioural effect of these drugs. These results could help to explain the impact of NOS inhibitors on BDNF expression.

Despite the evidence mentioned above that NO might regulate BDNF levels in stress and depression, evidence about NOS inhibitors' effects in promoting recovery of impaired synaptogenesis and dendritic branching in stressed animals is scarce. However, it is known that NO is critically involved in establishing and activity-dependent refinement of axonal projections during the later stages of development [175]. Under physiological concentrations, NO signal downstream, either through sGC activation or through nitrosylation to promote the growth of presynaptic filopodia that rapidly leads to the formation of new synaptic contacts in vitro experiments [176]. Conversely, high levels of NO, as in nerve injury, can produce the opposite effect, with reduced synaptogenesis through cGMP-dependent and

S-nitrosylation-mediated mechanisms [176]. Although this can be blocked by treatment with NOS inhibitors [176], and since inhibition of NO synthesis in adult rats increase hippocampal expression of synaptophysin [177], it is not known whether blocking NO synthesis may prevent a stress-induced decrease in synaptogenesis and dendritic arboring. However, this seems likely, since PSD-95 promotes synaptogenesis and multi-innervated spine formation through nitric oxide signalling [178]. However, further research is needed, and the question is open for investigation. A proper answer would contribute to a better understanding of NO's role in stress-induced neuroplasticity related to neuropsychiatric disorders.

Another important neuroplasticity factor affected by NOS inhibitors is neurogenesis, which has been exhaustively reviewed elsewhere [179, 180]. Only a brief overview is presented here. Neurogenesis is the process of neural stem cells (NSCs) to foster newborn neurons in replacement for damaged neurons or maintain function. Neurogenesis has attracted significant interest, and although somewhat controversial in humans, it has been suggested that neurogenesis may be linked to recovery from clinical depression [181–183], and even in a controversial paper, it may be a prerequisite for antidepressant response [184]. In the brain, neurogenesis has been observed in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus (DG) [183, 185].

Interestingly, it has also been demonstrated that the subventricular zone is surrounded by NOS1 positive neurons [186], and cells expressing NOS1 also have been identified in neuronal precursors in DG [187], suggesting that NOS1 could participate in the regulation of neurogenesis. Indeed, it has been demonstrated that the NOS1-mediated suppressing on neurogenesis effect may be caused by NO generated from neurons, not from NSCs [188]. Besides, evidence that the subcellular localization of NOS1 in neurons and NSCs seem to be distinct, implying that the role of NOS1 in neurons and NSCs is different [188]. It has also been demonstrated that inhibition of NO synthesis with 7-NI increases the proliferation of neural precursors isolated from the postnatal mouse subventricular zone [189]. However, another report has demonstrated that NOS1 inhibition with 7-NI enhanced progenitor cells' proliferation in the dentate gyrus. The antidepressant-like effect of this drug was dependent on this neurogenic effect [190]. These results are in line with findings using a NOS1 knockout mouse line, where the number of new cells generated in neurogenic areas of the adult brain, the olfactory subependyma, and the dentate gyrus was strongly augmented, indicating that division of neural stem cells in the adult brain can be negatively controlled by NO [191]. It has also been reported that the NOS1 inhibitor L-VNIO or deletion of the NOS1 gene could affect the differentiation of NSCs into neurons and astrocytes [188]. Specifically, it was found that NOS1 could facilitate differentiation of hippocampal neural progenitor cells [192], suggesting that NOS1 in NSCs is essential for neurogenesis. In the DG of the hippocampus, NSCs forms into granule neurons contributing to neuroplasticity, learning, and memory. Impairments in these cognitive functions have been observed in NOS1 transgenic mice, suggesting that NOS1 affects differentiation of NSCs in the DG [94]. High levels of the NOS1 are found in granule neurons in the DG [193], and NO generated from NOS1 in these neurons may therefore be speculated to negatively

govern granule neuronal precursor proliferation and further reduces differentiation of granule neuronal precursors. Given these observations, it is possible to speculate that the behavioural effects of NOS inhibitors observed in animals under exposure to chronic stress might involve positive regulation of hippocampal neurogenesis.

One of the unique physiological properties of NO is its function as a retrograde messenger, influencing synaptic properties, such as LTP and LTD [194, 195]. Such processes are crucial in synaptic homeostasis, and, conversely, affecting NO levels may virtually affect the plasticity and homeostasis of all known synapses [196, 197]. NO is likely to play a significant role in diseases where synaptic dysfunction, such as depression, is essential. NO has been shown to mediate local activity-dependent excitatory synapse development and spine dynamics [198]. A change in NO levels during development has been shown to promote axon pruning in a cGMP-independent mechanism and enable a switch between neuronal degeneration and regrowth [199].

Nitric Oxide and Neuroinflammation

An essential role for neuroinflammation in depression pathophysiology has been proposed since increased levels of inflammatory mediators (e.g., IL-1, IL6, TNF, among others) have been described in the brain of animals repeatedly exposed to stress and in the blood and the brain of depressed patients [200, 201]. Moreover, the response to antidepressants is usually associated with decreased levels of proinflammatory cytokines. In contrast, administration of such molecules and other immunological stimuli triggers depressive-like behaviour in animals and depressive episodes in humans [202–204] (references). Furthermore, anti-inflammatory drugs induce antidepressant effects in animal models and are effective as add-on therapy to antidepressant medication in patients [205].

Increased proinflammatory cytokines can trigger iNOS expression, as mentioned above (Fig. 15.1), and lead to increased NO levels in different brain regions. Excessive NO can then promote nitrosative stress and further microglial activation and neuroinflammation [206]. Inhibition of iNOS activation during stress could then attenuate neuroinflammation and protect against its effects on brain plasticity and behavioural adaptation.

Conclusion

Although the studies cited in the current chapter utilize different methodologies, from a preclinical genetic approach to human postmortem material, the role of NO in depression pathophysiology is unquestionable. The information reviewed indicates that NO derived from different sources can influence several mechanisms associated with depression neurobiology, such as the HPA axis activation, neuroinflammation, and neuroplasticity (Fig. 15.2). Furthermore, dysfunctional regulation of NO synthesis has been described in the blood and the brain of depressed patients and

stressed animals, providing an essential anatomical substrate for a NO role in depression. This is further corroborated by evidence from animal models indicating that inhibition of iNOS or nNOS promotes antidepressant effects associated with ameliorating stress-induced impairments in neuroplasticity and neuroinflammation. Therefore, the NO system continues to be an exciting approach in the future development of antidepressants.

On the other hand, increased iNOS expression contributes to increased reactive oxygen and nitrogen species (RONS) and oxidative stress. As in inflammatory conditions, activated microglia can also promote iNOS expression and increased NO levels, further aggravating neuroinflammatory conditions. NO can regulate BDNF levels directly or indirectly by the mechanisms mentioned above and therefore impact synaptic plasticity. Dysfunctional neural circuits under such conditions can then predispose the brain to depressive behaviour. Conversely, inhibition of iNOS or nNOS can promote antidepressant effects.

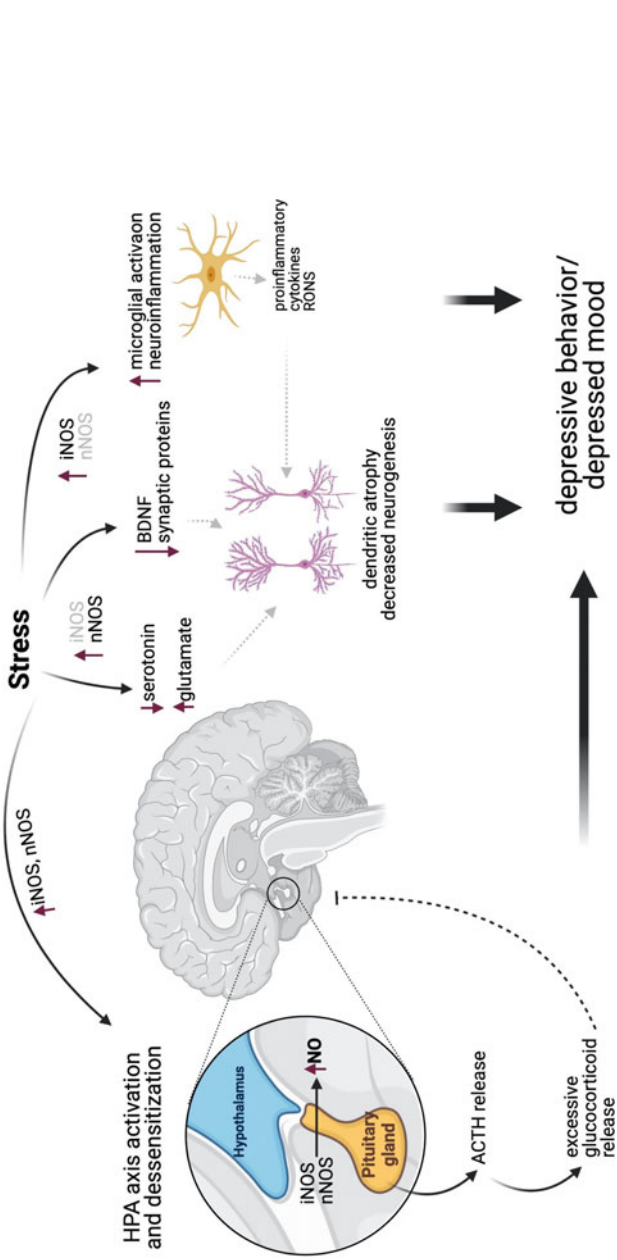

Fig. 15.2 Summary of the mechanism modulated by NO in depression neurobiology. Exposure to stress increased the expression of both iNOS and nNOS in different cell types (glial and neuronal, respectively) in several brain regions involved with depression neurobiology. Increased levels of NO can trigger HPA axis activation and glucocorticoid release. No can also down-regulate glucocorticoid receptors (GR) in the hippocampus and contribute to the HPA hyperactivation and hypercortisolemia often described in depressed patients. NO can also decrease serotonin levels by different mechanisms and increase glutamate release and neurotoxicity, with significant participation of nNOS

Acknowledgements This study was financed by Aarhus University Research Foundation (AUFF starting grant to SJ), and Independent Research Fund Denmark (grant 8020-00310B to GW).

Disclosures GW reports having received lecture/consultancy fees/research support from H. Lundbeck A/S, Servier SA, Astra Zeneca AB, Eli Lilly A/S, Sun Pharma Pty Ltd, Pfizer Inc, Shire A/S, HB Pharma A/S, Alkermes Inc, Mundipharma International Ltd., J&J Inc., and Jannsen Pharma A/S. SJ reports no conflicts of interest.

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Chapter 16
Nitric Oxide in Parkinson's Disease: Insights into Research and Therapeutics

Bhupesh Vaidya and Shyam S. Sharma

Abstract Parkinson's disease (PD) is characterized by the symptoms of motor and cognitive deficits. There are several approaches available for the treatment of PD, but most of them are only aimed at providing symptomatic relief. Pathophysiological mechanisms involved in the aetiology of PD are complex. Several mechanisms like oxidative stress, inflammation, apoptosis, play important role in the pathophysiology of PD. There are various reports implicating the involvement of NO in PD. In this book chapter we have highlighted the importance of nitric oxide and its targeting for the development of new therapeutics against PD. It also emphasizes the molecular partners and downstream pathways affected by the increased levels of NO and nitric oxide synthase (NOS) in the in vitro and in vivo PD models has also been emphasized. Additionally, in-depth insights into the clinical evidence are also provided to support and warrant these preclinical findings. Although most of the studies have observed a direct correlation between increased levels of NOS and neuronal death in PD, few scattered reports have found otherwise. Utilizing this evidence, several of the nNOS and iNOS inhibitors and antioxidants demonstrated to relieve the effects of nitrosative stress in PD have also been discussed.

Keywords Nitric oxide · Major depression · Neuroinflammation · Neuroplasticity · iNOS · nNOS

Introduction

PD is a neurodegenerative condition characterized by symptoms of muscle rigidity, tremors, bradykinesia, and akinesia [1]. This is also accompanied by non-motor symptoms of cognitive decline, depression, and sexual dysfunction [2]. Amongst the different dopamine signaling pathways, it's the nigrostriatal pathway which undergoes maximum degeneration leading to loss of tyrosine hydroxylase positive neurons

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_16

in the substantia nigra pars compacta and the striatum [3]. As it is a progressive neurodegenerative condition, first symptoms appear only after eighty per cent of the dopaminergic neurons have undergone neurodegeneration [4]. There are several in vitro and in vivo models which have been made using knock out or knockdown approaches and/or toxins such as MPTP, 6-OHDA, rotenone, paraquat and maneb for understanding the PD pathophysiology [5].

Role of different molecular pathways and signaling molecules have already been elucidated with the help of these models but with little translational success [6]. Hence, there is a need to look for new mechanisms which could serve as therapeutic targets in PD. Nitric oxide (NO) is one such target which could be explored more extensively in PD at the clinical stages. Synthesis of NO is controlled by nitric oxide synthase (NOS) [7]. Expression of the different isoforms of NOS including neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) is widespread in the brain with nNOS being the most abundant amongst them [8].

nNOS serves as a common interneuronal marker in the nervous system because of its presence in the interneurons. It is expressed both in the GABAergic and glutamatergic interneurons of the ventral tegmentum and substantia nigra pars compacta [9]. nNOS mediated nitric oxide–soluble guanylyl cyclase–cyclic GMP signaling in the striatum was observed to control the locomotor activity besides opposing the inhibitory effects of D2 dopaminergic receptor activation on the corticostriatal transmission [10]. Additionally, co-localization studies have shown that nitrergic terminals are located in the close proximity of the tyrosine hydroxylase positive terminals suggesting the role of nitric oxide in the dopamine release and/or metabolism [11, 12].

iNOS is not normally present in the CNS but owes its presence to any inflammatory response or toxic insult [13]. Under such conditions, it gets expressed both in the neurons as well as the glial cells like microglia and astrocytes [13–15]. iNOS levels are closely correlated with the degeneration of dopaminergic neurons and the expression of tyrosine hydroxylase in PD. Hence, its role has been widely explored and illustrated in PD with the help of different model systems [16].

eNOS is another NOS isoform which is majorly present in the endothelial cell, but some expression has also been reported in the brain [17]. Owing to its lower levels in the CNS, its physiological relevance with respect to the dopaminergic system is limited and hence has little pathophysiological significance in comparison to the other two isoforms [18].

Molecular Targets of Nitric Oxide in PD

There are different genetic and environmental factors which alone or in combination are responsible for the onset and progression of PD. Mutations in several genes like PINK1, PRKN, DJ-1, LRRK2, SNCA, GBA and HLA has been linked to the pathogenesis of PD. Nitric oxide in close association with these mutations contributes

to the PD pathogenesis. Some of the byproducts of these genes which owe a part of their pathophysiological relevance to nitric oxide have been listed below (Fig. 16.1).

PINK1: Mutations in Phosphatase and Tensin homolog (PTEN)-induced Kinase 1 (PINK1) is known to be responsible for the autosomal recessive forms of familial PD [19]. Loss of PINK1 activity causes mitochondrial dysfunction mediated by inhibition of complex 4 activity by mitochondrial associated chaperons like Hsp90, Hsp60 and LRPPRC [20, 21]. A study done on PINK1 null dopaminergic cell lines demonstrated that complex 4 activity could be rescued by the restoration of the NO signaling using optimum doses of sodium nitroprusside or ginsenoside Re. However, higher doses of these compounds didn't show enough rescue of the complex 4 activity suggesting that there is a fine line which demarcates the neuroprotective effects of NO in PD [20]. These results were further confirmed when optimum levels of NO and nNOS were found to regulate the PINK1 dependent translocation of Parkin for the induction of mitophagy in the PINK1 null dopaminergic cell lines [22]. On the other hand, PINK1 deficient mixed astroglia/microglia cultures showed elevated levels of NO and COX-2 mediated by the increased expression of iNOS upon exposure with lipopolysaccharides (LPS)/IFN-γ. LPS and IFN-γ further led to upregulation of the TGF-β1, which was responsible for the increased iNOS expression specifically in the astrocytes [23]. Additionally, it has also been reported that NO causes S-nitrosylation of the PINK1 at cysteine 568, resulting in its diminished kinase activity and altered Parkin translocation to the mitochondria leading to reduced mitophagy [24, 25].

Alpha-synuclein: Another gene which is responsible for early-onset cases of familial PD is SNCA which codes for alpha-synuclein [26]. A missense mutation in SNCA gene is known to be responsible for the Lewy body pathology seen in PD, Alzheimer's disease (AD) and Lewy body dementia [27]. A study done in SH-SY5Y cell lines looked at the nitration pattern of α-synuclein in the presence of a nitrating agent $NaNO_2$. Nitrated α-synuclein formed amorphous cytotoxic aggregates besides increasing the production of NO via iNOS induction. These cellular changes in totality led to increased caspase 3 expression and hence apoptotic cell death in the SH-SY5Y cell lines [28]. Similar results were obtained in the presence of the intracellularly generated nitrate using N-propyl-1,3-propanediamine/NO in the HEK 293 cell lines [29]. Recently a new model system making using of controlled iNOS expression with the help of bicistronic α-syn-IRES-tTA adeno-associated virus (AAV) was developed to study the nitration pattern and its effects on α-synuclein aggregation. Studies done on this model system demonstrated the dose-dependent increase in α-synuclein aggregation with the increasing iNOS expression suggesting the possible role of homeostatic machinery in maintaining the optimum levels of NO in the brain [30]. Moreover, neuron to neuron and neuron to glial propagation of α-synuclein is also well established as a pathological change in PD. In order to mimic this in the model systems, the effect of extracellular administration of α-synuclein on NOS expression has been investigated by several researchers [31–33]. They have provided useful insights into mechanisms of neuronal death in PD as a result of nitrosylation of important proteins like Parkin, GAPDH, HSP-70 and DJ-1 [33]. Additionally, it has also been shown that α-synuclein mediated increase in iNOS expression is responsible for the impaired axonal transport, mitochondrial

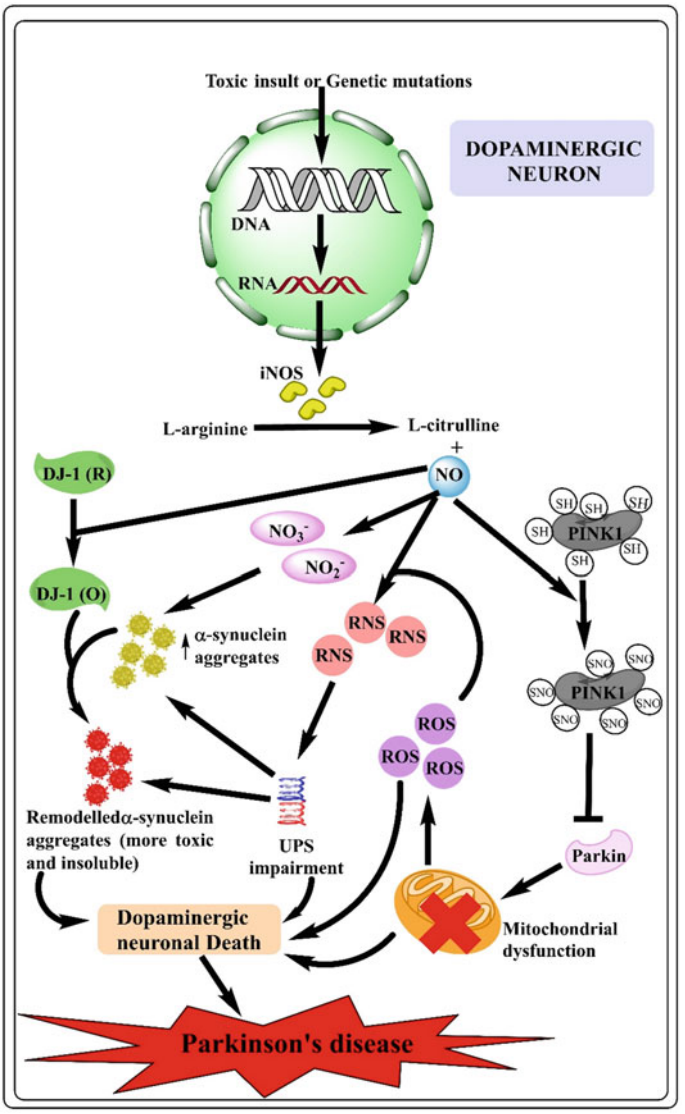


Fig. 16.1 Molecular targets of nitric oxide in PD: Toxic insults from neurotoxins like MPTP, 6-OHDA, paraquat and rotenone or genetic mutations in PINK1, PRKN, SNCA, DJ-1 and LRRK2 lead to increased synthesis of iNOS. Latter further stimulates a cascade of events which contribute to PD pathology. Nitric oxide generated as a result of increased iNOS expression leads to increased S-nitrosylation of PINK1 and alters the Parkin activity. This results in impaired mitophagy of the damaged mitochondria and generation of the ROS. The ROS species can then combine with the NO to form the RNS contributing towards the impairment of the ubiquitin proteasomal system. This leads to the increased aggregation of the alpha-synuclein into Lewy bodies. Furthermore, increased NO levels under the pathological conditions leads to increased levels of the oxidized form of DJ-1, which remodels the already present alpha-synuclein aggregates into the more toxic and insoluble aggregates. These events in totality contribute to the neuronal death and aggravate the pathophysiology of PD

dysfunction, neurite retraction and inflammatory response specific to the dopaminergic neurons [34, 35]. Methamphetamine induced dopaminergic neurotoxicity was also observed to induce changes resulting in nitration of α-synuclein at tyrosine 9 residue, which coincided with activation of PARP and caspase 3 enzymes. These molecular changes were reversed following inhibition of NOS, suggesting a critical role of latter in the pathophysiology of PD [36]. Besides its role in the PD pathology in the central nervous system, its effect on the enteric nervous system has also been investigated. It has been observed that α-synuclein aggregates were present in several neuronal subtypes in the enteric nervous system, but it was poorly colocalized with nNOS [37]. Thus, it is likely that NOS plays a critical role in the Lewy body formation in CNS in PD with no possible involvement in the enteric nervous system.

DJ-1: DJ-1 is another candidate gene which is linked to the cases of the familial PD [38]. It codes for DJ-1 protein which is responsible for regulation of oxidative stress, mitochondrial function and proteasome system. In the sporadic cases of PD, its increased oxidation resulted in the disruption of cellular antioxidant machinery and progressive neuronal death [39]. Furthermore, it was also demonstrated that though partially oxidized DJ-1 inhibited primary nucleation and α-synuclein aggregation cascades, it remodelled the mature α-synuclein aggregates rendering them more neurotoxic. In the similar study, nitric oxide levels measured by Diaminofluorescein-FM diacetate dye through Fluorescence-activated cell sorting (FACS) showed a two-fold increase in cells having remodelled α-synuclein aggregates than the ones having the mature fibrils [40]. Moreover, a recent report suggested that moderate levels of NO can S-nitrosylate DJ-1 at Cys106 and the latter then served as a nitrate donor to PTEN in the transnitrosylation reaction which led to inhibition of PTEN's phosphatase activity. This accorded neuroprotection from the increased phosphatase activity of PTEN, which is otherwise involved in increased neuronal death in PD [41]. Apart from these studies, the effects of DJ-1 mutations on NO production have also been studied with respect to the glial cells, including astrocytes. Exposing a transgenic zebrafish model, Tg (gfap:egfp-2A-flag-zDJ-1 (TgDJ-1) to MPP^+ led to an increased iNOS expression in the astrocytes. However, DJ-1 overexpression inhibited iNOS induction, besides protecting other cellular proteins from undergoing S-nitrosylation [18]. Similar findings were obtained in the primary astrocytic cultures where *DJ-1* knockout generated ten times more NO inside the astrocytes in comparison with the astrocytes of the wild type littermates. The basal levels were however restored once the *DJ-1* was reintroduced using a lentiviral transfection. The changes observed in the *DJ-1* knockout were accompanied by the induction of iNOS and MAPK activation [42]. Use of MAPK inhibition was observed to rescue the LPS induced iNOS expression, which suggested a possible correlation between these pathways [42, 43]. Nevertheless, there is a contradictory report which has looked at mRNA levels of iNOS in the $DJ\text{-}1^{+}/^{+}$ and $DJ\text{-}1^{-}/^{-}$ astrocytes with no significant difference between them [43]. This difference pointed out at the possibility that the iNOS expression might be regulated at the translational levels without any change in the levels of the transcription products.

LRRK2: Mutations in other PD specific genes such as leucine-rich repeat kinase 2 (LRRK2) is also accompanied by pathological changes associated with increased

iNOS expression [44]. Missense mutations in the LRRK2 are responsible for the late-onset autosomal dominant form of PD [45]. It regulates immune responses in the brain by microglia and modulates TNF secretion and induction of nitric oxide synthase [46]. Besides this, LRRK2 also interacts with α-synuclein and latter increased the iNOS expression in the myeloid cells of the substantia nigra, which overexpressed LRRK2 [47].

Above studies point out the effects and interactions of NO and NOS with other molecular targets in PD.

Nitric Oxide and Nitrosative Stress in PD

Nitrosative stress is known to play a critical role in the neurodegeneration associated with PD. Hence, its role has been investigated thoroughly in different PD models. MPTP injected mice showed increased expression and activity of iNOS in the glial cells [48]. Moreover, a significant increase in the levels of iNOS mRNA was also observed, suggesting the transcriptional regulation of this process following MPTP administration. This was later confirmed with the help of $iNOS^{-}/^{-}$ phenotypes which were more resistant to the neurotoxic effects of MPTP [49]. In some of the earlier studies, reducing or knocking out nNOS also accorded protection to the dopaminergic neurons from MPTP besides making the phenotype resistant to the effects of the latter [50]. A recent study involving iPSCs (Induced pluripotent stem cell) looked at the effects of nitrosative stress on other critical proteins involved in PD. It was demonstrated that under the influence of mitochondrial toxins, paraquat and maneb, there was increased nitrosative stress which led to S-nitrosylation and inhibition of the myocyte enhancer factor 2C (MEF2C) transcription activity. This event further inhibited transcription of PGC1α, which is a known master regulator of mitochondrial activity [51]. It provided new insights into mechanisms of mitochondrial dysfunction caused by nitrosative stress.

Reduction in the glutathione (GSH) levels has been invariably shown in a number of studies with respect to PD models [52, 53]. In line with the same, a report showed that inhibition of mitochondrial GSH transport yielded the neurons more susceptible to oxidative and nitrosative stress. The same study found increased apoptosis following inhibition of dicarboxylate (DIC)-dependent mitochondrial GSH transport in primary cultures of rat cerebellar granule neurons [54]. Moreover, it led to the possibility that mitochondrial dysfunction could amplify the effects of nitrosative stress contributing to neuronal death in PD.

Other cellular targets of nitrosative stress include endoplasmic reticulum. NO was found to S-nitrosylate the ER stress sensors like IRE1α and PERK following treatment of SH-SY5Y cells with the MPP^{+}. This led to the downstream inactivation of eIF2α, which is an important mediator for autophagy [55]. Furthermore, increased nitrosative stress also affected the activity of the molecular chaperons in the sporadic cases of PD by S-nitrosylation of protein-disulphide isomerase. It led to a series of

events which aggravated the ER stress and led to an increased accumulation of the polyubiquitinated proteins [56].

Clinical Evidence for the Involvement of Nitric Oxide in PD

Levels of nitric oxide and accompanying nitrite level have been estimated in several clinical studies. Most of the earlier studies had suggested that there are no significant differences in the levels of NO or nitrates in the plasma and cerebrospinal fluid of the PD patients with respect to the control group. Additionally, no correlation was found between plasma nitric oxide levels of PD patients when compared on the basis of age, duration, onset and scores of the Unified Parkinson's Disease Rating scales and Hoehn and Yahr scale [57]. Similar results were obtained when nitrite and malondialdehyde levels were compared in the CSF of the PD patients [58, 59]. Moreover, none of the NO biomarkers, including nitrite, nitrate and cGMP showed any alteration in the levels following the onset of PD in the patients [60]. However, a study done on a smaller cohort in the state of West Bengal in India found that though there was an increase in the plasma nitrite levels in the PD patients, the difference became significant only after a longer duration of the onset of PD [61]. A study done on isolated neutrophils showed that NO was elevated both in the newly diagnosed and treated patients of PD following stimulation with phorbol myristate acetate (PMA) [62]. This result suggested that nitrate levels might play a critical role in the PD pathology, but the reduction in its levels isn't achieved by the currently available drug therapy. In line with these findings, most of the recent studies have observed an elevation in the levels of NO and associated products such as dimethyl arginine and peroxynitrite in the serum of the PD patients [63, 64].

The difference in the results observed across years may be due to the improved instrumentation and measures of quantification for the estimation of the NO. This discrepancy in the results obtained across studies could be one of the reasons there is no drug yet in the clinical trials aimed at targeting the NO levels. However, with more clear understanding and improved limit of detection, some are expected to reach the clinics soon.

Pharmacological Agents Targeting Nitric Oxide in Preclinical PD Models

Nitric Oxide Synthase Inhibitors

There has been little to no translational success with the inhibitors of the nitric oxide synthesis in PD patients. However, several of the specific nNOS and iNOS inhibitors

have been studied at the preclinical stages for their neuroprotective effects in the in vitro and in vivo models, as shown in Table 16.1.

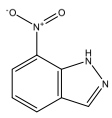
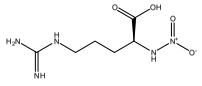
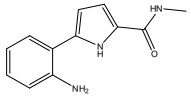
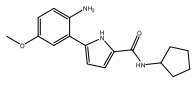
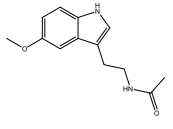
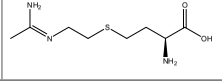
Though there are some contradictory reports related to the beneficial effects of nNOS inhibitors, the role of iNOS inhibitors has been well elucidated in PD. Administration of the nNOS inhibitor 7-nitroimidazole led to a significant reduction in the number of glial fibrillary acidic protein (GFAP) positive reactive astrocytes after MPTP administration [66] besides reducing the levels of isolectin B4 positive microglia cells in the striatum and substantia nigra of the C57BL/6 mice [66]. It also improved the motor performance of the animals in the rotarod test following the stereotaxic injection of 6-OHDA in the right medial forebrain bundle [65, 66]. Similarly, the beneficial effects of another nNOS inhibitor NG-nitro-L-arginine were also observed in the 6-OHDA model, where it accorded protection from the levodopa-induced dyskinesias [65]. These effects have also been validated in the higher mammals including baboons where administration of 7-nitroimidazole led to decrease in the reduction of TH^+ neurons in the substantia nigra as well as improvement in the cognitive performance [74].

On the contrary, there are reports which have attributed the protective effect of 7-nitroimidazole in the MPTP model to its inhibitory effects on MAO-B [75]. Moreover, a study was done on the ventral mesencephalic (VM) primary culture demonstrated that application of the selective nNOS inhibitor ARR17477 following MPP^+ treatment had no effect on the reduced number of TH-positive neurons. Furthermore, it didn't increase the number of GFAP- and OX-6-positive cells and hence did little to reduce the glial cell immunoreactivity [71].

On the other hand, the levels of iNOS were elevated in different models of PD including MPTP, MPP^+, Paraquat, 6-OHDA and maneb. Moreover, inhibition of iNOS using 1400 W in the same model system where nNOS inhibition was ineffective, conferred dose-dependent neuroprotection in the MPP^+ treated ventral mesencephalic (VM) primary culture besides reducing the glial cell immunoreactivity [71]. Other iNOS inhibitors which have been tested positive in the MPTP induced parkinsonism in mice include QFF205, QFF212 and melatonin [67–69]. Since melatonin also inhibits nNOS by the calcium calmodulin dependent mechanisms (IC_{50} of 0.1 μM in the rats), this may also be one of the factors responsible for its neuroprotective effect in PD [76]. GW274150 a selective iNOS inhibitor showed bell-shaped dose–response curve and protected TH^+ neurons in the nigrostriatal pathway [70]. Similar results were obtained with aminoguanidine which led to a reduction in the lipid peroxidation and protection from maneb and paraquat-induced neurotoxicity [72]. Furthermore, norfluoxetine which is the pharmacologically active metabolite of fluoxetine conferred neuroprotection by a reduction in the nitrate production by iNOS in the lipopolysaccharides treated cortical microglial culture [73].

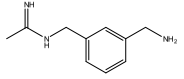
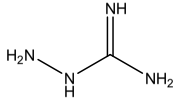
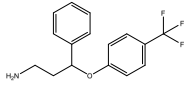
These results point out at the possible involvement of nitric oxide and NOS in the pathophysiology of PD. Modulation of the NOS activity in the brain by exogenous drugs is, however, still a translational challenge owing to the complex physiological action of NO in the central nervous system [77].

Table 16.1 Pharmacological Agents targeting nitric oxide synthase in PD

S. No.	Pharmacological agent	Structure	Class of drug	Model system	Outcome of study	References
1	7-Nitroindazole		nNOS inhibitor	MPTP in mice 6-OHDA in rats	• Reduced microglial activation • Reduced L-DOPA induced dyskinesias and improved motor performance	[65, 66]
2	NG-nitro-L-arginine		nNOS inhibitor	6-OHDA in mice	• Reduced L-DOPA induced dyskinesias	[65]
3	QFF205		iNOS inhibitor	MPTP in mice	• Reduced iNOS activation in cytoplasm and mitochondria	[67]
4	QFF212		iNOS inhibitor	MPTP in mice	• Reduced iNOS activation in cytoplasm and mitochondria	[67]
5	Melatonin (currently used for the treatment of insomnia)		iNOS inhibitor	MPTP in mice	• Accorded neuroprotection by inhibition of mitochondrial iNOS	[68, 69]
6	GW274150		iNOS inhibitor	6-OHDA in rats	• Reduced levels of inflammatory markers and increase in TH levels	[70]

(continued)

Table 16.1 (continued)

S. No.	Pharmacological agent	Structure	Class of drug	Model system	Outcome of study	References
7	1400W		iNOS inhibitor	MPP^+ in rat primary culture	• Reduced astroglial activation and improvement in TH levels	[71]
8	Aminoguanidine		iNOS inhibitor	Maneb and paraquat in mice	• Reduced lipid and protein peroxidation in the striatum	[72]
9	Norfluoxetine (currently used as an antidepressant)		iNOS inhibitor	Lipopolysaccharide in mice	• Reduced nigrostriatal neuronal death and microglial activation	[73]

Potential of Antioxidants Acting Through Nitric Oxide Pathway in PD

Besides reducing the nitric oxide levels with the help of NOS inhibition, the other most common approach which has been tried is the use of antioxidants to protect the cells in the CNS from the effects of nitrosative stress. A different class of chemicals, including polyphenolics, carotenoids, flavonoids, phenols, and amino acid derivatives, have been used as antioxidants for the protection from nitrosative stress. Table 16.2 gives an insight into some of the antioxidants which have been tried in the PD models but are yet to translate into clinics as relevant therapeutic strategies.

Many of the polyphenolic compounds have been tried in the in vitro and in vivo model systems to protect against nitrosative stress in PD. The most common being curcumin which has been used extensively in a variety of neurodegenerative disorders including PD, AD and Huntington's disease [53, 97, 98]. It owes a part of its beneficial effect on the reduction of nitrosative stress. Additionally, following curcumin administration immunohistochemistry analysis of the tyrosine hydroxylase positive neurons in the substantia nigra demonstrated that it prevented the loss of dopaminergic neurons [53]. Besides this, derivatives of curcumin have also been tried and tested in PD. Di-glutamoyl curcumin also protected against nitrosative stress, reduced the peroxynitrite levels and prevented mitochondrial dysfunction [78]. Another curcumin analog, EF-24 (3,5-bis(2-flurobenzylidene)piperidin-4-one) accorded protection from the rotenone-induced synphilin aggregation and nitrosative stress in the SH-SY5Y cell lines [79]. Furthermore, polyphenolics from green tea also conferred protection to dopaminergic neurons in the midbrain and striatum from elevated levels of NO, nitrate, nitrite, iNOS and protein-bound 3-nitro-tyrosine [80]. Other phenolic compounds such as Arbutin and Carvacrol have also been tested in the MPTP and 6-OHDA model of PD. These compounds showed improvement in the motor deficits besides providing relief from the oxidative and nitrosative stress. Estimation of the nitrite levels in the midbrain in these studies demonstrated the potential of these compounds to be useful in improving the biochemical parameters in PD [84, 88].

Other classes of compounds helpful in providing relief from nitrosative stress include carotenoids, flavonoids and monoterpenoids. Crocin, a carotenoid obtained from *Crocus sativus* is a commonly used dye which was also found effective in the 6-OHDA hemiparkinsonian model to improve the behavioural deficits and reduce the nitrite levels in the striatum [86]. A similar reduction was also observed with the use of flavonoids, quercetin and sesamin, which were tested in the Microglial (N9)-Neuronal (PC12) co-culture and chrysin in the 6-OHDA model [92].

A herbal extract from *Juniperus communis*, animal-derived products like melatonin and amino acid derivative L-carnitine have also been tried in the PD models and have been found effective in the reduction of nitric oxide and the associated products [89–91].

There has been significant progress in the study of antioxidants for the reduction of oxidative and nitrosative stress. However, due to variation in the dose and lack of

Table 16.2 Antioxidants targeting nitrosative stress in PD

S. No.	Antioxidant/plant extract	Source	Class of compound	Model system	Outcome of study	References
1	Curcumin	*Curcuma longa* (Zingiberaceae)	Polyphenol	MPTP in mice	• Restoration of TH and GSH levels	[53]
2	Di-glutamoyl curcumin	Artificial synthesis	Polyphenol	MPP^+ in N27 dopaminergic neuronal cultures	• Reduced mitochondrial damage and nitrosative stress	[78]
3	EF-24	Artificial synthesis	Polyphenol	Rotenone in SH-SY5Y cell line	• Prevention of nitrosative induced Lewy body aggregate formation	[79]
4	Green tea polyphenolics	*Camellia sinensis* (Theaceae)	Polyphenol	6-OHDA in rats	• Inhibition of nuclear translocation of NFκB and restoration of free radical scavenging capability of both the striatum and midbrain	[80, 81]

(continued)

Table 16.2 (continued)

S. No.	Antioxidant/plant extract	Source	Class of compound	Model system	Outcome of study	References
5	CAPE	*Populus* spp.	Polyphenol	Rotenone in mice	• Improvement of locomotor deficits and reduced inflammatory changes	[82, 83]
6	Arbutin	*Arctostaphylos uva-ursi* (Ericaceae)	Hydroquinone (Phenol)	MPTP in mice	• Improvement of motor function and reduced oxidative stress	[84, 85]
7	Crocin	*Crocus sativus* (Iridaceae)	Carotenoid	6-OHDA in rats	• Improvement in motor and cognitive function accompanied with reduced inflammation	[86, 87]
8	Carvacrol	*Thymus vulgaris* (Lamiaceae)	Monoterpenoid phenol	6-OHDA in rats	• Reduced amphetamine induced rotations and oxidative stress	[88]

(continued)

Table 16.2 (continued)

S. No.	Antioxidant/plant extract	Source	Class of compound	Model system	Outcome of study	References
9	*Juniperus communis*	Cupressaceae	Monoterpene hydrocarbons	Chlorpromazine in rats	• Improvement in motor deficits and increase in GSH levels	[89]
10	Melatonin	Synthesized by pineal gland	Acetamide	PD patients	• Reduced activity of enzyme COX-2 and lipoperoxides	[90]
11	L-Carnitine	Food products like milk, meat, poultry, and fish	Amino acid derivative	Lipopolysaccharides in SIM-A9 microglia cells	• Reduced inflammation and microglial activation	[91]
12	Quercetin	Food products like apples, onions, citrus	Flavonoids	MPP^+ in microglial (N9)-neuronal (PC12) co-culture system	• Reduced levels of pro-inflammatory cytokines and apoptosis	[92, 93]
13	Sesamin	*Sesamum indicum* (Pedaliaceae)	Flavonoids	MPP^+ in microglial (N9)-neuronal (PC12) co-culture system	• Reduced levels of pro-inflammatory cytokines and apoptosis	[92]

(continued)

Table 16.2 (continued)

S. No.	Antioxidant/plant extract	Source	Class of compound	Model system	Outcome of study	References
14	Chrysin	*Passiflora incarnata* (Passifloraceae)	Flavonoids	6-OHDA in mice	• Improved motor and cognitive performance accompanied with reduced inflammatory markers	[94]
15	2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside	*Fallopia multiflora* (Polygonaceae)	Glucoside	6-OHDA in PC-12 cells	• Increased cell viability and reduced apoptosis	[95]
16	Rutin	*Fagopyrum esculentum* (Polygonaceae)	Flavonoid	MPP^+ in SH-SY5Y cell line	• Reduced proteins, γH2AX and COX-2 accompanied with increased cell viability	[96]

stringent quality control of the herbal drugs and plant-derived products, not much has translated into the clinics. Though some of the antioxidants have undergone clinical trials for their beneficial effects, a relevant drug targeting nitric oxide associated nitrosative stress is yet to come out in the market [99].

Concluding Remarks and Future Directions

This chapter summarizes the key findings related to the role of nitric oxide in PD. There are several underlying mechanisms involved in the pathophysiology of PD but have failed to translate into the clinics as useful therapeutic strategies. In line with these lies the nitric oxide which has been extensively explored in the context of other risk factors in PD but demands more attention for it to be targeted in the clinics. Drugs reducing the iNOS expression work well in the preclinical stages and can be taken up for clinical trials for the management of PD and associated complications. Moreover, there are several dietary supplements available which work towards the reduction of the nitrosative stress and NO-mediated neuronal death in the PD models. However, most of these approaches suffer from lack of specificity and side effects related to the use of non-standardized dosage regimens. Therefore, there is a need for deeper insights so that therapies targeting nitric oxide could be taken up for the management of PD.

Acknowledgements We would like to acknowledge the financial support of Department of Pharmaceuticals, Ministry of Chemicals and Fertilizers, Govt. of India provided to SSS, NIPER SAS Nagar (Grant# 2019-2020-NPLC-SSSHARMA). Additionally, the fellowship support provided by The Council of Scientific and Industrial Research to BV is also acknowledged.

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Chapter 17
Stable Gastric Pentadecapeptide BPC 157 and NO-System

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Abstract Stable gastric pentadecapeptide BPC 157 is known with very safe profile when used to be in ulcerative colitis and now multiple sclerosis trial, lethal dose (LD1) not achieved. Its pleiotropic beneficial effects were largely combined with its particular modulatory effect on NO-system functions, providing that BPC 157 may counteract adverse effect of nitric oxide synthase (NOS)-blocker L-NAME and NOS-substrate, L-arginine. Previous review emphasized the large range of relationships between and NO-system. These relationships were described as those on (i) gastric mucosa and mucosal protection, following alcohol lesions, in cytoprotection course, NO-generation, and blood pressure regulation; (ii) alcohol acute/chronic intoxication, and withdrawal; (iii) cardiovascular disturbances, chronic heart failure, pulmonary hypertension, and arrhythmias; (iv) disturbances after hypokalemia and hyperkalemia, and potassium-cell membrane dysfunction and (v) complex healing failure, proved by the fistulas healing. Further studies revealed additional particular relations on sphincter function, free radicals induced injuries, bleeding, non-specific and specific NSAIDs-induced lesions, general anesthesia (thiopental)- and local anesthesia (lidocaine)-induced disturbances, rat models that resemble schizophrenia-positive symptoms and most importantly, with organs lesions, or with vessels occlusion, the effect on the vessels presentation, and recruitment of additional collateral pathways to bypass occlusion. All of the studies used the triple relationships L-NAME versus L-arginine versus L-NAME + L-arginine, all together, as an indicator how NO-system may be involved. In a series of more than 80 targets investigated, L-NAME and L-arginine exhibited the opposite, but also the parallel effects. We proposed the presentation of new additional receptor(s) to resolve the matching of NO agents commonly supposed that negatively or positively affected the NO system and BPC 157 central role.

Keywords Stable gastric pentadecapeptide · BPC 157 · NO-system modulation · L-NAME · L-Arginine

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_17

Stable Gastric Pentadecapeptide BPC 157

Stable gastric pentadecapeptide BPC 157 is known with very safe profile when used to be in ulcerative colitis and now multiple sclerosis trial, lethal dose (LD1) not achieved [1–17]. Its pleiotropic beneficial effects were largely combined with its particular modulatory effect on NO-system functions [7], providing that BPC 157 may counteract adverse effect of nitric oxide synthase (NOS)-blocker N(G)-nitro-L-arginine methylester (L-NAME) and NOS-substrate, L-arginine [7].

Of note, such modulatory effect since very beginning (i.e., BPC 157 antagonized L-NAME-induced hypertension as well as L-arginine-induced hypotension [18]) opposes the common concept (i.e., [19]). Generally, that NO-system common concept [19] mostly works based on the NOS-blocker effect alone, and NO-inhibition [19]. Much less frequently, that NO-system common concept was based on the NOS-substrate effect, and even less frequently on the combined application of the NOS-blocker and NOS-substrate, and their mutual antagonization (for review see, i.e., [7]). Thus, considering these obvious limitation in the studies, it is evident that commonly accepted concept [19], in general, may only assume the opposite effects of NOS-blocker and NOS-substrate, as well as their responses to antagonize each other's response. On the other hand, the modulatory effect ascribed to BPC 157 application that would provide an alike antagonization of both opposite adverse effects [7] was outside the commonly accepted NO-system concept (i.e., [19]) and required an additional approach. Thereby, we introduced the administration of L-NAME (for NOS-blockade), administration of L-arginine (for NOS-substrate), and L-NAME + L-arginine application, thus, NOS-blockade (L-NAME) versus NOS-over-function (L-arginine) versus NO-immobilization (NOS-blockade/NOS-over-function, L-NAME + L-arginine) [7], triple relationships L-NAME versus L-arginine versus L-NAME + L-arginine, all together, as an indicator how NO-system may be involved [7].

The additional important points may be that BPC 157 may interact in a particular way with dopamine system as well. This may be the counteraction of the adverse effects of dopamine agonists as well as the counteraction of the adverse effects of dopamine antagonists application and the counteraction of dopamine-neurotoxin (methyl-4-phenyl-1,2,3,6-tetrahydrophyridine) [20–24]. Also, a similar interaction is possible with prostaglandins system. BPC 157 largely counteracted toxicity of non-steroidal anti-inflammatory drugs [6], both non-specific and specific [6] as well as exhibited particular anti-inflammatory action of its own, i.e., both prevented and reversed adjuvant arthritis, periodontitis, or peritoneal adhesions in rats [6, 25–27].

Stable Gastric Pentadecapeptide BPC 157 and NO

Previously, we reviewed [7] the large range of relationships between stable gastric pentadecapeptide BPC 157 [1–17] and NO-system. These relationships were described depending on the target affected. An important point includes gastric

mucosa and mucosal protection, following alcohol lesions, in cytoprotection course, NO-generation, and blood pressure regulation [18, 28]. The next point was alcohol acute/chronic intoxication, and withdrawal [19]. Additional studies include cardiovascular disturbances, chronic heart failure, pulmonary hypertension, and arrhythmias [29–33]. Evidenced were also disturbances after hypokalemia and hyperkalemia, and potassium-cell membrane dysfunction [32–34]. Final point includes complex healing failure, proved by the fistulas healing, duodenocutaneous [35], colocutaneous [36], and esophagocutaneous [37]. Further studies revealed additional particular relations on the quite distinctive targets. Illustratively, studies revealed the sphincter function [28, 38–40], free radicals induced injuries [39–41], bleeding [42, 43], non-specific and specific NSAIDs-induced lesions [44, 45], general anesthesia (thiopental)- and local anesthesia (lidocaine)-induced disturbances [46, 47], and rat models that resemble schizophrenia-positive symptoms [20]. Likely, the most important point may be, with organs lesions, or with vessels occlusion, the effect on the vessels presentation, and recruitment of additional collateral pathways to bypass occlusion [27, 42, 48–50]. In addition, in the healing process, BPC 157 was shown to interact with many molecular pathways [15, 17, 37, 51–59].

It was also emphasized that BPC 157 is formed constitutively in the gastric mucosa, stable and present in human gastric juice [1–17]. Thereby, along with suggested significance of NOS and the basal formation of NO in stomach mucosa, greater than that seen in other tissues [19], BPC 157 may alone induce the release of NO in ex-vivo condition [18], even in the conditions that annihilate the effect of L-arginine [60]. Consequently, it may exhibit a general, effective competing with both L-arginine analogues (i.e., L-NAME) and L-arginine [7]. Finally, the most recent study demonstrates that BPC 157 can modulate the vasomotor tone of an isolated aorta in a concentration- and NO-dependent manner [55]. BPC 157 can induce NO generation likely through the activation of Src-Cav-1-eNOS pathway [55]. However, how this advantage of modulating NO-system (i.e., BPC 157 may counteract both L-NAME-induced hypertension and L-arginine-induced hypotension [18]) may be practically translated into an enhanced clinical performance still remains to be determined.

Particular Relationships Between the NO-System as a Follow Up of the NO-Agents Effects

Triple Relationships L-NAME Versus L-Arginine Versus L-NAME + L-Arginine, All Together, as an Indicator How NO-System May be Involved

Now, this review further revealed the particular relationships between the NO-system, taken as the main basic bodily system, and stable gastric pentadecapeptide BPC 157 [1–17], as a system that may interact with [7]. In principle, for the NO-system

interactions with other systems, we could claim, the more points identified where the two systems may interact, the closer may be their relationships [7]. Likewise, we could argue in vivo studies, the more agents employed (NOS-blockers, NOS-substrate, combination of the NOS-blocker and NOS-substrate), the more precise relationships to be defined [7]. At the end, with respect to the established significance of the NO-system, we can, also with suited addition, (re)-evaluate the real significance of the other system, BPC 157, employed in the noted interactions. In theory–practice application, the significance of the NO-system that may be estimated, depends on the effect(s) of the NO-agents, given alone and/or together—that may be seen on the one or more of the particular targets. These are the effect or no effect of NOS-blocker (L-NAME), the effect or no effect of NOS-substrate (L-arginine), the effect or no effect of their combination (L-NAME + L-arginine), and their ability or no ability to oppose (or not oppose) each other effect, the effects opposite or the effects parallel. This means triple relationships L-NAME versus L-arginine versus L-NAME + L-arginine, all together, as an indicator how NO-system may be involved [7]. Evidently, this triplet complex approach largely exceeds general presumption that holds the opposite relation between the NO synthase (NOS)-blocker and NOS-substrate, and that NO synthase (NOS)-blocker administration and NOS-substrate administration have to have the opposite effect. Contrarily, they may have also parallel effects. Thereby, it strongly opposes the use of only either NOS-blocker (i.e., NO studies are usually limited to the blunted generation of NO only (and thereby less precise [7]) or NOS-substrate, alone, to substantiate the particular involvement of NO-system [7]).

In general, NO-system, taken as the main basic bodily system, with tight interactions between NO-synthases, implies effective control of the entirety of the bodily functions, permanent maintenance of the close balance [7]. Together, this significance and complexity may overwhelm the common concept that beneficial or harmful effect of either NOS-blocker or NOS-substrate would exclude the possibility of the similar effect of other one. Thus, this triplet concept directly opposes general concept of the mandatory opposite effects holding that controversial is the existing application of the both L-NAME and L-arginine in the similar disease condition (i.e., [61]).

Both L-NAME and L-Arginine in the Similar Disease Condition

Of note, the list of possible "controversy" is appreciable. It includes, at least, cerebral ischemia [62, 63], and schizophrenia [64, 65] (including in the animal models resembling 'positive-like' symptoms of schizophrenia (i.e., amphetamine application [66–69])). Questioned were also the inhibition of endogenous NO production in severe sepsis [70–72] and administration of exogenous NO in acute lung injury [73, 74]. In gastrointestinal tract, for instance, L-NAME blocks the diarrheal effect of castor oil while worsens the apparent injury to the mucosa [75].

Recently, using the above described triplet complex approach [20], we rationalize in the further schizophrenia therapy, the evidenced use of both NOS-inhibitor, L-NAME, and NOS-substrate, L-arginine [64, 65, 76]. This would anticipate distinctive patterns of the disease that would be responsible to either NOS-blocker or NOS-substrate administration. We used the rat models that resemble "positive-like" symptoms of schizophrenia [20], acutely [66–69] and chronically [77], to induce sensitivity [77]. Also, we employed also the rat extrapyramidal symptoms models [23, 78], known to be related to the more severe psychiatric symptoms [79]. Two distinctive patterns, "L-NAME non-responsive, L-arginine responsive" and "L-NAME responsive, L-arginine responsive" were identified [20]. These responses were suggested to indicate two distinctive presentations of the NO-pathways [20]. In apomorphine-, and MK-801-induced effects, methamphetamine chronic-sensitivity, and haloperidol-induced catalepsy, L-arginine (counteraction) and L-NAME (no effect), when combined (L-NAME + L-arginine), exhibited opposite effect since antagonize each other effect ("L-NAME non-responsive, L-arginine responsive"). Contrarily, in amphetamine-effect, L-arginine (counteraction), L-NAME (counteraction) exhibited a parallel effect, and when combined (L-NAME + L-arginine), exhibited no antagonization of each other, rather a persisting effect ("L-NAME responsive, L-arginine responsive").

L-NAME and L-Arginine May Have Both Opposite and Parallel Effect; BPC 157 Effect

Indeed, L-NAME and L-arginine may also have a parallel effect [20, 34, 38, 42, 49, 50]. In addition to amphetamine-effect [20], this parallelism occurs with quite distinctive models (myosis, atropine-mydriasis [38], huge magnesium over-dose [34], ischemic/reperfusion colitis [49], duodenal congestion lesions [50], cecum perforation [42], and L-NAME and/or L-arginine interaction with other systems (i.e., acetylcholine) [38]). In all these experiments, the consistent beneficial outcome with BPC 157 co-administration shows that BPC 157 therapy effect is regularly present in BPC 157 + L-NAME + L-arginine-animals [20, 34, 38, 42, 49, 50]. This congruent point is also demonstrated in other studies [80, 81].

Unfortunately, the similar studies using described L-NAME, L-arginine, and L-NAME + L-arginine triplet complex application, are generally lacking [7]. Thereby, using that triplet complex approach (L-NAME versus L-arginine versus L-NAME + L-arginine complex application), we going to summarize the evidence coming from all of our NO-studies, including more than 80 targets investigated (Tables 17.1 and 17.2). The evidence for these conclusions is, in brief, that a series of most of twenty L-NAME, L-arginine, and L-NAME + L-arginine complex administration has particular order of potency on the particular targets investigated (Table 17.1). In contrast, this same series of L-NAME, L-arginine, and L-NAME + L-arginine complex effects has an entirely different order of potency on other targets (Table 17.2).

Table 17.1 Relationships between NO-system and gastric pentadecapeptide BPC 157, with the opposite effect of L-NAME and L-arginine, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157. With respect to the control values, the effects were assessed, if not otherwise specified, as increase (↑), decrease (↓) or no change (/). Increase (↑) and decrease (↓) were assessed as response (R), no change (/) as non-response (NR). With combined administration of L-NAME and L-arginine (L-NAME + L-arginine), when opposite effect of L-NAME and L-arginine did counteract each other's responses, the effect was assessed as specific. When opposite effect of L-NAME and L-arginine did not counteract each other's responses, the effect was assessed as nonspecific. When both L-NAME and L-arginine showed no effect (/)—the effects were assessed to be not NO-system related (Not related)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Drmic et al. (2018) Counteraction of perforated cecum lesions in rats: effects of pentadecapeptide BPC 157, L-NAME and L-arginine [42]	Perforated cecum lesion	↓	/	↓	/	↓	/	↓	Not related
	Vessel presentation	↑	↓↓	↑	↓	↑	Counteraction to control values	↑	L-NAME R, L-arginine R *Opposite, specific*
	Bleeding	↓	↓	↓	↑	↓	Counteraction to control values	↓	L-NAME R, L-arginine R *Opposite, specific*
Sucic et al. (2019) Therapy of the rat hemorrhagic cystitis induced by cyclophosphamide. Stable gastric pentadecapeptide BPC 157, L-arginine, L-NAME [40]	Hemorrhagic cystitis	↓	↑	↓	↓	↓	↑	↓	L-NAME R, L-arginine R *Opposite, specific*
	Increased leak point pressure	Reversed to normal	/	Reversed to normal	/	Reversed to normal	/	Reversed to normal	Not related

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Lojo et al. (2016) Effects of diclofenac, L-NAME, L-arginine, and pentadecapeptide BPC 157 on gastrointestinal, liver, and brain lesions, failed anastomosis, and intestinal adaptation deterioration in 24 h-short-bowel rats [45]	Gastrointestinal lesions	Reversed below control values	↑	Reversed below control values	/	Reversed below control values	Reversed to control values	Reversed below control values	L-NAME R, L-arginine NR *Opposite, specific*
	Liver lesions	Reversed below control values	↑	Reversed below control values	↓	Reversed below control values	Reversed to control values	Reversed below control values	L-NAME R, L-arginine R *Opposite, specific*
	Brain lesions	Reversed below control values	↑	Reversed below control values	↓	Reversed below control values	Reversed to control values	Reversed below control values	L-NAME R, L-arginine R *Opposite, specific*
	Failed anastomosis healing	Reversed up to control values	↑	Reversed below control values	/	Reversed below control values	Reversed to control values	Reversed below control values	L-NAME R, L-arginine NR *Opposite, specific*
	Intestinal adaptation deterioration	Reversed below control values	↑	Reversed below control values	/	Reversed below control values	Reversed to control values	Reversed below control values	L-NAME R, L-arginine NR *Opposite, specific*
Drmic et al. (2017) Celecoxib-induced gastrointestinal, liver and brain lesions in rats, counteraction by BPC 157 or L-arginine, aggravation by L-NAME [44]	Gastric lesions	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Liver lesions	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Brain lesions	↓↓	/	↓↓	↓↓	↓↓	Counteraction to control values	↓↓	L-NAME NR, L-arginine R *Opposite, specific*
Djakovic et al. (2016) Esophagogastric anastomosis in rats: improved healing by BPC 157 and L-arginine, aggravated by L-NAME [48]	Esophagogastric anastomosis esophagitis lesions	↓	↑	↓	↓	↓	↓	↓	L-NAME R, L-arginine R *Opposite, nonspecific*
	Esophagogastric anastomosis gastric lesions	↓	↑	↓	↓	↓	↓	↓	L-NAME R, L-arginine R *Opposite, nonspecific*

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
	Esophagogastric anastomosis anastomosis strength	↓	↑	↓	↓	↓	Counteraction to control values	↓	L-NAME R, L-arginine R *Opposite, specific*
	Esophagogastric anastomosis "esophageal sphincter" function	↑	↓	↑	↑	↑	Counteraction to control values	↑	L-NAME R, L-arginine R *Opposite, specific*
	Esophagogastric anastomosis pyloric sphincter function	↑	↓	↑	↑	↑	Counteraction to control values	↑	L-NAME R, L-arginine R *Opposite, specific*
	Esophagogastric anastomosis lethal outcome	None	Increased	None	None	None	None	None	L-NAME R, L-arginine R *Opposite, nonspecific*
Stupnisek et al. (2015) Pentadecapeptide BPC 157 reduces bleeding and thrombocytopenia after amputation in rats treated with heparin, warfarin, L-NAME and L-arginine [43]	Tail amputation spontaneous bleeding for 20 min, fall in platelet count without any failure of coagulation parameters	Less bleeding	Less bleeding	Less bleeding	More bleeding	Less bleeding	Less bleeding	Less bleeding	L-NAME R, L-arginine R *Opposite, nonspecific*
		Counteracted prolonged APTT-, TT-values	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	Not relevant
	Heparin + tail amputation extensive bleeding and blood loss, prominent fall in platelet count, drastic prolongation of PT-, APTT-, TT-values	Less bleeding	No effect on bleeding	Less bleeding	More bleeding	Less bleeding	Counteraction to control values	Less bleeding	L-NAME NR, L-arginine R *Opposite, specific*
		Counteracted prolonged APTT-, TT-values	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	Not relevant

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
	Warfarin + tail amputation extensive bleeding and blood loss, prominent fall in platelet count, drastic prolongation of PT-, APTT-, TT-values	Less bleeding	No effect on bleeding	Less bleeding	More bleeding	Less bleeding	Counteraction to control values	Less bleeding	L-NAME R, L-arginine R *Opposite, specific*
		Counteracted thrombocytopenia	No effect on thrombocy-topenia	No effect on thrombocy-topenia	Counteracted thrombocy-topenia	No effect on thrombocy-topenia	No effect on thrombocy-topenia	No effect on thrombocy-topenia	Not relevant
		No counteraction of prolonged PT	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	Not relevant
Luetic et al. (2017) Cyclophosphamide induced stomach and duodenal lesions as a NO-system disturbance in rats: L-NAME, L-arginine, stable gastric pentadecapeptide BPC 157 [41]	Stomach ulcer	↓	↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
	Duodenal ulcer	↓	↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
Zemba et al. (2015) BPC 157 antagonized the general anaesthetic potency of thiopental and reduced prolongation of anaesthesia induced by L-NAME/thiopental combination [46]	Thiopental anesthesia	↓	↑↑↑	Counteraction to control values	/	Counteraction to control values	↑	↓	L-NAME R, L-arginine NR *Opposite, nonspecific*

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Zemba Cilic et al. (2020) Pentadecapeptide BPC 157 counteracts L-NAME-induced catalepsy. BPC 157, L-NAME, L-arginine, NO-relation, in the suited rat acute and chronic models resembling 'positive-like' symptoms of schizophrenia [20]	Apomorphine-induced disturbances	↓↓	/	↓	↓	↓	Counteraction to control values	↓	L-NAME NR, L-arginine R *Opposite, specific*
	MK-801-induced locomotion, stereotyped sniffing and ataxia disturbances	↓↓	/	↓	↓↓	↓	Counteraction to control values	↓	L-NAME NR, L-arginine R *Opposite, specific*
	Haloperidol-induced catalepsy	↓↓	/	↓	↓↓	↓	Counteraction to control values	↓	L-NAME NR, L-arginine R *Opposite, specific*
	Methamphetamine-induced disturbances	↓↓	/	↓	↓	↓	Counteraction to control values	↓	L-NAME NR, L-arginine R *Opposite, specific*
Skorjanec et al. (2015) Duodenocutaneous fistula in rats as a model for "wound healing-therapy" in ulcer healing: the effect of pentadecapeptide BPC 157, L-nitro-arginine methyl ester and L-arginine [35]	Duodenal defect	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Skin defect	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Fistula	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Mortality	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Balenovic et al. (2009) Inhibition of methyldigoxin-induced arrhythmias by pentadecapeptide BPC 157: a relation with NO-system [31]	Arrhythmia	↓	↑↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
	Mortality	↓	↑↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
Cesarec et al. (2013) Pentadecapeptide BPC 157 and the esophagocutaneous fistula healing therapy [37]	Esophageal defect	↓↓	↑↑	↓↓ below control values	↓	↓	Counteraction to control values	↓↓ (below control values)	L-NAME R, L-arginine R *Opposite, specific*
	Skin defect	↓↓	↑↑	↓↓ below control values	↓	↓	Counteraction to control values	↓↓ (below control values)	L-NAME R, L-arginine R *Opposite, specific*
	Esophagocutaneous fistula leaking	↓↓	↑↑	↓↓ below control values	↓	↓	Counteraction to control values	↓↓ (below control values)	L-NAME R, L-arginine R *Opposite, specific*
	Lower esophageal sphincter pressure	↑↑	↓↓	↑↑ up to control values	↑↑	↑↑	↑↑	↑↑ up to control values	L-NAME R, L-arginine R *Opposite, nonspecific*
	Pyloric sphincter pressure	↑↑	↓↓	↑↑ up to control values	↑↑	↑↑	↑↑	↑↑ up to control values	L-NAME R, L-arginine R *Opposite, nonspecific*
	Mortality	Absent	/	Absent	Absent	Absent	Absent	Absent	L-NAME NR, L-arginine R *Opposite, specific*

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Klicek et al. (2008) Pentadecapeptide BPC 157, in clinical trials as a therapy for inflammatory bowel disease (PL14736), is effective in the healing of colocutaneous fistulas in rats: role of the nitric oxide-system [36]	Colon defect	↓	↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
	Skin defect	↓	↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
	Colocutaneous fistula	↓	↑	↓	/	↓	Counteraction to control values	↓	L-NAME R, L-arginine NR *Opposite, specific*
Becejac et al. (2018) An endogenous defensive concept, renewed cytoprotection/adaptive cytoprotection: intra(per)-oral/intragastric strong alcohol in rat. Involvement of pentadecapeptide BPC 157 and nitric oxide system [28]	Tongue lesions	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Esophagus lesions	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Stomach lesions	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Duodenum lesions	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Lower esophageal sphincter pressure	↑	↓	↑	↓	↑	Counteraction to control values	↑	L-NAME R, L-arginine R *Opposite, specific*
	Pyloric pressure pressure	↑	/	↑	/	↑	/	↑	Not relevant

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Barisic et al. (2013) Mortal hyperkalemia disturbances in rats are NO-system related. The life saving effect of pentadecapeptide BPC 157 [33]	Serum electrolytes values	/	/	/	/	/	/	/	Not relevant
	Survival and life-saving potential	Complete survival	Accelerated lethal outcome	Complete survival	Postponed lethal outcome	Complete survival	Counteraction to control values	Complete survival	L-NAME R, L-arginine R *Opposite, specific*
	Arrhythmias	↓↓↓	↑	↓↓↓	↓	↓↓↓	Counteraction to control values	↓↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Hypertension	↓↓↓	↑	↓↓↓	↓↓↓	↓↓↓	Counteraction to control values	↓↓↓	L-NAME R, L-arginine R *Opposite, specific*
	Muscular disability	↓↓↓	↑	↓↓↓	/	↓↓↓	Counteraction to control values	↓↓↓	L-NAME R, L-arginine NR *Opposite, specific*
	Sphincteric failure lower esophageal	Increased pressure	Decreased pressure	Increased pressure	Increased pressure	Increased pressure	Counteraction to control values	Increased pressure	L-NAME R, L-arginine R *Opposite, specific*
	Sphincteric failure pyloric	Increased pressure	/	Increased pressure	Increased pressure	Increased pressure	Counteraction to control values	Increased pressure	L-NAME NR, L-arginine R *Opposite, specific*

(continued)

Table 17.1 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Sikiric et al. (1997) The influence of a novel pentadecapeptide, BPC 157, on N(G)-nitro-L-arginine methylester and L-arginine effects on stomach mucosa integrity and blood pressure [18]	Stomach lesions	↓↓	/	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME NR, L-arginine R *Opposite, specific*
	Blood pressure	Normotension	Hypertension	Normotension	Hypotension	Normotension	Normotension	Normotension	L-NAME R, L-arginine R *Opposite, specific*
	In vitro, in gastric mucosa from rat stomach tissue homogenates, BPC 157, given in the same dose (100 microM) as L-arginine, induced a comparable generation of NO. But, BPC 157 effect could not be inhibited by L-NAME, even when L-NAME was given in a tenfold (100 versus 1000 microM) higher dose than that needed for inhibition of the L-arginine effect. NO synthesis was blunted when the pentadecapeptide BPC 157 and L-arginine were combined								L-NAME R, L-arginine R *Opposite, specific*
Cesar et al. (2020) Bowel adhesion and therapy with the stable gastric pentadecapeptide BPC 157, L-NAME and L-arginine in rats [27]	Parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats, increased adhesion formation	↓↓	↑	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Opposite, specific*

Table 17.2 Relationships between NO-system and gastric pentadecapeptide BPC 157, with the parallel effect of L-NAME and L-arginine, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157. With respect to the control values, the effects were assessed, if not otherwise specified, as increase (↑), decrease (↓) or no change (/). Increase (↑) and decrease (↓) were assessed as response (R), no change (/) as non-response (NR). With combined administration of L-NAME and L-arginine (L-NAME + L-arginine), when parallel effect of L-NAME and L-arginine did counteract each other's responses, the effect was assessed as specific. When parallel effect of L-NAME and L-arginine did not counteract each other's responses, the effect was assessed as nonspecific. When both L-NAME and L-arginine showed no effect (/)—the effects were assessed to be not NO-system related (not related)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Amic et al. (2018) Bypassing major venous occlusion and duodenal lesions in rats, and therapy with the stable gastric pentadecapeptide BPC 157, L-NAME and L-arginine [50]	Duodenal lesions	↓↓	↓	↓↓	↓	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R, *Parallel, specific*
	Vessels presentation	↑↑	/	↑↑	/	↑↑	/	↑↑	Not related
Medvidovic-Grubisic et al. (2017) Hypermagnesemia disturbances in rats, NO-related: pentadecapeptide BPC 157 abrogates, L-NAME and L-arginine worsen [34]	Muscle weakness	↓	↑	Decreased to control values	↑	Decreased below control values	Counteraction to control values	Decreased below control values	L-NAME R, L-arginine R, *Parallel, specific*
	Muscle lesions	↓	↑	Decreased to control values	↑	Decreased below control values	Counteraction to control values	Decreased below control values	L-NAME R, L-arginine R, *Parallel, specific*
	Brain lesions	↓	↑	Decreased to control values	↑	Decreased below control values	Counteraction to control values	Decreased below control values	L-NAME R, L-arginine R, *Parallel, specific*
	Hypermagnesemia	↓	↑	Decreased to control values	↑	Decreased below control values	Counteraction to control values	Decreased below control values	L-NAME R, L-arginine R, *Parallel, specific*

(continued)

Table 17.2 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
	Hyperkalaemia	↓	↑	Decreased to control values	↑	Decreased below control values	Counteraction to control values	Decreased below control values	L-NAME R, L-arginine R, *Parallel, specific*
Stupnisek et al. (2015) Pentadecapeptide BPC 157 reduces bleeding and thrombocytopenia after amputation in rats treated with heparin, warfarin, L-NAME and L-arginine [43]	Tail amputation spontaneous bleeding for 20 min, fall in platelet count without any failure of coagulation parameters	Counteracted thrombocytopenia	Counteracted thrombocy-topenia	Counteracted thrombocytopenia	Counteracted thrombocy-topenia	Counteracted thrombocy-topenia	Counteracted thrombocy-topenia	Counteracted thrombocy-topenia	L-NAME R, L-arginine R *Parallel, nonspecific*
		Counteracted prolonged APTT-, TT-values	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	Not relevant
	Heparin + tail amputation extensive bleeding and blood loss, prominent fall in platelet count, drastic prolongation of PT-, APTT-, TT-values	Counteracted thrombocytopenia	Counteracted thrombocy-topenia	Counteracted thrombocytopenia	Counteracted thrombocy-topenia	Counteracted thrombocy-topenia	Counteracted thrombocy-topenia	Counteracted thrombocy-topenia	L-NAME R, L-arginine R *Parallel, nonspecific*
		Counteracted prolonged APTT-, TT-values	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	No effect on coagulation parameters	Not relevant
Kokot et al. (2016) NO system dependence of atropine-induced mydriasis and L-NAME- and L-arginine-induced miosis: reversal by the pentadecapeptide BPC 157 in rats and guinea pigs [38]	Normal pupil miosis	/	↑↑	↓	↑↑	↑	↑	↑	L-NAME R, L-arginine R *Parallel, nonspecific*
	Atropine-induced mydriasis	↓↓↓	↓	↓↓↓	↓	↓↓↓	↓↓	↓↓↓	L-NAME R, L-arginine R *Parallel, nonspecific*

(continued)

Table 17.2 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Belosic Halle et al. (2017) Class side effects: decreased pressure in the lower oesophageal and the pyloric sphincters after the administration of dopamine antagonists, neuroleptics, anti-emetics, L-NAME, pentadecapeptide BPC 157 and L-arginine [39]	Decreased lower esophageal sphincter pressure	Increased to normal values	↓↓	Increased to normal values	↓	Increased to normal values	Counteraction to control values	Increased to normal values	L-NAME R, L-arginine R *Parallel, specific*
	Decreased pyloric sphincter pressure	Increased to normal values	↓↓	Increased to normal values	↓	Increased to normal values	Counteraction to control values	Increased to normal values	L-NAME R, L-arginine R *Parallel, specific*
Zemba Cilic et al. (2020) Pentadecapeptide BPC 157 counteracts L-NAME-induced catalepsy. BPC 157, L-NAME, L-arginine, NO-relation, in the suited rat acute and chronic models resembling 'positive-like' symptoms of schizophrenia [20]	Amphetamine-induced disturbances	↓↓	↓	↓	↓	↓	No antagonization	↓	L-NAME R, L-arginine R *Parallel, nonspecific*
Duzel et al. (2017) Stable gastric pentadecapeptide BPC 157 in the treatment of colitis and ischemia and reperfusion in rats: new insights [49]	Arcade vessels	↑	↓	↑	↑	↑	Counteraction to control values	↑	L-NAME R, L-arginine R *Parallel, specific*
	Pale areas	↓	↑	↓	↑	↓	Counteraction to control values	↓	L-NAME R, L-arginine R *Parallel, specific*

(continued)

Table 17.2 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Boban-Blagaic et al. (2006) The influence of gastric pentadecapeptide BPC 157 on acute and chronic ethanol administration in mice. The effect of N(G)-nitro-L-arginine methyl ester and L-arginine [82]	Acute alcohol intoxication	Improved behavior	Aggravated behavior	Aggravated behavior	Aggravated behavior	Aggravated behavior	Aggravated behavior		L-NAME R, L-arginine R *Parallel, nonspecific*
		↑ improved temperature	↓ aggravated temperature	↓ aggravated temperature	↓ aggravated temperature	↓ aggravated temperature	↓ aggravated temperature		L-NAME R, L-arginine R *Parallel, nonspecific*
		↓ ethanol in blood	↓ ethanol in blood	↓ ethanol in blood	↓ ethanol in blood	↓ ethanol in blood	↓ ethanol in blood		L-NAME R, L-arginine R *Parallel, nonspecific*
		↑ survival	↓ survival	No effect on survival	↓ survival	No effect on survival	↓ survival		L-NAME R, L-arginine R *Parallel, nonspecific*
	Withdrawal	↓	↓	↓	↓	↓	↓		L-NAME R, L-arginine R *Parallel, nonspecific*
Grabarevic et al. (1997) The influence of BPC 157 on nitric oxide agonist and antagonist induced lesions in broiler chicken [30]	Pulmonary hypertension syndrome	↓↓	↑↑	↓	↑	↓↓	↑	↓	L-NAME R, L-arginine R *Parallel, nonspecific*

(continued)

Table 17.2 (continued)

Relationships between NO-system and stable gastric pentadecapeptide BPC 157, represented with administration of L-NAME and administration of L-arginine, alone and together, as well as with stable gastric pentadecapeptide BPC 157

Study	Target	BPC 157	L-NAME	L-NAME + BPC 157	L-Arginine	L-Arginine + BPC 157	L-Arginine + L-NAME	L-Arginine + + L-NAME + BPC 157	Response
Lozic et al. (2020) In relation to NO-system, stable pentadecapeptide BPC 157 counteracts lidocaine-induced adverse effects in rats and depolarisation in vitro [47]	Lidocaine-induced local anesthesia via intraplantar application	↓↓	↑	↓↓	↑	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Parallel, specific*
	Lidocaine-induced axillary block	↓↓	↑	↓↓	↑	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Parallel, specific*
	Lidocaine-induced spinal (L4–L5) intrathecal block	↓↓	↑	↓↓	↑	↓↓	Counteraction to control values	↓↓	L-NAME R, L-arginine R *Parallel, specific*
Cesar et al. (2020) Bowel adhesion and therapy with the stable gastric pentadecapeptide BPC 157, L-NAME and L-arginine in rats [27]	Parietal peritoneum excision with an underlying superficial layer of muscle tissue in rats, failed vasculature	Abundant vessels in and near defect	Weakening of blood vessel disappearance	Abundant vascular vessels in and near the defect	Weakening of blood vessel disappearance	Abundant vessels in and near defect	Counteraction to control values	Abundant vessels in and near defect	L-NAME R, L-arginine R, *Parallel, specific*

Delineation of Both Opposite and Parallel of Effects of L-NAME and L-Arginine

As in theory long-ago established (see i.e., [83]), we should consider that the variations in activity could be due to (a) quantitative differences in potency, (b) qualitatively different effects or (c) differences due entirely to the experimental methods used. However, if the last two factors are controlled as much as possible by the selection of the same dose for L-NAME, L-arginine, and L-NAME + L-arginine complex and by using suitable experimental techniques, then the variations in activity are presumably due to actual differences in the receptors involved.

These would further delineate both opposite and parallel of effects of L-NAME and L-arginine (Tables 17.1 and 17.2). Few targets in the studies that revealed, however, no effect of either L-NAME or L-arginine, may be related to limitation of the 5 mg/kg of L-NAME and 100 kg mg of L-arginine, applied intraperitoneally, as used standards.

Mostly, opposite effects of L-NAME and L-arginine, when both L-NAME and L-arginine, given alone, exhibit innate activity, appear as specific effect, since combined L-NAME and L-arginine may antagonize each other effect to the level of the control values (Table 17.1). However, it may be that either of NO-agents, L-NAME or L-arginine, may also have a hidden effect. That activity would be apparent as the effect only when given together, as the antagonization of the previous innate activity of the either L-NAME or L-arginine. Finally, it may be that the opposite effects of L-NAME and L-arginine, when given together (L-NAME + L-arginine) may not antagonize each other response, or at least no antagonization to the extent of the control values, thereby, they appear to be nonspecific (Table 17.1). Thus, for the opposite effect of L-NAME and L-arginine, we can envisage both simple effect (when inhibition (L-NAME) and stimulation (L-arginine) appear to be balanced, and thereby, mutual antagonization may occur), and composed effect (when either inhibition (L-NAME) or stimulation (L-arginine) prevails, while the other remains hidden, and may appear only on the background of the prevailed effect). Possibly, opposite effects of L-NAME and L-arginine, that may not antagonize each other's response, providing however the known specificity of both L-NAME and L-arginine, may suggest the presentation of the particular NO-targets, inhibitory and stimulatory, that can be likely separated. Commonly, the antagonization only to the control level, leaves the remained disturbances out of the functioning of the NO-agents, and thereby, this may suggest the NO-system function particularly in the more disturbed conditions [7]. Illustrative example is when L-arginine may antagonize only the worse lesions, those L-NAME-induced lesions [31, 45].

Likely, parallel effects of L-NAME and L-arginine should resolve the matching of NO agents commonly supposed that negatively or positively affected the NO system (Table 17.2). Of note, parallel effects of L-NAME and L-arginine, when both L-NAME and L-arginine, given alone, exhibit innate activity, appear as specific effect, since combined L-NAME and L-arginine may antagonize each other effect to the level of the control values. Also, as may be seen with the opposite effects of L-NAME and L-arginine, the alternative possibility appeared that these parallel effects of L-NAME

and L-arginine, when given together (L-NAME + L-arginine), may not antagonize each other response. Thus, if the variations in activity would be presumably due to actual differences in the receptors involved, we can suggest the presentation of the particular "pre-synaptic" inhibitory receptors. Providing the inhibitory role of L-NAME, the inhibition of these inhibitory receptors by L-NAME, may be a particular point. Vice versa, "inhibition of inhibition", may indirectly result with NO-release stimulation, thereby a comparable effect to that of L-arginine. Possibly, that NO-release may antagonize the further effect of L-arginine administration. We could speculate that these "pre-synaptic" inhibitory receptors may have a complex role in the controlling of the NO-system function that should be further determined.

Finally, we can theorize that BPC 157, behaving as an insurmountable antagonist of similar potency for the adverse effects of various L-NAME and L-arginine regimens, activating whatever of their specific receptors (including those suggested "pre-synaptic"), may act by modulating a mechanism common to those activated receptors. Two isozymes, neuronal NOS (nNOS) and endothelial NOS (eNOS) are Ca^{2+} dependent and constitutively expressed. The third NOS isoform, inducible NOS (iNOS) is Ca^{2+} independent and inducible by, for example, bacterial enterotoxins, cytokines and following intestinal injury [84].

Thus, it may be that in addition to the reported essential effect on eNOS [55], BPC 157 may modulate the calcium effect as well. This may be likely, since BPC 157 may counteract the adverse effect of both hyperkalemia and hypokalemia both in vivo, as well as in vitro, their opposite effect on the membrane potential [32, 33]. Finally, as an additional clue, may be that BPC 157 acts as stabilizer of cellular junction [17]. Via increasing tight junction protein ZO-1 expression, and transepithelial resistance [17], it significantly mitigated indomethacin-induced leaky gut syndrome [17]. Likewise, there were inhibited the mRNA of inflammatory mediators (iNOS, IL-6, IFNγ and TNF-α), increased expression of HSP 70 and 90, and antioxidant proteins, such as HO-1, NQO-1, glutathione reductase, glutathione peroxidase 2 and GST-pi [17].

Conclusion

Stable gastric pentadecapeptide BPC 157 pleiotropic beneficial effects, largely combined with its particular modulatory effect on NO-system functions were already reviewed [7]. Since beginning, this goes outside of the common NO-system concept [19] (opposite effects of NOS-blocker and NOS-substrate, which should antagonize each other's response to be NO-specific) providing that BPC 157 may counteract adverse effect of NOS-blocker L-NAME and NOS-substrate, L-arginine. Resolving this modulatory role needs demonstration how NO-system is working, based on the activity of the NO-agents. Purposefully, we emphasized in vivo studies. Obviously, the more agents employed (NOS-blockers, NOS-substrate, combination of the NO synthase-blocker and NO-synthase-substrate), the more precise relationships to be defined [7]. Thereby, we suggest that triple relationships L-NAME versus L-arginine versus L-NAME + L-arginine, should be further standard. This triplet complex

concept may oppose the common simple concept that beneficial or harmful effect of either NOS-blocker or NOS-substrate would exclude the possibility of the similar effect of other one. Introduction of the L-NAME versus L-arginine versus L-NAME + L-arginine approach can identify also parallel effect of NOS-blocker and NOS-substrate administration in addition to their prevalent opposite effects. Thereby, using that triplet complex approach (L-NAME versus L-arginine versus L-NAME + L-arginine complex application), we summarized the evidence coming from all of our NO-studies, including more than 80 targets investigated (Tables 17.1 and 17.2). The evidence for these conclusions is, in brief, that a series of most of twenty L-NAME, L-arginine, and L-NAME + L-arginine complex administration has particular order of potency on the particular targets investigated (Table 17.1). In contrast, this same series of L-NAME, L-arginine, and L-NAME + L-arginine complex effects has an entirely different order of potency on other targets (Table 17.2). Likewise, we can envisage that in all these experiments, the consistent beneficial outcome with BPC 157 co-administration shows that BPC 157 therapy effect acts as an insurmountable antagonist of similar potency for the adverse effects of various L-NAME and L-arginine regimens.

At the end, with respect to the established significance of the NO-system, we can, also with suited addition, (re)-evaluate the real significance of the other system, BPC 157, employed in the noted interactions. Starting from the initial specific NO-system related effect, NO-system blocking (L-NAME) and the NO-(over)-stimulation (L-arginine), all of the studies used the L-NAME versus L-arginine versus L-NAME + L-arginine complex application, all together, as an extended indicator how NO-system really works, may be an essential key. This summary provides a series of more than 80 targets investigated. L-NAME and L-arginine exhibited mostly the opposite, but also the parallel effects. Most of the opposite effects can antagonize each other's response (thereby, specific within the scope of the common NO-system concept [19]), but not all (thereby, these effects remain outside of the scope of the common concept [19], and need further explication). The same relations were noted for the parallel effects, which are entirely outside of the scope of the common concept [19]. Therefore, using the Ahliquist's approach [83], we proposed the presentation of new orchestrated additional pathways and the presentation of the new additional receptor(s) to resolve the matching of NO agents commonly supposed that negatively or positively affected the NO system and BPC 157 central role.

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Chapter 18
Exploring the Gastroprotective, Ulcer Healing and Chemopreventive Properties of Nitric Oxide-Releasing Nonsteroidal Anti-inflammatory Drugs

Jolanta Majka and Tomasz Brzozowski

Abstract Nitric oxide (NO) is a pleiotropic endogenous mediator in the gastrointestinal (GI) tract that is produced by the three NO-synthase (NOS) enzymes; neuronal (nNOS, NOS1), inducible (iNOS, NOS2), mainly involved in the inflammation response and endothelial (eNOS, NOS3), which regulates blood flow and mucosal defense against damage. NO contributes to the maintenance of mucosal integrity, gastrointestinal protection and ulcer healing, and therefore, the NO-based therapies have recently been proposed. The non-steroidal anti-inflammatory drugs (NSAIDs) exert anti-inflammatory, analgesic, anti-pyrogenic and chemopreventive properties, but their use is limited due to serious side effects such as GI-bleedings, mucosal erosions, and even gastric and duodenal ulceration. Thus, the strategy of incorporating a NO-releasing molecule into an NSAID has been shown to abolish the gastric side effects of native NSAIDs, presenting with lower gastric toxicity despite inhibiting both, prostaglandin cyclooxygenase (COX)-1 and COX-2 activity in the stomach. For example, new adducts of NO donor drugs that inhibit COX (CINODS, such as NO-aspirin, NO-ibuprofen and NO-sulindac) have shown better tolerability, less gastrointestinal damage and reduced hepatic toxicity of the parent drugs, also providing better cardiovascular safety compared to selective COX-2 inhibitors (coxibs). Moreover, such adducts prompted research into the anti-carcinogenic potential of NO-NSAID, as these novels NO-NSAID adducts can inhibit cell proliferation either directly or through their effect on COX isoenzymes. Here we review some of the most promising recent advances in NO-NSAID physiology and pharmacology, focusing on the protective and chemopreventive mechanism of these novel NO-NSAID prodrugs. Hopefully, this new class of NO-releasing anti-inflammatory agents could offer a new therapeutic and chemopreventive approach to counteracting the gastrointestinal adverse effects associated with NSAIDs.

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_18

Keywords Non-steroidal anti-inflammatory drugs · Nitric oxide-releasing nonsteroidal anti-inflammatory drugs · Gastroprotection · Ulcer healing · Chemoprevention

Therapeutic potential of NSAIDs despite the gastrointestinal (GI) risk associated with acute and chronic use of NSAIDs such as aspirin (ASA) are known. These agents have been recognized for their antipyretic, analgesic, and anti-inflammatory properties, but the main limitations in their clinical use are serious side effects such as induction of acute hemorrhagic erosions and micro-bleeding, stress ulcers aggravation and prolongation of the healing of existing gastric ulcers [1–4]. Currently, NSAIDs are considered a second risk of peptic ulcer upper gastrointestinal tract after *Helicobacter pylori* infection. The main representative of NSAIDs, ASA is increasingly used for prophylaxis against thrombosis, especially to prevent relapse in patients with a heart attack infarction and angina, but also in healthy adults to prevent myocardial infarction [5]. Following the breakthrough discovery by Vane et al., the beneficial effects of ASA are attributed on its ability to irreversibly inhibit the enzyme cyclooxygenase-1 (COX-1), thanks to which preventing the formation of the pro-aggregate vasoconstrictor thromboxane A2, and inducible prostaglandins (PGs) derived from COX-2 that are considered pro-inflammatory arachidonic acid metabolites [6].

NSAIDs account for 8% of prescriptions worldwide and are the most used in humans over 65.7 years of age. Interestingly, distal to the second part of the duodenum, damage to the mucosa of the small intestine (erosions, ulcerations, and mucosal effusions) are observed in the up 70% of people taking NSAIDs as detected by video capsule endoscopy. Such damage to the mucosa can lead to occult bleeding from the small intestine as well 10–15% of iron deficiency anemia patients are suspected of ingesting NSAIDs. In addition to the "cytoprotective" inhibition of COX-1 derived PG which is essential mechanism by which NSAIDs cause adverse gastrointestinal events, the direct local damage of these drugs has also been postulated [5, 7]. Because stomach producing gastric acid is closely related to pathogenesis of these side effects, a commonly recommended strategy preventing gastrointestinal side effects of NSAIDs is the simultaneous administration of gastric acid secretion inhibitors such as proton pump inhibitors (PPIs) and H_2 receptor antagonists (H_2RA) [8, 9]. In particular, the effectiveness of PPIs in reducing the incidence gastrointestinal side effects of NSAIDs are nowadays widely accepted. However, recently concerns have been raised about an increased risk of fractures, infections, the higher prevalence of dementia and kidney disease with long-term use of PPIs [10].

The GI damage associated with the use of all NSAIDs is related to their ability to inhibit COX-1 activity, thereby inhibiting PG formation, and shifting the arachidonate cascade for the overproduction of vasoconstrictor leukotrienes (LT) such as LTC4 and LTD4 [8]. In addition, NSAIDs have been shown to impair the protective lines of mucosal defense by reducing the secretion of protective mucus and bicarbonate in the stomach and small intestine. However, apart from them inhibitory effect

on the production of arachidonate cascade products, NSAIDs-induced an impairment of the digestive tract resulting in mucosal damage through various mechanisms unrelated to simple ones as the obvious inhibition of COX enzymes. This includes the non-ionic diffusion of low pK_a NSAIDs such as ASA into the surface epithelial cells, which retain this factor in ionized form, thus inhibiting oxidative phosphorylation and resulting in a potent fall in the cell viability. Moreover, the endogenous PG deficiency caused by NSAIDs causes several important pathogenic events, for instance evoking gastric hypermotility associated with magnifying of NSAIDs-induced harmful effects in the stomach.

Furthermore, the ingestion of NSAIDs is associated with increased expression and release of tumor necrosis factor alpha (TNF-α) which promotes cell apoptosis and triggers activation adhesion molecules and leukocyte recruitment leading to microvascular abnormalities and the formation of acute changes in the GI tract mucosa [11–13]. Moreover, it was proposed that inhibition of the extracellular regulated kinase (ERK) pathway and the inhibition of nuclear transcription factor kappa B (NFκB) play a key role in NSAIDs gastropathy [14].

A New Class of Anti-inflammatory Drugs Containing the NO Moiety and Stomach Protection. Evidence from Preclinical Studies

A new class of NSAIDs has emerged in recent years developed by NicOx (Sophia Antipolis, France) by adding an NO moiety to native NSAID [9, 11, 12, 15, 16]. NO has a number of effects in the GI tract that can counteract the loss of protective prostanoids caused by NSAIDs. NO increases: (1) secretion of protective gastric mucus, (2) increases blood flow to the gastric mucosa, (3) supports the repair and removal of toxins, and (4) reduces the interaction of neutrophils with microcirculation in the stomach, and (5) may also aid the healing of gastric ulcers [12–14, 17]. Earlier studies have shown that endogenous NO which is released from the vascular endothelium, sensory afferent nerve or gastric epithelium works with PG in a maintenance mechanism integrity and microcirculation of the gastric mucosa [18–21]. Therefore, a strategy has been introduced of the inclusion of a NO-releasing molecule in an NSAID that can alleviate gastric side effects their parent drugs, including their primary representative native ASA. For example, NO-releasing ASA (NO-ASA) was found to exhibit less gastric toxicity despite inhibiting both the activity of COX-1 and COX-2 in the gastric mucosa [12–14, 17]. Rationale for this strategy is that the NO released from this derivative has a beneficial effect on the gastric mucosa by increasing the defensive capacity of the mucosa and preventing pathogenic events including decreased leukocyte adhesion to endothelium, decreased microcirculation of the mucosa and reduced secretion of protective mucus and bicarbonate secretions caused by this parent drug [13, 20, 21–23] (see Fig. 18.1). This concept has been proven on human volunteers as adding the group donating NO to aspirin

resulted in the creation of a new chemical formulation, which maintained COX-1 and platelet inhibitory activity, almost avoiding damage to the GI tract [22]. A new series of compounds have been introduced which consist of NSAIDs (e.g., flurbiprofen, diclofenac, ketoprofen) combined with a NO that can be released from each of these agents. With the introduction of these new pro-drugs, GI side effects observed with the ingestion of the conventional NSAIDs have been significantly reduced. This was due to the beneficial topical protective effects of NO on the gastric mucosa. Although these NO-releasing NSAIDs keep their anti-inflammatory properties comparable to those of the parent NSAID, they exert significantly reduced gastropathy. The significantly reduced GI side effects caused by conventional NSAIDs, are limited because of the local protective action of NO on the gastric mucosa [24–26] (Fig. 18.1).

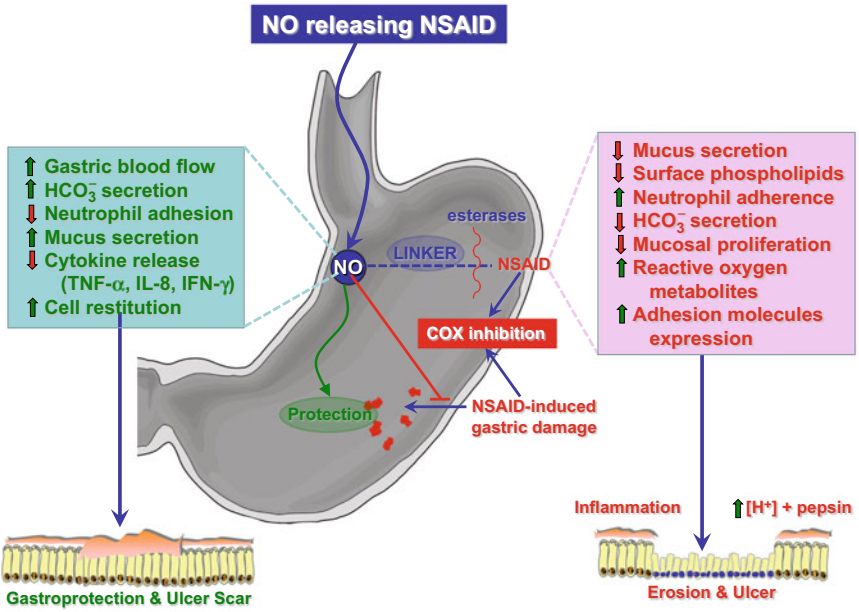

Fig. 18.1 The simplified scheme of beneficial action of nitric oxide (NO)-releasing (NSAID) in the stomach. Conventional NSAID can induce gastric mucosal damage by inhibition of cyclooxygenase (COX) enzymes and a profound inhibition of endogenous prostaglandins (PG). These arachidonate metabolites have been shown to influence many physiological processes including gastric mucosal integrity, mucosal defense, and gastric microcirculation. The PG deficiency caused by NSAID leads to the impairment of major lines and factors of mucosal defense resulting in formation macro- and microscopic gastric damage. NO released from NSAID due to activity of esterase's affords protection via improvement of gastric blood flow, mucus and bicarbonate secretion, enhancement of mucosal repair and attenuation of local mucosal and systemic inflammation

NO-Releasing NSAIDs Versus Conventional NSAIDs in the Mechanism of Gastric Ulcer Healing

The development of a NO-releasing NSAID constructed by adding a nitroxy-butyl moiety to ASA or naproxen, alleviated the side effects of native NSAIDs while preserving anticoagulant activity comparable to their parent NSAID [5, 6, 12–14, 17]. These drugs were shown to not only prevent mucosal damage but may also contribute to the mechanism of acceleration of ulcer healing process. The endogenous NO released by capsaicin and NO derived from the L-arginine, the substrate for NO synthase (NOS) [18, 23] and finally the NO donor, glyceryl trinitrate [24], exhibited stomach protection and accelerated ulcer healing. This was of a foremost importance since longer administration of NSAIDs was reported to (1) enhance the ulcerative response to various stimuli, and (2) impair healing pre-existing ulcerations [3, 7, 12, 18]. This is the detrimental effect of administered conventional NSAIDs *per os* or even parenterally [7] ascribed to direct damage to the surface epithelium, excessive activation of white blood elements leading to disorders in microcirculation of the GI tract, the enhancement of the gastric motility and reduction in mucosal generation of gastroprotective PGE_2 [17, 22–24]. In studies of chronic ulcers [13, 27], treatment with ASA or naproxen daily administered *per os* for 15 days caused the expected delay in ulcer healing and decreased both GBF at the ulcer margin while inhibiting of PGE_2 synthesis in the gastric mucosa. In contrary, the treatment with NO-ASA and NO-naproxen did not delay the healing rate of chronic gastric ulcers and failed to impair the GBF at the ulcer margin compared to the vehicle-administered control values. Treatment with NS-398, a highly selective COX-2 inhibitor that has prolonged itself ulcer healing and a reduction in gastric blood flow (GBF) at the edge of the ulcer reversed the beneficial effects NO NSAIDs and enhanced the harmful effects of native ASA and naproxen on ulcer healing and GBF at the margin of ulceration. The specific COX-2 inhibitors have been widely proposed as an attractive therapeutic development in treatment of rheumatoid arthritis and osteoarthritis, in part, because it has been shown to be spare the COX-1 isoform responsible for the protection of the GI tract. Moreover, Coxibs do not induce peptic ulcers or bleedings but, paradoxically, appear to have a far less beneficial effect on healing pre-existing ulceration than simple NO-releasing NSAID, blocking both COX-1 and COX-2, through the mechanism involving a local NO release to counteract PG deficiency [18, 28]. In case of chronic stomach ulcers, the beneficial effects of the NO released from NSAIDs such as ASA, is attributed to an increase in GBF in the mucosa, especially on the edge of the ulcer, and enhancement of angiogenesis [27]. In addition, COX-1 mRNA was detected in intact gastric mucosa on the edge of the ulcer and in the mucosa treated with NO-ASA, NO-naproxen, and their parent NSAID. In contrast, COX-2 mRNA was not detected in the mucosa of the vehicle (control)-treated animals but was elevated already at the edge of the ulcer in rats treated for 15 days with vehicle and both, native ASA, and naproxen or their NO derivatives. This suggests that increased mucosal COX-2 expression at the edge of the ulcer contributes to the healing of gastric ulcerations and that NO released from gastric sparing NO-NSAIDs

can compensate for PG deficiency caused by NSAIDs with the NO component. The importance of COX-2 in ulcer healing has recently been highlighted by the observation that COX-2 mRNA expression is increased after induction of chronic gastric ulcer suggesting that COX-2 and PG expression derived from COX-2 may be key in mechanism of ulcer healing [27, 29–32]. It is therefore rational to assume that PGs that originate from COX-2 isoform upregulated at the edge of the ulcer may be of immense importance in mechanism of gastric ulcer healing [32, 33]. This view is supported by the fact that treatment with NS-398 delayed ulcer healing caused by classic NSAIDs while this was not a case when NO-releasing NSAIDs and NO donors such as SNAP or GTN have daily been administered in rats with chronic gastric ulcerations. This conclusion is consistent with the observation of Salvemini et al. [34] that NO-releasing NSAID may activate the COX-2 pathway, suggesting that the COX-2 products can mediate this beneficial effect of NO-NSAIDs on ulcer healing.

The Importance of NO-NSAIDs in Protecting the Stomach Against Experimental Ethanol Damage, Stress, and Ischemia–Reperfusion

As mentioned above, the NO-releasing NSAIDs themselves show only minimal or no ulcerative properties in the gastrointestinal tract, despite they exert a strong anti-inflammatory and analgesic effect like that of native NSAIDs [9, 15]. Brzozowski et al. [18] showed that ASA and naproxen are themselves ulcerative stomach, and their destructive effect was strongly intensified by adding an exogenous acid that was used in their research to mimic the natural fate of both NSAIDs under strongly acidic conditions in the stomach. Unlike conventional NSAIDs, NO-NSAIDs did not alter the stomach after acidification and had no adverse effect on GBF in the stomach. Moreover, both ASA and naproxen inhibited PGE_2 production, confirming previous observations that suppression of COX and the subsequent deficiency of endogenous PG in the gastric mucosa may at least partially explain their harmful effects in the stomach. Unlike parent drugs ASA or naproxen, their NO-releasing derivatives ASA- and NO-releasing naproxen which were themselves devoid of ulcerative properties, failed to induce the damage in the stomach after acidification, despite their continued ability to inhibit PGE_2 production like that observed with their parent drugs. In addition, the pretreatment with NO-ASA and NO-naproxen mitigated the injury caused by ethanol and increased GBF. These effects were counteracted by ODQ, an inhibitor of NO-dependent guanylate cyclase, but not by the inhibition of NO synthase resulting from L-NNA administration [26, 35]. However, a significant amount NO metabolites were detected in the gastric contents of rats administered with these NO-releasing NSAIDs [26]. Moreover, SNAP which is a potent NO donor that by itself afforded gastroprotection against ethanol-induced gastric damage when added to native ASA or naproxen, has provided protection and improvement of GBF

comparable to NO-releasing NSAIDs. This indicates that, indeed, the NO released from these compounds plays a key role in this protection and the accompanying increase in GBF.

Interestingly, NO-ASA was relieved acute gastric changes caused not only by concentrated ethanol—classic, widely used substances that causes damage the stomach, but also those that appear in the gastric mucosa, that has been exposed to other experimental ulcerative agents such, for instance, water immersion and restraint stress or ischemia–reperfusion (I/R) [17, 27, 28]. This protective effect of NO-ASA against stress and I/R injury was accompanied, as in the case of ethanol, by an increase in GBF and a significant increase in the content of nitrite/nitrate in the stomach lumen. Concomitant treatment of SNAP or GTN, both known as NO donors, added to the native ASA to mimic the gastric fate of NO released from NO-NSAID in combination with I/R and exposure to stress also caused protection, followed by the rise in GBF and an increase in luminal release of NO similar to that observed with NO-NSAID-induced protection against ethanol lesions [18]. Specific suppression in the stress or I/R model of gastric lesions of the NO-sensitive guanylyl cyclase pathway by pretreatment with ODQ completely reversed NO-releasing ASA-induced protection and the rise in GBF.

NO-releasing NSAIDs can counteract two events that occur after the inhibition of PG synthesis by NSAIDs, namely decreased blood flow in the stomach and increased adherence of neutrophils to the vessel's gastric microcirculation endothelium [7, 27–29].

Other mechanisms have also been suggested as critical in the pathogenesis of experimental NSAIDs gastropathy [29, 30, 33]. For example, Fiorucci et al. [36] showed that administration of ASA caused an increase in the rate of apoptosis through upregulation caspase system mediated by TNF-α and the administration of NO-derivatives of NSAIDs counteracted these apoptotic effects. Moreover, a single administration of NSAIDs led to acute activation of cysteine proteases in the stomach, while prolonged NSAID exposure resulted in sustained upregulation gastric cysteine endoproteases involved in apoptosis [36]. Although apoptosis means a significant event in the regulation of gastric mucosa turnover, mediators involved the mechanisms of initiation and performance of gastric apoptosis are unknown. One of the potent extracellular modulators of pro-apoptotic caspases that play a role in regulation of gastric cell apoptosis in rats treated with NSAIDs may be TNF-α. It was shown that the damage to the gastric mucosa caused by oral administration of NSAIDs is related to TNF-α-dependent activation of ICE-like cysteine proteases and that NO-ASA can protect the gastric mucosa by inhibiting these key endopeptidases in the caspase cascade [36, 37]. Thus, a NO-NSAID such as NO-ASA can spare gastric mucosa and inhibit caspase activity, at least in part, through a cGMP-dependent pathway an effect mediated by NO release from NO-NSAID. These authors [36] proposed that the activation of the ICE/caspase-1 pathway by native ASA it is the rate-limiting the process of maturation and the pro-inflammatory secretion cytokines such as IL-1β. Thus, IL-1β and ICE-1 such as cysteine endopeptidase modulation by NO-ASA may explain the beneficial effects of NO derivatives NSAIDs in the stomach. Consistent with this hypothesis, other studies have documented that the

native ASA intensified stress-induced gastric damage and decreased GBF linked with an increase in plasma formation IL-1β and TNF-α levels and these effects were eliminated in rats pretreated with NO-releasing ASA [36, 37]. This suggests that expression of IL-1β and TNF-α is suppressed and this effect plays a significant role in the mechanism of protective action of this NO-ASA that releases NO on the gastric mucosa. Previous research has shown that development of bleeding erosions in the stomach caused by cold restraint stress in rodents were accompanied by an increase in lipid peroxidation and neutrophil-dependent myeloperoxidase (MPO) activity, as well as a decrease in non-protein sulfhydryl levels in the gastric mucosa [38, 39]. Harmful effects of ASA and others NSAIDs in the stomach may involve the activation of reactive oxygen metabolites (ROM). It is well known that ROMs are engaged in the pathogenesis of experimental gastric ulcer formation in animal models evoked by I/R, stress and NSAID administration including ASA and indomethacin. ROM [40–44] has been reported to play a vital role in the pathophysiology of I/R-induced and NSAID-induced acute changes in the stomach from treatment with strong oxygen scavengers such as superoxide dismutase (SOD), catalase, or dimethyl sulfoxide (DMSO) capable of providing mucosal protection and significantly reduced the severity of these deleterious changes [39–42]. According to these reports, the exposure to ASA can induce focal ischemia that leads to increased formation of ROM [43, 44]. Stress ulcers are defined as acute micro bleedings in the stomach occurring as complications in seriously ill patients after burns, sepsis, major surgery, or CNS trauma [45]. Among the various stress models used in animals the most reproducible results can be obtained under cold stress and restraint technique which appear to act synergistically in the formation of gastric ulceration [46]. Recent study showed that the damaging effects of ASA are attributed to the amplification in ROS as determined by the chemiluminescence test [43, 44]. In addition, ASA increased the content of MDA in the mucosa and decreased gene expression and activity SOD and GPx in the gastric mucosa, suggesting suppression of key mucosa antioxidant enzymes along with increased levels of pro-inflammatory cytokines such as IL-1β and TNF-α play an important role in the damaging activity of this NSAID and furthermore, these cytokines may increase the stress-induced damage to the stomach.

Interestingly, this increase in the production of ROM by the mucosa favoring the activation of neutrophils, an increase in lipid peroxidation and a decrease in the expression and activity of SOD and GPx were in part weakened by NO-releasing ASA, suggesting that this new generation of a "safer" NSAID adduct, namely a NO-NSAID, can counteract the adverse effect classic NSAID on the gastric mucosa such as aggravation of acute and chronic gastric injury evoked by variety of ulcerogens [36]. This is confirmed by the fact that GBF was elevated, and luminal NO production was increased in NO-releasing ASA-treated animals compared to those treated with native ASA. Thus, it is now clear that native NSAIDs exacerbate ulcerative stress processes, but NO-releasing NSAIDs protect against stress-induced gastric damage not only due to excessive NO release, which can compensate for PG deficiency caused by native NSAIDs, but also due to inhibition of excessive ROM synthesized in the gastric mucosa in response to classic NSAIDs [36, 37]. Moreover, Brzozowski's

group [27] found it for the first time that NO-ASA accelerates mucosal repair and regeneration after cold stress and restraint-induced damage, and that this beneficial effect was accompanied by an increase in GBF. In contrast, native ASA prolonged the regeneration of the stress-induced gastric mucosal damage with a simultaneous reduction in GBF in the stressed-gastric mucosa, indicating that the NO-releasing ASA derivative is superior to native ASA in mucosal repair of stress-induced changes in the stomach [27]. Interestingly, an increase in the number of activated neutrophils and lipid peroxidation were inhibited by NO-releasing ASA, revealing mechanism by which a new generation of these NSAID adducts could be counteracted the harmful effect of classic NSAIDs on the gastric mucosa. It should be mentioned, however, that NO-ASA protection was observed in their studies only after topical but not parenteral administration [27] and further studies are needed to check if this compound will be effective also after systemic administration.

The compared the mechanisms underlying the negligible gastrotoxicity of NO-releasing NSAIDs versus conventional NSAIDs tend to be complicated and still not fully understood.

Recently, an association has been demonstrated between NO and the heat shock protein (HSP) family [47]. HSPs as chaperones are essential for important cellular events such as protein folding, and translocation [48]. In general, HSP expression, particularly HSP 70, is induced in cell response to exposure to stressful events such as exposure to heat, heavy metals, chemicals, and other pathophysiological stressors. HSPs are also involved in the defense mechanisms of the GI mucosa [49, 50]. Byrne et al. [50] reported that NO induces HSP 70 in a concentration dependent manner in gastric mucosal cell culture. However, whether this effect might contribute to the gastroprotective properties of NO-releasing NSAIDs has been little investigated, and there is no information available as to whether NO-releasing NSAIDs can affect the expression of HSP 70 in gastric mucosa under stress and regeneration of this mucosa after stressful stimuli. Recently, Konturek et al. [49] concluded that NO-ASA increased the expression of HSP 70 mRNA in the gastric mucosa of rats subjected to water immersion and restraining stress. The fact that NO-ASA, unlike ASA, increased the expression of HSP 70 in the gastric mucosa indicates that the NO released from NO-ASA has a stimulating effect on the expression of HSP 70. Byrne et al. [50] also reported that the NO donor, S-nitroso-N-acetyl-penicillamine induced HSP 70 in gastric mucosa cells, and this effect was abolished by the NO scavenger. Consistent with these results, Xu et al. [51] confirmed that NO released from various NO donors, including sodium nitroprusside or SNAP, can activate HSP 70 in cultured vascular smooth muscle cells. Induction of HSP 70 by NO donors was associated with the activation of heat shock transcription factor 1 (HSF1), indicating that the response was regulated at the transcription level [51]. Since HSP 70 has been shown to play a vital role in gastric mucosa defense and ulcer healing, HSP 70 induction by NO-ASA may be an important mechanism underlying the gastroprotective properties of this drug [48–50]. Increased HSP 70 expression is an early step in stomach response to acute, stress-induced damage to the gastric mucosa which is consistent with previous observations that stress overexpressed HSP 70 [52]. On the other hand, native ASA, which potentiated stress-induced damaging changes in

gastric mucosa, significantly suppressed this HSP-70 response. This indicates that suppression of HSP 70 expression by ASA may be one of the mechanisms that may contribute to the development of NSAID gastropathy. HSP 70 activation may play a key role in preventing NO-ASA-induced stress damage to the stomach and this effect appears to be consistent with previous observations that induction of HSP 70 by mild total body warming or pretreatment with geranylgeranyl acetone (GGA) enhanced mucosal defenses and reduced the number of stress-induced gastric lesions [53, 54]. In summary, NO-releasing ASA, as opposed to native ASA, is beneficial in suppressing stress-induced lesions compared to classical ASA and contributes to the healing of these lesions by reducing stress-induced oxidative damage in gastric mucosa cells. The antioxidant effect of NO-ASA may also result from the inhibition of leukocyte adhesion to the epithelium and the increased production of HSP 70, which acts as an endogenous gastroprotective against protein denaturation induced by lipid peroxidation. This evidence confirmed that one of the important pathways mediating the protective effects of NO-ASA is the upregulation of HSP 70 in the gastric mucosa and a strong inhibition of lipid peroxidation-induced ROM production and release.

Anticarcinogenic and Chemopreventive Potential of NO-Releasing NSAIDs

The prototypes of this class of anti-inflammatory agents exhibiting non-selective profile versus COX isoenzymes, for instance, COX-inhibiting NO-donating drugs (CINODS) and named “napro-CINODS” have been shown to reduce systemic blood pressure. These agents revealed better tolerability and enhanced cardiovascular safety than selective COX-2 inhibitors (Coxibs), while causing less gastrointestinal damage than its parent drug, naproxen [55]. These and other properties of this adduct prompted the investigations on the anticarcinogenic potential of NO-NSAIDs. Among prodrugs evaluated for this purpose were i.e., NO-ASA, NO-ibuprofen and NO-sulindac which opens new avenues for the therapeutic approach against different cancers. These compounds inhibited the growth of cultured human colon cancer cells much more effectively than their parent compounds in vitro [56]. For example, NO-releasing ASA (NCX 4016) attenuated aberrant crypt foci, a precancerous lesion, in a rat model of colorectal cancer and this effect was superior to that observed with parent ASA [56]. Similar greater efficacy of NCX 4016 was observed in a panel of colorectal cancer cell lines in vitro as documented by this prodrug evident cell perturbations and growth inhibition [57]. This higher antiproliferative activity of NCX 4016 compounds compared to the parent ASA was confirmed by Williams et al. [58], using another panel of human colon cancer lines.

The mechanism of colon cancer chemoprevention by NO-NSAIDs should be further elucidated but it is postulated that these agents inhibit proliferation directly and through their effect on COX isoenzymes. Another mechanism this anticancer

action of NO-NSAIDs may involve the stimulation of cell death by induction of apoptosis and through other forms of cell death. Moreover, NO-NSAID was shown inhibit the expression NF-κB, one of the members of transcription factor family, and this effect led to an increase in cell loss. It has been postulated that NO released from NO-NSAID can prevent the degradation of the cytoplasmic NF-κB inhibitor, IκBα [57, 58]. Due to this action of NO released from NO-NSAID, NF-κB is unable to translocate to the nucleus to activate the process of several gene transcription responsible for inflammation and carcinogenesis. Moreover, these prodrugs have been shown to suppress the expression of inducible NOS (NOS2), which is thought to contribute to enhancement of inflammation and colon carcinogenesis [56–58]. It is also not excluded that this action of NO-NSAIDs on NOS2 might augment the inhibitory effect of ASA on NF-κB. Besides local inhibition of cell proliferation, more systemic action of these compounds can account for their chemopreventive effects.

It is of interest that low dose of ASA recommended prophylactically to prevent cardiovascular diseases failed to protect patients from colon cancer [59]. Thus, NO-NSAIDs, recognized for their effectiveness against colon cancer, may provide double protection against coronary artery disease and colon cancer [56]. Besides inhibition of NOS2, and NF-κB, other pathways such as the suppression of ß-catenin/an activator of T-cell factor (TCF) signaling, and upregulation of COX-2 expression have been implicated in the chemopreventive action of NO-NSAIDs.

Further studies are needed to explain the role of the induction of these pathways in the chemopreventive activity of NO-ASA against colon cancer. Interestingly, the administration of ATB-346, the new prodrug naproxen-releasing hydrogen sulfide (H_2S), has been shown to inhibit colon prostaglandin synthesis and whole blood thromboxane synthesis as effectively as naproxen, but did not cause any damage to the GI tract [60]. Moreover, it was proven that ATB-346 exerted a better chemopreventive effect against colon cancer than naproxen while sparing the damage to GI tract usually associated with the use of the parent NSAID [60]. Thus, ATB-346 may therefore be an attractive agent for the chemoprevention of colon cancer and possibly cancers of other tissues as well. Recently, Kashif's group [61] studied the role of NOSH-aspirin (NBS-1120), a novel hybrid that releases NO and H_2S, the gaseous molecules known to exert profound GI tract protective action against a variety of damaging agents [62]. This novel hybrid was designed as a safer alternative to conventional NSAIDs and even NO-NSAIDs to compare the gastrointestinal safety, anti-inflammatory, analgesic, antipyretic, antiplatelet and chemopreventive properties of ASA and NBS-1120 administered orally to rats at equimolar doses [61]. They revealed that ASA increased plasma levels of TNF-α to a much greater extent than NBS-1120, and that NBS-1120 was more effective than ASA as a chemopreventive agent in inhibiting tumor growth and tumor mass depending on the dose [61]. It was concluded that for gastrointestinal safety reasons, NOSH-ASA may represent an alternative pharmacological approach that may also prove an enhanced cardiovascular and renal safety profile [61]. Thus, this NOSH-aspirin hybrid is a good and promising candidate as a chemopreventive agent deserving of great attention for the further identification of its molecular targets in vitro and in vivo.

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Chapter 19
The Role of Nitric Oxide in the Etiopathogenesis of Preeclampsia

Huma Quasimi, Arunabha Ray, and Md. Iqbal Alam

Abstract Preeclampsia (PE) is a severe pregnancy complication that presents mostly after twenty weeks of gestation. It can be diagnosed by increased systolic blood pressure >140 mmHg (hypertension), edema, and proteinuria along with other complications. In recent years the new onset of preeclampsia is defined as thrombocytopenia, renal insufficiency, neurological complications, liver involvement, fetal growth restriction, etc. Preeclampsia/ eclampsia (PE/E) poses a significant public health problem in India and the developing world accounting for 11.71% of total pregnancies in India as per the Federation of Obstetrics and Gynaecological Society of India (FOGSI, 2010 survey). PE complicates around 2–10% of all pregnancies resulting in increased perinatal morbidity and mortality. Several mechanisms have been included in the pathophysiology of PE, including oxidative stress, inflammation, maternal endothelial dysfunction, liver dysfunction along with other systemic disturbances but the exact mechanism could not be deciphered owing to the heterogeneous nature of the disease. Nitric Oxide (NO) is the key regulator of PE. In PE, defective remodeling of spiral arteries occurs. The poorly perfused placenta is the site of origin of oxygen free radicals and lipid peroxides which also activate endothelial cells leading to the down-regulation of NO, the release of nitroso precursors of NO as well an increase in oxidative stress. In this chapter, we are focusing on the role of NO in the pathophysiology of PE and its interplay with oxidative stress.

Keywords Pre-eclampsia · Oxidative stress · Nitric oxide · Hypertension · Inflammation · Glutathione

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_19

Introduction

Preeclampsia (PE) is a serious pregnancy condition that often manifests after 20 weeks of pregnancy. It is diagnosed by elevated systolic blood pressure of more than 140 mm Hg (hypertension), edema, and proteinuria [1]. The recent criteria of PE suggest that new-onset proteinuria may be replaced by new-onset thrombocytopenia, renal insufficiency, neurological problems, liver involvement, or fetal growth limitation [2]. According to the Federation of Obstetrics and Gynecological Society of India, preeclampsia/eclampsia (PE/E) affects 11.71 percent of all pregnancies in India, making it a serious public health issue in the developing world (FOGSI, 2010 survey) [3]. PE causes complications in 2–10% of pregnancies, which increases perinatal morbidity and death [4]. It is classified into early onset (34 weeks gestation) and late-onset (>34 weeks gestation) according to gestational age, with early onset being less common and having a greater risk of maternal morbidity, perinatal death, and severe morbidity of the newborn than the later onset condition [5]. Oxidative stress, inflammation, maternal endothelial dysfunction, hepatic dysfunction, and other systemic disturbances have all been included in the pathophysiology of PE, although the precise mechanism has not been yet determined due to the heterogeneous nature of the disease [6]. PE development occurs in two stages: the stage one is asymptomatic which occurs at the time of placental invasion and differentiation (placental phase) whereas; the stage two is symptomatic which occurs at the maternal interface (maternal phase). At the time of normal placentation, the embryonic cytotrophoblast properly invades the uterine wall, the myometrium, and spiral arterioles which transforms the maternal spiral arteries into a large capacitance and low resistance vessels. PE develops in two stages: the first stage is asymptomatic and happens during the placental phase (when the placenta invades and differentiates), and the second stage is symptomatic and happens at the maternal interface (maternal phase). The uterine wall, the myometrium, and spiral arterioles are appropriately invaded during normal placentation by the embryonic cytotrophoblast, which changes the maternal spiral arteries into high-capacitance, low-resistance vessels. However, this process is dysregulated in preeclampsia [7–10]. The incomplete invasion of the cytotrophoblast confines it to the surface decidua layers, depriving the placenta and developing fetus of adequate oxygen and nutrition. The result of this deficit is a drop in the uteroplacental perfusion pressure and local ischemia [11]. The second stage of the illness, maternal dysfunction, is carried by abnormal placental invasion [12]. Numerous bioactive substances are released abnormally and into the maternal circulation as a result of chronic placental hypoperfusion. These circulating chemicals affect endothelial cells, causing endothelial dysfunction, generalized multi-system vasospasm, decreased plasma volume, oxidative stress, and a hyper-inflammatory state. PE is an antiangiogenic state because it exhibits increased expression of anti-angiogenic proteins like soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble Endoglin (sEng) and decreased expression of pro-angiogenic molecules like vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and transforming growth factor (TGF-β) [13–15].

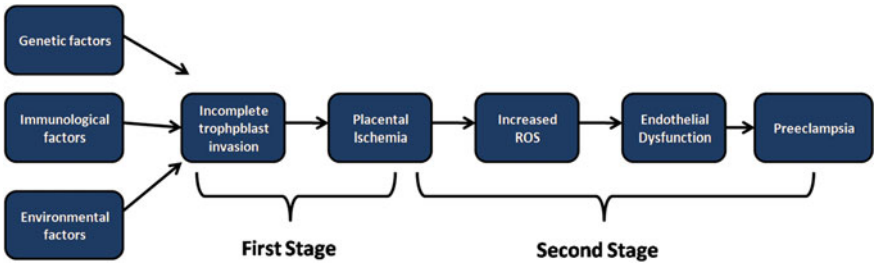

Fig. 19.1 Two stages of preeclampsia. Placental ischemia caused by incomplete trophoblast invasion causes the formation of humoral agents such as ROS, which are then released into the bloodstream. These variables impair the function of the vascular endothelium, which eventually results in preeclampsia

An early stage of preeclampsia development is characterized by defective trophoblast invasion [16]. However, it is still unknown why trophoblast invasion is blocked, leaving open the question of whether it is the cause of or a symptom of another underlying issue. Additionally, genetics, environmental variables, and a changed immune response during the maternal–fetal interphase are thought to be involved, albeit each patient may respond differently to each of these [17]. It is hypothesized that the start, severity, clinical symptoms, and course of the condition are all influenced by the mother's sensitivity to placental abnormalities. In contrast to PE linked to maternal metabolic syndrome, the most current ideas link placental phenotype to shallow trophoblastic invasion and limited fetal development [18]. The alternative phenotype, which is mostly brought on by oxidative stress, placental villi congestion, and decidual lesions, is linked to normal fetal development and low-grade maternal inflammation [19] (Fig. 19.1).

PE can develop as a result of immunological mismatches between the mother and the fetus, which results in inadequate placentation (first stage), which in turn triggers a cascade of immunological agents into the mother's blood, including cytokines, chemokines, anti-angiogenic factors, ROS, etc. [20]. These factors are released, which causes generalized endothelial dysfunction (second stage), followed by clinical issues such as hypertension, proteinuria, eclampsia, HELLP syndrome (hemolysis, increased liver enzymes, and a low platelet count), fetal growth limitation, etc. These factors are released, which causes generalized endothelial dysfunction (second stage), which causes several clinical issues such as hypertension, proteinuria, eclampsia, HELLP syndrome (hemolysis, increased liver enzymes, and a low platelet count), fetal growth limitation, etc. (Fig. 19.2) [21, 22].

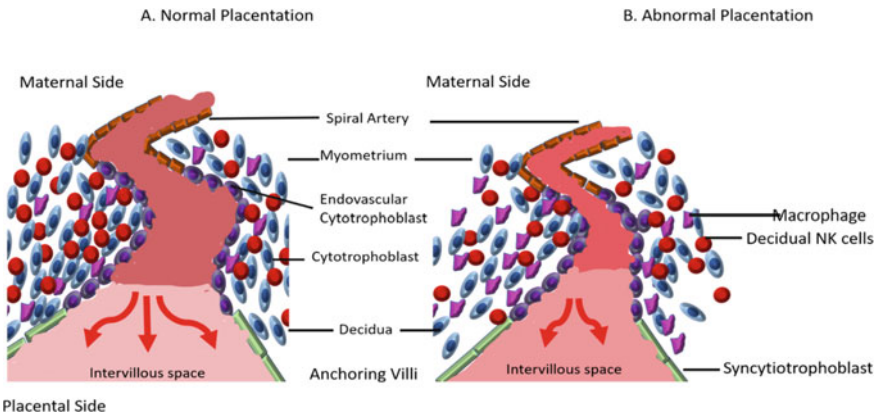

Fig. 19.2 Placentation defect in PE. At 15–16 weeks of pregnancy, normal placentation (**a**) and defective placentation (**b**) are shown. The placenta and the maternal decidua are connected by the anchoring villi. When the placenta develops in a healthy pregnancy, cytotrophoblasts (blue) cross the placental-maternal bridges to infiltrate the maternal decidua and nearby spiral arteries. The remodeling of the arterial wall results from the penetration of the arterial wall and the replacement of maternal endothelium (yellow). They come into contact with NK cells (red) and macrophages (purple) in the deciduas, which promote cytotrophoblasts' complete invasion of the myometrial segments (**a**). Significant spiral artery remodeling is encouraged by this mechanism. This invasion is interrupted (**b**) with poor arterial remodeling in the early stages of preeclampsia

Role of Nitric Oxide in Preeclampsia

Nitric oxide (NO) is a modulator of vascular endothelial hemostatic activities. According to several experts, the primary malfunction in preeclampsia is caused by a relative lack of NO and an excess of peroxynitrate. The combination of a NO and peroxynitrate deficiency can cause a cascade of physiological and serological mechanisms in preeclampsia, including hypertension, increased glomerular filtration rate, proteinuria, platelet dysfunction, increased thromboxane and endothelin levels, and a decrease in prostacyclin levels [23]. Nitric oxide (NO) is an important regulator of placental blood flow. Through its specific angiogenic and vasculogenic capabilities, it actively participates in cytotrophoblast endovascular invasion and placental development [22]. In preeclampsia, abnormal NO generation in the fetoplacental unit may lead to vasoconstriction of the placental bed, improper placental perfusion, and associated maternal effects, such as hypertension and systemic vascular resistance (SVR), etc. [6, 12, 22, 24].

NO works as a transmitter for the humoral, metabolic, and mechanical factors-controlled endothelium-dependent regulation of vascular tone. In addition, NO blocks the activation of platelet aggregation, diminishes the toxicity of superoxide ions, and functions as an anticoagulant and anti-atherogenic agent [24]. It also promotes embryo survival, tissue remodeling, immunosuppression, and vasoregulation, all of which are significant regulators of placental nutrition delivery [25]. Since the human feto-placental vasculature lacks autonomic innervation, it has effects that

are either autocrine or paracrine and affect several physiological aspects of pregnancy. Particularly, NO is the primary vasodilator that controls platelet adherence and aggregation in the intervillous space, trophoblast invasion and death, placental bed vascular resistance, and fetoplacental vascular reactivity. The role of NO in angiogenesis, where it helps create functional capillaries from pre-existing vasculature, and in vasculogenesis, where it plays a role in the de novo development of arteries from pluripotent precursor cells, is well documented. VEGF, or vascular endothelial growth factor, is a crucial component in these processes. Its expression, which is dependent on the beginning of vasculogenesis, is mediated by NO release.

A vascular endothelial condition called preeclampsia is characterized by an inadequately perfused placenta and widespread endothelial dysfunction [26]. Endothelial dysfunction gets worse as gestation progresses because the mother cannot adjust to the physiological stress of pregnancy. Oxygen free radicals and lipid peroxides are produced in the poorly perfused feto-placental unit, where they also activate endothelial cells and inhibit the production of nitroso precursors of NO, and increase oxidative stress. Endothelial dysfunction gets worse as gestation progresses because the mother cannot adjust to the physiological stress of pregnancy. Oxygen free radicals and lipid peroxides are produced in the poorly perfused feto-placental unit, where they also activate endothelial cells and inhibit the production of nitroso precursors of NO, and increase oxidative stress [22, 24].

Placental preeclampsia is classified as either mild or severe, whereas maternal preeclampsia is frequently classified as late or mild. These interactions between maternal and placental pathophysiological variables result in a variety of clinical manifestations, including maternal inflammation, vascular dysfunction, and pro-coagulation pathway activation.

Nitric Oxide Basics

Joseph Priestly discovered nitric oxide in 1772 and named it nitrous air. The identification of cell signaling and other significant physiological, neurological, and immunological processes resulted from the discovery of the NO pathway. Nearly all biological and therapeutic systems interact with NO, a straightforward biological molecule. The primary regulator of maternal and fetal hemostasis throughout pregnancy is NO, which also controls changes in the mother's cardiovascular system, the growth and development of the fetus, and the fetus' adaptation to extrauterine life [24, 27].

The bioavailability of nitric oxide is low, and it leaks from its source cells into nearby target cells. Nitric oxide binds to the heme group of cytosolic guanylate cyclase, which activates the enzyme and speeds up the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate by 50–200 fold (cGMP). Increased cGMP levels cause vascular smooth muscle to relax since they promote the binding of free calcium intracellularly and prevent platelet aggregation and adherence to vascular endothelial surfaces [22, 24, 28].

A family of enzymes called nitric oxide synthases produce it from L-arginine (NOS). NOS are enzymes that catalyse the conversion of L-arginine into L-citrulline through a system that depends on calcium, calmodulin, and other elements (Fig. 19.3). There are three NOS isoforms: NOS 1, NOS 2, and NOS 3. Each has unique properties and expression patterns. Because it was initially isolated and cloned from neuronal tissue, NOS1 is also known as nNOS, while NOS3 is known as eNOS because it was first discovered in endothelial cells. NOS 1 and 3 are known as constitutive isoforms (cNOS), which primarily function in cell signaling and produce low levels of NO. They are activated by an increase in tissue calcium concentration. However, macrophages produce an inducible (iNOS) and calcium-independent version of NOS2 that is dormant until stimulated by lipopolysaccharide [29]. Greater NO production from NOS2 can be cytotoxic. However, the endothelial isoform of NOS (ecNOS) is detectable in the healthy placenta, restricted to the endothelium of the umbilical cord, chorionic plate, and stem villous arteries. NOS1 and 2 are not generated by the human placenta [30]. Due to their angiogenic and vasculogenic capabilities, villous cytotrophoblasts play a significant role in cytotrophoblast endovascular invasion, which is a crucial characteristic of effective placentation [31–33]. S-nitrosylation is a mechanism by which NO can control protein activities. When compared to normotensive women, preeclampsia patients exhibit significant changes to the placental S-nitroso-proteome [34]. Asymmetrical dimethyl arginine (ADMA), an amino acid that is normally present in tissues and cells and circulates in plasma, inhibits the action of NOS [35]. Protein arginine methyltransferases (PRMT) catalyze the synthesis of ADMA by methylating the guanidine nitrogens of arginine with one or two methyl groups. Type 1 PRMT is responsible for the synthesis of (asymmetrical dimethylarginine) ADMA by adding two methyl groups to one of the guanidine nitrogens of arginine, whereas Type 2 PRMT is responsible for the synthesis of symmetrical dimethylarginine (SDMA) by methylating both of the guanidine nitrogens to form a symmetrical molecule. ADMA and L-NMMA, but not SDMA, can reduce NOS activity. ADMA and L-NMMA are detected in human plasma and urine, however, ADMA is expressed 10 times more than L-NMMA [36].

Oxidative/Nitrosative Stress

ROS and RNS are extremely reactive substances that are created by biological redox reactions during normal cell metabolism. ROS and RNS mediate the oxidation and reduction of virtually all biomolecules [37–40]. Oxidative and nitrosative stress are generated, respectively, by an increase in ROS and RNS production or a lack of antioxidant mechanisms. When cellular ROS/RNS production exceeds antioxidant capability, these highly reactive chemicals become lethal [41–43]. ROS, RNS, and lipid peroxides are responsible for a wide range of disorders, including preeclampsia, diabetes, cataracts, cancer, Bechet's disease, and rheumatoid arthritis [44, 45].

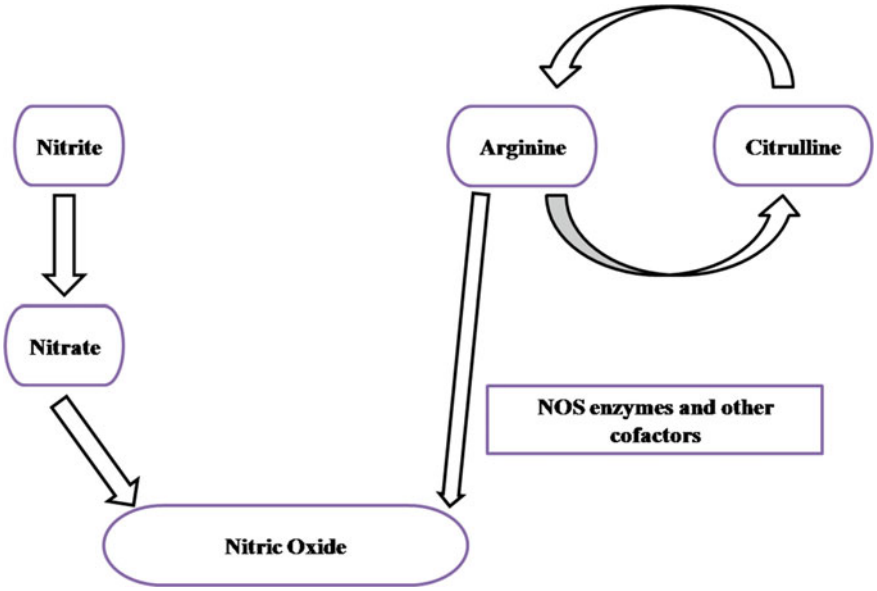

Fig. 19.3 Mechanism of NO synthesis. NO is synthesized from L-arginine by the action NOS which converts it into citrulline and NO

Oxidative Stress in Preeclampsia

Oxidative stress is caused by a poorly perfused placenta, which causes leukocytes and platelets to adhere to endothelial cells, triggering the production of cytokines and anti-angiogenic proteins [46]. This adhesion is essential for triggering inflammation, which is then followed by widespread vasoconstriction and increased resistance in the placental circulation. Reactive Oxygen Species (ROS) is one of the primary mechanisms orchestrating PE-associated endothelial dysfunction. Inflammation, generalized vasoconstriction, and increased resistance in the placental circulation are all brought on by decreased uteroplacental blood flow. PE, which is characterized by vasoconstriction and limited anticoagulant activity, has impaired circulatory homeostasis due to vascular endothelial dysfunction. The endothelial dysfunction linked to PE is greatly influenced by ROS [47]. The etio-pathogenesis of preeclampsia is still unknown, but a few theories, such as anomalous trophoblast invasion of uterine vascular system, aberrant NO levels caused by oxidative stress, immunologic insufficiency between maternal and fetal tissues, pregnancy-related cardiovascular and inflammatory response maladaptations, genetic predisposition, and so on, are thought to be possible triggers. Many recent studies suggest that oxidative stress is one of the primary causes of preeclampsia among the possible causes of PE. In preeclampsia, the balance between the oxidant and antioxidant systems is disrupted. As a result of oxidative stress, there is an increase in capillary permeability, proteinuria and edema, microvascular coagulation, thrombocytopenia,

lipid-laden macrophage foam cells, and atherosclerosis in PE. Trophoblast apoptosis is increased in preeclamptic women's placentas because free radicals promote trophoblast apoptosis. It is proposed that free radicals enter the systemic circulation and travel through the circulatory system components via vascular endothelial cells, producing broad oxidative damage. According to research, thrombolytic dysfunction caused by endothelial cell injury may be responsible for the pathophysiology of preeclampsia. Based on these data, it is possible to conclude that endothelial cell activation orchestrates the inflammatory response in preeclampsia.

When compared to normotensive pregnant women, preeclamptic women have reduced NO levels in their endothelial cells. Several studies have shown a correlation between nitric oxide and blood pressure management during pregnancy. Preeclamptic women excrete less cGMP, a secondary messenger of NO, in their urine than normotensive pregnant women [48]. NO is a powerful vasodilator that helps to maintain vascular tone, regulate blood pressure, recruit thrombocytes, and adhere to endothelial cells [49]. Along with the placenta, maternal leukocytes and endothelium are important contributors to free radical formation. By virtue of its vasculogenic properties, NO is generated locally and is crucial for promoting cytotrophoblast invasion. It is speculated that the reduced formation of NO account for abnormal perfusion of the placenta in PE. However, some researchers demonstrate that preeclamptic placenta can normally synthesize ecNOS and the level of formation of NO is also comparable in preeclamptic as well as the normal placenta. Henceforth, it can be deduced that PE is a condition of normal placental expression of ecNOS and normal production of NO, whose activity is reduced abnormally. This paradox can be explained by considering that the relative activity of NO in a given tissue/organ depends on its rate of synthesis and degradation.

It is well established that low-resistance vascular remodeling does not exist in PE. As a result of the hypoxia in the intervillous gap, the blood supply to the placenta is reduced, resulting in aberrant placentation. The hypoxic preeclamptic placenta stimulates the release of syncytiotrophoblast microparticles (STBM) [50]. Placental hypoxia and STBM collaborate to form damage-associated molecular patterns (DAMPs), which aid in the activation of immune cells such as neutrophils and dendritic cells. This activation causes the release of pro-inflammatory cytokines such as tumor necrosis factor- (TNF-), which, in conjunction with neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, promotes oxidative stress. Recent research indicates that in PE, elevated advanced glycation end products (AGEs) interact with receptors (RAGE), activating NADPH oxidase [51].

Role of Nitric Oxide in Normal Pregnancy

NOS catalyzes the guanidino nitrogen atom of L-arginine, resulting in the synthesis of NO as well as the activation of guanylate cyclase, resulting in an increase in cyclic guanosine monophosphate (cGMP) within the cell. cGMP relaxes smooth muscle cells, dilates endothelial cells, deaggregates platelets, and has anti-inflammatory

properties [52, 53]. Under physiological conditions, Ca2+ dependent eNOS leads to the constitutive production of NO in the endothelium. This NO prevents tissue damage, hence, is very beneficial in the peripheral circulation. eNOS is inhibited by inflammatory cytokines which in turn causes vasoconstriction in the peripheral circulation [54].

iNOS, which is Ca2+ independent, plays a key role in inflammation but is not expressed in the endothelium under normal conditions. Increased levels of inflammatory cytokines such as IL-1, IL-6, TNF-, and interferon (INF-) promote the development of iNOS in the vascular endothelium, resulting in excessive NO generation [55]. The iNOS isoform is instrumental in leukostasis as it upregulates ICAM-1 expression resulting in vascular dysfunction [56]. Increased NO expression may result in increased inflammation and other immunological reactions, leading to multiple organ failure in PE. NO is an essential physiological mediator of the renin-angiotensin system via AT subtype 2 (AT2), which acts in a counter-regulatory manner to the effects mediated by AT1. AT2 activation starts a vasodilator cascade that includes the NO/cGMP pathway by boosting NOS mRNA and protein, which leads to NO generation. On the contrary, the AT1 signaling pathway inhibits NO generation in the endothelium, resulting in vasoconstriction. L-arginine antagonists, such as ADMA, also limit NO production, resulting in hypertension [57, 58].

Role of NO in the Pathogenesis of Preeclampsia

eNOS and iNOS are predominantly expressed on syncytiotrophoblasts and endothelial cells in the placenta during pregnancy [59]. In normal pregnancy, increased estrogen levels drive endothelium-dependent vasodilation mediated by NO, which is generated and secreted by activated eNOS [60]. eNOS-derived NO maintains vascular smooth muscle relaxation, which leads to enhanced uterine blood flow and uterine myometrial quiescence [61]. Blood pressure is somewhat lowered in the middle of a normal pregnancy due to enhanced flow-mediated dilatation (FMD). FMD caused by shear stress induces vascular eNOS activation and temporally raised NO levels, resulting in easy vessel dilatation during normal pregnancy [62]. Vascular eNOS-derived NO can prevent inflammation by suppressing the expression of adhesion molecules such as ICAM-1, vascular cell adhesion molecule-1, E-selectin, and P-selectin [63]. In PE, the expression of adhesion molecules that promote inflammation is increased, causing inflammation in the systemic vasculature and placenta, resulting in uteroplacental perfusion failure. When compared to their normal counterparts, patients with PE have reduced NO concentrations in their serum during the first trimester [64]. Low levels of NO are thought to disrupt vascular dilatation and development in early pregnancy, resulting in poor placentation [64]. However, eNOS-derived vascular dilatation was reduced in PE patients due to FMD disruption. The lower bioavailability of NO explains this disparity. For example, when there is an overabundance of both NO and ROS, ROS quickly scavenge NO and

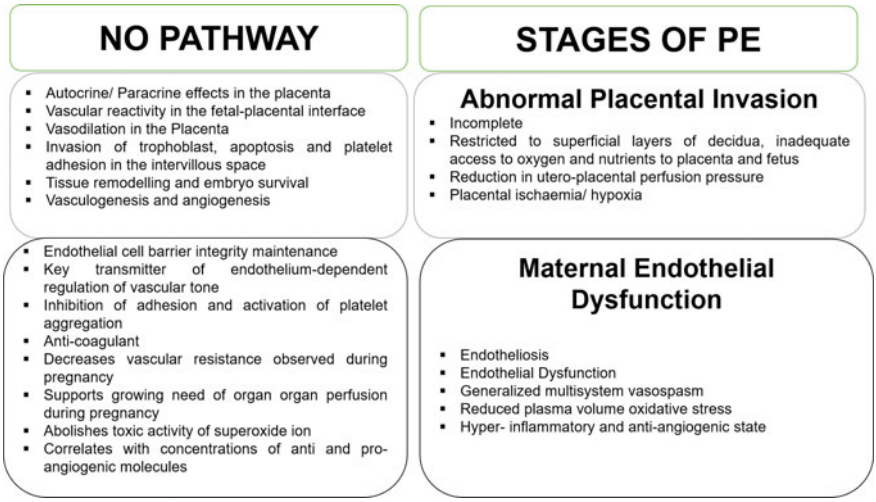

Fig. 19.4 NO pathway and its effects on different stages of Preeclampsia

generate ONOO anions, reducing vascular NO availability [65]. Although the interplay of NO and ROS has the potential to modulate endogenous vascular tone during healthy pregnancy [66]. The imbalance of NO and ROS may have a role in the pathophysiology of PE. The production of ONOO is caused by the rapid absorption of NO by ROS. High quantities of ONOO oxidize and degrade DNA, proteins, and lipids, but low levels of ONOO interfere with NO, prostaglandins, calcium ions, MAP kinase, and NF-B signaling pathways [67]. Furthermore, ONOO causes eNOS uncoupling by oxidizing tetrahydrobiopterin (BH4) to trihydrobiopterin (BH3), impairing eNOS activity [68]. BH4 balances NO and ROS in the vascular endothelium, and an imbalance in its amount causes hypertension. Furthermore, ONOO can cause permanent nitration of tyrosine residues on other proteins, impairing phosphorylation and causing enzymatic malfunction (Fig. 19.4).

ADMA and Preeclampsia

Asymmetrical dimethyl arginine (ADMA) is an endogenously generated NOS inhibitor whose exact origin is uncertain. ADMA levels are generally kept reasonably low by a demethylating enzyme called dimethylarginine dimethylaminohydrolase (DDAH), which is also found in NOS-containing cells [69]. Increased ADMA levels have been linked to hypercholesterolemia, congestive heart failure, atherosclerosis, end-stage renal disease, thrombotic microangiopathy, and preeclampsia. The oxidation of low-density lipoprotein (LDL) and TNF-reduces DDAH activity within cells. It has also been discovered that oxidized LDL and TNF-levels remain increased in preeclampsia [70]. The binding of synthetic or endogenously generated arginine

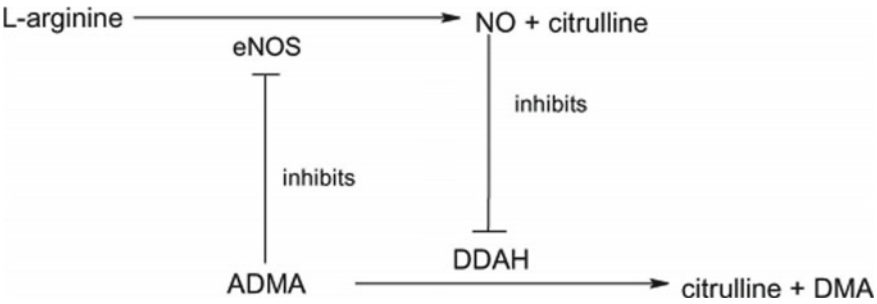

Fig. 19.5 The feedback loop of NO. NO is generated in response to the changes in the redox environment which further switches off NO synthesis [35]

analogs to the NOS enzyme's arginine binding site neither prevents nor stimulates the synthesis of O2−. ADMA competes with L-arginine for cellular uptake, which may result in lower L-arginine concentrations or a lower L-arginine/ADMA ratio inside the cells, resulting in O2− the production via NOS. If ADMA were simply an inhibitor of NOS, increasing it should result in a decrease in NO and ONOO− production. However, other research shows that when ADMA is present in larger concentrations, the production of NO and ONOO− increases. Preeclampsia is an excellent illustration of such a condition. Even when ADMA is increased, NOS and NOS activity are known to rise (Fig. 19.5).

Antioxidant Defense System in Preeclampsia

Oxidative stress in the placenta of preeclamptic women increases as early as 8–10 weeks of gestation. To compensate for the oxidative stress caused by ROS and RNS, aerobic cells have developed a defense mechanism comprising enzymatic and non-enzymatic components that quench the flux of ROS and RNS. Glutathione (GSH), vitamin C, vitamin E, carotenoids, coenzyme Q, and various antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione-S-transferases (GSTs) all play important roles in regulating ROS systems. It has been well documented that the activity of antioxidants such as vitamins E and C, GSH, and SOD is altered in PE. Important antioxidant enzymes in PE include glucose-6-phosphate dehydrogenase, glutathione peroxidase (GPx), and glutathione S-transferase [71].

Vitamins (Vitamin C, E)

Vitamin C, commonly known as ascorbic acid, is a necessary nutrient that also functions as an antioxidant, protecting the organs from oxidative damage. The level of plasma ascorbate is generally reduced during pregnancy, which is further reduced in the plasma and placenta of PE patients [72]. Ascorbate is oxidized to ascorbyl radical, which then oxidizes to dehydroascorbate, resulting in tissue harm caused by oxidative stress. In PE patients, plasma levels of oxidized ascorbate are elevated, which is regarded to be a key factor in vascular dysfunction. By giving an electron, vitamin E decreases peroxynitrite (ONOO). Vitamin E levels in serum and placental tissue are significantly lower in severe PE, indicating an increase in oxidative stress. Vitamin C, also known as ascorbic acid, is a vital nutrient that also acts as an antioxidant, preventing oxidative damage to the organs. During pregnancy, the level of plasma ascorbate decreases, which is further lowered in the plasma and placenta of PE patients. In this situation, there is a lot of stress [73].

Superoxide Dismutase (Mn-SOD, CuZn-SOD, EC-SOD)

SOD catalyzes the superoxide radical's dismutation into oxygen (O_2) or hydrogen peroxide (H_2O_2) $2O_2 + 2H + O_2 + H_2O_2$. Mn-SOD, CuZn-SOD, and EC-SOD are three kinds of SOD. Mn-SOD is constitutively expressed in the mitochondria and scavenges superoxide radicals, whereas CuZn-SOD is produced in the cytoplasm and released into the extracellular space. EC-SOD, on the other hand, is only generated by a few cells, such as vascular smooth muscle cells, and is found in the extracellular matrix of the vascular wall and placenta. As an antioxidant, SOD can react with NO as well as ROS, generating powerful ONOO since NO has three times the affinity for superoxide as SOD [74]. SOD expression is raised during normal pregnancy, while SOD activity and CuZn-SOD mRNA expression are lowered in PE, resulting in higher oxidative stress in the placenta of PE patients. The role of EC-SOD in modifying vascular function in resistance vessels is crucial. According to research, the Ala40Thr SNP, a mutant carrier of the EC-SOD, may enhance the risk of severe prenatal growth restriction-complicated PE. SOD is also observed to be reduced in erythrocytes from PE patients [75].

Catalase

Catalase is an enzyme that degrades hydrogen peroxide into water and oxygen ($2H_2O_2$ $2H_2O + O_2$) and is critical for lowering ROS levels. Hydrogen peroxide works as a cellular messenger in insulin signaling pathways at low concentrations but produces toxicity in pancreatic cells at high concentrations. Furthermore, catalase

is a key enzyme in the breakdown of hydrogen peroxide in erythrocytes, and its lack causes increased hydrogen peroxide synthesis in many organs, such as type 2 diabetes mellitus. During pregnancy, catalase levels rise with gestational age, reaching a peak at 12 weeks. In contrast, catalase activity in PE patients' erythrocytes is significantly reduced, which is necessary for metabolizing hydrogen peroxide in systemic circulation [76].

Glutathione Peroxidase (GPx)

GPx is an enzyme that lowers lipid hydroperoxide and hydrogen peroxide and protects organs from oxidative stress (2Glutathione + H_2O_2 Glutathione disulfide + $2H_2O$). So far, eight GPx isoforms have been discovered, with GPx1 being the most abundant in several organs. Normal pregnant women's placentas have increased GPx activity, whereas PE patients' placentas have decreased GPx activity and mRNA (GPx1, GPx3, GPx4). The erythrocyte GPx is lower in people with PE but higher in patients with HELLP (hemolysis, elevated liver enzymes, and low platelet count). GPx insufficiency may have a role in the pathogenesis of PE since its decreased activity leads to the formation of lipid peroxides and thromboxanes, both of which are elevated in the PE placenta [77].

Oxidative Stress Biomarkers for Preeclampsia

Biomarkers are molecules that can be reliably examined and analyzed as indicators of normal biological, pathogenic, or pharmacologic reactions to treatment intervention. There are various in vitro markers of oxidative/nitrosative stress, but most of them have drawbacks such as being insensitive/specific or requiring intrusive procedures. Because ROS/RNS are highly reactive and have a very short half-life, measuring them in cells/tissues or body fluids is extremely difficult. Because ROS/RNS byproducts (e.g., nitrate/nitrite) are more stable molecules, they can be detected directly and/or indirectly, including lipid peroxidation end products and oxidized proteins. To be employed as diagnostic tools, these molecules must meet certain criteria such as oxidation stability, detectable concentration, specific oxidation pathway, and correlation with disease severity. Upregulation of iNOS occurs in the placenta as a result of increased O2− production, resulting in elevated amounts of OONO−. OONO− causes peroxidation of membrane lipids and MDA, as well as the production of conjugated dienes. MDA levels in maternal plasma, placental tissue, and erythrocytes are higher, and the severity of the disease correlates with MDA levels in both serum and erythrocytes of PE pregnancies [78]. The plasminogen activator inhibitor-1 (PAI-1) level rises in all groups during pregnancy. Plasma concentrations in the preeclampsia group were substantially greater than in the low-risk group. They discovered that the PAI-1/PAI-2 ratio is considerably higher in women who acquire PE later in life [79].

Blood GSH concentrations are an indicator of glutathione status in tissues, and blood measurements of both reduced GSH and glutathione disulfide (GSSG) are thought to be helpful indicators of oxidative stress status in humans. High GSH concentrations and GSSG: GSH ratios have been found in the blood of patients suffering from a variety of illnesses, including preeclampsia, breast cancer, lung cancer, and coronary heart disease [80]. Oxidative stress biomarkers in maternal serum, such as pregnancy-associated plasma protein A and placental growth factor (PlGF), change during the first trimester of pregnancies, especially in those with preeclampsia [81]. Some oxidative stress markers have various impacts on the placenta. Biomarkers such as sFlt-1 demonstrate placental dysfunction by inhibiting the effects of vascular endothelial growth factor and PlGF and modifying endothelial tissue maintenance. PlGF is an angiogenic factor that is considerably reduced in preeclamptic pregnancies. Soluble endoglin is an anti-angiogenic protein that inhibits capillary tube development and induces vascular permeability and hypertension. Activin-A and inhibin-A are placental-derived endocrine hormones that provide early warning of pre-eclampsia. Endothelial activity is indicated by cell adhesion molecules such as vascular cell adhesion molecule-1 and E-selectin. In preeclampsia, plasma nitrotyrosine, an indication of peroxynitrite exposure, is found in placental vascular endothelium. C-reactive protein (CRP) and Pentraxin-3 are inflammatory markers that are elevated in early pregnancy, indicating preeclampsia. When syncytiotrophoblasts undergo oxidative stress, anti-angiogenic factors -sFlt-1 and soluble endoglin are released into the maternal circulation, resulting in endothelial dysfunction, hypertension, and proteinuria in PE patients.

Conclusion

Preeclampsia is an illness with an unknown cause. However, advances in the understanding of the pathogenesis of PE have not been converted into treatment options. Oxidative stress is caused by an imbalance between ROS and anti-oxidants and may contribute to the development of PE via vascular dysfunction (Fig. 19.6). Current therapies include hypertension medications, magnesium sulfate, accelerated deliveries, and aspirin, among others. As a result of poor penetration of cytotrophoblast into the uterine myometrium and altered spiral artery remodeling, placental hypoxia, enormous generation of ROS, and decreased bioavailability of NO occur, leading to the development of clinical symptoms in women with PE. The formation of cytotoxic ONOO may be a characteristic of vascular injury in PE, confirming the theory that an imbalance of ROS and NO causes vasodilatory dysfunction in PE. A variety of circumstances might result in a reduced L-arginine/ADMA ratio, which can set off a never-ending cycle of NOS dysfunction. Early L-arginine supplementation may be an effective way to balance this ratio, thereby preventing the dysfunctional loop. Its overall efficiency may be improved by combining it with antioxidants. Although therapy with vitamins C and E alone reduced maternal symptoms, no significant perinatal benefits were found. However, antioxidant supplementation is likely to

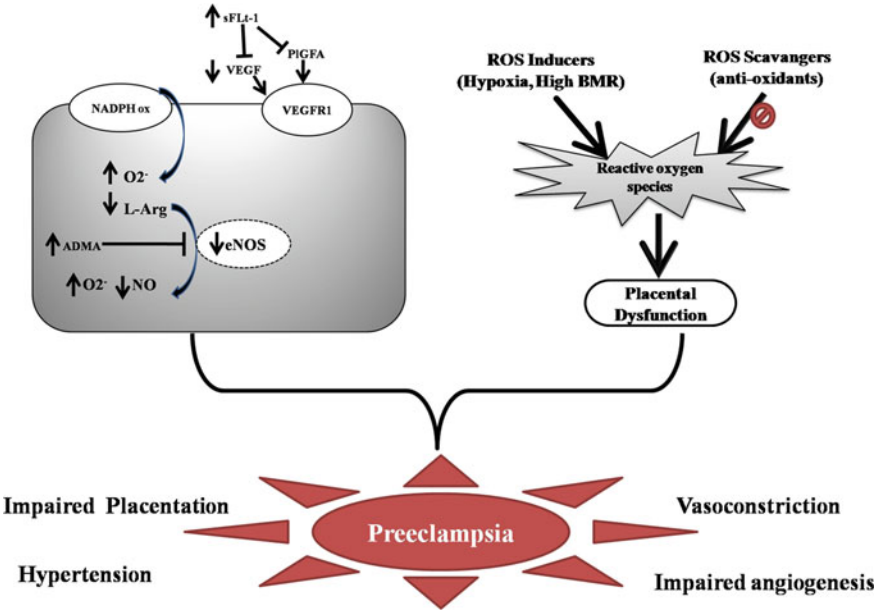

Fig. 19.6 NO and oxidative stress as the precursors of Preeclampsia

prevent the occurrence of PE or repair the future manifestation of PE. To determine the involvement of ROS and NO in the etiology of PE and to assess the efficacy of antioxidants on PE, extensive research is required. Understanding the essential role of NO and its interaction with oxidative stress induction could be used to develop better treatment and prevention techniques.

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Chapter 20
Therapeutic Prospects of Nitric Oxide as an Anti-teratogen

Palanivel Gajalakshmi, Pavitra Kumar, Suvendu Giri, and Suvro Chatterjee

Abstract A physical, chemical or biological agent that causes malformation of the embryo of foetus is referred to as teratogen. Foetal abnormalities have become a serious concern considering the fact that out of 131.4 million babies born every year worldwide, approximately 5% of live births are reported to have birth defect/s. Nitric oxide participates in various biological processes associated with early embryonic development and interference in its pathway causes foetal abnormalities. In our previous studies, we found that exogenously supplemented nitric oxide protected embryos against thalidomide- and cadmium-mediated teratogenicity in experimental models. In this chapter, our aim was to summarize the recent knowledge about teratogens, their mechanisms and possible potential anti-teratogenic compounds with thalidomide, cadmium and nitric oxide in focus. We envisage that nitric oxide delivery to embryo will protect the embryo from teratogenic effects.

Keywords Teratogen · Nitric oxide · Thalidomide · Cadmium · Chick embryonic model · Developmental defects

Introduction

Teratology (from Greek, teratos, monster) is the science that involves the study of abnormalities of physiological development, congenital malformations and their causes. An agent, a physical, either chemical or biological is called as a teratogen when it is capable of disturbing the development of the embryo or foetus. Reports

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_20

showed that adverse effect of teratogens on prenatal development caused 2.7 million neonatal deaths, worldwide and these deaths constitute for 45% of the deaths of children under 5 years [51]. The etiology behind birth defects could be multifactorial, although considerable evidence exists for both genetic and environmental factors.

Most structural defects caused by teratogenic exposures occur during the embryonic period, when critical developmental events are taking place and the foundations of organ systems are being established [10]. Different organ systems have different periods of susceptibility to teratogens. Teratogens induced most common structural anomalies such as growth retardation, microcephaly, microphthalmia, tachycardia, cardiac failure, hepatosplenomegaly, thin upper lip, brachydactyly of the fifth finger, auditory, vestibular, retinal, and other neurologic dysfunction in children.

Teratogens were classified under five major categories in general [28]. The present chapter is focussed on cadmium (Cd), as an environmental teratogen and thalidomide as a drug teratogen.

Cadmium as an Environmental Teratogen

Cadmium is a major environmental pollutant and listed as a potential teratogen in humans by the United Nations Organization. The reported sources of cadmium are seafood and tobacco smoke and has a long half-life, ranging from 75 days to 26 years [33]. Animal-based studies are widely known compared to human studies with respect tofetal growth restriction upon maternal cadmium exposure. A murine-based study indicates the time window as late gestational period at which maternal cadmium exposure caused reduction in fetal weight and length through inhibition of placental progesterone synthesis [48]. An increased DNA methylation pattern was observed in inflammatory signalling genes (TNFAIP2, ACOT7, and RORA), which perturbed inflammatory processes, and impaired placental function and development from human placentae upon higher cadmium exposure [9].

Our laboratory has demonstrated that cadmium exposure to cultured endothelial cells induced significant reduction in nitric oxide (NO) production by impairing phosphorylation of endothelial nitric oxide synthase enzyme and is capable of attenuating angiogenesis in an egg yolk angiogenesis model [25]. In addition, cadmium exposure to endothelial cells interfered in functions of endothelial cells such as tube formation, cell migration and disturbed the subcellular actin polymerization, which could be the reason for cadmium-mediated inhibition of cellular migration and angiogenesis [21]. We have also observed that cadmium exposure to developing chick embryo caused multiple birth defects. Interestingly, we observed the ill effects of cadmium can be negated by addition of exogenous NO through a NO donor and restore normal vascular and embryonic development, thus serving as a protective molecule [45]. Further, we have shown that instead of using synthetic NO donor, beetroot juice (natural source of NO) can be a best alternative for attenuating the effects of cadmium and can serve as a dietary supplement for pregnant women [2].

Thalidomide as a Drug Teratogen

Thalidomide was developed by ChemieGrünenthal (West Germany) in 1957 and was used as a sedative over 60 years ago. However, its use was banned in 1964 due to its teratogenic effect. A broad range of birth defects was reported, including malformations of the limb, ear, eye, internal organs, face, genitalia, and central nervous system upon exposure to thalidomide during early pregnant period [44]. Stunted limb growth in humans is a well-known teratogenic effect of thalidomide. Later in 1998 and 2006, thalidomide was accepted by the Food and Drug Administration (FDA) for the treatment of leprosy and multiple myeloma, respectively [29] after realising its potential to treat multiple diseases, though its prohibited to be used by pregnant women.

Thalidomide is an immunomodulatory agent, which arrests angiogenesis [5]. In our several independent studies, we found that thalidomide reduced tip cell formation in an ex vivo model [19], caused structural anomalies during cardiac development [23], targeted fibroblast growth factor receptor 2 [38] and altered transcriptome profile of developing chick embryo [45]. We have previously shown that mechanism of anti-angiogenic activity of thalidomide involves inhibition of nitric oxide-sGC-cGMP pathway in endothelial cells [26] and exogenous supplement of NO offered partial protection against thalidomide-induced limb [37] and eye deformities [22] in chick and zebrafish embryo models.

Prevention of Birth Defects

Currently, the following are the measures taken to prevent congenital malformations. 1. Increase the folic acid intake during pregnancy [3]. 2. Reduce the intake of sugar during gestation [4] 3. Regular visit to gynaecologist 4. Avoid alcohol, smoking cigarettes, other drugs, infections, and fever during pregnancy 5. Healthy lifestyle and the right prenatal nutrient balance is an important environmental factor in the growth of a healthy child. However, it is not always possible to strictly follow the above procedure due to the fact that pregnant mothers have increased appetite and tend to take carbohydrate rich foods and also due to day today activities. Therefore, one should think of a new approach to negate the teratogenic effects of both genetic and environmental factors. Since NO is a critical signalling molecule for embryo development [43], it could be repositioned to test its anti-teratogen potentials.

Nitric Oxide and Teratogenicity

Several studies have demonstrated that NO participates in various biological processes associated with early embryonic development. Preimplantation murine embryos were found to be regulated by NO and these embryos when cultured with

NOS inhibitor at very early stage were developmentally delayed or nonviable and this was the first evidence which demonstrated that NO participates in implantation and it is necessary in early embryonic development in mice [12]. Further studies, demonstrated that appropriate NO concentration is important for embryonic development either too little or too much amount of NO had adverse effect [35]. Earlier studies demonstrated that maternal exposure of nonspecific NOS inhibitor during pregnancy caused growth retardation and hind limb defects in rat embryo model [32]. NOS inhibition impaired vascular integrity, and caused vasoconstriction, dysmorphogenesis in the developing embryo and over production of reactive oxygen species is associated with L-NAME mediated limb defects [1]. Knockout (KO) mouse models were used to study the role of specific NOS isoforms during embryonic development. eNOS knock-out caused focal acute hemorrhages in the distal limbs and limb deformities, growth retardation, placental abnormalities, cardiovascular malformation, and neonatal death [42]. Whereas iNOS and eNOS deficiency mice reported to affect reproduction, fertilization, early embryo development and neuronal development [49].

Nath and co-workers provided the first evidence that exogenous NO is being capable of protecting embryos against teratogenic assaults. They showed that inhibition of NO production during blood island stage using murine embryo culture affected the yolk sac vasculogenesis and exogenous NO supplementation via NO donor at this stage reduced the high glucose induced oxidative stress and protect murine embryos from glucose mediated vasculopathy [30]. Exogenous NO supplementation via a NO donor, Dean NONOate restored the endogenous NO level, eNOS phosphorylation and cGMP production and ameliorated copper deficiency mediated abnormal embryo and yolk sac vascular development [50]. Consistent with this strong background, we hypothesized to study the protective role of nitric oxide against thalidomide and cadmium mediated teratogenicity.

Scope of Nitric Oxide as an Anti-teratogen

It is clear from the literature that cadmium and thalidomide are potential teratogens and the teratogenic effect could be recovered by addition of NO. It is an established fact that NO is a second messenger and has a half-life of few seconds. It is good to add NO as a supplement in susceptible population. However, one has to design strategies to add NO in limited quantity and slow release form. One can think of NO based drugs. Isosorbide, nitroglycerin, nitroprusside, amyl nitrite are some of the medicines containing nitrates to treat cardiovascular diseases. Natural dietary source of nitrate are beetroot juice, fennel seeds, etc. To treat teratogenicity, NO based drugs can be designed by synthetic chemists and biologists. Such an attempt is worthwhile because our laboratory has demonstrated that NO has the potential to reduce teratogenic effects of thalidomide and cadmium.

Methodology

Drug Administration to the Chicken Embryos

Fertilized brown leghorn chicken eggs were purchased from the Poultry Research Station, Potheri, Chennai and were incubated at 37 °C in a sterile humidified incubator. The chick embryos were staged based on the previously described Hamilton and Hamburger (HH) stages of chick embryo development [14]. All the experiments were performed on chick embryos between the HH stages of 1–38. All treatments were administered as a single dose to the embryos via a hole made with a sterile needle in the air sac of each egg.

Different concentrations (0, l, 10, 20, 40, 80, 160 μg) of thalidomide (99% purity) were dissolved in 200μL DMSO and the volume made up to 1 ml using 1X PBS. 50μL of final volume for each concentration was applied to chick embryos through a hole in the air sac made using a sterile needle. Most of the treatment was administered as a single dose at HH stage 8 for all the experiments except those mentioned otherwise. Different indicated concentrations of thalidomide analogs; pthalimide, lenalidomide and teratogens; mercuric chloride, lithium chloride and ethanol were also applied to chick embryos at HH stage 8 through the air sac. Similarly, different concentrations (0.01, 0.1, 1, 10 and 100 μM) of SpNO were added at HH stage 8 after 30 min of thalidomide treatment. 10 μM of SpNO was added at HH stage 8 after 30 min of thalidomide or thalidomide analogs or teratogens treatments. For the morphometric analysis, all treated embryos were dissected at HH stage 37 and the images were taken by using canon 10X optical zoom camera to examine the drugs effect on embryonic development.

Morphological Analysis

Vehicle control, Cd, spNO and Cd + spNO treated HH-26 (5th day) and HH-37 (11th day) staged embryos were dissected out to examine the morphological effect of the respective treatments on embryo development. Images were taken using an Olympus camera (Olympus India Pvt Ltd, New Delhi, India) attached with a stereo microscope. Additionally a digital chick heartbeat monitor was used to measure the heart beats per minute (bpm) of these embryos according to manufacturer instructions (Avian Biotech International UK). The HH-37 staged embryos, were weighed (wet weights) and measured (crown-rump length) to observe the differences between the growth rates of the embryos i.e. normal versus retarded growth. The embryos at this stage can be visibly checked to see if their external organs (beak, eyes and limbs) have developed normally while embryos having reduced height and weight were identified as retarded growth.

Histopathology Study of Eye

Brown leghorn eggs were treated with vehicle control, thalidomide, SpNO and thalidomide plus SpNO as described above. Eyeballs from each group of the embryos were dissected out at HH 8 stage and were fixed in 10% formalin. Histology sections were stained with hematoxylin and eosin.

Transcriptome Sequencing and Analysis

Brown leghorn eggs were treated with vehicle control orthalidomide as described previously and the total RNA was isolated from whole embryo of stage HH29 (pre-treated with vehicle orthalidomide) using TRIzol® method. The transcriptome sequencing was carried out using Illumina HiSeq 2500 platform and the data has been submitted to GEO (GSE69159). To align the sequence reads to the reference genome of chicken (Galgal4) downloaded from Ensembl Release 75 databases, TopHat (v2.0.8) was used with default parameters. Then, to identify the differentially expressed genes, Cuffdiff (v2.2.0) program was used. To study the GO Biological process (BP), GO Molecular Function (MF) and GO KEGGPathways enriched, we used DAVID and GeneCodis, online modular enrichment tools [16]. Using STRING v10 tool, we constructed the protein-protein interaction (PPI) network.

Results

To Study the Anti-teratogenic Effect of NO Against Teratogens

To investigate whether NO recovery is global to the other known teratogens, HH stage 8 chick embryos were treated with different teratogens such as, thalidomide (40 μg/mL), mercuric chloride (50 μM), ethanol (5%), lithium chloride (10 μM) and cadmium (10 μM) followed by 10 μM of spNO treatment after 30 min. Best concentration of each teratogen was standardized separately (Data not shown). Embryos were dissected at HH stage 37 and subjected to the morphological analysis to check the effect of treatments on the embryonic development and percentage of abnormal embryos was scored, embryos with any deformities are considered as abnormal embryos. 44, 62, 76, 46 and 72% of embryos were abnormal in thalidomide, mercuric chloride, ethanol, lithium chloride and cadmium treatment respectively. SpNO rescued 98 and 92% embryos from thalidomide and cadmium mediated teratogenicity respectively but SpNO did not rescue embryos from other teratogens as shown in Fig. 20.1.

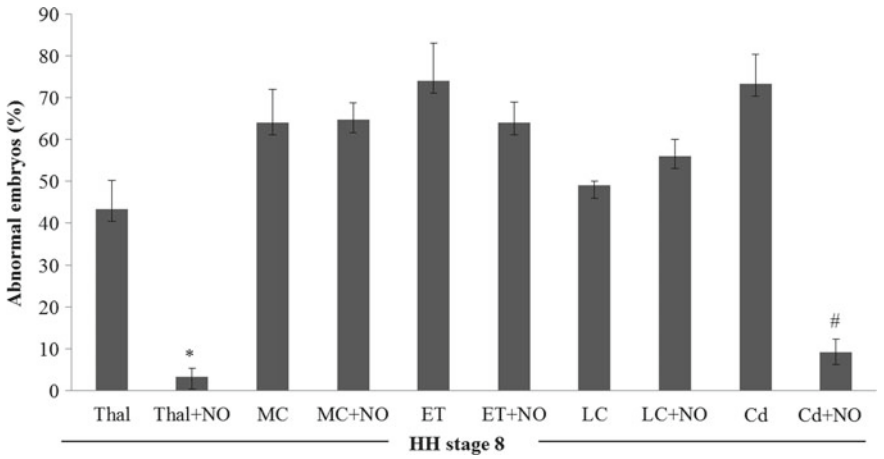

Fig. 20.1 Recovery effect of SpNO against other teratogens treated embryos. SpNO rescued embryos from thalidomide and Cd mediated teratogenicity but did not rescue other teratogens induced deformities. Chick embryos (n = 50 eggs) were treated with teratogens such as thalidomide (Thal); 40 μg, Mercuric chloride (MC); 50 μM, ethanol (ET); 5%, Lithium chloride (LC); 10 μM and cadmium (Cd); 10 μM followed by addition of spNO (NO) 10 μM after 30 min. Embryos were dissected at HH stage 37 to check abnormal embryos. *p < 0.05 versus Thal. #p < 0.05 versus Cd. Results are expressed as mean ± S.E of three experiments

Nitric Oxide Recovers the Eye Structural Integrity Affected by Thalidomide

Thalidomide caused structural and functional eye deformities in chick embryo as shown in our previous work [22]. Histological assessment showed loss of tissue integrity in eyes under thalidomide treatment together with loss of pigmented epithelium while SpNO and thalidomide + SpNO group were similar to that of vehicle control group (Fig. 20.2).

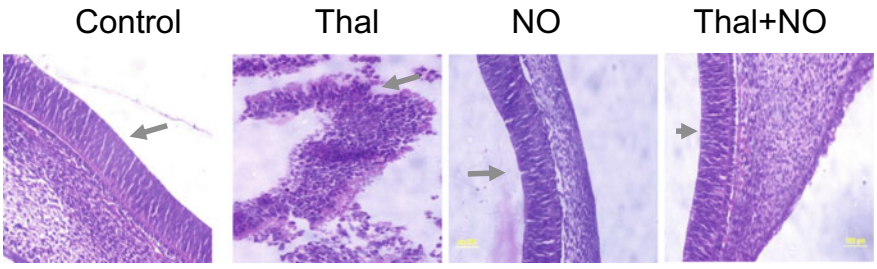

Fig. 20.2 Histopathological assessment of eye isolated from 6th day old chick embryo pre-treated with the corresponding treatments as single dose at HH8 stage. Arrows indicate the pigmented epithelium, which was deformed under thalidomide treatment alone. Morphology is shown in Kumar et al. [22]

Enrichment of Pathways Based on Transcriptome Sequencing Modulated by Thalidomide and Thalidomide-Nitric Oxide Combination

The transcriptome sequencing was carried out using Illumina HiSeq 2500 platform and the data was submitted to GEO (GSE69159) Ingenuity pathway analyzer QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGENRedwoodCity www.qiagen.com/ingenuity) was used to analyse differentially expressed genes while comparing thalidomide group with thalidomide+SpNO group. Upregulated genes under thalidomide+SpNO were clustered as 'size of body', 'oxidation of lipid', 'transport of lipid', 'synthesis of lipid' whereas downregulated genes were clustered as 'inflammation of organ' and 'apoptosis of epithelial cells organ' (Fig. 20.3).

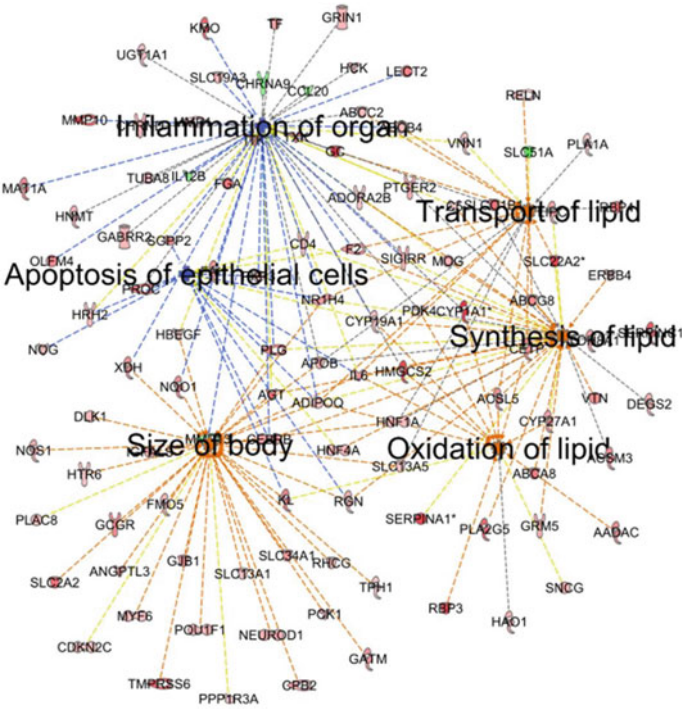

Fig. 20.3 Functional enrichment of differentially enriched genes in thalidomide versus thalidomide-NO comparison obtained from a transcriptome analysis of 6-day-old embryo pretreated with thalidomide orthalidomide+SpNO. The cluster connected with blue strings indicates the inhibitory function while the cluster connected with orange strings indicates the activated function. Inflammation of organ and apoptosis of epithelial cells were inhibited whereas size of body, transport of lipid, synthesis of lipid and oxidation of lipid were activated under thalidomide+SpNO treatment

Discussion

There are very few successful supplements/drugs to prevent the birth defects. One of those is FDA approved 400 μg of folic acid intake daily for childbearing women [3]. However, folic acid levels are associated with neural tube defects in developing embryos. Co-administration of beta cyclodextrin reduced abnormalities and showed protective effects against tolbutamide-induced abnormalities in fetuses [18].

NO mediated signaling pathway plays significant role during embryogenesis. In rat model, maternal exposure of NOS inhibitor during pregnancy leads to retarded growth and deformed embryos [32]. Earlier, disturbed nitrite/nitrate content had been reported in the maternal blood, which later caused retarded fetal growth [6]. NOS inhibition causes the overproduction of ROS, which mediates organ defects in embryo [1, 8]. NOS inhibition in the presence of teratogenic agent (valproic acid) aggravated the teratogenic outcome of the embryo [40]. Knock out mice of eNOS showed placental anomalies, abnormal reproductive features, limb defects, retarded growth and fetal death [31, 42].

Given the fact that NO signaling is important for developmental process, inhibition of NO synthesis leads to amplification of the teratogenic effects of several teratogens including thalidomide and cadmium. In addition, NO supplementation proved to reduce the teratogenic effects of thalidomide [20, 37], valproic acid [40, 41], cadmium [25, 46], and copper deficiency [7, 50].

The chemical nature of nitric oxide (·N=O or ·NO) is very unstable as it possesses an unpaired electron in its' antibonding π*2px/π*2py molecular orbital. It has a very short half-life and disappears rapidly in physiological solutions particularly in the presence of hemoglobin [13, 39]. NO can be produced by various nitric oxide synthase (NOS) enzymes and it can be stored as comparatively stable nitrite or nitrate salt in our physiology. There is a transient moment for it to be produced by NOS or nitrite reductase and to react on its target binding. Therefore, NO release dynamics are very relevant in this context. In order to attenuate teratogenicity by applying NO donor, it is very important to understand the NO release kinetics of individual NO donor along with the treatment time and doses. The release pattern should be in an extremely controlled approach for optimal effectiveness of NO-derived therapeutic strategy.

The selection of doses and chemistry of NO donors are key factors in a NO-dependent therapeutic strategy [27]. We have tested NO donors with half-life ranging from seconds to several hours for their efficacy in modulating angiogenesis. The study identified the best as spermine NONOate (spNO) with a half-life 39 s. We have used spNO as an effective anti-teratogen molecule for all our follow up studies, and observed a strong anti-tertogenic property of the spNO [37]. Further, the chemistry of NO is regulated by its ability to react with other radical species such as superoxide anion resulting in the production of cytotoxic peroxynitrite ($ONOO^-$) and its affinity to coordinate with transition metals [15, 17]. On one hand, physiological level of nitric oxide is essential for cellular homeostasis and even low levels of exogenous nitric oxide modulated cardiogenesis [24] whereas, on the other hand,

excess NO may undergo various nitration, nitrosylation, and nitrosation reaction and thus can hamper the regular biological process and molecular functions of a cell [47]. Overdose of NO can induce nitrosative stress, which is associated with an abnormality in cardiac function starting from hypertension to congestive heart failure [34]. Even excess production of NO due to other pathological factors in physiology can lead to cardiovascular dysfunctions [11]. Our laboratory [36] has shown that the level of NO in yolk has a modulatory effect in embryonic heart development. Gene expression of heart developmental marker including Pitx2, BMP4, Noggin, Shh had altered under high dose of NO. We have also demonstrated that excess levels of NO can cause situs inversus by impairing the migration of cardiac progenitor cells involving BMP4-SMAD1 associated pathway. Therefore, we have performed a series of standardization experiments to obtain optimal concentration of NO (through SpNO-NO donor) to protect the chick embryo from teratogenic effects of thalidomide and cadmium. Our results have shown that 10 μm is the best concentration of exogenous NO [46].

Yet another point to be considered is about the stage of pregnancy at which NO supplementation will help prevent teratogenic effects. Our earlier works have shown that ultra-low levels of Cd interfere with endothelial functions and specifically angiogenesis [25]. Depending on the above hint, we hypothesized that Cd interferes with early angiogenesis. Veeriah et al. [46] demonstrated that the severity of Cd-mediated effects observed at the HH-8 stage coincides with the formation of blood islands and cardiogenesis in the developing embryo. These results in turn parallel our previously published work, which showed that the exposure of classical teratogenic drug thalidomide to HH-8-staged embryos was detrimental inhibiting early vasculogenesis and subsequent embryo development.

Blood islands are the precursors of early blood vessels in the embryo; the time of their formation is an extremely delicate and dynamic phase, which ultimately defines the vasculature of area vasculosa and embryo development. Results demonstrate that Cd exposure at HH-8 stage caused significant deformations of area vasculosa vascular network of embryos. In the present work, we noticed the loss of tissue integrity in the form of deformed eyes under the treatment of thalidomide while SpNO and thalidomide + SpNO group recovered the effects (Fig. 20.2).

In these experimental models, we observed that embryos had a phase "window of susceptibility" during which they are more susceptible to teratogens. Embryos were less susceptible to teratogens once the heart start beating and the circulation is established. This could be due to the increase in the number of total and differentiated cells and dispersion of teratogenic metabolites due to the newly established circulatory system. This phase in an embryo could be termed as "window of susceptibility" Although, these studies were from experimental models, there could be an analogous phase in human embryonic development during which embryos are the most susceptible for teratogenic assaults. Thus, we observed that the most sensitive window (Fig. 20.1) at which the teratogens, thalidomide and cadmium exert their maximum effects is HH-8-staged embryos and supplementation of low dose of NO at HH-8 staged embryos could prevent teratogenic effects of thalidomide and cadmium significantly [37].

Thalidomide treatment reduced body size in developing chick embryos as shown by Veeriah et al. [45]. It also induced apoptosis in various cell types such as cardiomyocytes [23] and endothelial cells [37]. In our transcriptome data obtained from 6th-day old chick embryo pre-treated with thalidomide or thalidomide + spNO, we observed that upregulated genes under thalidomide + spNO were clustered as 'size of body' indicating that spNO could help to recover the thalidomide-induced reduction in the size of the embryo. The downregulated genes in the similar comparison were clustered as 'apoptosis of epithelial cells' showing that spNO could prevent thalidomide-induced apoptosis and hence provide protection against thalidomide-mediated teratogenicity. Other pathways that SpNO altered were lipid metabolism, transport, and 'inflammation of organ' (Fig. 20.3).

Summary

Teratogens are toxic substance which causes the adverse effect to the developing embryos resulting in structural and functional abnormalities or embryonic death. We proposed NO as an anti-teratogen. Present study offers the evidences that NO exerts anti-teratogenic role against thalidomide and Cd. Exogenous NO supplementation via a NO donor to thalidomide exposed embryos protected embryonic eyes from damages. Based on these observations we propose that NO supplementation during early stage of pregnancy may shield developing embryos from teratogenic assaults and boost embryonic growth.

Acknowledgements The authors are thankful to Dr. Vimal Veeriah for his contribution in the data used in Fig. 20.1. P.G. thanks UGC for post-doctoral fellowship to women. SG is grateful to the Department of Science and Technology, Government of India for the INSPIRE Fellowship.S.C. acknowledges a Grant from University Grants Commission—Faculty Recharge Programme, (UGC-FRP), Government of India.

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Chapter 21
Ruthenium Nitrosyl Complexes: Photoinduced Delivery of NO to Different Biological Targets

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Abstract Nitric oxide (NO) molecule participates in various biological events such as vasodilation, neurotransmission, antioxidant, and immune responses. In living organisms, it is generated as a side product by nitric oxide synthase (NOS) enzyme via conversion of *L*-arginine to *L*-citrulline. The physiological role of NO is concentration-dependent, which is crucially important to obtain the desired effects in biosystem. Coordination complexes of NO with transition metals, especially ruthenium (Ru), have gained increasing interest for the past few decades. So far, several ruthenium nitrosyl complexes have been developed as NO carriers, and their photochemical properties are well documented in the literature. Most of the ruthenium nitrosyl (Ru–NO) complexes are found photolabile in nature and release NO molecule in the presence of the light of suitable wavelength. The photoreleased NO can stimulate various biological targets in different in-vitro and in-vivo models. The main motif of this book chapter is to cover majority of the light sensitive Ru–NO complexes, and the photochemical properties of these complexes, quantification, delivery and application of NO molecule for various biological targets are discussed at a length.

Keywords Photolability · Ru–NO complexes · NO delivery · Biosystem

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_21

Introduction

Nitric oxide (NO), a small gaseous molecule, has been considered as a highly toxic gas for an unlimited period (during pre-1980s) because of insufficient awareness of its biological significance. It is a diatomic free radical and acts as a strong field ligand for different transition metals. Louis J. Ignarro was the first to reveal the importance of this molecule in biosystems under physiological conditions during the 1980s [1]. Later, NO was selected as a *Molecule of the Year* in 1992 [2]. In the year 1998, three American scientists Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad, were awarded Nobel Prize in Physiology or Medicine for demonstrating the signalling properties of NO molecule.

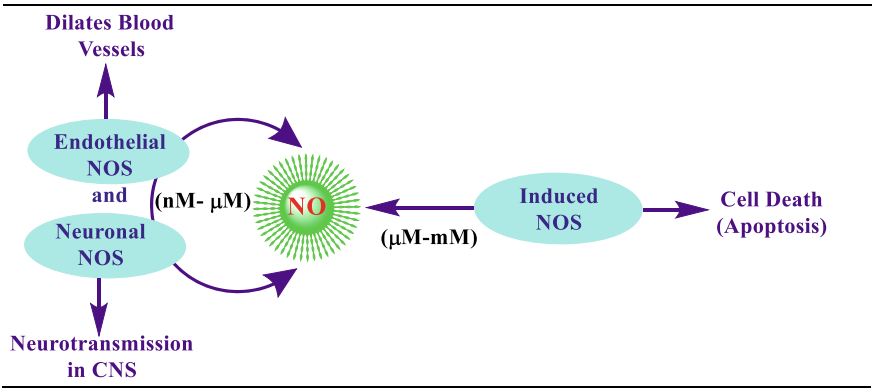
In living systems, nitric oxide is synthesized by a heme-containing metalloenzyme named nitric oxide synthase (NOS) [3, 4]. Three types of NOS enzymes have been characterized and purified: (i) endothelial NOS or e-NOS [5]; (ii) neuronal NOS or n-NOS [6] and (iii) inducible NOS or i-NOS [7]. These enzymes could easily be differentiated on the basis of their sensitivity towards Ca^{2+} stimulation, the separate genes that are cloned or sequenced on an organ localization.

Endothelial and neuronal NOSs are considered as constitutive NOSs (c-NOSs) enzymes due to their presence as normal constituents in healthy living cells. They produce NO on-demand in short periods of time. These enzymes generate NO at nM to μm level, which induces changes in targeted cells with the activation of soluble guanylyl cyclase [8]. Neuronal NOS is dispersed in the brain as well as in the spinal cord within the central nervous system (CNS) [9]. Nitric oxide molecule has been reported as a neuronal messenger in the central nervous system by Snyder et al. in 1991 (Scheme 21.1) [10].

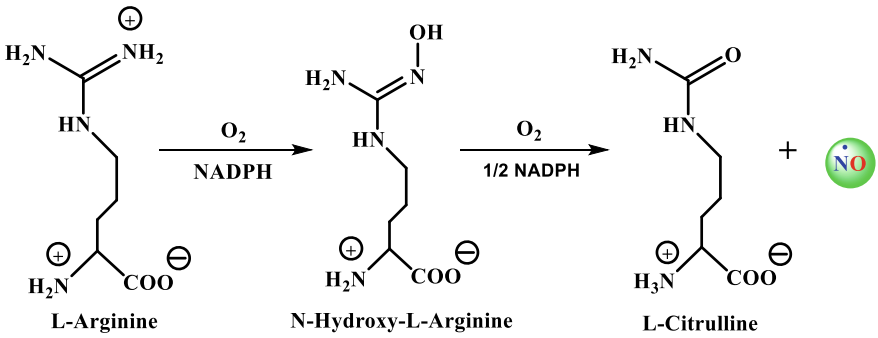
Endothelial NOS isoenzyme exists in the endothelium of human arterioles, arteries, and veins and regulates the blood vasodilatation in case of high BP. Many reports have shown that the NO molecule plays essential functions in memory-related events [11].

Inducible NOS holds a tightly bound *calmodulin,* and so it is Ca^{2+}-independent isoenzyme [12]. This isoenzyme is activated from the products of any infection, which may include bacterial exotoxins and endotoxins [13]. It generates NO at very high concentrations (upto mM level), which exhibits cytostatic property in infected or tumor cells (Scheme 21.1) [14]. The large amount of NO produced by i-NOS is toxic and may show different side effects towards normal cells. Interaction of NO with the enzymes containing Fe or S centers may agitate the respiration cycle in mitochondria [15].

Nguyen et al. [16] demonstrated that the high concentration of NO can also cause DNA damage. Furthermore, NO with superoxide ion interaction leads to the generation of highly stable toxic reactive intermediates such as hydroxyl and peroxynitrite anions [17].



Scheme 21.1 Nitric oxide production from different NOSs and its biological functions



Scheme 21.2 Biosynthesis of NO by NOS enzyme

Synthesis of NO in the Biosystem

The NOS enzyme produces NO molecule with *L*-arginine conversion into *L*-citrulline (Scheme 21.2) [18] in which the substrate oxidation occurs via two succeeding oxidative steps [19]. During this reaction, an intermediate *N*-hydroxy-*L*-arginine is formed which has been characterized well. *L*-citrulline can also be converted back to *L*-arginine for maintaining a continuous NO generation, as reported by Hecker et al. [20].

Biological Significance of NO

Rather than being merely a toxic molecule, the biosynthesized NO is found to be an essential component in several biological events such as vasodilator in blood vessels

[21, 22], immune response [23, 24], a neurotransmitter in CNS [25] and apoptosis (a programmed cell death) in tumor cells [26, 27] and the physiological functions of nitric oxide are found dependent on its concentration produced by NOS in the living organisms.

Cardiovascular Function of NO

Bioactivity of NO was first observed in cardiovascular systems where NO acted as a vasodilator. It helped in blood vessels relaxation, in the case of patients suffering from high blood pressure [1–3, 21, 22]. In the cardiovascular region, endothelial nitric oxide synthase enzyme produces NO with the conversion of amino acid arginine to citrulline. The blood vessels are dilated with the help of a physiological amount of NO via an activation of guanylyl cyclase enzyme.

Function of NO in CNS

Although NO production occurs in different tissues all over the human body, the brain is considered to be the major source of NO. It is because of the wide distribution of both soluble guanylyl cyclase (sGC) enzymes and NOS in the central nervous system. The role of NO in CNS's is tremendous, which may include the stimulation and maintenance of synaptic plasticity to the sleep control, appetite, body temperature, and neuro-secretion. In the nervous system, n-NOS is found in astrocytes and cerebral blood vessels. It becomes important to note that the physiological concentration of NO is neuro-protective. However, a large amount of it is toxic to neurons [28, 29].

NO Function in Programmed Cell Death (Apoptosis)

A large amount of NO in the human body is produced by i-NOS enzymes. The interaction of biosynthesized NO with superoxide ions results in oxidative injury and cell death. Therefore, a large concentration of this small molecule is utilized to induce cell death in tumor cells. NO also participates in several redox events to synthesize highly toxic compounds (mostly reactive nitrogen species), causing cellular death.

Owing to the essential functions and high demand for NO in biosystems, a number of exogenous organic and inorganic NO carriers have been designed and developed during the past two decades [30–33]. Such molecules may deliver NO on demand to different biological targets such as heme based proteins such as myoglobin, haemoglobin and cysteine-S.

Table 21.1 Some well known NO donating agents and their biological significance [34]

	NO donating agents	Physiological function	Clinical name
1	GTN (Glyceryl trinitrate)	Vasodilation for treatment of angina pectoris	Nitroglycerin
2	ISMN (Isosorbide mononitrate)	Dilation of blood vessels	Ismo, Imdur
3	ISDN (Isosorbide dinitrate)	Dilation of blood vessels	Isordil, BiDil
4	NONOates (Diazeniumdiolates)	Cardiovascular treatments	–
5	SNP (Sodium nitroprusside)	Blood flow regulation	Nitropress
6	RBS (Roussin's black salt)	Inhibitor for bacterial growth	–
7	RRS (Roussin's red salt)	Bactericidal agent	–

Exogenous Organic NO Carriers

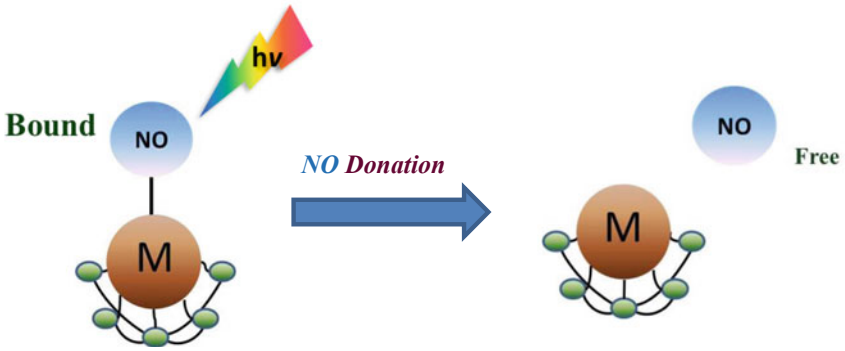
Many organic compounds, including nitrates and nitrites, nitrosothiols, and diazeniumdiolates, have been investigated as potential NO donors [30, 34]. Some diseases such as hypertension and angina pectoris are treated using well known NO donor drugs glyceryl trinitrate (also known as nitroglycerin, GTN) and isosorbide mononitrate (ISMN). A literature survey revealed that GTN having three nitrate groups releases one molar equiv. of NO via enzyme activation [35, 36] (Table 21.1).

Diazeniumdiolates (NONOates) are often used to improve the neuro-transmission process in CNS. NONOates usually provide 2.0 molar equiv. of NO on impulsive decomposition under the physiological environment. Several NONOates have been investigated to treat cardiovascular diseases but still not in use at the clinical level [37].

The majority of organic-based NO donor drugs have a flaw in that they release NO spontaneously at physiological pH and temperature. Moreover, many of them were found insoluble in an aqueous medium. Hence, their low solubility in water, NO leakage under moderate conditions, and sensitivity towards UV light make them non-specific for PDT. Therefore, the syntheses of an extensive range of molecular NO donating drugs are in demand due to the versatile roles of NO in biological processes.

Exogenous Inorganic NO Carriers

Due to the tremendous biological significance of NO, the coordination chemistry of this molecule with different transition metals has been extensively investigated during the past [37–39]. Several light-sensitive metal-NO complexes have been constructed for a rapid release of NO in the presence of UV and/or visible light of suitable wavelength (see Scheme 21.3).



Scheme 21.3 Schematic representation of photoactive metal nitrosyl complexes

Such complexes have shown potential applications as NO donating drugs in PDT of cancerous cells. Nitric oxide complexes of iron such as sodium nitroprusside $Na_2[Fe(CN)_5NO]$ and Roussin's salts are well-known NO-releasing agents that donate NO under UV light illumination (Fig. 21.1) [30, 40]. However, the sensitivity of these iron nitrosyl complexes towards high intensity UV light makes them non-specific for PDT of cancer cells. Moreover, the use of complex $Na_2[Fe(CN)_5NO]$ as NO drug was limited to an extent due to its toxic effects linked with the products after photorelease of NO.

In this endeavour, several other light-activated metal nitrosyl complexes that may deliver NO on demand have been developed by different research groups [30, 39, 41]. Majority of Fe based nitric oxide compounds are found less stable under physiological conditions.

Mascharak and his group constructed [42, 43] a pentadentate carboxamido based complex $[Fe(PaPy_3)(NO)]^{2+}$, which could release NO in the presence of visible light of low intensity. However, this complex was found unstable in aqueous media and was not utilized for biological applications. Nitric oxide complexes with other metals such as Mn [44, 45], Cr [41, 46] and Mo [41] have also been reported, but only a few of them were found suitable for PDT of cancer cells.

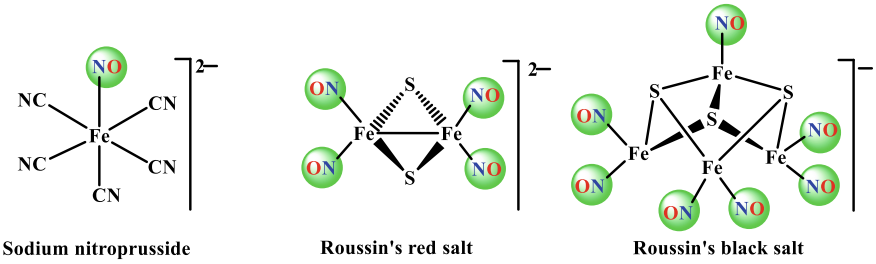


Fig. 21.1 Iron-based inorganic NO carriers utilized at the clinical level

Ruthenium Nitrosyl (Ru–NO) Complexes

Over the past few decades, ruthenium complexes have been extensively studied due to their immense photochemical and photophysical properties [47–49] and tunable redox properties [50, 51]. Such properties demand their applications in the research area of material chemistry, catalyses, medicines, and biology. A majority of ruthenium complexes have been investigated in DSSC [52, 53], artificial molecular synthesis [54, 55], and catalytic organic transformation reactions [56, 57]. Therefore, ruthenium-based coordination complexes are gaining increasing attention of researchers in different research areas. Several ruthenium complexes have been utilized as NO donors and scavengers [30–34], anti-cancer [58–60], anti-microbial, and antimalarial agents [61, 62], and their medical applications are well documented. Gray and group pioneered the use of ruthenium-based photosensitizers to probe the electronic properties in modified proteins (CytP-450s) [63].

Nitric oxide complexes of ruthenium (Ru-NO complexes) are found extraordinary stable under the physiological environment. In the last two decades, many photosensitive Ru–NO complexes have been developed for their potential applications as NO donating molecules under visible light and NIR light suitable for PDT [30–33]. Mascharak and group pioneered the work on metal nitrosyl complexes and their biological applications [64–72]. They have extensively investigated the photochemistry of Ru–NO, Mn–NO, and Fe–NO complexes. They developed a variety of Ru–NO complexes which exhibit strong absorption in the visible region (400–600 nm).

Tfouni et al. [73] demonstrated the biological applications of auxiliary ammine based light-activated ruthenium nitrosyl complexes formulated as $[Ru(NH_3)_4(NO)(X)]^{3+}$ (where X was NH_3, pyridine, imidazole, or $P(OEt)_3$). The effect on NO photolabilty with the variation of X group present at the position *trans* to NO, was investigated in UV light (λ_{max} 300–370 nm). The photolysis experiments were carried out in acidic aqueous solutions. Under acidic conditions, all the complexes were found to be soluble in water.

Porphyrin Based Ruthenium Nitrosyl Complexes

Nitric oxide molecule participates in many biological processes by targeting heme proteins, for instance, myoglobin, Cyt-c oxidase, and guanylyl cyclase enzyme [74, 75].

Ford and group [76, 77] demonstrated the photochemical properties of Ru–NO complexes based on porphyrin scaffold (Fig. 21.2). These complexes served as potential photolabile NO-releasing agents under visible light irradiation. Ford's group has developed a number of such complexes, and their kinetic studies were performed during the light-induced release of NO. Most of the porphyrin-based Ru–NO complexes have been found non-specific for biological applications due to the

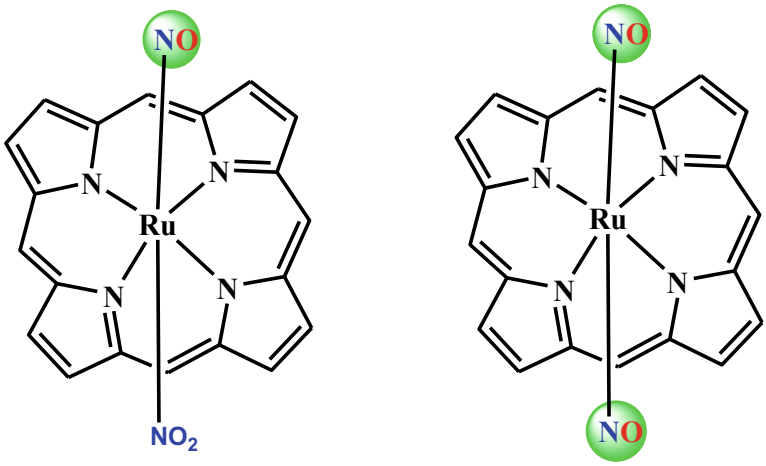


Fig. 21.2 Chemical drawings of Ru–NO complexes **1** and **2**

recombination of NO molecule released during photolysis experiments. Many non-heme-based Ru–NO complexes have been developed to overcome this ambiguity of NO recombination in this endeavour.

Polypyridyl Ligands Based Ruthenium Nitrosyl Complexes

In 2005, Chanda et al. [78] reported several polypyridyl based ruthenium-NO complexes of the type $[Ru(trpy)(L^{1-4})(NO)]^{3+}$ (**3**) (trpy = terpyridine, L^1 = 2-(2-pyridyl)-benzoxazole, L^2 = 2-(2-pyridyl)-benzothiazole, L^3=2-(2-pyridyl)-benzimidazole, L^4 = 1-methyl-2-(2-pyridyl)-1*H*-benzimidazole) (Fig. 21.3). With the variation 2-(2-pyridyl)-azole based auxiliary ligands (L^1 to L^4), the electrophilic effect of nitric oxide has been scrutinized in this report. However, these nitrosyl complexes were not examined to check the photochemical reactivity of NO molecule.

Lahiri, Kaim [79, 80] and the group developed a series of light-activated Ru–NO complexes derived from polypyridyl based ligands. The structural elucidation of these complexes was performed using NMR spectroscopy and X-ray diffraction studies. The electrochemical measurements of reported nitrosyl complexes exhibited one e- reduction of Ru–NO moiety. Meyer and co-workers [81, 82] constructed two bipyridyl based *cis*- and *trans*- derivatives of complex $[Ru(bpy)_2(NO)Cl]^{2+}$ (**5–6**) (where bpy = bipyridine) (Fig. 21.4). The photochemical behavior of NO has been investigated under UV light irradiation in organic solvents. Upon UV light irradiation, NO was immediately released and the solvent coordinated photochemical products, namely $[Ru^{III}(bpy)_2(MeCN)Cl]^{2+}$ and $[Ru^{II}(bpy)_2(MeCN)Cl]^{+}$ have been isolated.

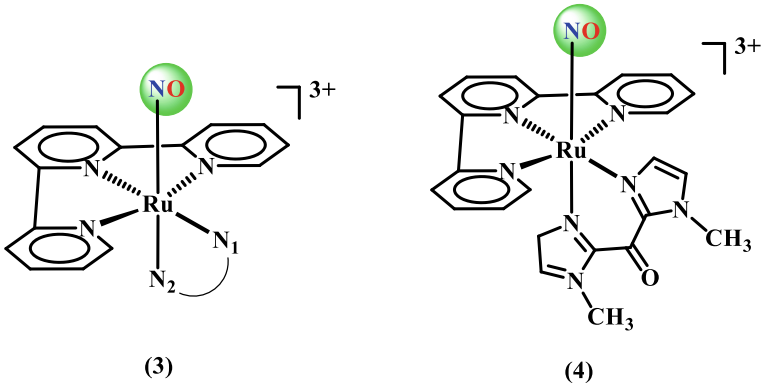


Fig. 21.3 Chemical drawings of ruthenium nitrosyl complexes **3** and **4**

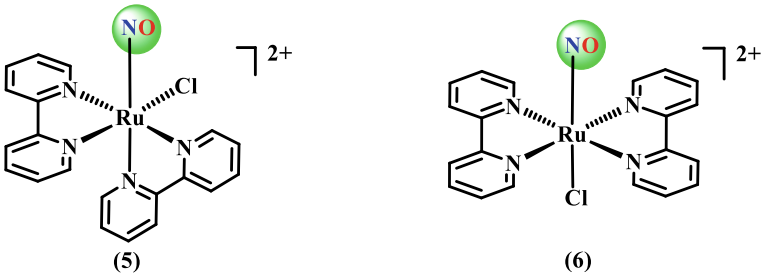


Fig. 21.4 Chemical drawings of ruthenium nitrosyl complexes **5** and **6**

It has been perceived that the majority of photochemical products of Ru–NO complexes result in Ru(III) species. However, some nitrosyl complexes, especially derived from polypyridyl ligands, may also contain Ru(II) after photolysis experiments. The presence of substantial σ donation and π accepting behavior of neutral pyridine ligands may cause considerable stability to Ru(II) species in the photoproducts after photorelease of NO molecule.

Sauaia et al. [83, 84] synthesized binuclear ruthenium nitric oxide complexes formulated as $[Ru(NH_3)_4(L)(pz)Ru(NO)(bpy)_2]^{5+}$ (**7**) in which a pentaammine Ru(II) complex is linked with bipyridine based Ru–NO complex via a pyrazine linker (Fig. 21.5). These complexes were investigated for their application as NO-releasing agents under visible light irradiation. The intense absorption peak near 530 nm has been observed in UV-visible spectra of these complexes. Laser-flash photolysis measurements have been performed to investigate the photochemical behaviour of nitrosyl complexes in acidic buffer solution of pH 4.5.

In the year 2012, Kumar et al. [85] reported a series of polypyridyl based photolabile Ru–NO complexes. The photocleavage of NO molecule was carried out under

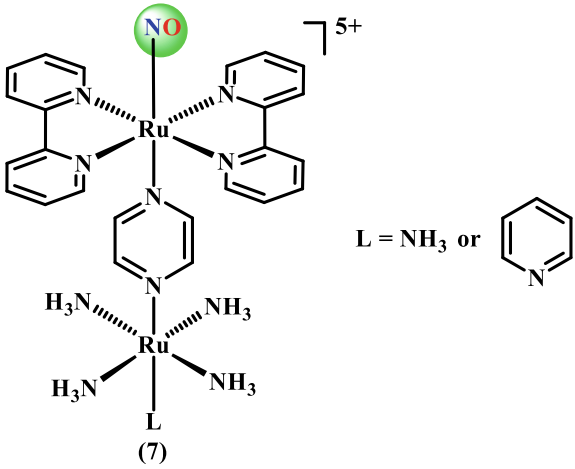


Fig. 21.5 Chemical drawing of pyrazine linked ruthenium nitrosyl complex **7**

UV light of low intensity (λ_{max} ~ 302 nm), and the photo released NO has been trapped by reduced myoglobin. Recently, Malfant and coworkers [86, 87] synthesized ruthenium nitrosyl complexes (**8–10**) based on $[Ru(tpy)(bpy)(NO)]^{3+}$ type core (Fig. 21.6). The photochemical, optical, and electronic properties of these nitrosyls were investigated with a variety of different substituents on pyridine fragments of ligands. TD-DFT calculations were also performed to understand the intramolecular based charge transfer transitions in reported nitrosyl complexes. Ru–NO complexes were found sensitive to visible light, and photoreleased NO when irradiated at $\lambda_{max} = 436$ nm.

In a recent report, Fraix et al. [88] attached a Ru(II)-polypyridyl based photosensitizer (PSs) with bipyridine based NO photodonor (NOPD) to obtain trinuclear molecular assembly (**11**) (Fig. 21.7). They utilized it as one of the most suitable strategies to make therapeutic action more effective towards tumor and bacterial diseases. This strategy also led to the reduction of unnecessary side products. The main focus was to develop the synergic influence stemming from the manifold therapeutic moieties serving with different mechanisms. This strategy may pave the way for interesting avenues to novel therapies which are directly controlled under the light. Such types of PS-NOPDs may display potential applications in therapeutic research areas such as antibacterial and antitumor research fields.

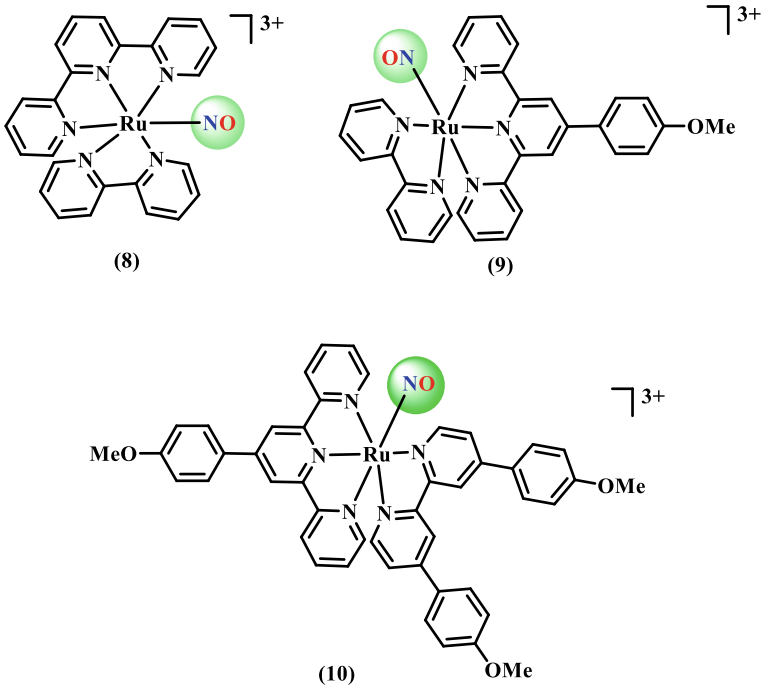


Fig. 21.6 Chemical drawings of ruthenium nitrosyl complexes **8–10**

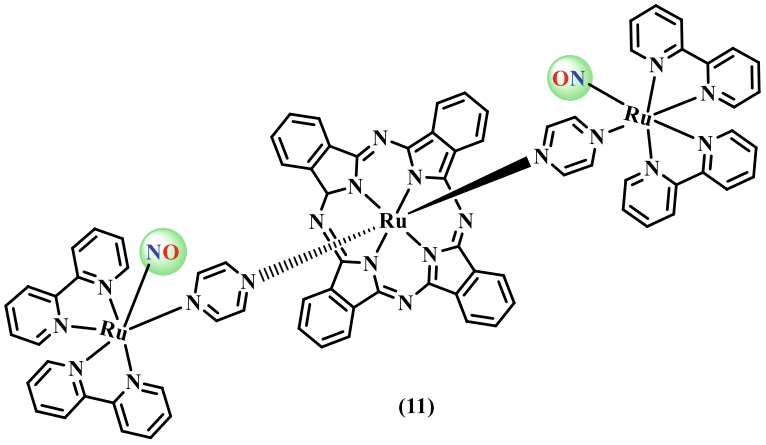


Fig. 21.7 Chemical drawing of PS-NOPD ensemble **11**

Imine and Carboxamido Ligand-Based Ruthenium Nitrosyl Complexes

Works et al. constructed [88] many non-heme based model Ru–NO complexes of type **12** (where X stands for chloride ion, nitrite ion, or water) (Fig. 21.8). These complexes were derived using tetradentate salen (bis-(salicylidene)-ethylenediamine) ligand. The photolytic behaviour of these nitric oxide complexes has been investigated in both aqueous and organic-based solvents. However, some nitrosyl complexes exhibited a reversible binding of NO during the photolytic experiments under UV light irradiation. Authors have established that the rate of recombination of NO was fast in organic solvents such as THF and toluene; however, the reversibility of NO was slow in acetonitrile solution.

To prevail over this setback of NO recombination, Koch et al. [89] reported a polymer matrix incorporated with salen based Ru–NO complex for a controlled lightinduced delivery of NO (Fig. 21.8). Ruthenium nitrosyl complex **13** containing *O*-phenylvinyl moiety has been synthesized, which was covalently linked into the polymer matrix (methacrylate polymer). The photolytic behavior of the hybrid material was quite similar to the parent salen based nitrosyl complex **13**. Upon exposure to UV light of low intensity ($\lambda_{max} = 370$ nm), NO was photo released from the polymer-based material and was trapped by reduced myoglobin under physiological conditions.

Mascharak's group [90, 91] developed polypyridyl based light-sensitive Ru–NO complexes $[(SBPy_3)Ru(NO)]^{3+}$ (**14**) and $[(PaPy_3)Ru(NO)]^{2+}$ (**15**) (Fig. 21.9). These complexes were found sensitive to high-intensity UV light and were quite suitable for their applications in biological media. The influence of strong σ-donating –CO–NH-group onto the photochemical nature of NO molecule has also been demonstrated in the report. Due to the presence of σ-donor carboxamido nitrogen, the product of $[(PaPy_3)Ru(NO)]^{2+}$ was stabilized in Ru(III) oxidation state. On the other hand, the photoproduct of nitrosyl complex $[(SBPy_3)Ru(NO)]^{3+}$ contained Ru(II) center under the same reaction conditions. The photoliberated NO has successfully been delivered to heme-enzymes, i.e., myoglobin and cytochrome *c* oxidase enzyme. The amount of photoinduced NO was monitored with the help of a nitric oxide sensing device.

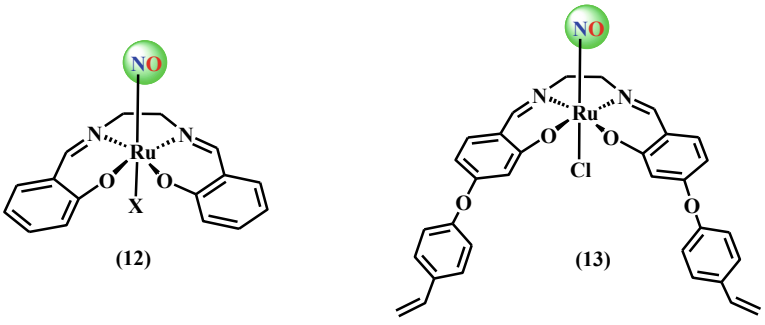


Fig. 21.8 Chemical drawings of salen based ruthenium nitrosyl complexes **12** and **13**

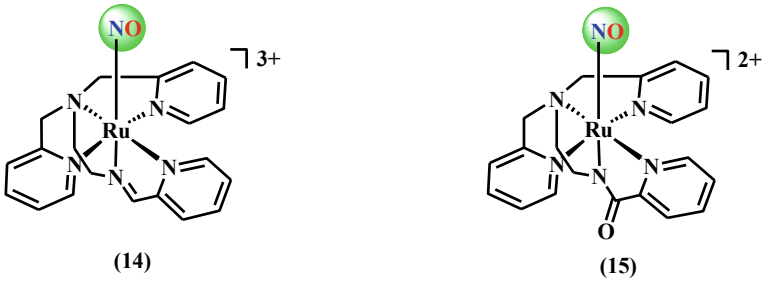


Fig. 21.9 Chemical drawings of imine based ruthenium nitrosyl complexes **14** and **15**

During photolysis experiments of different ruthenium nitrosyl complexes, it has been observed that the photolytic cleavage of NO is directly influenced by the substituents present on the ligand framework. This group has established this fact by measuring the amount of photoreleased NO in the presence of different substituent(s) on the ligands [66, 67]. The sensitivity of many nitrosyl complexes towards the low-intensity light in visible and/or NIR region has been investigated with the alteration of the substituent(s), donor sites, or the extent of conjugation in the ligands. Because of their sensitivity in visible and near-infrared red light, Ru–NO complexes were reported as potential candidates for PDT in cancerous cells.

The same group described a report on carboxamido based Ru–NO complex [Ru(Me_2bpb)(NO)Cl] (**16**) (Fig. 21.10). Complex **16** was investigated for its applications in photodelivery of NO under UV light illumination [33, 66]. In particular, the focus was aimed at the sensitization of this Ru–NO complexes towards the visible region. To do this, an extended conjugation was employed in the ligand framework of complex **16** to obtain a new Ru–NO complex **17**, which was found to be more sensitive to low-intensity visible light.

Mascharak's group [92] has successfully coordinated an intense red resorufin dye with the Ru center of nitrosyl complex **17** (Fig. 21.11). Substitution of Cl atom with resorufin dye resulted in complex **18**, which displayed an absorption band near

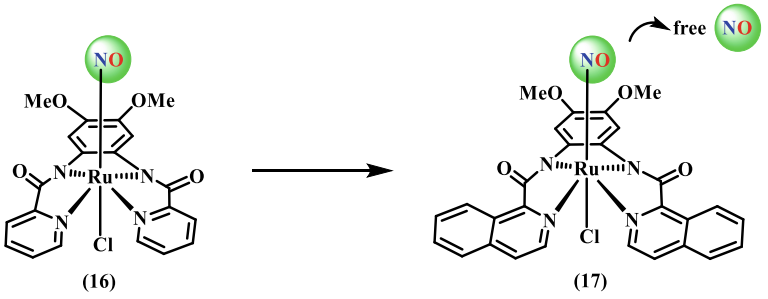


Fig. 21.10 Employment of extended conjugation in carboxamido based Ru–NO complex **16**

500 nm in its UV-visible spectrum. The resultant nitrosyl complex was highly sensitive towards low-intensity light in the visible region and released NO when illuminated with $\lambda_{max} \geq 455$ nm. A high quantum yield value in the visible area has been ($\phi \sim 0.05$ at λ_{max} 500 nm) observed due to the conjugation of Resf dye in nitrosyl complex. The amount of NO released during the photolysis experiment has been measured using a NO sensitive electrode device. The Resf dye-based nitrosyl complex **18** has also been investigated for its application in the cellular matrix as it displayed strong luminescence due to the conjugation of Resf dye. To induce programmed cell death, NO molecule was photodelivered towards cellular targets under physiological conditions.

In the year 2014, Ghosh et al. [93] developed two imine functionality based light activated ruthenium nitrosyl complexes (**19–20**) (Fig. 21.12). These complexes were found sensitive towards visible light and photoreleased NO upon UV and visible light exposure. The photolysis experiments have been investigated using absorption spectral studies, myoglobin trap and Griess reagent assay experiments.

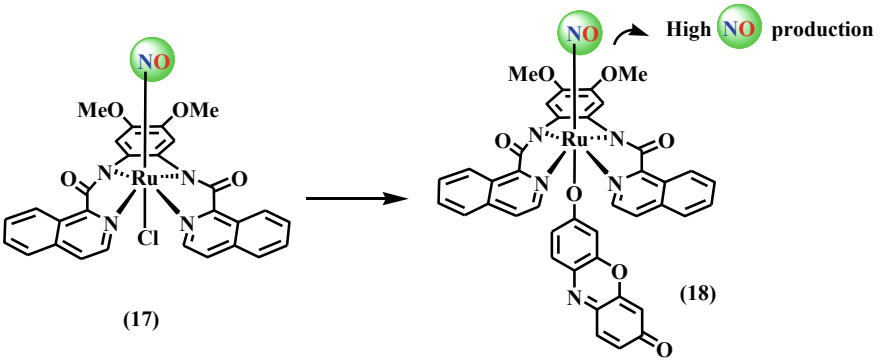


Fig. 21.11 Incorporation of luminescent bright red resorufin dye into ruthenium complex **18** to enhance its sensitivity towards visible light

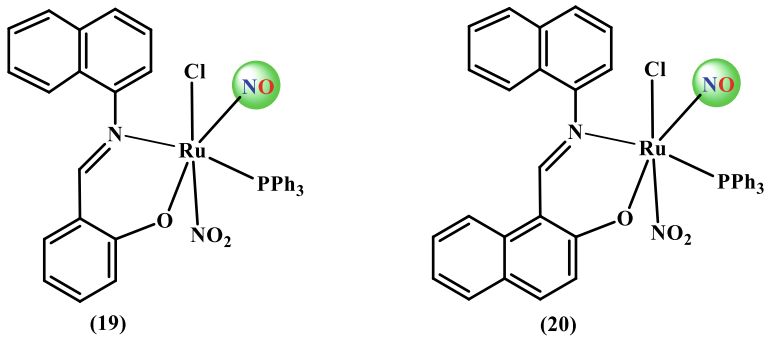
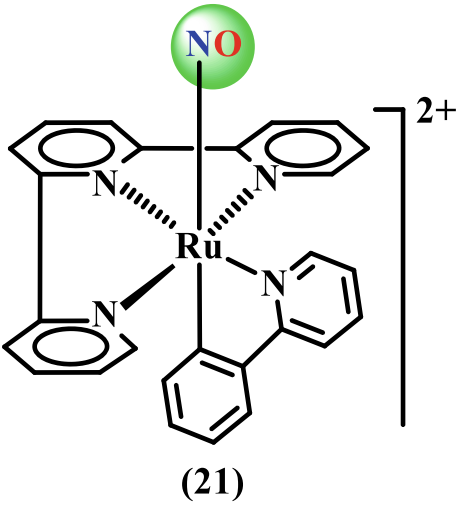


Fig. 21.12 Chemical drawing of synthesized ruthenium nitrosyl complexes **19** and **20**

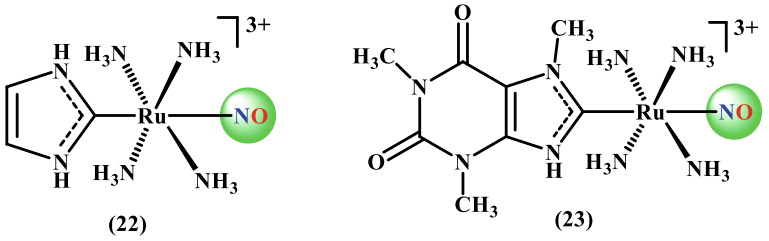
Organometallic Ru–NO Complexes

Hadadzadeh et al. [94] reported a terpyridine based organometallic Ru–NO complex $[Ru(phpy)(trpy)(NO)]^{2+}$ (**21**) in the year 2002 (Fig. 21.13). Complex **21** was characterized using standard spectroscopic techniques such NMR, FT-IR, and electronic absorption studies. However, this complex has not been employed for photochemical studies of NO in this report. In 2008, Holanda et al. [95] demonstrated several photolabile organometallic Ru–NO complexes formulated as $[Ru(L)(NH_3)_4(NO)]^{3+}$ (**22–23**) (L = imidazole or caffeine) (Fig. 21.14). The differential-pulse voltammetric, as well as electronic absorption studies, were performed to investigate the photolytic cleavage of NO from reported nitrosyls. The photoproducts having metal in +3 oxidation state were established using the electron paramagnetic resonance (EPR) technique.



(21)

Fig. 21.13 Chemical drawing of organometallic ruthenium nitrosyl complex **21**



(22) (23)

Fig. 21.14 Chemical drawings of imidazole based ruthenium nitrosyl complexes **22** and **23**

Ghosh and group [96–98] have synthesized a series of photoactive ruthenium nitric oxide complexes to perform the photolysis experiments for NO dissociation under light illumination (**24–27** in Fig. 21.15). The photochemical behavior of NO was observed with different substituent(s) in the ligand frames of these Ru–NO complexes. Nitric oxide reactivity and scavenging studies with different ruthenium complexes were investigated and the photoreleased NO was transferred to reduced myoglobin. The amount of liberated NO from nitrosyl complexes has been detected by using Griess reagent assay. Amount of the free NO as well as other reactive oxygen and/or reactive nitrogen species was also estimated by DPPH radical quenching assay using UV-visible spectrophotometer. Majority of these Ru–NO complexes were found sensitive for the liberation of NO in the presence of visible light of electromagnetic radiations. The liberation of nitric oxide was also investigated through myoglobin trapping experiment under physiological conditions.

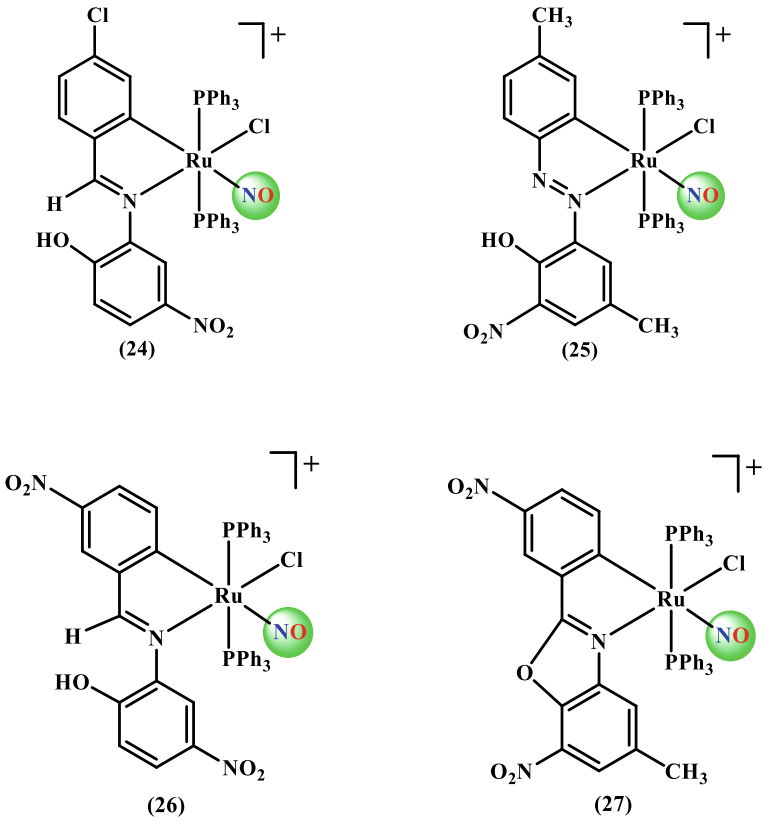


Fig. 21.15 Chemical drawing of ruthenium nitrosyl complexes **24–27**

Conclusions

The present book chapter covers photolabile ruthenium nitrosyl complexes used for light-induced delivery of NO molecule under UV and visible light illumination. We have considered several types of NO donors, such as carboxamido, imine, polypyridyl, and organometallic scaffold-based Ru–NO complexes. The biological significance of photoreleased NO has been discussed at length, and its applications encompass various research domains such as medicines, biology, and analytical chemistry.

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Chapter 22
Nitric Oxide Regulation in Microparticles

Abhinav Singh, Himalaya Singh, and Jagavelu Kumaravelu

Abstract Nitric oxide (NO) has been widely shown to have a varied roles physiologically. Nitric oxide presence in microparticle has been gaining importance due its circulatory in nature and representing the parental cell characteristics. Microparticles possess several organelles, functional signaling mediators, mitochondria, mRNA and cytokines which contributes in the normal physiology as well in the disease progression. Endothelial microparticles respond to variety of stimulants and acts as vector for communicating with distant cells leading to efficient signaling. Here we describe the various roles of microparticles in different physiological settings and its method to evaluate it both in vitro and in vivo using pertinent techniques. Current trends in research lead to the understanding of NO signaling in EMP generation and its role in liver and cardiovascular health and disease has opened up new avenues. Furthermore, the application of microparticles as drug delivery, and targeted therapeutics is the way forward.

Keywords Endothelial cells · Microparticles · Mitochondria · Angiogenesis · Myocardial ischemia · Cardiovascular diseases · Inflammation · Vascular biology

Introduction

Before 1980 Nitric oxide [1] is described as an air pollutant and its role in human physiology is been widely explored after the acetylcholine treatment in endothelial cells resulted in the release of a highly diffusible species which is described as endothelial-derived relaxing factor (EDRF) by Furchgott and Zawadzki in 1980 [2]. Ignarro, Palmer, and colleagues in 1987 [3, 4] called it nitric oxide (NO), which is a diatomic, cell-permeable molecule and stimulates its receptor guanylatecyclase

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A. Ray and K. Gulati (eds.), *Nitric Oxide: From Research to Therapeutics*,
Advances in Biochemistry in Health and Disease 22,
https://doi.org/10.1007/978-3-031-24778-1_22

(sGC) in vascular smooth muscle [4]. In 1992, NO was crowned as the "Molecule of the Year" for various roles as a signaling molecule in the cardiovascular system.

NO role is widely explored in vitro models where only limited role is explored, unlike test tube chemistry where a single NO molecule is studied in a regulated condition, whereas in vivo NO chemistry is much more complex. Identification of NO as a player in human physiology lead to ascertain several other novel roles like NO and its targets and NO diffusion in the cellular membrane. In the body, NO is generated by an enzyme called Nitric Oxide Synthase (NOS), which has three isoforms; neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and third Endothelial NOS (eNOS or NOS3) [5]. Primarily oxidation of L-arginine by NOS enzyme gives NO and citrulline, iNOS, and nNOS are mostly found in the cytosol, whereas eNOS by palmitoylation and myristoylation binds to the membrane. With the advance in research the understanding of NO signaling in EMP generation and its role in liver and cardiovascular health and disease has opened up new avenues.

Endothelial Microparticles

Most cells release some small microparticles during overt cell activation due to inflammation or apoptosis. Injury to vascular endothelial lining releases a variety of inflammatory markers and microvesicles. Endothelial microparticle (EMPs) generated from monolayer vascular endothelial cells range from 100 nm to 1 μm diameter and express CD31, CD34, CD51, CD62E, and CD146 markers. Vesicles released or shed from plasma membrane having a size less than 1 μm diameter are characterized as microparticles [6]. Early reports regarding microparticle were during the 1940s by Chargaff and West [7] they reported it as a subcellular particle-like structure resembles a pro-coagulant factor in human serum. The advent of electron microscopic techniques revealed the functional behavior of subcellular structure by facilitating thrombin generation in the same manner as platelets [8]. The role of platelet microparticle in disease progression was initially reported in 1975 in 21 idiopathic/autoimmune thrombocytopenic purpura patients [9]. Further evidence was provided through in vitro studies that activated platelets released MP bind to the vascular wall [10] (Fig. 22.1).

The microparticles are mainly composed of membranes, cytoplasmic contents (RNA, lipids, fragments of organelles, transcription factors, etc.) which characterized the source of the microparticle. MPs are mainly released from endothelial cells or from blood cells, which makes them a heterogeneous population [11]. MPs freely circulate in the blood and can be a reason for many pathological disorders. The basic mechanism of MPs generation to date is through apoptosis, necrosis, inflammation [12]. MPs are considered as the markers of endothelial cell damage and are considered to initiate platelet activation [13]. EMPs are without a nucleus and small size vesicles which are released from cells in the response of stimuli, for example, ischemia, hypoxia, inflammation, and prothrombotic or pro-apoptotic factors. Once microparticles enter into circulation these modulate several biological and pathophysiological

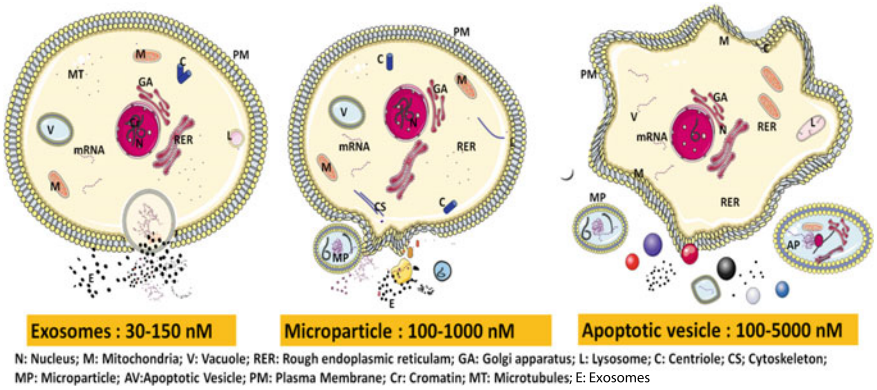

Fig. 22.1 Classification of extracellular vesicles from parental cell. These vesicles contain varied cytosol proteins, mRNA, microRNA, degraded proteins, genetic material, phospholipids and cellular organelles of the parental cells. Apoptotic vesicles harbor DNA, microRNA, phospholipids and degraded proteins

mechanisms of various diseases. In the 1940s microparticles was considered as cell dust particle shows procoagulant activity because of its negatively charged phosphatidylserine membrane, it attracts clotting protein such as prothrombin, Factor VII, IX, and X (Table 22.1 and Fig. 22.2).

Nitric Oxide and Endothelial Microparticles

NOS in EMP Mediated Inflammation

EMPs generation could be induced by several stimulants like IL-1, LPS, TNFα, calcium ionophore, plasminogen activator inhibitor-1, and C-reactive protein (CRP). Interestingly, eNOS decoupling mechanisms might participate, under certain conditions, in the production of EMPs emphasizing the common complementary relationships between endothelial microparticle and NO-dependent endothelial dysfunction [16, 17]. Several studies have reported that altered function of eNOS can cause endothelial dysfunction and release microparticles in the blood circulation. Patients with cardiovascular disorders have a high level of EMPs [18]. Microparticle causes transcellular delivery of arachidonic acid and activates platelets and EC resulting in increased binding of monocytes to endothelial cells [19]. Similarly, neutrophil-derived MPs exposure on endothelial cells causes the release of pro-inflammatory cytokines like IL-6, monocyte chemotactic protein, IL-1β, TNFα, and IL-8 from the endothelial cells [14]. Microparticles also contains partial cell membrane from parental cells, which act as ligands and promotes the adhesion of inflammatory

Table 22.1 Classification of cells producing Microparticles and its target

Type of cell producing MPs	Target cells	Molecules involved in the pathogenesis of inflammation mediated by MPs	References
Cytokines			
Endothelial	Various inflammatory cells	IL-1β and TNF-α	[14]
Leukocyte	Endothelial	IL-6 and MCP-1	
T Cells		IL-8, TNF-α and IL-1β	
Adhesion molecules			
Monocyte	Endothelial	Intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin	[15]
Lipids			
Platelets	Endothelial	Thromboxane A2 and cyclooxygenase	

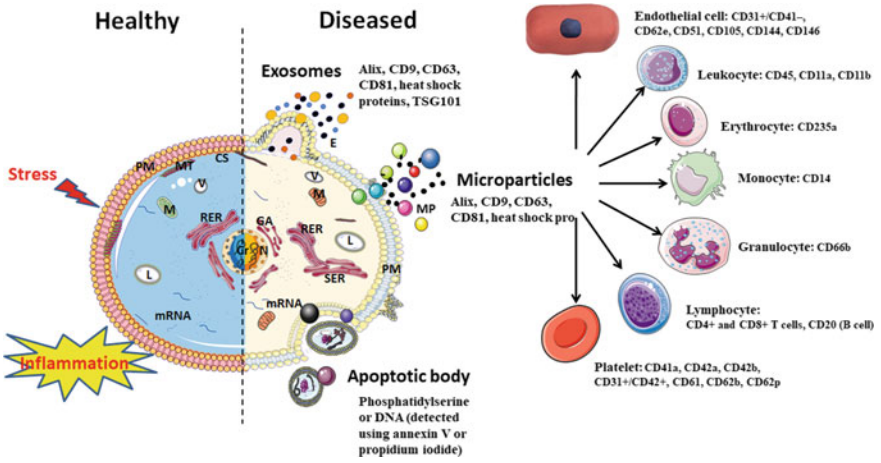

Fig. 22.2 Schematic representation of healthy and diseased cell, showing released microparticles and its surface biomarkers

cells to the endothelial layer through increased expression of E-selectin, intercellular adhesion molecule-1 (ICAM), vascular cell adhesion molecule-1 (VCAM) [15, 20]. Bacterial infections cause high circulatory levels of MPs which activates the endothelial cells resulting in ERK1/2 phosphorylation and activation of ICAM1, VCAM1, E-Selectin, and NFkB (Nulcear Factor) pathway through IL-1β and NLRP3 inflammasomes [15].

NO in EMP Mediated Vascular Function

Damaged endothelial cells or apoptotic endothelial cells results in the microparticle formation comprising a dynamic storage pool of bioactive molecules. MPs have been shown to signal dynamically and transfer proteins, mitochondria, nucleic acids, and mRNA [21, 22]. EMPs were reported to play a dual role in many diseases like myocardial infarction, cardiac hypertrophy, liver cirrhosis, atherosclerosis, and diabetes. Patients with portal hypertension have reduced NO levels and eNOS activity resulting in skewed hepatic vascular tone due to inconsistency in the intrahepatic vasodilators and vasoconstrictors [23] (Table 22.2).

EMP in Cardiovascular Diseases

In the heart, the role of MPs are widely explored in comparison to other diseases. Endothelial dysfunction is known to induce EMP generation and vice-versa. Patients with valvular heart disease (VHD) undergoing cardiac surgery have increased pro-inflammatory protein resulting in impaired endothelial function and vasodilation [35, 36]. A proteomics study showed EMP generated through different stimulus has distinct protein compositions [37, 38]. In bicuspid aortic valve [13] disease patients, the circulating EMPs positive for CD31 and CD62E were increased resulting in aortic stenosis [39]. Similarly, reactive oxygen species are known to induce endothelial dysfunction. A subunit NADPH, p22phox is detected in EMPs which plays an active role in superoxide formation and ROS generation [19]. In hypertension, excessive generation of oxidative stress and ROS formation are linked with MPs. Patients with heart valvular disease have impaired endothelium-dependent vasodilation by uncoupling and inhibiting endothelial nitric oxide synthase (eNOS) exacerbated by the released EMP. A study done in rats and mice showed that microparticles generated from endothelial cells, when injected in animals induce acute lung injury, inhibit angiogenesis, and impaired vasodilation [40]. Cardiovascular diseases have reduced neovascularization, EMPs were shown to possess neovascularization factors like PMP, plasmin formation from endothelial cells might activate tubulogenesis [41].

Table 22.2 Techniques used for EMPs characterization in research settings

S. no			References
1	**General characterization**	Microscopy Bead-based flow cytometry Western blot (mainly for cell culture media) Multiplex bead-based platforms Surface Plasmon resonance Fluorescence scanning	[24] [25] [26] [27] [28] [29]
2	**Quantative**	**Particle number** High-resolution bead-based flow cytometry Cryo-electron microscopy **RNA quantification** Bioanalyzerpico chip Quant-iTRiboGreen RNA Assay Quantitative reverse transcription polymerase chain reaction (qRT-PCR) **Total protein count** Colorimetric assays Fluoremectric assays Protein stain on SDS-PAGE **Specific protein count** ELISA Bead-based flow cytometry Aptamer- carbon nanotubes colorimetric assays	[24] [30] [31]
3	**Single vesicle characterization**	**High-resolution imaging technique** Confocal microscopy Transmission electron microscopy Scanning electron microscopy Cryo-electron microscopy Atomic force microscopy Super-resolution microscopy **Estimation of biophysical features** Resistive pulse sensing Nanoparticle tracking analysis High resolution flow cytometry Asymmetric flow field fractionation Raman spectroscopy	[15] [24] [32] [33] [34]

EMP in Hepatic Diseases

Stressed hepatic cells and other cells shed MPs into the extracellular-biological fluid and circulation, their presence in large quantity makes them compelling entities for liquid biopsy or 'fluidome'. Extracellular vesicles from the circulation system are attractive biomarkers for estimating disease pathology including alcoholic and non-alcoholic steatohepatitis, viral hepatitis B and C, liver steatosis and cirrhosis, primary

hepatocarcinoma, and acute liver failure by analyzing contents of extracellular vesicles, the severity of the disease can be predicted, which helps in the diagnosis and prognosis of liver diseases [42]. Biologically active hedgehog ligands present in microparticles induces hepatic sinusoid endothelial cell remodeling during chronic cholestatic liver injury [43]. Similarly, liver cirrhosis patients shows an elevated circulating levels of pan-leukocyte-derived CD11a(+) MPs, leuko-endothelial-derived CD31(+)/41(-) MPs, erythrocyte-derived CD235a(+) MPs and lymphocyte-derived CD4(+) MPs. Inflammatory cell markers such as CD4(+) or CD8(+) T cells, CD14(+) monocytes, and iNK cells were highly expressed on plasma MPs of NAFLD and other liver disease patients [44, 45]. A similar phenomenon is observed when the neutrophils are treated with MPs from alcoholic hepatitis patients triggers more TNFα production, ROS generation, and cytokines regulation in vitro condition [45]. Steatotic hepatocytes in NAFLD and NASH shows an increasing number of hepatocyte derived MPs in blood circulation which are internalized by the endothelial cells and activates angiogenesis in mice [46]. Concurrently, portal myofibroblasts (PMFs) releases MPs containing VEGF-A, which promotes vascular remodeling by activating VEGFR2 in endothelial cells leading to angiogenesis by the release of proangiogenic MPs [47]. The noninvasive biomarkers of hepatic cirrhosis like cytokeratin-18 (CK-18) and soluble CK-18 fragments are carried by microparticles in blood circulation and could be used in liquid biopsy [48, 49].

EMP as Double Edge Sword

Beneficial	Detrimental
Microparticles acts as vectors to exchange biological information between cells (intercellular communication) [50, 51]	"Microparticles from polymorphonuclear leukocytes contain the functionally active anti-inflammatory protein annexin 1, and annexin 1–containing microparticles inhibit the interaction between leukocytes and endothelial cells and in an animal model" [52]
Microparticles release protects against the external stimuli or stimuli faced by cells [53]	Problems persists in optimizing the isolation protocols for ExMVs and their characterization
The microparticles play a role in "cellular waste management" because they contain increased concentrations of chemotherapeutics, oxidized phospholipids, or caspase 3 [50, 51, 54]	Centrifugation is absolute for MP recovery from blood sample. Rigorous centrifugation may cause artificial shedding of cell particles
	MPs rapidly gets cleared from pheripheral blood, and thus there is a need to develop strategies to extend their life span so that they can fulfill their therapeutic goals [55]

Potential Action of NO During Diseases

The oxidation of L-arginine by calcium-dependent NOS results in NO production and eNOS is a major isoform which facilitates the physiological NO production. Imbalance in NOS production cause dysregulated NO synthesis resulting in reduced NO bioavailability and may lead to superoxide generation causing oxidative stress and endothelial dysfunction [56, 57]. Circulating endothelial microparticles (EMPs) generated from various cells in varied diseases play an important role in causing inflammation, vascular injury or acts as trans-cell messengers. MPs comprises cytosolic and nuclear proteins, RNA, miRNA, and the surface receptors of their parental cell. The specific cell signature of MPs is characterized by its origin and these signatures are delivered to another cell via circulation and can cause inflammation. [58, 59]. Numerous studies, shows that MPs in various body fluids including circulation, urine [60], saliva [61], bile [62], synovial fluids [63], semen [64], atherosclerotic plaques [65], liver tissues [66], and lungs are playing role in disease pathology and regulation, MPs were detected in the circulatory system in abundance from above all mentioned biological fluids. MPs generation by phospholipid asymmetry is one of the known mechanism, when stimulated there is loss of active phospholipid transmembrane enzymatic imbalance of floppase, flippase and scramblase. The cellular activation by any stimulus or apoptosis results in sudden release of intracellular calcium by the endoplasmic reticulum resulting in changes in transmembrane steady state. This eventually activates the cytosolic enzyme calpain, which leads to cytoskeleton filaments cleavage resulting in blebbing and shedding of membrane-derived EMPs into the circulation [67, 68] (Table 22.3).

Table 22.3 Effects of NO concentration on physiological activities [16]

NO concentration (nm)	Physiological results
<1–30	Promotes cell survival and proliferation
30–60	Protects cells from apoptosis
100	Protects tissue from injury
400	Mediates cell cycle arrest
>1000	Apoptosis and full cycle arrest, kills bacterial biofilms

Role of NO Mediated Cellular EMP Generation and Functions

Cell Migration

Microparticles have been shown to play an important role in the cell migration, an integral part of many cellular mechanisms like angiogenesis. And endothelial cells harbor metalloproteinase that deliberately carry out proteolytic activity essential for cell migration which is a crucial step for the angiogenesis.

A recent study demonstrates accumulation of gelatinase B in human microvascular endothelial cells. These gelatinases are matrix metalloproteinase's that degrades the collagen of the basement membrane produced by endothelial cells. Since, microparticles contain matrix metalloproteinases (MMP's) which focalizes proteolytic activity necessary for migration and neovascularization [69, 70]. Microparticles also harbor their natural inhibitors in their latent forms, tissue inhibitors of MMP's (TIMP's). These findings suggest that gelatinase B secreted from endothelial cells during angiogenesis locally degrades the basement membrane and the ability of endothelial cells to accumulate gelatinase B enables cells to carry out proteolytic activity which is essential in cell migration processes [71].

In contrast, microparticles in atherosclerotic plaque containing inflammatory factors induce the monocyte adhesion to the endothelial cells and enhances the transendothelial migration which is responsible for promoting the development of plaque [72]. During the tube-formation phenomenon as well, the cell migration of endothelial cells is also promoted by the platelet derived microparticles which is inhibited in response to the inhibitors VEGF and heparanase [73]. However, the process of cell migration was disrupted in an in-vitro setting, while in the presence of growth factors such as VEGF and bFGF-2, endothelial derived microparticles were found to be involved in disruption of tube formation in endothelial cells [74].

Angiogenesis

Various recent studies have established a link between endothelial microparticles and angiogenesis where endothelial microparticles at known pathophysiological conditions impair angiogenesis. Very megre information is available regarding the role of endothelial derived microparticles in the vascular functions and the impact of microparticles in angiogenesis has not been meticulously investigated.

Angiogenesis regulation by MPs was first shown in platelets, which are known carriers of angiogenesis factors and platelet mediated particles (PMPs) were possessing similar bioactive lipids to initiate tubulogenesis in human endothelial cells. This ability of microparticlesis linked to the activation of PI3K and ERK1/2 pathways [73]. Reports also suggests that with the involvement of factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF),

platelet-derived growth factor (PDGF), heparanase platelet based microparticles enhance endothelial cell migration and tubule formation [75]. This has been well validated when platelet microparticles were been injected locally after the induction of myocardial ischemia in rats happen to increase the angiogenesis in the ischemic region and this effect was eliminated with the VEGFR inhibitors and by inhibiting heparanase [76].

Conversely, Lymphocyte-based microparticles possess the ability to induce the release of Nitric Oxide (NO) from the endothelial cells which may contribute to its pro-angiogenic properties. This may however may lead to the oxidative stress related with the decreased release of NO which is associated with the development of the anti-angiogenic effects [77]. On the other hand leukocyte based microparticles derived from apoptotic human T-cells are reported to develop anti-angiogenic effects by repressing the formation of sprouts in microvessels of aortic ring in-vitro and corneal neovascularization, whereas under in-vitro condition the effect observed due to the downregulation of phosphorylation of ERK1/2, VEGFR-2 expression and production of Reactive Oxygen Species (ROS) [76]. Similarly, microparticles derived from T-lymphocytes reduced the migration of endothelial cells [78].

Angiogenic effect observed in microparticles derived from endothelium involves the activation of matrix metalloproteinase and remodelling of extracellular matrix. Since, endothelial cells express the microparticles that contain matrix metalloproteinases which focalizes proteolytic activity necessary for neovascularisation. But high levels of endothelial microparticles can induce the production of ROS leading to endothelial dysfunction [71, 79]. This is also involved in regulation of differentiation of progenitor cells despite of production of ROS and over expression of NADPH oxidase [80]. Endothelial progenitor cell based microparticles express various adhesion molecules that are involved in internalization, once internalized it activates the eNOS and PI3K/Akt signalling pathway. This also favours the regeneration during ischemic insult [22]. Macrophage based microparticles also happens to promote angiogensis inside human atherosclerotic lesions regions via CD40 ligand that induces endothelial cell proliferation [65].

Inflammation

Inflammation triggers the activation of coagulation pathways and happens to increase various procoagulant factors, diminishes fibrinolytic responses and inhibits endogenous anticoagulants. The microparticles are mixed population of small fragments of membrane shed from different kind of cells. And one of the primary sources of circulating microparticles is the endothelium and microparticles acquired from blood are regarded as the biomarkers of the vascular inflammation and injury. Many documented studies have reported that microparticles are involved in the endothelial dysfunction which is believed to be caused by the disturbed release of nitric oxide (NO) from vascular endothelial cells and cause the remodelling of the vascular tone [81]. Nitric oxide (NO) is the chief endothelium-derived relaxing factor which plays

a significant role in maintaining vascular tone and also in the inhibition of inflammation, coagulation and oxidative stress [82]. Microparticles can have an effect on both the pro-inflammation and anti-inflammation processes in endothelial cells. Phospholipase A2 binds with microparticles as their preferred site of binding and subsequently triggers the inflammatory processes and platelet aggregation by releasing lysophosphatidic acid [83, 84].

It has been reported that platelet-derived microparticles increases cylcooxygenase-2 enzyme by transporting arachidonic acid and elevates the expression of ICAM-1 which induces membrane-linked signaling cascade [85, 86]. Microparticles aids the interaction between leukocytes and endothelium, whereas leukocyte based microparticles contributes in releasing various endothelial and monocytic cytokines including IL-6, IL-8, TNF-α. Altogether, these events contribute to the processes of inflammation and dysfunction of the endothelium and may also facilitate the development and progression of the atherosclerosis.

Upon evaluation of plasma concentration of patients suffering from arteriosclerosis, elevated levels of IL-6 was observed that can be associated with increased expression of platelet derived microparticles and P-selectin [87]. In addition to that platelet derived microparticles assists the recruitment of a number of immune cells like NK cells, T-lymphocytes, B-lymphocytes, monocytes [88]. The pro-inflammatory effect on the other hand is possibly associated with the activation of the receivers of platelet-activating factor (PAF) by the oxidized phospholipids recruited on the lymphocytes and endothelial cells [89]. A study shows leukocyte based microparticles when incubated with HUVEC's resulted in a de novo synthesis of cytokines like IL-8, IL-6 and adhesion molecules suggesting that microparticles can be the agonists of the inflammation [90].

A latest study shows that contraction induced by arachidonic acid and methacholine in aorta and rabbit pulmonary arteries was exasperated by platelet-derived microparticles which was blocked by inhibitors of thromboxane receptor and thromboxane synthase suggesting that platelet-derived microparticles may be involved in regulation of the vascular tone and possibly in the development of the inflammatory diseases [91]. In another recent finding carried out in murine aorta, the apoptotic microparticles derived from T-cells induced expression of inflammatory genes in smooth muscle cells via activation of NF-κB pathway and this leads to excessive over-production of the Nitric Oxide (NO) and prostacyclin due to the vascular hyporeactivity that is further linked with the induction of expression of pro-inflammatory enzymes such as iNOS and COX-2 and due to this microparticles during the inflammatory diseases upholds the vascular dysfunction [92]. Similarly, platelet-derived microparticles expresses considerable amount of an inflammatory cytokine, RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted) and elicits monocyte adhesion by depositing it on endothelial cells.

Oxygen Radical (O_2^-)

Amongst the various responses elucidated by endothelial derived microparticles in endothelial cells is the production of reactive oxygen species (ROS) which commonly is observed to be produced via mitochondrial and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. And redox-sensitive processes of endothelial microparticles have an effect on endothelial cells for which underlying mechanisms is still not very clear. There are several studies that have demonstrated the association of endothelial NO and oxidative stress. Microparticles have an effect on the vascular tone by impairing the release of NO from the endothelial cells. This can be related to the limited activity of eNOS that depends on the ERK1/2, PI3K and NF-κB signalling pathways. This has been validated experimentally in aorta of mice which was induced with T-lymphocyte derived microparticles, which demonstrates that acetylcholine-induced endothelial relaxation occurs as a result of diminished nitric oxide (NO) and enhanced ROSproduction [93].

Likewise, a study demonstrated that microparticles derived from activated monocytes had a detrimental effect on endothelial function as when the microparticles are incubated with endothelial cells resulted in the generation of NO which does not have any effect on production of superoxides. Hence, the conclusive remarks include that microparticles via activation of calveolin-1, ERK1/2 and PI3K can induce nitrosative stress by elevating the nitration of numerous proteins in endothelial cells [94].

Similarly, Endothelial Microparticles (EMP's) impairs endothelium-dependent relaxation in aorta of rat due to over-production of superoxide in aortic rings that might contribute in diminishing the NO bioavailability [19]. EMP's also possesses traceable amounts of nicotinamide adenine dinucleotide (phosphate) oxidase NADPH oxidase and superoxide as well. EMP's also elevates the expression cell adhesion specific proteins [95]. Upon, in-vivo injection of mice from the patients suffering from metabolic syndromes, it was observed that expression of eNOS was reduced and impairment in the endothelium-dependent relaxation in aorta. It happens to induce vascular dysfunction as a result of increase in NO and ROS production and by modified cyclooxygenase metabolites and MCP-1 (monocyte chemotactic protein-1) via Fas/Fas-ligand pathway.

Recently, a study investigated the formation of endothelial based microparticles is stimulated by Ang-II that is activated via Rho kinase and NADPH oxidase. Endothelial cell includes numerous lipid rafts that contribute to the production of the microparticles and is a redox-sensitive effect. Other signalling molecules that contribute in the production of these microparticles include p38 mitogen-activated protein kinase, IL-1, MAPKAPK2 [20, 96]. Ang-II triggers the generation of microparticles via angiotensin-I receptor through redox-sensitive signalling pathway and stimulates the oxygen radical production and translocation of Rho kinase which is extremely detrimental to the endothelial cells [97, 98]. Microparticles in turn exhibit pro-oxidative response in endothelial cells via an EGFR- dependent pathway.

One hypothetical basis for superoxide formation by microparticles is uncoupling of eNOS which results in a switch leading to formation of superoxide from NO-producing enzyme [99]. Additionally, inhibition of eNOS partially hinders the release of superoxide induced by microparticles suggesting regulatory role of the enzyme in generation of superoxide of by endothelial cells. Altogether, these revelations gives an idea of endothelial based microparticles being a significant pathogenic factor.

Cytotoxicity

Microparticles imitate the profile of the cells from which they are derived and according to their activation potential, they are considered as biomarkers. Likewise, in cardiovascular abnormalities including dysfunctions such as atherosclerosis, coronary artery disease, hypertension, the circulating levels of endothelial derived microparticles represents the activation or dysfunction of the endothelial cells [100–102]. There are studies demonstrating where microparticles represents as essential biomarkers of endothelial injury induced by inhibitors of VEGF and significant mediators of Endothelin-1 mediated pro-inflammatory and redox-signalling relevant in endothelial cells and these may contribute to cardiovascular complications produced as a result of administration of inhibitors of VEGF.

The release of endothelial microparticles occurs in activation of various pathways. Few of these also contribute in the progression of atherogenesis especially the inducers of apoptosis are responsible for the functional alterations in the vascular system ultimately leading to pathogenesis of atherosclerosis. Hence therapies having direct effect on the microparticles may be an important step towards the development of therapeutics for treating cardiovascular based diseases [103]. There are studies that demonstrate that endothelial based microparticles levels are positively associated with the extent of functional and morphological abnormalities [104].

NO Formation in EMP

A recent investigation puts forward microparticle induced dysfunction in healthy blood vessels by having an effect on the endothelial NO transduction pathway. Nitric oxide derived from endothelium is majorly involved in relaxations in response to acetylcholine. Microparticles though have an inhibitory effect on these relaxations which alters the NO transduction pathway in patients suffering from myocardial infarction without having any effect on expression of eNOS. Hence circulating microparticles may be involved in exaggerating myocardial ischemia [105].

Another study suggested that elevated generation of superoxide induced by endothelial based microparticles plays a crucial role in impaired endothelial relaxation. And not only vascular responses are restored but production of NO as well

regardless of the source of superoxide (Endothelium-derived microparticles impair endothelial function).

Nitric oxide production from the endothelium depends on the regulation of calcium and its binding to the eNOS. An investigation on monocyte derived microparticles found that it does not have any effect on the eNOS expression and phosphorylation. However, Akt phosphorylation was found to be increased. Conversely, monocytic microparticles decreases the caveolin-1 expression and amplifies the phosphorylation on Tyr14 while the production of NO increases. Generalising the whole concept, it suggests decreasing the expression of caveolin-1 reduces the capacity of eNOS to sequester and hence resulting in more release of NO [106].

Targets and Physiological Function of NO in EMP

Microparticles are derived from various kinds of cells and depending on the parental cell microparticles expresses different cellular proteins on the surface and intracellularly. Tumor necrosis factor-α, interleukin-1 (IL-1), lipopolysaccharides triggers the release of endothelial cell derived microparticles [107, 108]. However, quite meagre knowledge is available regarding the circulating endothelial microparticles apparently due to restricted number of endothelial markers for instance CD62E for E-selectin and CD31 [109, 110].

In the pathological states such as acute coronary syndrome, disruption of atherosclerotic plaques, significant numbers of microparticles are released suggesting its role in the thrombus formation [111]. Furthermore, the role of endothelial microparticles in interaction with the monocytes elicits procoagulant activity and this effect to some extent depends on the interaction between ICAM-1 present on endothelial microparticles and α_2 integrins present on monocytic cells and thus representing that endothelial microparticles are capable of developing interactions with leukocytes [112]. It is evident from another study that platelet derived microparticles have the ability to enhance the interaction of leukocytes and endothelial cells and hence platelet derived microparticles also play role in the formation of atherosclerotic plaque and is associated with the vascular damage [113].

Previous studies have also shown the role of interaction between leukocytes, platelets and endothelial cells in vascular inflammation, as demonstrated by incubation of endothelial cells with leukocytes derived microparticles, leads to de-novo synthesis of cytokines and adhesion molecules. That implies microparticles as an inflammatory agonist and stimulator of release of chemotactic mediators [14].

Further, it was found that circulating microparticles in the patients suffering from myocardial death caused endothelial dysfunction by which can be linked to the impairment in transduction of nitric oxide (NO) pathway without inducing any changes in the expression of endothelial nitric oxide synthase (eNOS). This may have a role in exaggerating vasodilator responses contributing in amplification of myocardial ischemia [105].

More recently a study was carried out focussing on treatment of T-lymphocyte derived microparticles and endothelial cells can modify gene expression implicated in vessel relaxation. This can be associated with reduced levels of NO (Nitric Oxide) and prostacyclin demonstrating ability of microparticles to reduce eNOS and to induce overexpression of caveolin-1. Interestingly, patients of diabetes and HIV infection can reduce eNOS since they contain a population of T-cell derived microparticles [106].

NOS Inhibitors in EMP and Therapeutic Implications

Studies demonstrate expression of adhesion molecules involved in the progression of inflammation due to amplified circulating endothelial microparticles [114] and is also engaged in vascular calcification [115]. Another implication of endothelial microparticles is associated with the hypertension due to endothelial dysfunction that develops due to the deficiency of nitric oxide [116]. Furthermore, angiotensin-II is related with the endothelial dysfunction through endothelial microparticles induced inflammation and oxidative stress, this given an idea about the correlated effect of endothelial microparticle generation with vascular pathology [95]. Another significant factor associated with cardiovascular pathology and vascular function is angiogenesis. Endothelial microparticles play a significant role in regulating angiogenesis under variable antigenic expression [117].

Hence, microparticles are pertinent targets to achieve therapeutic outcomes. Likewise, PPAR agonists such as rosiglitazone are an excellent approach to target microparticles to treat inflammatory disorders, also it induces NFκB associated with vascular dysfunction and evokes an elevation in proinflammatory proteins [118]. Inhibition of TNF-α also is involved in dysregulated microparticle production by activating NFκB which also happens to offset vascular dysfunction [119]. Similarly, calcium channel blockers are suggested for patients with type 2 diabetes since they are involved in attenuating microparticle responses [120].

There are investigations demonstrating the association of dysregulation of morphogen sonic hedgehog pathways leads to tumor and erectile dysfunction due to down streaming of targets such as vascular endothelial growth factor (VEGF) and NO-synthase suggesting morphogen sonic hedgehog modulates regulation of these targets [121, 122].

Even in determining the extent of pulmonary hypertension microparticles has proven to be an integral tool since microparticles bearing endoglin, chemoattractant protein-1 and vascular cell adhesion molecule-1 are amplified in cases of pulmonary arterial hypertension. Similarly, patients suffering from coronary artery disease also display elevated levels of CD144 EMP levels and analogous is the case with metabolic syndromes [123].

Altogether, these results give an idea that microparticles could be shows potential targets for the treatment of cardiovascular disorders.

Drug Mediated Effect

Microparticles are now emerging as a promising novel class of cell-derived therapeutics because of its protein, lipids, and nucleic acid carrying capacity. As microparticles emerging from almost all types of cells they can carry their parent cell content and can deliver active molecules, peptides, hormones, growth factors, inflammatory factors, and microRNA in endocrine or paracrine fashion by circulation [117, 124]. Endothelial derived MPs plays an important role in wound healing, tissue repair, and regeneration by angiogenesis.

Reduced eNOS-derived NO bioactivity can cause endothelial dysfunction and leads to atherosclerosis, for impairment of endothelium-dependent vascular dysfunction, a natural compound salidroside (SAL) attenuates endothelial cell senescence by decreasing the expression of inflammatory cytokines and increasing the expression of SIRT3. In a study, apolipoprotein E-deficient ($ApoE^{-/-}$) mice show alleviated atherosclerosis by eNOS activation and NO production through AMPK-dependent activation of PI3K/Akt pathway. SAL is in clinical trial phase and its role in MPs production still not clear [125]. Atorvastatin treatment significantly protected MI-RP injury and IRS by increasing NOS expression through Akt dependent pathway in insulin resistance syndrome (IRS) rats fed with 60% high fructose diet. Atorvastatin treatment reversed endothelial dysfunction, insulin resistance, and oxidative stress, it also showed improvement in systemic inflammatory load and infarct size in the heart [126] (Table 22.4).

Conclusion

It is apparent from the foregoing discussion that microparticles induces both the positive and negative impact on the interacting cells. These are shown to have an important role in wound healing, tissue repair, and regeneration. Similarly microparticles can also induce inflammation depending on the parental cell characteristics and the content it carries. It has been implicated in several diseases both as a causative agent and protective agent. During sepsis and inflammation profound changes in physiological functions are attributed to variety of mediators, including microparticles containing NO and high circulatory levels of microparticles. These microparticles are emerging as novel drug delivery vehicles as they can deliver most molecules including hormones, miRNA and others. Further investigations into the NO processing and its generation in microparticles, and how microparticles induces NO mediated signaling in both acute and chronic condition of disease are required to completely understand the microparticle and molecule.

Table 22.4 Drug mediated effect & therapeutic implications

Drug	Approved by FDA	Mediator(s)	Action mechanism	Possible side effect	References
Salidroside	Phase II clinical trial	SIRT3 and eNOS	Activation of antioxidants and reduces inflammatory cytokine production	Increases cytokine production by Th1 and Th2 and therefore activates immune response	[125]
Pitavastatin	Yes	miR-155 and eNOS	Increases NO production and reduces inflammatory response	Causes endothelial cell death through activation of apoptosis ROSs production by JNK and p38-MAPK signal activation	[127]
Atorvastatin	Yes	Akt and eNOS	Reduces inflammatory Response Reverse MI-RP injury and IRS increasing NOS expression		[126]
Pravastatin	Yes	Angiotensin II	Reduces ROS production by targeting angiotensin II	May increase endothelin-1 expression that inhibits NO production	[55]

Acknowledgements The authors' research was funded by Council of Scientific and Industrial Research Govt. of India MLP0277, MLP2042 (CSIR-FIRST) and DST SERB, Govt. of India—EMR/2017/003730.

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