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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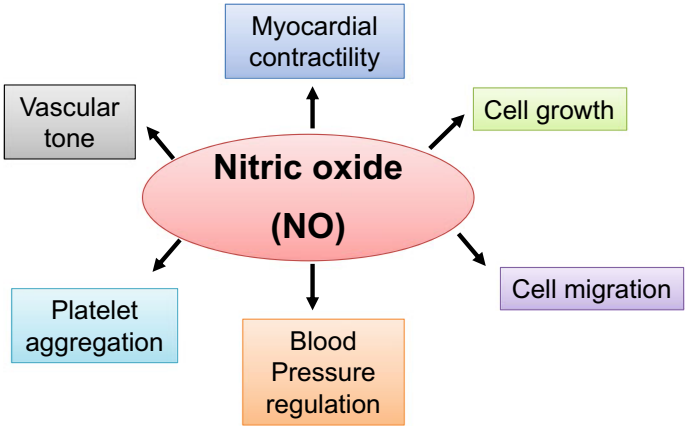

Fig. 2.1 Role of nitric oxide (NO) in the regulation of physiological functions

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

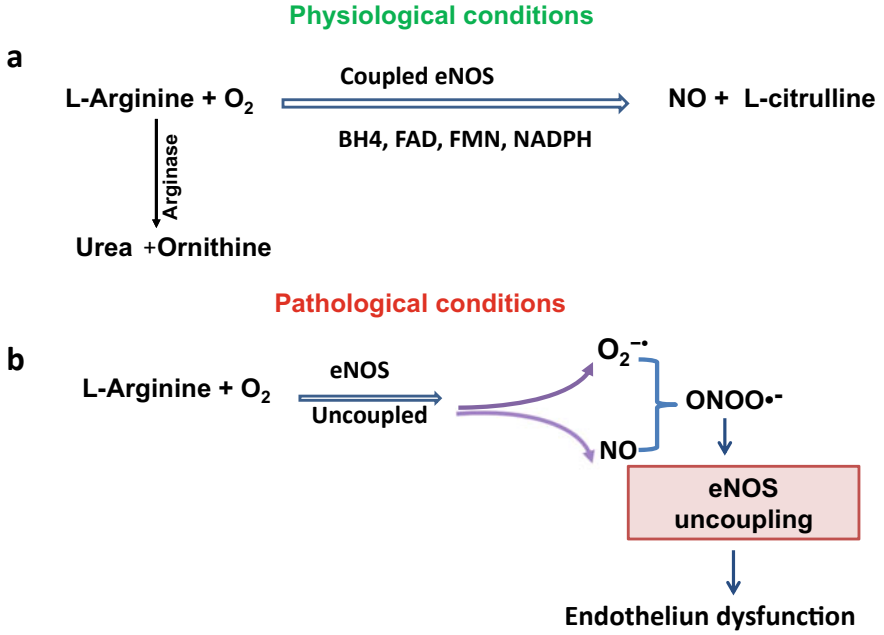

Fig. 2.2 Nitric oxide synthesis **a** Under physiological conditions, nitric oxide is synthesized by the oxidation of L-arginine to L-citrulline, a reaction catalyzed by nitric oxide synthase (NOS) and requires the presence of several cofactors including flavin mononucleotide (FMN), flavin adenine dinucleotide (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].

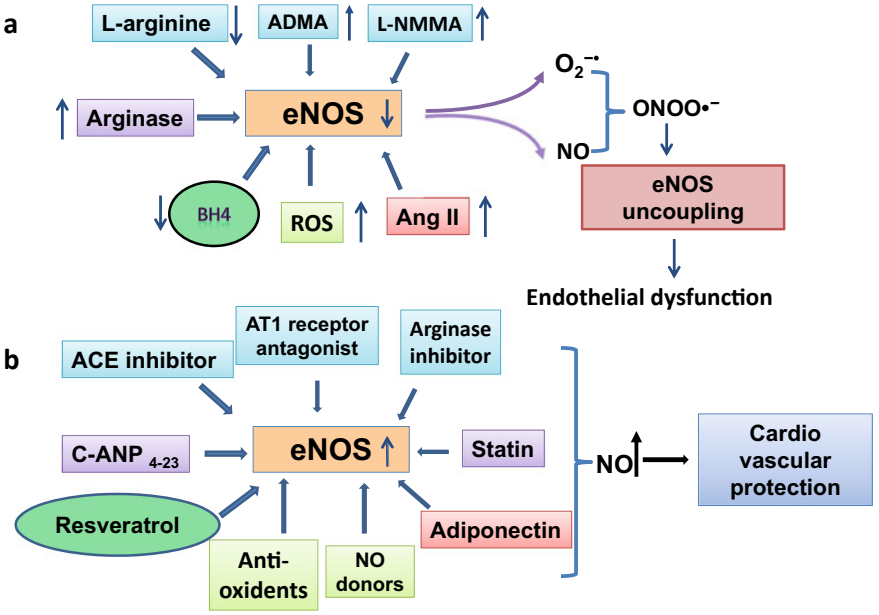

Fig. 2.3 **a** Factors that may contribute to reduced bioavailability of nitric oxide leading to endothelial dysfunction and pathogenesis of cardiovascular diseases. Several factors that could reduce NO availability are; the reduced levels of substrate L-arginine, BH4, an essential cofactor and augmented 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

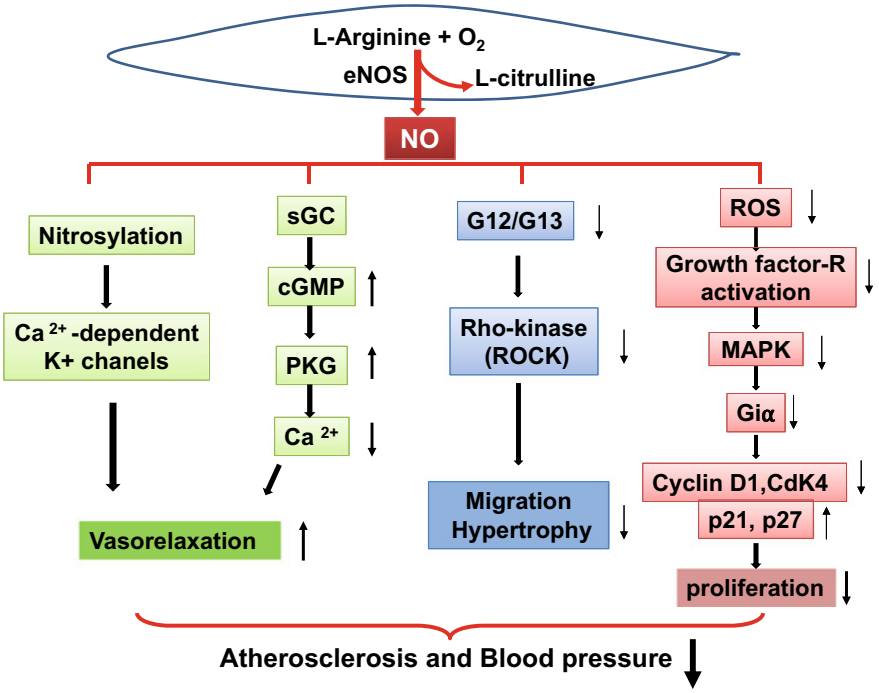

Fig. 2.4 Signaling mechanisms implicated in NO-mediated cardiovascular protection. NO induces vasorelaxation through S-nitrosylation-induced activation of Ca^{2+}-dependent K^+ channels as well as via sGC/cGMP/protein kinase G-dependent pathway. The beneficial effects of NO on remodeling of the vessels 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.

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