R E V I E W

Vascular nitric oxide: formation and function

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Abstract: Nitric oxide (NO) is a structurally simple, highly versatile molecule that was originally discovered over 30 years ago as an endothelium-derived relaxing factor. In addition to its vasorelaxing effects, NO is now recognized as a key determinant of vascular health, exerting antiplatelet, antithrombotic, and anti-inflammatory properties within the vasculature. This short-lived molecule exerts its inhibitory effect on vascular smooth muscle cells and platelets largely through cyclic guanosine monophosphate-dependent mechanisms, resulting in a multitude of molecular effects by which platelet activation and aggregation are prevented. The biosynthesis of NO occurs via the catalytic activity of NO synthase, an oxidoreductase found in many cell types. NO insufficiency can be attributed to limited substrate/cofactor availability as well as interactions with reactive oxygen species. Impaired NO bioavailability represents the central feature of endothelial dysfunction, a common abnormality found in many vascular diseases. In this review, we present an overview of NO synthesis and biochemistry, discuss the mechanisms of action of NO in regulating platelet and endothelial function, and review the effects of vascular disease states on NO bioavailability.

Keywords: platelets, endothelium, vasculature

Introduction

Nitric oxide (NO) is a structurally simple molecule that exerts effects on a wide variety of actions in the vasculature. NO was first discovered in 1980 by Furchgott and Zawadzki[1] as an endothelium-derived relaxing factor, it has been recognized as a key determinant of vascular homeostasis, regulating several physiological properties of the blood vessel, including vasodilation, vascular permeability, and antithrombotic properties.[2] The bioavailability of NO represents a central feature of the normal vascular phenotype required for maintaining vasodilator tone and inhibiting platelet activation, thereby preventing thrombosis and its clinical vascular consequences. Decreased production or increased metabolism of NO may lead to NO insufficiency within the vasculature and its pathobiological consequences. This review begins with an overview on NO's ability to modulate vascular tone and platelet activity, discusses the biochemical pathways involved in metabolism of NO, briefly summarizes factors controlling NO synthase (NOS) activity, discusses the impact of vascular pathologies on NO and vice versa, and comments on pharmacological therapies for restoring vascular NO and their therapeutic utility.

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Vascular actions of nitric oxide

Vascular tone

NO is a major regulator of vascular homeostasis, acting in various ways to regulate vascular tone. Low levels of NO produced by the endothelial cell play a key role maintaining vasorelaxation by exerting its effects on vascular smooth muscle cells (VSMCs), the contractile state of which defines vascular tone. NO produced within the cytosol of endothelial cells diffuses rapidly into adjacent VSMCs, exerting paracrine effects by activating soluble guanylyl cyclase to increase the synthesis of 3,5-cyclic guanosine monophosphate (cGMP). NO also decreases cytosolic Ca^{2+} concentration through inhibition of voltage-gated Ca^{2+} channels[3] and activates protein kinases that phosphorylate proteins in the sarcoplasmic reticulum, as well as Ca^{2+}-dependent potassium channels.[3] Reduction in cytosolic Ca^{2+} concentration results in inhibition of calcium-calmodulin myosin light chain kinase complex formation in the VSMC, promoting vasorelaxation.[4]

Low levels of NO released by endothelial cells are critical for the maintenance of basal vascular tone. The synthesis of endothelial NO is increased in response to biochemical stimuli, including thrombin, adenosine diphosphate (ADP), serotonin, acetylcholine, and bradykinin; as well as mechanical stimuli, including shear stress and cyclic strain. Studies have previously shown that inhibition of NO synthesis in the vasculature may lead to hypertension[5] or ischemic stroke,[6] likely, in part, through its effects not only on vascular tone, but also on thrombotic potential.[7]

In addition to the ability of NO to regulate basal vascular tone, NO has also been found to influence stimulated endothelial release of tissue-type plasminogen activator (t-PA). Tissue-type plasminogen activator acts as the primary activator of endogenous fibrinolysis by converting the inactive proenzyme plasminogen to the active enzyme plasmin. Studies regarding the release of t-PA have thus far been conflicting, with some studies showing an inhibitory effect of NO on t-PA release[8,9] and another showing the opposite.[10] The role of NO in modulating t-PA still remains unclear; however, a possible explanation for the inhibitory effect of NO on t-PA release may be attributed to acute NO deprivation invoking a protective response resulting in greater endothelial t-PA release. Conversely, a possible explanation supporting NO-induced t-PA release as seen in Giannarelli's study may be due to the lower concentration of L-N^G-monomethyl arginine citrate, a NOS inhibitor, 1 mg/min, compared with the 2 previously mentioned studies by Smith et al[8] and Pretorius and Brown,[9] 5 mg/min and 3 mg/min, respectively. Further

studies are needed to elucidate NO's role in endothelial t-PA modulation.

Platelet function and formation

NO plays a key role in maintaining vascular integrity and blood flow by modulating platelet–endothelial interactions.[11] Although platelets play an important role in hemostatic plug formation and wound healing, hyperreactivity can lead to vascular thrombosis and its clinical sequelae.[7] Platelets normally circulate in an inactive state, suppressed by 3 biochemical systems: prostacyclin, ecto-AD(T)Pase/CD39, and NO.[12] Prostacyclin, the main product of arachidonic acid metabolism in endothelial cells,[13] inhibits platelets via cAMP-dependent mechanisms that include direct inhibition of Ca^{2+} mobilization and granule release.[14] Ecto-AD(T)Pase is responsible for inhibition of platelet function by decreasing ADP-induced platelet aggregation[15] and also indirectly by inhibiting the thromboxane A_2 (TXA_2) receptor[16] (see later).

Calcium plays a central role as a second messenger in platelets. Concentrations within resting platelets are maintained between 50 and 100 nM, and upon activation, can achieve levels as high as 1 mM.[17] An increase in Ca^{2+} concentration within platelets leads to cytoskeletal rearrangement, shape change, and platelet granule secretion, ultimately resulting in aggregation.[12] Platelet-associated Ca^{2+}-binding proteins include alpha-helix, loop, second alpha-helix (EF-hand) proteins, and Ca^{2+}-phospholipid binding proteins.[18] Calmodulin is an EF-hand protein that binds to alpha-granules and is associated mechanistically with the Ca^{2+}-calmodulin-induced phosphorylation of myosin light chain contributing to platelet granule secretion.[19,20] Synaptotagmin, a Ca^{2+}-phospholipid binding protein, has been associated with the regulation of dense granule secretion,[21] further contributing to platelet activation and aggregation.

NO exerts its inhibitory effects on platelets via cGMP production and a secondary Ca^{2+}-adenosine triphosphatase (ATPase)-dependent refilling of Ca^{2+} stores.[22] Increased NO-stimulated guanylyl cyclase activity results in decreased intracellular Ca^{2+} levels through the inhibition of receptor-mediated Ca^{2+} release from the dense tubular system, an increased rate of Ca^{2+} extrusion, and decreased Ca^{2+} entry from the extracellular environment. In addition, NO increases sarcoplasmic reticulum Ca^{2+}-ATPase activity via cGMP-dependent protein kinase G-mediated phosphorylation of phosphlamban,[23] which results in lower Ca^{2+} available for participating in activation/aggregation mechanisms.

NO's ability to regulate cGMP is the principal determinant of its platelet inhibitory actions. The mechanisms by which cGMP-associated inhibition of platelets occurs include not only a decrease in intracellular Ca^{2+} concentration by the mechanisms described earlier, but also impaired expression of the active (fibrinogen binding) conformation of glycoprotein (GP) IIb/IIIa and decreased platelet-bound fibrinogen. For hemostatic plug formation to take place, platelets must interact with one another by forming crosslinks with cell surface receptors, thereby leading to aggregation. Fibrinogen, a bivalent molecule, bridges adjacent activated platelets via the platelet GPIIb/IIIa integrin receptors. Cyclic GMP reduces the total number of conformationally active GPIIb/IIIa receptors on the platelet surface via inhibition of thrombin-mediated activation of phophoinositide 3-kinase[24] and increases in the dissociation constant between fibrinogen and the GPIIb/IIIa receptor, resulting in unfavorable conditions for platelet aggregation. The resulting decrease in fibrinogen binding has been shown to be dose-dependent and strongly correlated with NO-dependent cGMP production.[25] TXA_2, an eicosanoid produced via arachadonic acid metabolism within the platelet, contributes to platelet aggregation by mediating platelet GPIIb/IIIa expression. The TXA_2 receptor serves as a substrate for cGMP-dependent protein kinase and cGMP itself,[16] leading to impaired receptor function, thereby representing another mechanism by which NO inhibits platelet activation. Furthermore, cGMP regulates the expression of the platelet (and endothelial) surface adhesion molecule P-selectin, in part, through the down-regulation of protein kinase C (PKC).[26]

NO has also been shown to modulate megakaryocyte apoptosis. Megakaryocytes are responsible for platelet production, and factors affecting their viability may ultimately affect platelet formation (thrombopoiesis). Battinelli and Loscalzo[27] investigated the role of NO on apoptosis in megarkaryocyte cell lines and found that NO induced apoptosis. They reported that both endogenous and exogenous sources of NO regulated apoptosis in Meg-01 and human erythroleukemia megakaryocytoid cell lines via modulation of the Bcl-2 family of proteins. This same group further investigated the role of NO-induced apoptosis on platelet formation by exploring the role of NO in the terminal stages of megakaryocytopoiesis. They found that NO-induced apoptosis in megakaryocytes resulted in platelet production.[28] Thus, NO has effects on platelet production from megakaryocytes as well as on platelet function itself.

Nitric oxide synthesis and biochemistry
Nitric oxide synthesis
NO synthesis occurs in a range of cell types and tissues within the vasculature, including platelets, macrophages, and the vascular endothelium.[29] A class of enzymes known as NOS includes neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), all sharing between 50% and 60% sequence homology.[30] All 3 isoforms catalyze a 5-step electron oxidation of the terminal guanidino nitrogen atom of L-arginine to produce L-citrulline and NO.[31] Enzyme activity requires flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH_4), Ca^{2+}-calmodulin, and heme, which serve as cofactors, along with nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, which serve as cosubstrates.[32]

NO is an uncharged radical molecule approximately 70 times more soluble in hydrophobic environments than in water, enabling it to diffuse readily across cell membranes. Owing to its relatively short half-life, NO acts primarily in local environments. After synthesis, NO diffuses across biological membranes into specific target cells where it stimulates guanylyl cyclase to produce cGMP from guanosine triphosphate (GTP).[33] Biological responses mediated by cGMP second messenger pathways described earlier are the principal, but not exclusive, action of NO.[34]

The 3 NOS isoforms are characterized by their site of synthesis, pattern of expression, and Ca^{2+} dependency: NOS I or nNOS is expressed primarily in neurons; NOS II or iNOS, initially isolated from cytokine-induced macrophages, is now recognized as located in macrophages, neutrophils, platelets, and VSMCs, as well as in other nonvascular cells; NOS III or eNOS is constitutively expressed in endothelial cells.[35] Each NOS isoform is comprised of an N-terminal oxygenase domain and a C-terminal reductase domain with a calmodulin recognition sequence between the 2.[36] NOS activity is regulated by changes in intracellular Ca^{2+} concentration that affect the binding of calmodulin for efficient electron transfer from NADPH to the heme iron, which then catalyzes NO synthesis.[37] The constitutively expressed NOS isoforms (nNOS and eNOS) are membrane-associated and bind calmodulin in a rapidly reversible and strongly Ca^{2+}-dependent manner to regulate their activity.

When endothelial cells are activated by a receptor-mediated agonist, such as acetylcholine or bradykinin, or mechanical stimuli, such as shear stress, Ca^{2+} is transiently released from intracellular stores via a second messenger cascade involving inositol triphosphate, resulting in Ca^{2+}-calmodulin complex-dependent activation of eNOS. NO production

by eNOS is transient, paralleling intracellular Ca^{2+} levels.[38] Endothelial NOS produces NO in the nanomolar range, a concentration that decreases with decreasing intracellular Ca^{2+} concentration, associated with the cytoprotective effects of NO and effective regulation of vascular tone. Unlike eNOS and nNOS, iNOS is less susceptible to changes in intracellular Ca^{2+} concentration, binding more avidly to calmodulin at the low Ca^{2+} concentrations found in resting cells.[39] NO production by iNOS is regulated at the transcriptional level, stimulated mainly by cytokines and microbial products (endotoxin),[40] resulting in NO production in the micromolar range; these concentrations have been associated with mitochondrial membrane pore transition and caspase activation, resulting in apoptosis and necrosis.[41] These elevated levels of NO are associated with the general cytotoxic effects of NO.

Nitric oxide bioreactivity

NO can exist in 3 closely interrelated redox forms: the free radical (NO·), nitrosonium (NO^+) resulting from a 1-electron oxidation of NO·, and nitroxyl anion (NO^-) resulting from a 1-electron reduction of NO·.[42] Each redox form has distinct chemical properties and reactivity. The major metabolic product of NO in aqueous solutions is nitrite. These NO species (NO_x) (reactive nitrogen species) and, in some cases, NO itself, can interact with molecular oxygen, thiols, reduced hemoproteins, and redox metals that conspire to determine its half-life and metabolism in biological systems. Some of these interactions include the reaction of NO with heme iron that leads to guanylyl cyclase activation resulting in cGMP formation; and the reaction of nitrosonium with –SH group-containing biomolecules in the presence of molecular oxygen to produce S-nitrosothiols (RSNO). S-nitrosothiols are relatively stable compared with NO and may represent a storage pool for bioavailable NO.[43,44] S-nitrosoalbumin accounts for the majority of RSNO in human plasma, and, along with other S-nitrosothiols, protects NO from inactivation with an approximate half-life ranging from 15 to 40 minutes in circulating blood. Cysteine is the sole thiol source in proteins and also constitutes the reactive thiol residue of glutathione. S-nitrosoglutathione (GSNO) possesses an approximate half-life of 8 minutes in the circulating blood. Biological actions of RSNO may be associated with heterolytic as well as homolytic mechanisms of decomposition resulting in 1 of the 3 NO redox forms. Heterolytic pathways of RSNO decomposition have been associated with many biological activities of NO.[45] The substantially slower reaction rates between superoxide and RSNO compared with superoxide and NO suggest that the RSNO stabilizes NO protecting it

from oxidative inactivation and thereby increasing its bioavailability.[46] In addition, as many S-nitrosoproteins[47] form via *trans*-S-nitrosation from GSNO, GSNO reductase[48] is an important regulator of the bioavailability of (RS)NO.

The ability of NO to form a complex with hemoglobin (Hb) is a recent emerging concept unveiling a vital physiological function in the setting of hypoxic vasodilation. Hb becomes S-nitrosylated on a specific cysteine residue on the β-chain (βCys93) as red blood cells become oxygenated in the lungs, resulting in SNO-Hb formation. This complex, SNO-Hb, exists in 2 conformational states, the R-state (relaxed and unreactive state, high oxygen affinity Hb conformation) and the T-state (tense reactive state, low oxygen affinity Hb conformation) in which SNO-Hb can rapidly react with thiols and elicit a vasodilatory response within the vasculature.[49,50] Dysfunction in this pathway has been shown to be associated with congestive heart failure,[51] diabetes,[52] and pulmonary hypertension.[53] This SNO-Hb paradigm, recently reviewed by Allen et al,[54] could have therapeutic implications for diseases involving abnormal microcirculatory perfusion.

NO can react with molecular oxygen and several reactive oxygen species (ROS), as shown in Figure 1, including superoxide anion ($O_2^{·-}$), hydrogen peroxide (H_2O_2; indirectly), and hydroxyl radical (·OH), to limit its bioactivity. Both endothelial
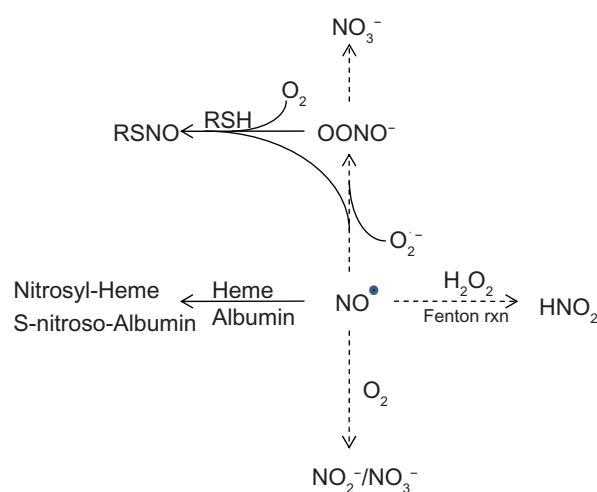
Figure 1 Biological reactions of nitric oxide. The free radical nitric oxide (NO·) can react with many constituents within the vasculature that affect its bioavailability. NO· can undergo oxidative inactivation (reactions shown in dashed lines) to form nitrite (NO_2^-) and nitrate (NO_3^-). Other biologically relevant mechanisms include indirect interaction with hydrogen peroxide (H_2O_2) products (via Fenton chemistry) resulting in nitrous acid (HNO_2) formation. Another key reactive oxygen species in the vasculature contributing to NO insufficiency is superoxide (O_2^-) which reacts with NO to form peroxynitrite ($OONO^-$). S-nitrosothiols (RSNO) formation via $OONO^-$ interaction with thiols, and nitrosyl-heme/S-nitroso-albumin formation represent ways in which NO can be protected from oxidative inactivation, thereby increasing overall bioavailability.

cells and macrophages are sources of these ROS. NO reacts with O_2^{-} to form peroxynitrite $(OONO^-)$[55] at an almost near-diffusion-limited rate of 6.7×10^9 M^{-1} s^{-1}. This rate of peroxynitrite formation exceeds the rate constant for the reduction of superoxide to hydrogen peroxide by superoxide dismutase, the principal antioxidant responsible for O_2^{-} metabolism.[56] Among its deleterious effects are included loss of bioactive NO and direct cytotoxic effects resulting in apoptosis or necrosis.[57]

Through the reduction of H_2O_2 via Fenton chemistry, a hydroxyl radical, $^{\cdot}OH$, can react readily with NO, decreasing its bioavailability. ROS in the vessel wall can react with polyunsaturated fatty acid species in the low density lipoprotein (LDL) particle, initiating lipid peroxidation, which results in the formation of oxidized LDL.[58] Oxidized LDL has been shown to limit NO bioavailability by a number of mechanisms. For example, Chen et al[59] investigated the role of the L-arginine-NO pathway in oxidized LDL-mediated platelet activation, and found that oxidized LDL decreased L-arginine uptake, eNOS expression, cGMP production, and subsequent NO (nitrite) production.

Nitric oxide synthase regulation

The activity of NOS is regulated by a number of transcriptional and posttranslational mechanisms as shown in Figure 2. Some modes of regulation are specific to a particular NOS isoform, whereas others are shared between them. Factors regulating NOS activity affect NO bioavailability and, ultimately, target cell function and phenotype. One major factor regulating NOS activity is feedback inhibition by NO itself. NOS isoforms are hemoproteins, readily binding NO to their heme prosthetic groups.[60] NO binds reversibly to ferric or ferrous heme proteins within seconds after the initiation of NO synthesis, resulting in temporary eNOS inactivation.[60,61] These inactive complexes have the ability to reactivate, producing NO when NO dissociates from the heme iron. Studies have shown that 70%–90% of iNOS and nNOS are present in their inactive ferrous-NO forms during steady-state catalysis,[60,61] resulting in NOS activity that is only a fraction of its maximal activity. Ferrous-NO can react with O_2 to participate in NO generation; ferrous-NO complex formation results in NO synthesis rates proportional to the O_2 concentration throughout the physiological range due primarily to NOS–NO complex dissociation, which itself is a function of O_2 concentration rather than the intrinsic affinity of the NOS heme iron for O_2.[60]

NOS activity can also be regulated by BH_4 levels. BH_4 is a critical cofactor required for NO synthesis owing to its ability to couple heme iron reduction to NO synthesis.

Subsaturating levels of BH_4 have been shown to result in NOS inhibition by NO,[62] occurring over several minutes. Possible mechanisms of inactivation include uncoupled O_2 reduction to superoxide that occurs in the absence of sufficient BH_4, dimer dissociation,[62] or NO binding to heme.[63] Dimerization of NOS is essential for its biological activity, and BH_4 interacts with residues on both subunits of the dimer and also forms hydrogen bonds with the heme site. Dimerization activates NOS by sequestering heme from solvent, creating favorable conditions for L-arginine and BH_4 binding, and also allows electrons from reductase domain flavins to transfer to the oxygenase domain heme.[64,65] NO can inhibit expression of NOS, limiting assembly of dimeric NOS by preventing heme insertion and decreasing heme availability.[66]

Another cofactor essential for NO biosynthesis via NOS is the substrate, L-arginine. L-arginine is converted to L-citrulline and NO via a 5-step electron oxidation of the terminal guanidino-nitrogen atom of L-arginine. An experiment of nature leading to L-arginine deficiency also supports the importance of this amino acid in NO and vascular homeostasis. In the genetic disorder, lysinuric protein intolerance, a mutation in the *SLC7A7* gene leads to impaired L-arginine uptake by cells.[67] The resulting deficiency of L-arginine transport led to significantly reduced NO synthesis, impaired endothelium-dependent dilation,[68] and enhanced platelet activation and intravascular coagulation,[69] highlighting the importance of endogenous L-arginine in NO production and its key vascular actions.[70]

Administration of L-arginine has proven to be protective for endothelial function in animal studies.[71] Both acute and chronic administration of L-arginine in cholesterol-fed animals results in improvement of endothelium-dependent dilation.[72] The beneficial effects of L-arginine administration have also been demonstrated in other disease states, such as hypertension,[73] coronary atherosclerosis,[74] and transgenic-knockout sickle mice.[75] L-arginine supplementation in hypercholesterolemic rabbits resulted in attenuation of aorta intima/media thickening and increased eNOS expression compared with controls.[76] A randomized, double-blind, placebo-controlled study administering oral L-arginine to patients with heart failure for 6 weeks resulted in improved forearm blood flow, increased distances during a 6-minute walk test, as well as improved arterial compliance compared with controls.[77] Most of the previously mentioned studies showing beneficial effects of L-arginine supplementation were short-term studies. Long-term administration of L-arginine in patients with peripheral artery disease showed the contrary data. Wilson et al[78] conducted a randomized clinical trial of oral

Endothelial cell
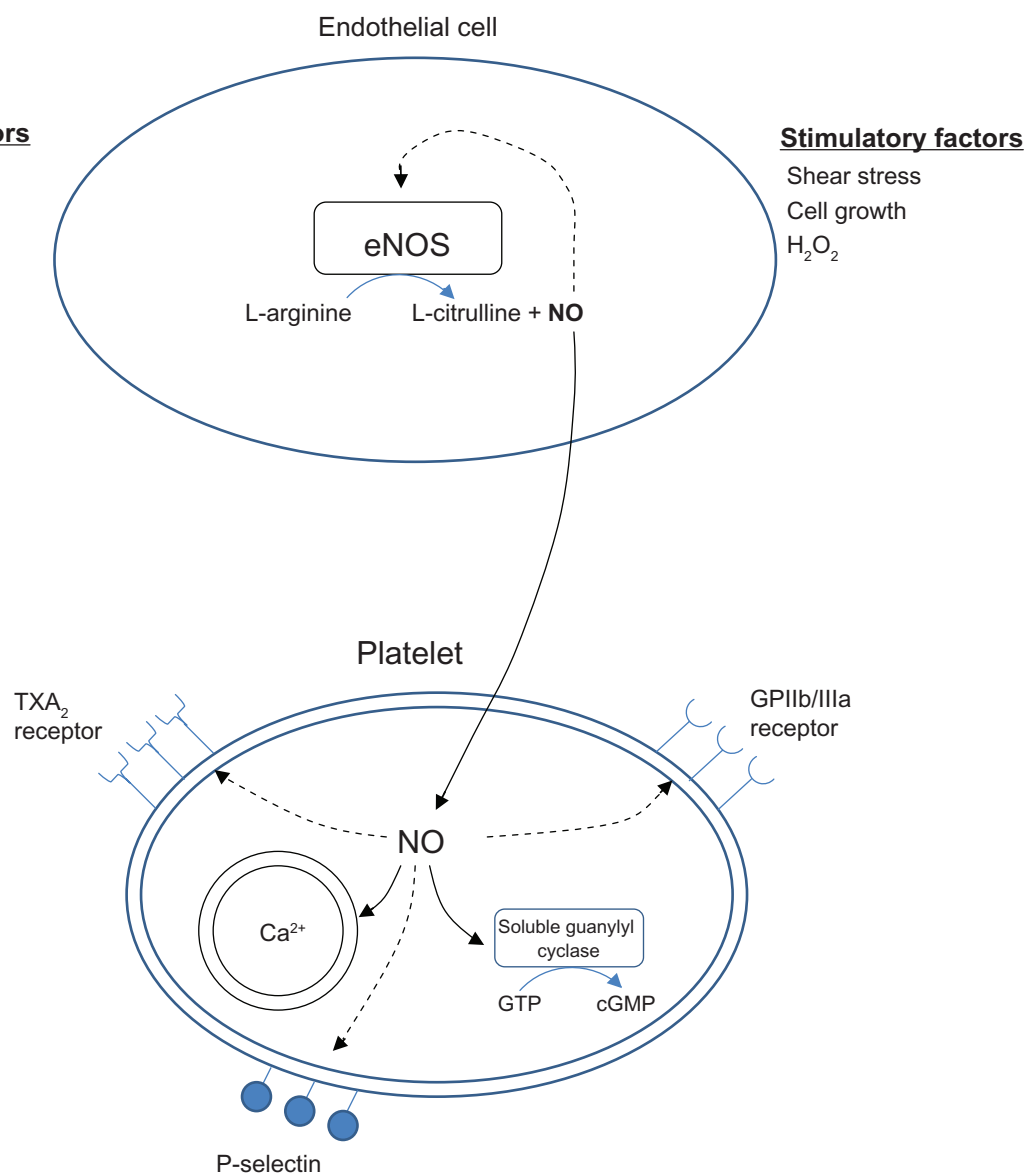
Figure 2 Factors and actions affecting NO and platelet function. Nitric oxide (NO) exerts inhibitory effects (dashed lines) on platelets through a variety of mechanisms. The predominant pathway involves generation of NO from L-arginine by endothelial nitric oxide synthase (eNOS) and cyclic guanosine monophosphate (cGMP) formation via guanylyl cyclase activation by NO. Nitric oxide inhibits platelet activation and aggregation by decreasing intracellular Ca^{2+} concentration, glycoprotein (GP) IIB/IIIa expression, and platelet association with fibrinogen. cGMP, formed from NO's catalytic effect on guanylyl cyclase, also inhibits platelets by decreasing thromboxane A_2 expression as well as expression of the platelet surface adhesion molecule, P-selectin. Endothelial NOS activity is regulated by several transcriptional, posttranslational, and physiological factors that either result in inactivation or upregulation, ultimately affecting NO bioavailability. Factors decreasing eNOS (dashed lines) activity include NO itself, via negative feedback, reduced substrate and/or cofactor bioavailability, hypoxia, and oxidized low density lipoprotein (LDL). By contrast, laminar shear stress, cell growth, and H_2O_2 have been shown to increase eNOS activity (solid lines), cGMP formation, and bioavailable NO.

L-arginine vs placebo for 6 months in patients with intermittent claudication due to peripheral artery disease. They found that long-term L-arginine supplementation did not increase NO synthesis or improve vascular reactivity.[78] In another clinical study involving patients with stable coronary heart disease, L-arginine supplementation failed to show any significant improvement of endothelium-dependent vasodilation or blood flow as compared with placebo.[79] L-arginine supplementation has also been shown to have no significant effect on endothelial function, blood flow, markers for oxidative stress, or exercise performance in patients with coronary heart disease and stenosis[80] as well as no benefit in patients with coronary artery disease after the onset of myocardial infarction with respect to death, reinfarction, or recurrent myocardial ischemia.[81] The mechanism by which L-arginine administration exerts its beneficial effects in various disease states is unclear; however, future benefits occurring via the enhancement of vascular NO synthesis has been supported

by corresponding upregulation of the NO-cGMP pathway in endothelial cells of the myocardium.[82]

In addition to limited cofactor availability affecting eNOS activity and NO synthesis, a naturally occurring inhibitor, asymmetric dimethylarginine (ADMA), was first identified in 1992.[83] ADMA is synthesized by methylation of arginine residues via N-methyltransferases, which utilize S-adenosylmethionine as a methyl group donor. It is released when the methylated arginine residues are degraded into their amino acid components during hydrolytic protein turnover.[84] The physiological effects of ADMA were first characterized in vascular rings where ADMA inhibited NOS resulting in decreased NO synthesis and vasoconstriction.[83] Plasma ADMA concentrations are elevated in many disease states, including hypercholesterolemia,[85] hyperhomocysteinemia,[86] hypertension,[87] pulmonary hypertension,[88] stroke,[89] and diabetes mellitus.[90] A recent study by Ding et al[91] identified a novel 4-nucleotide deletion/insertion variant in the dimethylarginine dimethylaminohydrolase I (*DDAH1*) gene, an important gene in the major pathway for ADMA clearance, associated with increased susceptibility to thrombotic stroke and coronary heart disease. Overexpression of DDAH1 has been shown to protect against cerebral arteriole hypertrophy and to improve vascular smooth muscle function.[92] In a study investigating the effects of ADMA on shear stress-dependent dilation of skeletal muscle arterioles, Toth et al[93] showed that ADMA inhibited shear stress-induced NO release and vasodilation, and, instead, stimulated O_2^{-} release, favoring the development of increased shear stress, increased vascular resistance, and, ultimately, hypertension.

The association between plasma ADMA concentration and endothelial dysfunction contributing to cardiovascular disease has lead to its emerging as a novel risk factor and potential pharmacotherapeutic target. Clinical studies involving L-arginine supplementation in hypercholesterolemic subjects have shown improved endothelial function,[94] improved vasomotor responses to methacholine,[95] and improved flow-mediated dilation[96] in the brachial artery. Studies involving L-arginine supplementation in subjects with arterial hypertension have also shown increases in forearm blood flow,[77] improvement in coronary small-vessel endothelial function,[97] and decreased blood pressure via increased NO production.[98]

In addition to posttranslational mechanisms regulating NOS activity, there are many transcriptional and post-transcriptional regulators of the enzyme. Posttranslational regulators of eNOS include phosphorylation by several kinases regulating protein–protein interactions and the avail-

ability of cofactors and substrates,[99] localization of eNOS to the caveolae, and dissociation from caveolin-1.[100] Other posttranslational modifications regulating eNOS expression include myristoylation and palmitoylation, which are required for the targeting of eNOS to the caveolae.[101,102]

Several physiological and pathophysiological stimuli have been indentified that affect eNOS mRNA expression and stability. Stimuli shown to increase eNOS expression include shear stress, cell growth, and hydrogen peroxide. By contrast, hypoxia and oxidized LDL can decrease eNOS expression. One of the more potent regulators of eNOS expression is laminar shear stress. Both *in vivo*[103] and *in vitro*[104,105] studies have shown an upregulation in eNOS expression due to shear stress. Weber et al[106] conducted *in vitro* experiments using bovine aortic endothelial cells and found that shear stress increased expression of eNOS mRNA with long poly(A) tails resulting in a 3-fold increase in half-life compared with cells not exposed to shear (ie, cultured under static conditions). Their results support the concept that shear stress modulates eNOS mRNA stability and translation via increased 3′-adenylation due to a dose-dependent effect of shear on eNOS polyadenylation, also observed *in vivo*,[107] and shown to be dependent on the duration of shear stress. Laminar shear stress resulted in an early increase in eNOS mRNA expression and a sustained stabilization of eNOS mRNA, as evidenced by a 4-fold to 5-fold increase in transcription and an increase in mRNA half-life by more than 3-fold compared with cells cultured under static conditions.[108] Davis et al[108] identified two pathways involving c-src that lead to an increase in eNOS mRNA transcription and mRNA stability. The increase in eNOS mRNA transcription involved the Raf, Ras, and ERK 1/2 pathway, whereas the stabilization of eNOS in response to shear stress was completely abrogated by the c-src inhibitor PP1, but not altered by Ras or ERK inhibition, supporting the existence of separate pathways responsible for the increase in transcription and for mRNA stabilization.

More recent studies identified miR-21 as a regulator of eNOS activity and apoptosis in human umbilical vein endothelial cells (HUVEC). MicroRNAs are short RNA molecules averaging 22 nucleotides that bind to complementary sequences in the 3′-untranslated region (UTR) of mRNA transcripts, leading to posttranscriptional regulation (degradation of mRNAs) of genes involved in differentiation, proliferation, and apoptosis. Through the utilization of a TaqMan low density array and qRT-PCR, miR-21 was shown to be increased 5.2-fold in HUVECs exposed to unidirectional shear stress compared with cells grown under static conditions. HUVECs overexpressing miR-21 had

decreased apoptosis and increased eNOS phosphorylation and NO production.[109] miR-21 also suppressed phosphatase and tensin (PTEN), which is a tumor suppressor gene, and has been shown to play an important role in regulating apoptosis via antagonism of the PI3K/Akt pathway.[110] The authors concluded that miR-21 overexpression in HUVECs involves regulation of PTEN expression and subsequent changes in the PI3K/Akt/eNOS pathway.[109]

Cell growth has also been shown to be a potent stimulus for eNOS expression, both *in vitro*[111] and *in vivo*.[112] *In vitro* studies using bovine aortic endothelial cells (BAECs) revealed a 3-fold increase in eNOS protein level, a 6-fold increase in mRNA expression, and a 3-fold increase in eNOS activity in proliferating cells compared with confluent cells.[111] The authors concluded that these results were due either to an increase in transcription or a decrease in degradation of eNOS mRNA. In a follow-up study, Searles et al[113] investigated the factors responsible for changes in eNOS expression during cell proliferation. Using BAECs, they showed steady-state eNOS mRNA levels to be 4-fold higher in proliferating cells compared with confluent cells. A nuclear run-on analysis was performed to determine whether or not this was due to a change in transcription. The intensity of the bands for eNOS nascent transcripts was identical between proliferating and confluent cells, thereby providing evidence for growth-induced regulation of eNOS expression modulated by post-transcriptional mechanisms. By contrast, they observed a 3-fold increase in half-life in proliferating cells compared with confluent cells. Ultraviolet-crosslinking analysis revealed a 51-kDa protein expressed 3-fold higher in confluent cells compared with proliferating cells. This cytosolic protein was found to bind to a 43-nt *cis*-element in the proximal 3′-UTR resulting in destabilization of eNOS mRNA.[113]

Endothelial NO activity has also been shown to be regulated by ROS. Hydrogen peroxide, in particular, induces both an elevation of eNOS protein expression and enzyme activity. Studies have shown that adding H_2O_2 to endothelial cells stimulates eNOS activation by PI3-kinase-Akt-mediated phosphorylation of Ser1177 on eNOS.[114] Drummond et al[115] performed nuclear run-on and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole case studies to show a 3-fold increase in eNOS transcription and a 2.8-fold increase in eNOS half-life in cells exposed to H_2O_2. A subsequent study revealed that the induction of eNOS expression by H_2O_2 was Ca^{2+}-dependent. Immunocytochemical staining of BAECs revealed expression of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), and an in-gel kinase assay showed H_2O_2-induced autophosphorylation of CaM kinase II,

resulting in increased eNOS activity. Cai et al[116] were able to show that H_2O_2-induced activation of eNOS was dependent on the CaM kinase II/Janus kinase (JNK) 2 pathway. Hydrogen peroxide produced under shear stress has been shown to be regulated by PKC delta. Kumar et al[117] investigated the mechanism by which shear stress can stimulate eNOS activity via PI3 kinase/Akt signaling and phosphorylation of Ser1177. They found that shear stress decreased PKC delta activity, attenuating catalase activity and increasing H_2O_2 signaling, resulting in increased eNOS phosphorylation at Ser1177 and NO synthesis.[117] A recent study by Tian et al[118] investigated how H_2O_2 affects NO activity when endothelial cells are subjected to shear stress. Using a small interfering RNA for caveolin-1, they showed decreased eNOS localization to the plasma membrane as well as impairment of increases in NO in BAECs. Caveolin-1 enhances eNOS transport to the plasma membrane and appears to be involved in priming eNOS for activation under conditions of shear stress.[118]

Although factors such as shear stress, cell proliferation, and H_2O_2 have been found to increase both eNOS expression and stability, hypoxia has been associated with both upregulation and down-regulation of eNOS expression via transcriptional and posttranscriptional mechanisms.[119–122] In response to hypoxia, the systemic arteries will dilate, allowing more blood to be delivered to peripheral tissues, whereas vasoconstriction will take place in the pulmonary system resulting in higher pulmonary pressures. This same trend has been shown for eNOS expression in rats exposed to hypoxia. Rats subjected to 10% oxygen for 12 hours, 48 hours, or 7 days showed an 80% decrease in eNOS protein expression as well as eNOS mRNA expression compared with normoxic controls.[119] Exposure to hypoxia also resulted in impaired capacity of aortic segments to generate cGMP in response to stimulation by acetylcholine. Comparison of eNOS expression in the left and right lungs of normoxic and chronically hypoxic rats resulted in a decrease in eNOS protein expression (32% ± 7%) and eNOS mRNA expression (54% ± 13%) in hypoxic lungs compared with the normoxic lung from the same rat.[120] Thus, not only are there regional differences in eNOS expression in the setting of hypoxia, but the onset or duration of hypoxia may also affect eNOS expression. In lung biopsies of infants with pulmonary hypertension secondary to cardiac abnormalities (early stage of pulmonary hypertension), eNOS expression was found to be significantly increased in pulmonary vascular endothelial cells.[121] This increase in eNOS expression in early stages of pulmonary vascular disease may represent a compensatory mechanism to rising pulmonary pressures. However, in the

setting of chronic pulmonary hypertension, there is reduced eNOS expression.[122]

Endothelial NOS activity is not always simply overexpressed or inhibited by certain physiological or pathophysiological stimuli. As is the case with hypoxia, where regional differences and onset/duration of disease can affect eNOS expression, oxidized LDL has been shown to elicit both stimulatory and inhibitory responses in eNOS expression. Liao et al[123] treated human saphenous vein endothelial cells with increasing concentrations of native or oxidized LDL and found that oxidized LDL resulted in a time-dependent decrease in eNOS mRNA expression. This decrease in eNOS mRNA expression coincided with a 56% decrease in NOS activity. Nuclear run-on studies revealed the complex nature of eNOS regulation by oxidized LDL. During the first 6 hours of treatment, eNOS expression was decreased by 25%, followed by a 2.2-fold increase in the subsequent 18 hours. The results of this study indicate that oxidized LDL regulates eNOS expression through a combination of early transcriptional inhibition and mRNA destabilization.

Vascular diseases and nitric oxide
Atherosclerosis and thrombosis
In addition to promoting endothelial dysfunction, NO insufficiency has been shown to increase atherosclerotic burden *in vivo*. Under normal conditions, NO exerts atheroprotective effects through several pathways, including the reduction of endothelial cell apoptosis and activation, via inhibition of nuclear factor-kappa factor (NF-κB) and inflammatory gene expression.[124] Another way in which NO prevents atherogenesis is via the attenuation of leukocyte–endothelial cell adhesive interactions.[125] BH_4 deficiency, which is commonplace in vascular disease, has been linked to eNOS uncoupling in the vasculature, resulting in O_2^- production and NO insufficiency. A more recent study by Takaya et al[126] highlighted the importance of BH_4 availability in preventing eNOS uncoupling-induced atherosclerotic lesion progression. The authors compared atherosclerotic lesion progression between apolipoprotein E knockout (ApoE-KO)/eNOS-Tg mice treated with vitamin C and ApoE-KO mice crossed with mice overexpressing eNOS (eNOS-Tg) with mice overexpressing GTP-cyclohydrolase I (GCH-Tg) to produce ApoE-KO/eNOS-Tg/GCH-Tg mice. Atherosclerotic formation was increased in the ApoE-KO/eNOS-Tg mice compared with ApoE-KO mice. This finding was attributed to uncoupled eNOS-superoxide production and was reversed by overexpressing GTP-cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis.[126] Endothelial

dysfunction in hypercholesterolemia has been implicated in atherosclerosis progression. Several studies have shown a decrease of eNOS activity and expression,[127,128] as well as its reversal with L-arginine supplementation[129,130] in hypercholesterolemic models.

NO insufficiency, eNOS deficiency,[131] and a deficiency of the extracellular antioxidant enzyme plasma glutathione peroxidase (GPx-3) in patients with vascular disease and stroke represent further evidence of the prothrombotic consequence of NO insufficiency *in vivo*. The first direct evidence that an arterial thrombotic disorder can derive from NO insufficiency stems from a study conducted by Freedman et al[132] analyzing platelet inhibition by NO in 2 brothers with a cerebral thrombotic disorder of unknown etiology. The patients were found to have hyperreactive platelets, a 3-fold increase in plasma H_2O_2, and decreased GPx-3 activity. GPx-3 is an enzyme that protects cells from oxidative damage via the reduction of hydrogen peroxide and lipid hydroperoxides. GPx-3 is produced primarily in the renal promixal tubules and is the only isoform in the GPx family that is found in the extracellular space. During platelet activation, specifically, activation of the second messenger cascade and the cytoskeletal changes that occur, ROS are generated.[133] Hydrogen peroxide production as a result of collage-stimulated platelet activation has been shown to reach concentrations up to 1 mM.[134] GPx-3 maintains NO bioavailability by preventing the accumulation of ROS like H_2O_2, allowing NO to exert an inhibitory effect on platelets and preventing platelet-dependent thrombosis. Through platelet/plasma mixing experiments, it was determined that NO bioavailability was too low to limit platelet activation in patient plasma. After glutathione peroxidase was exogenously added to the patient plasma, the inhibitory effects of NO were restored. We concluded that decreased levels of GPx-3 resulted in increase ROS flux, limiting the ability of NO to inhibit further platelet recruitment to the growing platelet thrombus (which is partly due to platelet-derived NO[135]), resulting in a prothrombotic state. More recent studies have demonstrated an association between decreased GPx-3 activity in both arterial ischemic stroke[136] and cerebral venous thrombosis[137] patients.

Hypertension
Endothelial dysfunction in hypertension and diabetes mellitus appears to be due, in part, to an imbalance in ROS generation and a decrease in NO bioavailability. This decrease in NO bioavailability contributes to endothelial dysfunction, a common property in essential hypertension[138–140] and diabetes mellitus.[141,142] Endothelial NOS plays a prominent

role in regulating NO bioavailability, which contributes to endothelial function or the lack thereof, in various vascular disease states. In most situations in which endothelial dysfunction occurs as a result of increased oxidative stress, eNOS expression is upregulated rather than decreased.[143,144] This paradoxical increase in eNOS expression is likely due to increased H_2O_2 production by the endothelium, which upregulates eNOS via the PI3/Akt pathway. Upregulation of eNOS expression in the setting of endothelial dysfunction shows that this increase in NO synthesis may not be sufficient to overcome the deleterious effects or other factors that limit and decrease overall NO bioavailability.

Several studies have shown that eNOS uncoupling due to insufficient levels of the cofactor BH_4 can shift eNOS activity away from converting L-arginine to L-citrulline and NO production, toward generating $O_2^{-\cdot}$ via reduction of molecular oxygen.[145,146] Although several studies have shown that L-arginine supplementation improves endothelial dysfunction,[72,147,148] physiological concentrations in plasma (~100 μM)[149] far exceed the K_m of eNOS for L-arginine (~3 μM),[150,151] making substrate insufficiency an unlikely cause of eNOS uncoupling. As previously mentioned, BH_4 plays a critical role in eNOS activation that binds to heme and is involved in the electron transport-mediated release of NO and L-citrulline. Under conditions in which BH_4 is limited, eNOS cannot transfer electrons efficiently and, instead, reduces molecular oxygen resulting in $O_2^{-\cdot}$ production. One possible cause of BH_4 depletion may be attributed to its reactivity with $OONO^-$. Peroxynitrite is readily formed when NO reacts with $O_2^{-\cdot}$, which, in turn, can readily oxidize BH_4 to the BH_3 radical, resulting in eNOS uncoupling. Oxidative degradation of BH_4 by H_2O_2 and $OONO^-$ leading to the formation of 7,8-dihydro- L-biopterin, effectively competing for eNOS BH_4 binding sites, also results in eNOS uncoupling.

A major source of ROS found in hypertension can be attributed to activity of the NADPH-oxidases. NADPH-oxidase is a multisubunit enzyme that catalyzes the production of $O_2^{-\cdot}$ by the 1-electron reduction of O_2 using NADPH as the electron donor. An increase in NADPH-oxidase activity has been implicated with regulating vascular tone directly or indirectly by decreasing NO bioavailability through $O_2^{-\cdot}$ production.[152,153] Several studies have shown an increase in NADPH-oxidase-dependent $O_2^{-\cdot}$ production resulting in decreased NO bioavailability.[154,155] Superoxide dismutase, an antioxidant responsible for neutralization of $O_2^{-\cdot}$, was recently shown to prevent NADPH-oxidase-induced oxidative stress in both the early stages of diabetes mellitus and hypertension,[156] providing further evidence for redox imbalance contributing to endothelial dysfunction in these disorders.

Diabetes mellitus

As with hypertension, ROS generation and redox imbalance play a causative role in endothelial dysfunction in diabetes mellitus. Increased O_2^- levels have been reported in diabetic rats[157,158] as well as in diabetic hypertensive patients.[159] Hyperglycemia increases O_2^- generation in endothelial cells.[160] Possible mechanisms contributing to ROS generation under hyperglycemic conditions involve direct effects of glucose and free fatty acids on endothelial ROS production, as well as via the generation of advanced glycation end (AGE)-products.

The activation of NADPH-oxidase has been implicated as a source of increased ROS formation in endothelial cells exposed to hyperglycemia[161] as well as in hyperglycemic rats.[162] NADPH-oxidase activation appears to occur via increased diacylglycerol-mediated activation of PKC[161] and PI3-kinase-dependent activation of PKC,[163] both of which result in ROS production. Free fatty acids also stimulate ROS formation in endothelial cells[164,165] and appear to be associated with PKC activation.[161] Recent studies have shown a synergistic activation of glucose-6-phosphate dehydrogenase, the rate limiting enzyme in the pentose phosphate pathway, and NADPH-oxidase resulting in increased $O_2^{-\cdot}$ generation in type 2 diabetic rats.[166,162] AGE-products are formed as a result of nonenzymatic glycation and oxidation of proteins, lipids, and polynucleotides. The receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor that interacts with a variety of ligands. RAGE signaling includes the activation of NF-κB, which is associated with inflammation.[167] In vascular endothelial cells and monocytes, RAGE activation results in ROS formation via NADPH-oxidase activation. Although there are many possible causes of endothelial dysfunction in the setting of hypertension and diabetes, ROS generation, specifically $O_2^{-\cdot}$, appears regulated principally by NADPH-oxidase activation.

Pharmacotherapies and vascular nitric oxide

Effects of statins on nitric oxide

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are accepted as first-line agents for the prevention of cardiovascular diseases associated with atherosclerosis. Statins lower serum cholesterol by inhibiting the reduction of HMG-CoA to mevalonate, the rate-limiting step of liver cholesterol biosynthesis.[168] Statins

have been shown to reduce adverse cardiovascular events independent of the reduction of lowered serum cholesterol levels, suggesting additional beneficial effects of the drugs. These cholesterol-independent, pleiotropic effects of statins include improvement in endothelial function, stability of atherosclerotic plaques, and a decrease in oxidative stress and inflammation.[169] In endothelial cells, inactivation of RhoA by statins results in increased NO biosynthesis through increased expression of eNOS.[170] Recently, Nohria et al[171] demonstrated that high-dose atorvastatin inhibits the Rho/Rho-associated kinase pathway in patients with atherosclerosis, independent of cholesterol reduction.

Several studies have investigated the possible mechanisms by which statins increase NO bioavailability. One such study by Laufs et al[172] investigated whether statins can directly upregulate eNOS activity independent of a decrease in serum cholesterol levels. Human saphenous vein endothelial cells treated with oxidized-LDL resulted in decreased eNOS mRNA and protein levels (91% ± 4% and 67% ± 8%) after 72 hours.[172] Treatment with either simvastatin or lovastatin resulted in increased eNOS expression by almost 4-fold. Actinomycin treatment revealed that statins also increased the half-life of eNOS mRNA, supporting the concept that upregulation of eNOS by statins occurs via a posttranscriptional mechanism. Other studies showed that statins increased eNOS activity via activation of the PI3/Akt kinase pathway, resulting in upregulation of eNOS, increased cGMP production, and NO biosynthesis.[173,174]

Another mechanism by which statins restore endothelial function is through the inhibition of NADPH oxidase activity and uncoupled eNOS-dependent $O_2^{-\cdot}$ production.[175,176] Cholesterol is a large component of caveolae, which constitute plasma membrane microdomains and are ubiquitously present in endothelial cells.[177] Caveolin-1 is a 22 kDa protein constituent of caveolae which acts as a scaffolding protein that can modulate signaling of proteins and mediate transcytosis, transferring molecules from the lumen of blood vessels to the subendothelial space. Caveolae have been shown to be involved in the regulation of eNOS, which targets to endothelial caveolae via N-terminal myristoylation and plamitoylation, forming an inhibitory complex, that renders the enzyme inactive.[178] This inhibitory complex can be reversed through increased Ca^{2+} concentrations, leading to Ca^{2+}-calmodulin binding and caveolin displacement of eNOS.[179] A recent study by Suh et al showed that rosuvastatin improved endothelial dysfunction in spontaneously hypertensive rats: the statin resulted in increased NO_x levels, phosphorylation of eNOS

at Ser1177, and decreased caveolin-1 expression compared with controls.[180]

Nitrovasodilators and vascular nitric oxide

Nitrovasodilators have long been used in clinical settings to treat angina pectoris. Even though the physiological effects of nitrate administration is undeniably beneficial, the mechanisms through which it exerts its effects on the endothelium and the heart are not fully understood. A number of studies have implicated several enzymes, such as glutathione S-transferase,[181,182] oxidoreductases,[183] and mitochondrial aldehyde dehydrogenase,[184] in the bioactivation and conversion of nitroglycerin to NO. Despite the extensive number of studies conducted, none have been able to explain the observation that maximal nitroglycerin-induced vasorelaxation occurs within minutes of administration,[185] whereas NO produced from nitroglycerin bioactivation takes over an hour.[186] Bonini et al recently investigated the possible role for eNOS-mediated vasodilation in response to nitroglycerin administration. In their study, the authors showed a decrease in systolic and diastolic pressures immediately after sublingual nitroglycerin administration in rats. Treatment with NOS inhibitors, aminoguanidine and L-N^5-(1-iminoethyl) ornithine dihydrochloride, inhibited average diastolic arterial pressures and aortic ring relaxation compared with controls.[187] They also showed a time course of nitroglycerin-induced eNOS phosphorylation in HUVEC cells, which resulted in peak Ser1177-phosphorylation 5 minutes after treatment. Through their study, they suggested that eNOS modulation by nitroglycerin is involved in the immediate effects following nitroglycerin administration.

Conclusion

NO plays a versatile role in the vasculature, maintaining vascular tone, inhibiting or reversing platelet activity, and preventing endothelial dysfunction often seen in vascular disease states. This highly reactive molecule contributes to vascular hemostasis through a variety of different pathways, with the classical signaling heme group-binding reaction leading to activation of soluble guanylyl cyclase to produce cGMP as a central mechanism. Not surprisingly, NO exerts its inhibitory effects on platelets primarily, but not entirely, through cGMP generation, which, in turn, decreases intracellular Ca^{2+} concentration, GPIIb/IIIa expression, platelet fibrinogen binding, and platelet surface P-selectin expression. In the vasculature, an increase in ROS leading to NO insufficiency defines the pathobiological state of oxidant stress. Oxidant

stress can arise from pathophysiological stimulation of enzymatic sources and can result in a perturbation of normal endothelial and platelet function. Although NO inactivation is primarily due to deleterious interactions with ROS within the vasculature, other factors limiting cofactor or substrate bioavailability may result in eNOS uncoupling, resulting in decreased NO generation. Nevertheless, despite the increasing amount of information acquired since the discovery of endogenous NO 3 decades ago, many questions remain about the versatile nature of NO itself, and the complex interactions in which it is involved. With respect to potential new therapies intended to treat disease states, such as hypertension, diabetes mellitus, and atherothrombosis, associated with NO insufficiency, more studies, both basic and clinical, are clearly needed to elucidate the complex role NO plays in treating or preventing these common disorders.

Disclosure

The authors report no conflicts of interest in this work.

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