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Survey review

Vascular nitric oxide: Beyond eNOS

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ABSTRACT

As the first discovered gaseous signaling molecule, nitric oxide (NO) affects a number of cellular processes, including those involving vascular cells. This brief review summarizes the contribution of NO to the regulation of vascular tone and its sources in the blood vessel wall. NO regulates the degree of contraction of vascular smooth muscle cells mainly by stimulating soluble guanylyl cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), although cGMP-independent signaling [S-nitrosylation of target proteins, activation of sarco/endoplasmic reticulum calcium ATPase (SERCA) or production of cyclic inosine monophosphate (cIMP)] also can be involved. In the blood vessel wall, NO is produced mainly from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS) but it can also be released non-enzymatically from S-nitrosothiols or from nitrate/nitrite. Dysfunction in the production and/or the bioavailability of NO characterizes endothelial dysfunction, which is associated with cardiovascular diseases such as hypertension and atherosclerosis.

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1. Introduction

Nitric oxide (NO) is a free radical (1), generated naturally by electrical discharges [e.g. lightning) (2)], produced industrially as an important chemical intermediate (3), or released as an air pollutant from automobile engines or fossil fuel power plants (4, 5). Its potential endogenous production as an endothelium-derived relaxing factor (6) was first proposed independently by Robert Furchgott and Louis Ignarro in 1986 (7, 8) and confirmed in subsequent studies (9–12). It thus was the first gaseous molecule accepted to be a signaling mediator in the organism (13). It soon appeared that NO plays a key role in the physiological regulation of the cardiovascular system, since abnormalities in its productions and/or bioavailability accompany or even precede diseases such as hypertension, atherosclerosis and angiogenesis-associated disorders (14–17). NO also exerts physiological functions in the nervous and immune systems, contributing to the regulation of behavior, gastrointestinal motility, and defense mechanisms against infectious disease and tumors (18–24). This brief review focuses on the sources of NO in the blood vessel wall and its role in the regulation of vascular tone.

2. Components of the vascular wall

Blood vessels are composed of three layers: an intimal monolayer of endothelial cells, medial vascular smooth muscle and the adventitia or tunica externa (Fig. 1) (25, 26).

The intimal monolayer of **endothelial cells** covers the entire vascular tree, from the heart to the smallest capillaries, thus forming the interior surface of all blood vessels, which functions a barrier between the blood in the lumen and the surrounding tissues (25). The endothelium plays a modulator role in the basal and dynamic regulation of blood vessel diameter by releasing endothelium-derived NO (Fig. 2) (6,27–30) and contracting prostanoids and peptides (31–38), and by initiating endothelium-dependent hyperpolarizations (39–44).

The **vascular smooth muscle** layer mediates the constriction and dilatation of the blood vessels (25). Contractions of vascular smooth muscle cells can be initiated by mechanical (intraluminal pressure, stretch) or pharmacological activation (i.e., by binding of ligands to cell surface receptors), which increase the intracellular calcium concentration either by release from internal stores (sarcoplasmic reticulum) or by influx into the cell following opening of calcium channels in the plasma membrane (Fig. 3) (45–52). The intracellular free calcium ions bind to calmodulin and

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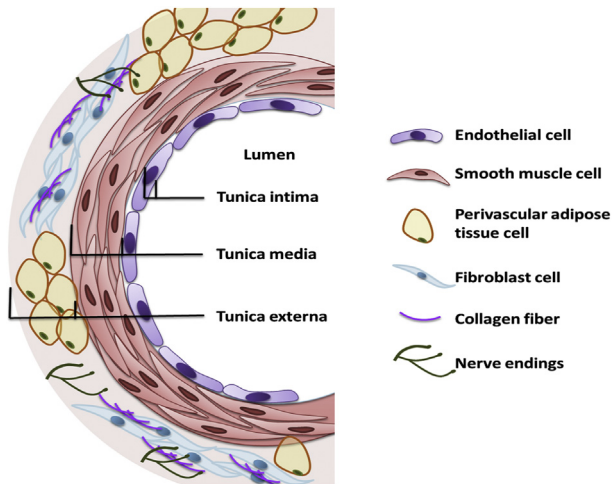E-mail address: swsleung@hku.hk (S.W.S. Leung).

Fig. 1. Structure of arterial wall. The arterial wall is composed of three layers: tunica intima (endothelial cells and internal elastic membrane), tunica media (vascular smooth muscle cells and external elastic membrane), and tunica externa (adventitia; contains perivascular adipose tissue cells, fibroblast cells, collagen fibers and nerve endings).

the calcium-calmodulin complex activates myosin light chain kinase (MLCK). MLCK phosphorylates the myosin light chain (MLC), which leads to cross-bridge formation between the myosin heads and the actin filaments, resulting in contraction of the smooth muscle cells (Fig. 3) (53–59).

The ***adventitia*** (Fig. 1) contains nerve endings, perivascular adipose tissue (PVAT) and connective elements (fibroblasts and collagen fibers) that insure adherence to the surrounding organs. Components of the adventitia are also involved in vascular development and remodeling (60–64), immune surveillance and inflammatory cell trafficking (65–70), and signal exchanges between the blood vessel and the tissue in which it resides (60, 69,71–74). In particular, the adipocytes of PVAT, like all fat cells, secrete adipokines which contribute to the regulation of vascular tone. For
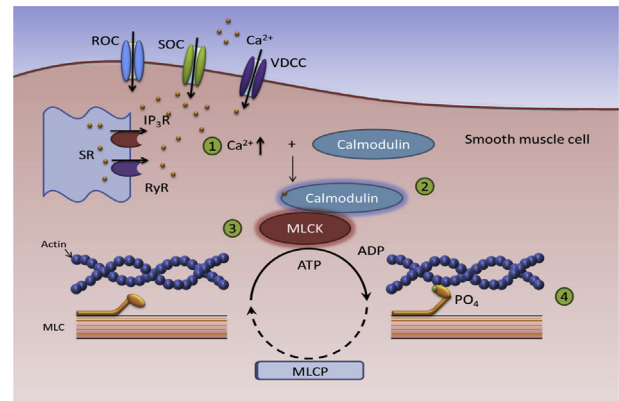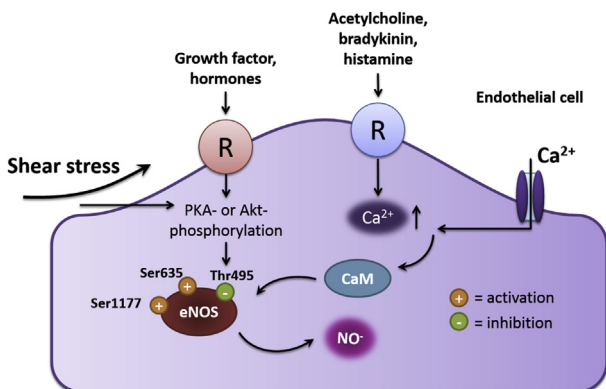

Fig. 2. Endothelial nitric oxide synthase (eNOS) can be activated in calcium-dependent or- independent ways. On the one hand, agonists, such as acetylcholine, bradykinin and histamine, act on specific receptors (R) on the endothelial cell membrane to increase the intracellular concentration of calcium, which binds to calmodulin (CaM) and leads to the activation of calmodulin-binding domain of eNOS to produce nitric oxide (NO). On the other hand, phosphorylation of eNOS independently of the calcium concentration is also important for the activation of the enzyme. Thr495 is an inhibitory site but Ser635 and Ser1179 are activation sites. The responses to hemodynamic shear stress and hormones are mediated mainly through this calcium-independent pathway.

Fig. 3. Increased intracellular calcium stimulates contraction of vascular smooth muscle cells 1. Mechanical or pharmacological activation increases the intracellular calcium (Ca^{2+}) concentration either from internal stores (sarcoplasmic reticulum, SR) or by influx into the cell following opening of calcium channels in the plasma membrane; 2. The intracellular free calcium ions bind to calmodulin and the calcium-calmodulin complex activates myosin light chain kinase (MLCK); 3. Activated MLCK phosphorylates the myosin light chain (MLC), which leads to cross-bridge formation between the myosin heads and the actin filaments; 4. Cross-bridge formation results in contraction of the smooth muscle cell. Calcium channels: Receptor-operated channel (ROC); store-operated channel (SOC); voltage-dependent calcium channel (VDCC). IP_3R: inositol 1,4,5-trisphosphate (IP_3) receptor-mediated calcium release. RyR: ryanodine receptor-mediated calcium release. MLCP: myosin light chain phosphatase.

example, in isolated rat aortic rings, the presence of PVAT decreases contractions to norepinephrine, an effect attributable to transferable vasodilator substances (75), termed "adipose-derived relaxing factors" (ADRF), which can cause direct relaxation of the vascular smooth muscle that they surround or, if and when they (e.g. adiponectin and angiotensin 1–7) reach endothelial cells, stimulate the production of NO (76–84). However, adipocytes also can produce adipokines (e.g. lipocalin-2) which can curtail this production (85, 86).

3. Sources of vascular nitric oxide

Although in the blood vessel wall NO is mainly produced from L-arginine by endothelial NOS (eNOS), other mechanisms of vascular NO production exist (87–90).

3.1. L-arginine and nitric oxide synthases

L-arginine is the first discovered and best-characterized source of NO as substrate for NOS (10,91–93). Three distinct genes encode NOS isozymes which catalyze the production of NO from L-arginine: neuronal NOS (nNOS or NOS-1), cytokine-inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3) (89,94,95). Triple NOSs null mice exhibit reduced endothelium-dependent relaxations (due mainly to blunted endothelium-dependent hyperpolarizations) and have a shorter life expectancy with accelerated appearance of cardiovascular diseases including hypertension, cardiac hypertrophy, diastolic heart failure, arteriosclerosis and myocardial infarction (96).

The production of NO from L-arginine by NOS requires the presence of various co-factors including tetrahydrobiopterin (BH_4), flavin adenine dinucleotide, flavin mononucleotide, calmodulin and iron protoporphyrin IX (haeme) (94,97). The three NOS isoforms are synthesized as monomers, and need to form dimers (Fig. 4) in order to bind BH_4 and the substrate L-arginine to catalyze NO· production (98). The monomers generate $O_2 \cdot{}^-$ instead of NO· from their oxygenase domain; a condition which is referred to as
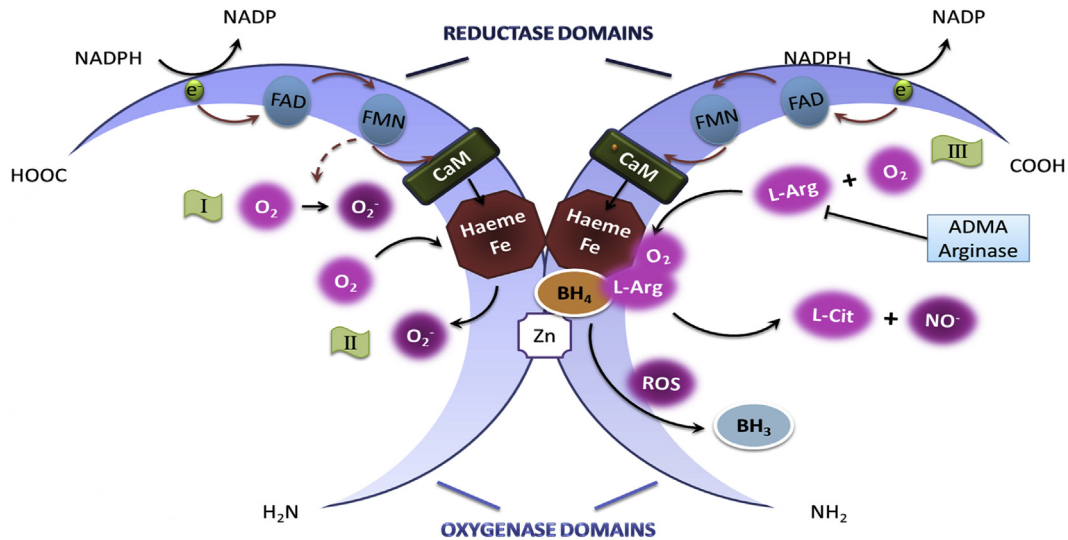
Fig. 4. Homodimers of nitric oxide synthase (NOS). I, as monomers, NOS produce O_2^- instead of NO^-; **II**, even as homodimers, significant O_2^- production will occur when the effective concentrations of L-arginine (L-Arg) falls below levels required to saturate the enzyme; **III**, when sufficient L-arginine and tetrahydrobiopterin (BH$_4$) bind to coupled NOS, the electron flux provided by nicotinamide adenine dinucleotide phosphate (NADPH) passes through flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM) and iron protoporphyrin IX (Haeme Fe), and is eventually used in NO production. BH$_4$ is essential for NOS coupling and reactive oxygen species (ROS) oxidize BH$_4$ to the BH$_3$· radical, leading to NOS uncoupling. The endogenous competitive inhibitor asymmetrical dimethyl arginine (ADMA) and arginase both reduce the L-arginine binding to NOS which decreases NO production.

NOS uncoupling (97). Compared to the actual protein presence of the NOS enzyme, its coupling is more important for the production of NO (99,100).

Neuronal NOS produces NO both in the central and peripheral nervous systems and thus plays a role in cell communication (19, 23, 24,101). NO produced by nNOS is implicated in the regulation of neuronal excitability and firing, in long-term potentiation or depression of synaptic plasticity, as well as in memory and learning processes. Moreover, NO produced by nNOS regulates the release of neurotransmitters such as acetylcholine, histamine and serotonin (102,103). Neuronal NOS is also expressed in cardiac and skeletal myocytes (104,105), smooth muscle and endothelial cells (106,107), the adventitial layer of penile arteries (108), and cells of macula densa in the kidney (109). A selective inhibitor of nNOS, S-methyl-L-thiocitrulline (SMTC), reduces the basal blood flow in the normal human forearm and coronary circulating, without affecting eNOS-mediated vasodilatation induced by acetylcholine or shear stress (110,111). *In vitro* experimental data implicates nNOS-derived NO in the local regulation of vascular tone independently of the central nervous system (112). Selective inhibition of nNOS by SMTC in explanted whole rat kidneys decreases afferent and efferent arteriolar diameters but does not affect vasodilatation in response to the endothelium-dependent vasodilator acetylcholine (113). Vinyl-L-N-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO; another selective inhibitor of nNOS) increases the arterial vasoconstriction and local norepinephrine concentration in response to perivascular sympathetic nerve stimulation in mesenteric arteries (114). However, nNOS inhibition does not reduce the vasoconstriction to exogenous norepinephrine, indicating that nNOS-derived NO may affect neurotransmitter release from perivascular sympathetic nerves (114). Neuronal NOS also participates in neurogenic vasodilation in the rat skin microvasculature (115). NO produced by nNOS not only initiates but also participate in maintenance of penile erectile (108,116−119) and decreased expression of nNOS is found in patients with erectile dysfunction (120). In arteries of spontaneously hypertensive rats (SHR) but not in normotensive Wistar-Kyoto (WKY) rats, nNOS is expressed in the smooth muscle cells and its activation is stimulated by angiotensin II (106). Acute hypoxia

increases nNOS expression but reduces its activity due to substrate deprivation, whereas chronic hypoxia induces increased synthesis and activity of nNOS which attenuate hypoxia-induced vasoconstriction (121,122). Vascular injury induces expression of nNOS in the intima and the medial smooth muscle cells and selective inhibition of this isoform augments the responses to various vasoconstrictors, suppresses the production of cyclic guanosine monophosphate (cGMP), and exacerbates neointimal formation (123). Furthermore, the expression of vascular nNOS is also up-regulated by stimulation with angiotensin II, platelet-derived growth factor or statins (123−125).

The expression/presence of **inducible NOS** is minimal under physiological conditions. However, this isoform is calcium-insensitive and continuously produces NO once it is expressed (126). Induction of iNOS occurs mainly during infection, chronic inflammation and in tumors (127). The activation of iNOS promoter requires interferon regulatory factor 1 and nuclear factor κ-light-chain-enhancer of activated B cells, explaining why expression of iNOS is characteristic of inflammation (128−136). Inflammation-induced iNOS expression in the endothelium but not in adventitia may contribute to vascular dysfunction by limiting the availability of BH$_4$ for eNOS (137). The production of iNOS within vascular smooth muscle cells following exposure to pro-inflammatory cytokines is a major cause of the hypotension, cardiodepression and vascular hyporeactivity in septic shock (138−142).

However, **eNOS** is the major isoform regulating vascular function (97,143). The activity of eNOS (Fig. 4), and thus the production of NO can be initiated/enhanced by several stimuli (Fig. 2) including shear stress (30,144−149), acetylcholine (150−153), bradykinin (154−157), histamine (158−162) and 17β-estradiol (163−167), in both calcium-dependent and -independent manners. Agonists, such as acetylcholine, bradykinin and histamine, act on specific receptors on the endothelial cell membrane to increase the intracellular concentration of calcium, which binds to calmodulin and leads to the activation of calmodulin-binding domain of eNOS (Fig. 4) (168−171). This facilitates electron flux from the reductase to the oxygenase domains of the enzyme for NO production (172). On the other hand, phosphorylation of eNOS independently of the

calcium concentration is also important for the activation of the enzyme because it facilitates the active flux of electrons from the reductase to the oxygenase domains (172). Phosphorylation is a post-translational modification, which adds a phosphate (PO_4^{3-}) group to eNOS by kinases; the phosphate, in turn, is removed by phosphatases (173–177). This modification alters the activity of eNOS and different sites of phosphorylation can have opposing effects (178). Thus, for eNOS, Ser1177 (or Ser1179 depending on the species) is an activation site and Thr495 is an inhibitory site. Protein kinase A (PKA) and protein kinase B (Akt) activate eNOS by phosphorylating Ser1177 in response to various stimuli (149,174). By contrast, hydrogen peroxide and bradykinin activate eNOS to produce NO by elevating both Ser1177 phosphorylation and Thr495 dephosphorylation (179,180). Increases in NO release induced by increases in hemodynamic shear stress and 17β-estradiol are mediated mainly through this calcium-independent pathway (149,163,168). In particular, shear stress induces phosphorylation of eNOS at Ser1179 and Ser635 in a PKA-dependent manner (144,181). In addition, herbal (e.g. aurantio-obtusin and puerarin) can regulate vascular tone by eNOS phosphorylation in an Akt-dependent manner (182,183).

3.2. S-nitrosothiols

S-nitrosothiols such as S-nitrosohemoglobin and S-nitrosoglutathione serve not only as downstream NO products regulating protein expression and function but also as sources of NO (87). In endothelial cells, S-nitrosothiols can be formed from exogenous S-nitrosothiol donors (i.e., S-nitrosoglutathione) or from endogenous NO produced by eNOS (184). In addition, NO groups can be transferred from a donor S-nitrosylated protein to an acceptor S-nitrosylation substrate in multi-protein complex situations (185). These S-nitrosoproteins, with a half-life of around one hour, exist mainly in the mitochondria and peri-mitochondrial compartment of endothelial cells (184). There is also a circulating pool of S-nitrosoalbumin in the plasma whose levels are positively related to NOS activity. Thus, NOS inhibition results in a decrease of S-nitrosoalbumin but an increase of low-molecular-weight S-nitrosothiols such as S-nitrosoglutathione. S-nitrosoglutathione can transfer its NO group to the reactive thiols (cysβ93) of hemoglobin, and the resulting S-nitrosohemoglobin can transfer the NO group to membrane-associated band 3 protein of erythrocytes; a process controlled by the oxygen level in the blood (186). S-nitrosothiols are stable compounds at 37 °C and pH 7.4 in the presence of transition metal ion chelators (187). The liberation of NO is promoted by trace amounts of transition metal ions (Cu^{2+} or Fe^{2+}) (188), flash photolysis or by the combination of the two (189). Dithiothreitol (thiol-preserving agent) and vitamin C (antioxidant) stimulate NO release from S-nitrosothiols to induce capillary morphogenesis (190). By releasing NO, S-nitrosothiols are more powerful vasodilators than nitroglycerin and potent inhibitors of platelet aggregation; the latter can be achieved at lower concentrations than those required to elicit vasodilatations (191,192).

3.3. Nitrite and nitrate

Nitrite and nitrate are not only the products of the metabolism of NO but also act as a reservoir (87). Indeed, under certain conditions, different enzymes [hemoglobin, myoglobin, xanthine oxidoreductase, mitochondrial cytochrome oxidase, aldehyde dehydrogenase 2, cytochrome P450 reductase and cytochrome P450] can catalyze the reduction of nitrite or nitrate to generate NO.

Hemoglobin is an iron-containing protein in the red blood cells, which carries and transports oxygen in the circulation system (193–196). It is an oxygen sensor and produces NO from nitrite

under hypoxic conditions. Hypoxia causes a quaternary structural change in hemoglobin from oxyhemoglobin to deoxyhemoglobin. When hemoglobin is 40–60% saturated with oxygen and at pH 6.4, it generates NO from nitrite at a maximal rate (197). Myoglobin is also an iron- and oxygen-binding protein which is present in muscle cells. Deoxymyoglobin reduces nitrite to NO at a rate 36 times faster than deoxyhemoglobin in vitro (198). As oxygen sensors, hemoglobin and myoglobin shift from being NO scavengers to NO producers in hypoxia; this perception of decreased oxygen concentrations then, by releasing NO, induces vasodilatation and increases the blood supply to the hypoxic tissues (199–201). The enzyme xanthine oxidoreductase also produces NO from nitrate and nitrite in anoxic tissues; this mechanism protects against ischemia-reperfusion injury (202,203). Likewise, mitochondrial cytochrome c oxidase produces NO from nitrite in the mitochondria under hypoxic conditions; this NO production increases with decreasing pH (204). The production of NO by the latter two enzymes may be important for the redistribution of blood flow to ischemic tissue (204,205). By contrast, aldehyde dehydrogenase 2 (ALDH2) effectively converts organic nitrate compounds, such as nitroglycerin, to NO under normoxic conditions (206,207). Both cytosolic and mitochondrial ALDH2 can catalyze the bioactivation of exogenous nitroglycerin; however, an endogenous substrate for this enzyme has not been identified (206,207). The activity and expression of ALDH2 in the vascular system are reduced in the case of tolerance to organic nitrates (208,209).

Cytochrome P450 reductase and cytochrome P450 induce NO release by reducing nitrate and nitrite, respectively (210). Nitrate (transformed to NO by cytochrome P450 reductase) has been identified as another endothelial source of NOS-independent NO, which is only present in SHR arteries but not in those of normotensive WKY rats (Fig. 5) (90). Indeed, in the presence of NOS and cyclooxygenase inhibitors, in aortic rings with endothelium of SHR, but not of WKY, contractions to phenylephrine and prostaglandin E2 were smaller than in preparations without endothelium; this endothelium-dependent depression of contractions was abolished when the downstream NO signaling pathway was inhibited [by NO scavengers and inhibitors of soluble guanylyl cyclase (sGC)]. Such endothelium-dependent, eNOS-independent depression of contractions was larger in preparations of 36 than 18 weeks old SHR, thus suggesting that the chronic elevation in arterial blood pressure plays a role in the development of NOS-independent NO release (90). However, in the absence of cyclooxygenase inhibition, the
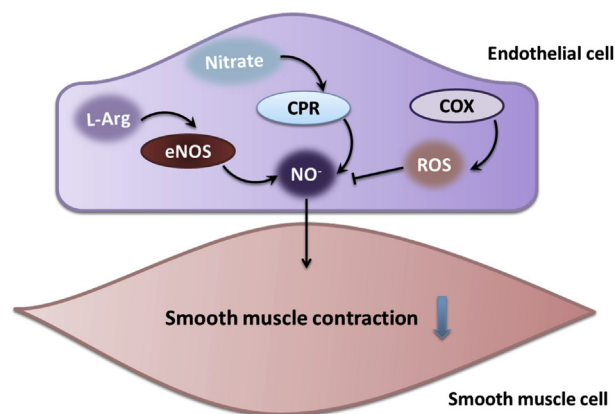

Fig. 5. Nitric oxide (NO⁻) produce by cytochrome P450 reductase (CPR) from nitrate. The endothelium-derived NO produced by CPR from nitrate is a supplement for NO produced by eNOS in arteries of the spontaneously hypertensive rat, and reduces the contractions to agonists. However, reactive oxygen species (ROS) significantly eliminate the NO production thus restoring the contractions.

endothelium-dependent, NOS-independent NO production was masked by reactive oxygen species (90), which supports the concept that oxidative stress play an important role in the endothelial dysfunction accompanying hypertension (211–215). The endothelium-dependent, cytochrome P450 reductase-mediated NO release from nitrate may serve as a compensatory mechanism to restore NO availability during endothelial dysfunction. Indeed, deficient production of NO by its canonical source (eNOS) is a feature of endothelial dysfunction associated with hypertension (216–219).

4. Nitric oxide and vascular tone

Endothelium-derived NO is a powerful vasodilator (6–11). It stimulates soluble sGC in the vascular smooth muscle cells to induce formation of cGMP (12,220–223). Cyclic GMP activates protein kinase G, which promotes the reuptake of cytosolic calcium into the sarcoplasmic reticulum, the expulsion of calcium out of the cell, and the opening of calcium-activated potassium channels (Fig. 6) (224–228). The intracellular concentration of calcium decreases and MLCK can no longer phosphorylate myosin and relaxation of the smooth muscle cells ensues (Fig. 6) (49,229–232). In addition, NO can affect cellular activity independently of sGC-activation (233). Thus, NO stimulates the sarco/endoplasmic reticulum calcium ATPase (SERCA) reducing the intracellular calcium concentration and causing relaxation of the smooth muscle; NO-derived peroxynitrite ($ONOO^-$) can directly enhance SERCA activity by S-glutathiolation (Fig. 6) (234). In the presence of an electron acceptor, NO reacts with the cysteine thiols to form S-nitrosylated proteins (235). Such S-nitrosylation reactions are specific in that not every cysteine-containing protein is S-nitrosylated and not
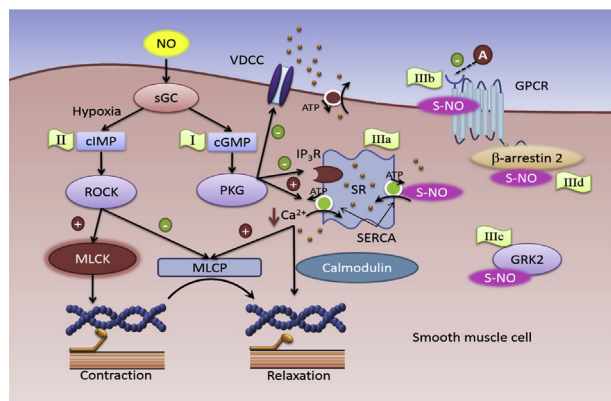
every cysteine residue in a protein becomes S-nitrosylated (185). Of particular importance for the tone of vascular smooth muscle, S-nitrosylation regulates the expression and functions of G protein-coupled receptors (GPCRs); this mechanism also plays a role in the regulation of vascular tone (185,236). For instance, NO and S-nitrosothiols modulate the activity of GPCR kinase 2 (GRK2) which phosphorylates β-adrenoceptors and induce receptor desensitization and internalization to curtail G-protein signaling. Thus, NO and S-nitrosothiols prevent the loss of β-adrenergic signaling *in vivo* by S-nitrosylating GRK2, attenuating GRK2-mediated phosphorylation of β-adrenoceptors and decreasing receptor desensitization and internalization (236,237). In the heart, activation of β_1 and β_2-adrenoceptors increases heart rate and atrial cardiac muscle contractility (238–241); by contrast, activation of these receptors in blood vessels induces vasodilatation (242–244). Thus, increased NO production with greater S-nitrosylation of GRK2 (preventing the loss of β-adrenergic signaling) may increase cardiac output and improve perfusion of target organs. GPCRs also can be S-nitrosylated directly by chemical NO-donors; such S-nitrosylation of muscarinic (245) or bradykinin receptors (246) disrupts their coupling to G proteins. Likewise, S-nitrosoglutathione inhibits α_1-adrenoceptor-mediated vasoconstriction and ligand binding (247). In addition, S-nitrosylation of cysteine 289 of the AT_1 receptor decreases its binding affinity for angiotensin II (248). Cytosolic β-arrestin binding to ligand-activated and GRK-phosphorylated GPCRs sterically impedes the interaction of G-proteins with activated GPCRs, resulting in GPCR signaling termination (249–251). Also, β-arrestin 2, can be S-nitrosylated on cysteine 410 by endogenous NO and S-nitrosogluthathione, which promotes binding of β-arrestin 2 to clathrin heavy chain/β-adaptin, thereby accelerating receptor internalization (252).

By contrast to its vasodilator effects, NO mediates hypoxic augmentation of contractions in coronary arteries, which is dependent on sGC but independent of cGMP (253,254): Indeed, acute hypoxia induces a transient further increase in tension in contracted coronary arterial rings, which can be abolished by L-NAME [L-N^G-Nitroarginine methyl ester, NO synthase (NOS) inhibitor] or ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, sGC inhibitor) but is restored by the NO-donor DETA NONOate. However, the cGMP levels do not, but those of cyclic inosine monophosphate (cIMP) increase under these conditions and inhibitors of protein kinase G (the canonical target of cGMP) do not inhibit the hypoxic augmentation, indicating a novel, cIMP-mediated signaling mechanism for NO different from the classic NO-sGC-cGMP-relaxation pathway (Fig. 6) (253–256).

5. NO deficiency

In patients with hypertension or prehypertension, and salt-sensitive hypertensive Dahl rats, the NO-mediated relaxation in response to acetylcholine is blunted (257–263). However, the relaxation of vascular smooth muscle to NO donors (such as sodium nitroprusside) is not altered (260–262,264,265). Thus, the reduced NO bioavailability can be attributed to a decreased NO production and/or an increased NO degradation. This conclusion is based on the evidence discussed in the following sections, taking mainly the SHR as an example of endothelial dysfunction.

5.1. Decreased NO precursors

L-arginine, the precursor of NO, is an essential amino acid for young mammals. In healthy human adults, L-arginine can be synthesized from L-citrulline by endogenous (*de novo*) synthesis (266). Therefore, decreased availability of L-arginine and L-citrulline can contribute to NO deficiency (266–270). Even when the L-arginine

Fig. 6. Regulation of vascular tone by nitric oxide (NO). NO regulates vascular tone by three different signaling pathways. **I**: NO stimulates soluble guanylyl cyclase (sGC) in the vascular smooth muscle cells to induce formation of cyclic guanosine monophosphate (cGMP). Cyclic GMP activates protein kinase G (PKG), which prevents the calcium influx from voltage-dependent calcium channel (VDCC) and calcium release mediated by inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R). PKG also acts on sarco/endoplasmic reticulum calcium ATPase (SERCA) to promote the reuptake of cytosolic calcium into the sarcoplasmic reticulum (SR). The intracellular calcium concentration decreases and calmodulin is inactivated which no longer able to activate myosin light chain kinase (MLCK). Calcium depletion also increases the activity of myosin light chain phosphatase (MLCP). The actin-myosin cross-bridge is broken and smooth muscle relaxation ensues. **II**: under hypoxic condition, soluble guanylyl cyclase produces inosine cyclic 3′,5'-monophosphate (cIMP) instead of cGMP, which activates Rho-associated protein kinase (ROCK) and inhibits MLCP, resulting in contraction. **III**: protein S-nitrosylated by NO. **IIIa**: S-nitrosylation increases the activity of SERCA which accelerates calcium depletion and induces relaxation. **IIIb**: G protein-coupled receptors (GPCR) can be directly S-nitrosylated by NO which impedes the binding of ligands for the receptor or G-protein coupling. **IIIc**: S-nitrosylation of G protein-coupled receptor kinase 2 (GRK2) prevents the desensitization and internalization of β-adrenoceptors. **IIId**: S-nitrosylation of β-arrestin 2 increases receptor internalization.

level is above the Km [concentration of substrate that allows half maximal rate of the enzyme-mediated reaction] for NOS, reduced NO generation can occur; this can be due to decreased competitive displacement by the endogenous competitive inhibitor asymmetrical dimethyl arginine [ADMA (Fig. 4), which is a metabolic by-product created during protein methylation in the cytoplasm of all human cells (268,271–273)]. This is defined as the arginine paradox: the dependence of cellular NO production on the exogenous L-arginine concentration despite the theoretical saturation of NOS enzymes by intracellular L-arginine (268). The plasma level of L-arginine is not significantly different in SHR and WKY, but L-arginine metabolism is impaired in the former under stress conditions and by nicardipine (a dihydropyridine type calcium channel blocker) (274,275). Long-term L-arginine supplementation improves endothelial function of small coronary arteries and attenuates cardiac hypertrophy in the SHR (276,277). L-citrulline, the precursor of L-arginine, can dilate retinal arterioles through NO- and prostaglandin-dependent pathways without significantly changing arterial blood pressure, heart rate and fundus blood flow (278). On the other hand, elevated levels of arginase, which catalyzes the transformation of L-arginine to ornithine and urea, compete with NOS for the available L-arginine thus reducing the production of NO and increasing the release of $O_2 \cdot^-$ (279,280). Inhibition of arginase reduces blood pressure and improves vascular function in the SHR (281,282).

5.2. Impairment of NO synthesis

A decreased NO production could be due to reduced eNOS expression/presence in adult and old (around 36 and 72 weeks) SHR (36,283). However, endothelial dysfunction is associated with an increase rather than a decrease of eNOS expression (284–286). The up-regulated expression of eNOS in situations of endothelial dysfunction is likely to be the consequence of an elevated production of hydrogen peroxide, which is a dismutation product of $O_2 \cdot^-$, and can increase the protein presence of eNOS by increasing the production and extending the half-life of its mRNA (287).

Uncoupling of eNOS has been reported in essential hypertensive patients with endothelial dysfunction (288), diabetes mellitus (289), or hypercholesterolemia (290) as well as in nitroglycerin-treated patients (291) and chronic smokers (292). In addition, eNOS uncoupling also can be observed in isolated blood vessels from animals with hypertension (99,100,293–295) or diabetes (296), and after induction of nitroglycerin tolerance (297). Several mechanisms contribute to the eNOS uncoupling causing endothelial dysfunction. Thus, vascular nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) plays a crucial role in eNOS uncoupling, as it produces ROS, which leads to oxidation of the critical co-factor BH_4 (298,299). Particularly, the direct reaction product of $O_2 \cdot^-$ and NO·, $ONOO^-$, also oxidizes BH_4 to the $BH_3\cdot$ radical (Fig. 4) (300,301). This reaction can be reversed by vitamin C (302). Oxidative stress disrupts the balance between de novo synthesis of BH_4 and its oxidation/degradation, thus leading to excessive depletion (303,304). For example, the BH_4 level is reduced in the plasma of SHR compared to WKY rats (305), in the aorta of insulin-resistant rats (306), and in DOCA-salt-treated hypertensive rats (298).

5.3. Increased NO degradation

NO can undergo a number of reactions under biological conditions. It is spontaneously inactivated in the presence of oxygen or $O_2 \cdot^-$ (307,308). It also can be scavenged by oxyhemoglobin to yield methemoglobin and inorganic nitrate (309). In addition, NO reacts with thiol groups in proteins to yield S-nitrosothiols (235,310). The

presence of $O_2 \cdot^-$ causes rapid and nearly complete inactivation of endothelium-derived NO (311–314). ROS produced by cyclo-oxygenases also inactivate the NO produced by cytochrome P450 reductase in SHR arteries (90). The biological half-life of NO varies in function of the oxygen tension and the $O_2 \cdot^-$ concentration (313,315–318). In the concentration range of 10–50 nM, NO has a half-life of about three to five seconds; in concentrations in excess of 300 nM, its half-life is longer than 30 s (308). Thus, the accelerated degradation of NO can worsen its deficient production.

6. Conclusions

NO is a free radical which not only is present in the environment but also can be produced in the body as a vital signaling molecule. In the vasculature, NO stimulates sGC to produce cGMP, decreases the intracellular concentration of calcium, causes relaxation of vascular smooth muscle and thus is a potent vasodilator. Besides activating sGC, NO reacts with cysteine thiols to form S-nitrosylated proteins, which increase the activity of SERCA and reduce the intracellular concentration of free calcium ions, also facilitating relaxation. S-nitrosylation of GRK2 prevents β-adrenoceptor from desensitization and internalization, enhancing the vasodilator response to catecholamines. However, NO also mediates hypoxic augmentation of contraction in coronary arteries, a response which depends on sGC but is independent of cGMP production. NO can be produced from L-arginine, S-nitrosothiols and nitrate/nitrite in the vascular wall by different enzymes under different conditions. Endothelial dysfunction does not simply result from a decreased NO production by eNOS, but can also involve a complex combination of decreased availability of L-arginine, enzyme dysfunction and increased degradation. Thus, when analyzing the role of NO in the vascular wall, different signaling pathways and sources, and alterations in bioavailability should be considered.

Conflicts of interest

The authors state no conflict of interest.

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