

Thirty Years of Saying NO

Sources, Fate, Actions, and Misfortunes of the Endothelium-Derived Vasodilator Mediator

Paul M. Vanhoutte, Yingzi Zhao, Aimin Xu, Susan W.S. Leung

Abstract: Endothelial cells control vascular tone by releasing nitric oxide (NO) produced by endothelial NO synthase. The activity of endothelial NO synthase is modulated by the calcium concentration and by post-translational modifications (eg, phosphorylation). When NO reaches vascular smooth muscle, soluble guanylyl cyclase is its primary target producing cGMP. NO production is stimulated by circulating substances (eg, catecholamines), platelet products (eg, serotonin), autacoids formed in (eg, bradykinin) or near (eg, adiponectin) the vascular wall and physical factors (eg, shear stress). NO dysfunction can be caused, alone or in combination, by abnormal coupling of endothelial cell membrane receptors, insufficient supply of substrate (L-arginine) or cofactors (tetrahydrobiopterin), endogenous inhibitors (asymmetrical dimethyl arginine), reduced expression/presence/dimerization of endothelial NO synthase, inhibition of its enzymatic activity, accelerated disposition of NO by reactive oxygen species and abnormal responses (eg, biased soluble guanylyl cyclase activity producing cyclic inosine monophosphate) of the vascular smooth muscle. Major culprits causing endothelial dysfunction, irrespective of the underlying pathological process (aging, obesity, diabetes mellitus, and hypertension), include stimulation of mineralocorticoid receptors, activation of endothelial Rho-kinase, augmented presence of asymmetrical dimethyl arginine, and exaggerated oxidative stress. Genetic and pharmacological interventions improve dysfunctional NO-mediated vasodilations if protecting the supply of substrate and cofactors for endothelial NO synthase, preserving the presence and activity of the enzyme and reducing reactive oxygen species generation. Common achievers of such improvement include maintained levels of estrogens and increased production of adiponectin and induction of silent mating-type information regulation 2 homologue 1. Obviously, endothelium-dependent relaxations are not the only beneficial action of NO in the vascular wall. Thus, reduced NO-mediated responses precede and initiate the atherosclerotic process. (*Circ Res.* 2016;119:375-396. DOI: 10.1161/CIRCRESAHA.116.306531.)

Key Words: atherosclerosis ■ calcium ■ endothelium ■ nitric oxide ■ reactive oxygen species

The historical demonstration by Furchgott and Zawadzki¹ that removal of the endothelium abrogates the in vitro vasodilator effect of acetylcholine has led to the identification 30 years ago of nitric oxide (NO) as a major endogenous local regulator of vascular tone.²⁻¹⁰ When the ability of endothelial cells to produce NO is blunted, the ensuing vascular dysfunction sets the stage for the occurrence of cardiovascular disease in general, and atherosclerosis in particular.¹¹⁻¹⁵ This review focuses, step-by-step, on the bioavailability (production, action, and disposition) of endothelium-derived NO as local regulator of vascular tone in health and disease. Obviously, there is much more than NO in the control of the degree of contraction of underlying vascular smooth muscle cells exerted by the endothelial cells. Indeed, they generate also prostacyclin and hyperpolarizing

signals (initiating endothelium-dependent hyperpolarization [EDH] and thus relaxation) and produce vasoconstrictor mediators (endothelium-derived contracting factors and the peptide endothelin-1); those have been discussed elsewhere in detail.¹⁵⁻²²

Endothelial Sources of NO

NO Synthase

Three NO synthase (NOS) isozymes, which are encoded by different genes, catalyze the production of NO from L-arginine: neuronal NOS (or NOS-1), cytokine-inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3).^{6,23-25} Although iNOS can be induced (by lipopolysaccharides and inflammatory cytokines) and neuronal NOS can be present in the blood vessel

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To limit the number of references cited, a large number of individual experimental studies were not included in the reference list as they were mentioned in an extensive recent review article (Vanhoutte et al.¹⁵ doi:10.1111/apha.12646) to which the readers are referred for more complete coverage of the literature, with the authors' apologies for the inconvenience.

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Nonstandard Abbreviations and Acronyms

ADMA	asymmetrical dimethyl arginine
Akt	protein kinase B
AMPK	AMP-activated protein kinase
BH₂	dihydrobiopterin
BH₄	tetrahydrobiopterin
Ca²⁺	calcium ions
cIMP	3'-5'-cyclic inosine monophosphate
CoA	coenzyme A
EDH	endothelium-dependent hyperpolarization
eNOS	endothelial NOS
ERK1/2	extracellular signal-regulated kinases 1/2
GRK2	G-protein-coupled receptor kinase 2
HDL	high-density lipoproteins
HO-1	heme oxygenase-1
iNOS	cytokine-inducible NOS
IP₃	inositol trisphosphate
LDL	low-density lipoproteins
NFκB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
NOX	nicotinamide adenine dinucleotide phosphate oxidase
PI3K	phosphoinositide 3-kinase
PK	protein kinase
PP	protein phosphatases
ROS	reactive oxygen species
sGC	soluble guanylyl cyclase
SIRT1	silent mating-type information regulation 2 homologue 1
SOD	superoxide dismutases
SR	sarco/endoplasmic reticulum
TLR	toll-like receptors
TRP	transient receptor potential
TRPV	TRP vanilloid subtypes

wall, in the rule, eNOS is the major source of endothelium-derived NO. Endothelial NOS is localized in caveolae, which are small invaginations of the plasma membrane containing caveolin-1 protein; the association of the caveolin-binding domain of eNOS with caveolin-1 results, on the one hand, in the localization of the enzyme to the caveolar microdomains where several signaling molecules (G-protein-coupled receptors, ion channels, protein kinases [PK], and tyrosine kinases, which also contain a caveolin-binding domain) are localized but, on the other hand, also causes a steric hindrance for the binding of calcium-calmodulin, which is important for the calcium-dependent activation of the enzyme (see Calcium-Dependent Activation of eNOS section of this article).^{25–28} With increased calcium-calmodulin formation (subsequent to increases in the intracellular calcium concentration; see Calcium-Dependent Activation of eNOS section of this article), eNOS associates with this complex, which displaces caveolin-1 thereby relieving the inhibitory effect of the latter.^{25–28} Thus, the dissociation from caveolin-1 promotes the translocation of eNOS to the cytosol where the enzyme can be optimally active because it has access to a milieu rich in oxygen, substrate, and cofactors and is more exposed to post-translational

modifiers (see Post-Translational Modulation of eNOS Activity section of this article); it also enhances the association of eNOS with heat shock protein 90, acting not only as a scaffold for kinases thereby facilitating the phosphorylation of the enzyme but also an enhancer of the displacement of caveolin-1 by the calcium-calmodulin complex (see Post-Translational Modulation of eNOS Activity section of this article; Figure 1).^{26–28}

The generation of NO from its precursor L-arginine by eNOS requires the presence of several cofactors, in particular, tetrahydrobiopterin (BH₄); although the protein eNOS is synthesized as monomer, it has to form dimers to bind BH₄ and L-arginine to achieve NO production.^{15,25,28–33} In the absence of BH₄ and L-arginine, the oxygenase domain of eNOS monomers generates superoxide anions instead of NO, a condition referred to as eNOS uncoupling.^{25,28,29,31} Compared with the actual total protein presence of the eNOS enzyme, the degree of dimer-formation and their coupling determines the production of NO. NO produced by eNOS contributes to the regulation of the endothelial BH₄-content because it prevents the degradation, via S-nitrosylation³³ (addition of NO to cysteine residues of proteins) of the enzyme dihydrofolate reductase that recovers BH₄ from oxidized dihydrobiopterin (BH₂; see Reduced Cofactor Availability section of this article).^{15,25,28–33}

Experiments in isolated blood vessels and endothelial cell cultures suggest that eNOS is constitutively active and responsible for the so-called basal release of NO in the absence of obvious external stimuli. The activity of the enzyme can be increased in both calcium-dependent and calcium-independent ways (Figure 1).

Calcium-Dependent Activation of eNOS

Certain neurohumoral mediators modify the calcium-dynamics (site and frequency of changes, as well as amplitude, duration, and spread of the signals) inside the endothelial cells; hence, they activate eNOS by increasing the intracellular concentration of calcium ions (Ca²⁺).^{15,25,29,34} These mediators bind to and activate specific receptors of the endothelial cell membrane. As in most cells, these receptors are coupled to the downstream intracellular events by G proteins. To judge mainly from the pharmacological evidence at hand (in particular, by determining the inhibitory effect of pertussis toxin, the established inhibitor of G_i proteins), the endothelial cell membrane receptors responding to certain agonists (eg, catecholamines, serotonin) are coupled to the activation of eNOS by G_i proteins. By contrast, endothelium-dependent relaxations caused by other mediators (eg, adenine nucleotides, bradykinin) are not prevented by pertussis toxin and have been attributed to coupling by G_q proteins (Figure 2, left).^{34–36} The G proteins are coupled to phospholipase C producing inositol trisphosphate (IP₃), which in turn binds to IP₃ receptors on the sarco/endoplasmic reticulum (SR) causing release of Ca²⁺ from this internal store.^{15,34,37–45} This process is facilitated by the presence of Ca²⁺-binding protein S100A1, which is coupled to IP₃ receptors.⁴⁶ The depletion of Ca²⁺ from the SR causes SR-transmembrane protein stromal interaction molecule 1 to oligomerize and redistribute to the interface between the SR and the cell membrane, where it activates store-operated calcium channels of the latter, including Orail 1, transient receptor potential (TRP; mainly the vanilloid

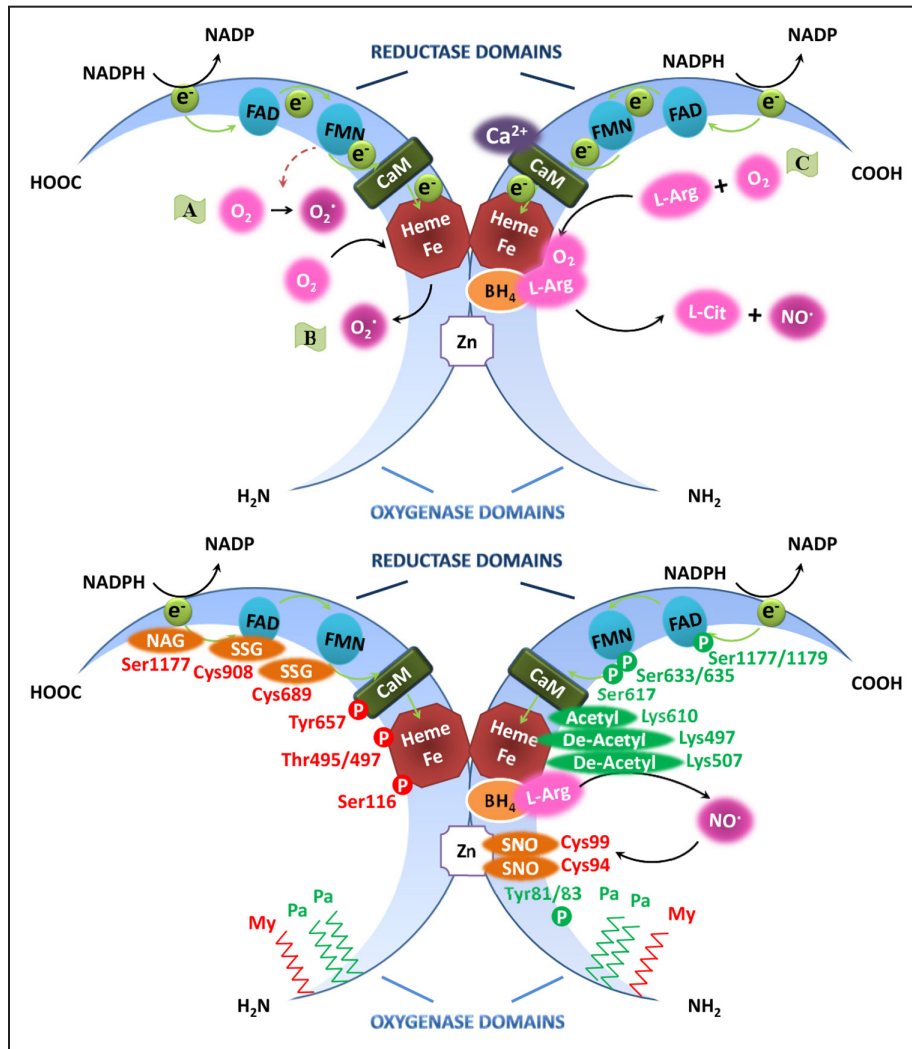


Figure 1. Endothelial nitric oxide synthase. **Top**, The production of nitric oxide (NO) by endothelial NO synthase (eNOS) requires not only the presence of precursor L-arginine (L-Arg) but also that of the cofactors tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM), and iron protoporphyrin IX (Heme Fe). Although the protein eNOS is synthesized as monomers, it has to form homodimers to produce NO: (A) as monomers, eNOS produce superoxide anions (O₂⁻) instead of NO; (B) even as homodimers, significant O₂⁻ production will occur when the effective concentrations of L-Arg is reduced below the levels required to saturate the enzyme; and (C) when sufficient L-Arg and BH₄ bind to coupled NOS, the electron (e⁻) flux provided by nicotinamide adenine dinucleotide phosphate (NADPH) passes through FAD, FMN, CaM, and Heme Fe and is eventually used in NO production. BH₄ is essential for eNOS coupling. The activity of the enzyme is increased when calcium ions (Ca²⁺) bind to calmodulin. **Bottom**, Principal post-translational modifications that enhance or reduce enzymatic activity (and thus favor or reduce endothelium-dependent relaxations, respectively) are indicated in green and red, respectively. Cyst indicates cysteine; My, N-myristoylation (at glycine 2); NAG, N-acetylglucosamine; P, phosphorylation; Pa, thiopalmitoylation (at cysteines 15 and 26); Ser, serine; SNO, S-nitrosylation; SSG, S-glutathionylation; Thr, threonine; and Tyr, tyrosine. Modified from Zhao et al²⁹ (Copyright ©2015, The Authors) and inspired by Förstermann and Münzel.²⁴

subtypes 1 and 4 [TRPV1 and TRPV4]) and T-type calcium (Ca_v3.1 and Ca_v3.2 subtypes) channels, triggering the entry of extracellular Ca²⁺ (capacitive calcium entry).^{40–45,47–49} In addition, certain G-protein-coupled receptors (eg, B₂-bradykinin receptors) are coupled to ADP-ribosylcyclase, the enzyme generating cyclic ADP ribose which then binds to ryanodine receptors of the SR; these receptors are also activated by Ca²⁺ released upon IP₃ receptors stimulation, thereby further enhancing the release of intracellular Ca²⁺ (calcium-induced calcium release).⁴¹ The capacitive calcium entry and calcium-induced calcium release account for a sustained increase in intracellular Ca²⁺ level. Although both IP₃ and ryanodine receptors are activated by increases in Ca²⁺ level in the lower

ranges (50–200 nmol/L and 1–10 μmol/L, respectively), they are inhibited at higher Ca²⁺ concentrations (feedback inhibition). The localized increased amounts of Ca²⁺ are taken up by the SR calcium-ATPase and the mitochondrial calcium uniporter.⁴⁵ The uptake of Ca²⁺ in the mitochondria then triggers the activation of the sodium/calcium exchanger in the mitochondria and hence rhythmic calcium release (oscillatory calcium signals) from these organelles, presumably causing a more harmonious calcium-dependent activation of eNOS; however, exaggerated (eg, in endothelial cells exposed to high cholesterol levels) oscillatory mitochondrial calcium signals may lead to reduced eNOS activity.^{45,47–49} Such mitochondrial Ca²⁺ release in turn activates IP₃ and ryanodine receptors, and

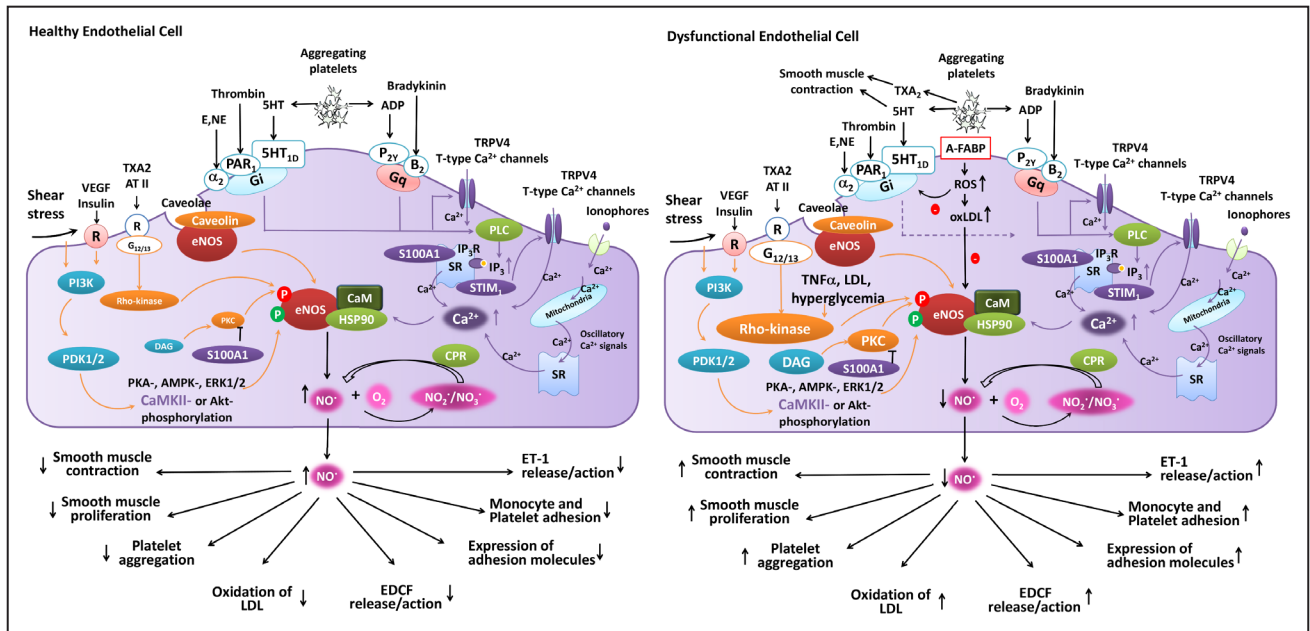


Figure 2. Production of nitric oxide (NO) by endothelial cells. **Left,** Healthy endothelial cells: endothelial NO synthase (eNOS) can be activated in calcium-dependent or calcium-independent ways. On the one hand, agonists (eg, acetylcholine, adenosine triphosphate, bradykinin, catecholamines, endothelin-1 [ET-1], histamine, serotonin, and thrombin) bind to specific receptors or open ion channels of the endothelial cell membrane to increase the influx of calcium (Ca^{2+}) and activate its release from intracellular stores (eg, by stimulating the formation of inositol 1,4,5-trisphosphate [IP_3]), in particular, the sarcoplasmic reticulum (SR) and mitochondria. The calcium ions bind to calmodulin (CaM) that leads to the activation of the CaM-binding domain of eNOS to produce NO; association of the calmodulin–eNOS complex with heat shock protein 90 (HSP 90) increases enzyme activity; increases in intracellular calcium concentration caused by ionophores (eg, A23187) also augment the production of NO by eNOS. On the other hand, increases in hemodynamic shear stress and agonists such as certain hormones (adiponectin and insulin) or growth factors (eg, vascular endothelial growth factor) by binding to their specific receptors initiate phosphorylation (P) of eNOS through activation of the phosphoinositide 3-kinase (PI3K)–phosphoinositide-dependent kinase-1/2 (PDK1/2) pathway stimulating kinases (protein kinase A [PKA], AMP-activated protein kinase [AMPK], Ca^{2+} /calmodulin-dependent protein kinase II [CaMKII]), extracellular signal-regulated kinases 1/2 [ERK1/2], and protein kinase B [Akt]). Phosphorylations that enhance or reduce enzymatic activity (and thus favor or reduce endothelium-dependent relaxations, respectively) are indicated in green and red, respectively. Endothelial cell membrane receptors are coupled to the activation of eNOS through different G proteins (Gi and Gq, inhibited or not, respectively, by pertussis toxin). In addition to eNOS, NO can be produced from nitrate by cytochrome P450 reductase (CPR). Once released, NO exerts a protective effect beyond direct control of vascular tone by inhibiting (downward arrows) many processes favoring vasospasm and initiation of atherosclerosis. **Right,** Dysfunctional endothelial cells: In regenerated endothelial cells the overexpression of fatty acid binding protein A (A-FABP) leads to increased production of reactive oxygen species (ROS) favoring the formation of oxidized low-density lipoproteins (ox-LDL) that interrupts (–) the Gi-mediated activation of eNOS. In addition, if Rho-kinase and protein kinase C (PKC) are activated the ensuing phosphorylations reduce eNOS activity. As a consequence, many processes are disinhibited (upward arrows) that favors the initiation of vasospasm and atherosclerosis. 5HT indicates serotonin (5-hydroxytryptamine); α , α -adrenergic receptor; AT II, angiotensin II; B, bradykinin receptor; DAG, diacylglycerol; E, epinephrine; EDCF, endothelium-derived vasoconstrictor prostanoids; G, coupling proteins; NE, norepinephrine; P, purinoceptor; PAR, protease-activated receptor; PLC, phospholipase C; R, receptor; TNF- α , tumor necrosis factor- α ; TRP, transient potential receptor; TRPV, TRP vanilloid subtypes; TX, thromboxane; S100A1, protein S100A1; STIM, SR-transmembrane protein stromal interaction molecule 1; and VEGF, vascular endothelial growth factor. Image derived from Vanhoutte et al¹⁴ and modified from Zhao et al²⁹ (Copyright ©2015, The Authors).

thus further Ca^{2+} release from the SR, at other sites (toward the perinuclear region) thereby spreading the calcium signals. Calcium entry through TRP and T-type calcium channels can also be activated directly by neurohumoral mediators, increases in shear stress and hypoxia. Ca^{2+} binds to calmodulin, together combining with the calmodulin-binding domain of eNOS; this facilitates the electron flux from the reductase to the oxygenase domains of the enzyme initiating NO production (Figure 1, top).^{24–26,29,37–49} In addition, the Ca^{2+} –calmodulin complex activates calcium/calmodulin-dependent PKs, facilitating post-translational modulation of eNOS activity (see Post-Translational Modulation of eNOS Activity section of this article). A similar calcium-dependent activation of eNOS can be achieved experimentally by the administration of calcium ionophores (eg, A23187; Figure 2). The basal activity of eNOS has been attributed to a calcium leak from internal stores (SR and mitochondria).^{37,45}

Post-Translational Modulation of eNOS Activity

Phosphorylation is a post-translational modification, whereby the addition of a phosphate group to eNOS modulates the activity of the enzyme; the phosphate, in turn, is removed by protein phosphatases (PP). Different sites of phosphorylation can have opposing effects. Thus, phosphorylation at Ser1177 (or Ser1179 depending on the species) activates while at Thr495 it inhibits the enzyme. In particular, PKA [activated by cAMP], PKB [Akt; activated by phosphoinositide 3-kinase (PI3K) and phosphoinositide-dependent kinase], AMP-activated PK [AMPK; activated by elevated AMP-levels, liver kinase B1 and calcium/calmodulin-dependent PK kinase β], calcium/calmodulin-dependent PK II (activated by increases in Ca^{2+} -concentration; see Calcium-Dependent Activation of eNOS section of this article), and extracellular signal-regulated kinases 1/2 (ERK1/2; activated by receptor-linked tyrosine kinase, the

small GTPase Ras and mitogen-activated PK kinase) phosphorylate eNOS at Ser1177 in response to various stimuli (eg, adiponectin, estrogens, increases in shear stress, insulin and irisin)^{25,27,28,50–52}; this process is also facilitated by protein S100A1.⁴⁶ By contrast, Rho-kinase (when activated by G_{12/13}-protein-coupled cell membrane receptors [eg, in response to angiotensin II, thrombin, or thromboxane A₂], tumor necrosis factor- α , low-density lipoproteins [LDL], or hyperglycemia) and PKC (β , δ , and ϵ subtypes; activated by accumulation of diacylglycerol and inhibited by binding with S100A1) phosphorylate eNOS at Thr495 and thus inhibit the enzyme; in particular, activation of eNOS by phosphorylation at Ser1177/Ser1179 is facilitated by simultaneous dephosphorylation of Thr495.^{25,27,28,50,51} Different phosphorylated sites are dephosphorylated by different PPs; the PP1 subtype dephosphorylates Thr495 and is activated by increases in Ca²⁺ or by PKA, whereas the PP2A subtype dephosphorylates Ser1177 on activation by various stimuli (eg, aldosterone, C-reactive proteins, and the bioactive lipid metabolite ceramide).^{25,27,28,50,51} Furthermore, phosphorylation at tyrosine residues of eNOS also modifies enzyme activity; the best characterized is Tyr657 phosphorylation by proline-rich tyrosine kinase (eg, in response to stimulation by angiotensin II, hydrogen peroxide, insulin, or shear stress) leading to reduction of eNOS activity and this process exerts a negative feedback to moderate NO-output thus preventing BH₄ depletion and eNOS uncoupling.^{25,27,28,50,51} Phosphorylation of eNOS at Tyr81 by Src kinase occurs in response to various stimuli (eg, acetylcholine, bradykinin, estrogens, and hydrogen peroxide); while it facilitates NO production, it does not affect the maximal activity of the enzyme (Figures 1, bottom, and 2).^{25,27,28,50,51}

In addition to phosphorylation, the activity of eNOS can also be regulated post-translationally by (1) thiopalmitoylation (addition of palmitoyl groups to cysteine residues), a process that is mediated by Asp-His-His-Cys-motif palmitoyl-acyltransferases (of which subtypes 2, 3, 7, 8, and 21 are present in the Golgi apparatus of endothelial cells) and depends on the N-myristoylation of eNOS (irreversible addition of a myristoyl group to Gly2 of the enzyme during protein translation that targets it to biological membranes to permit thiopalmitoylation). In particular, thiopalmitoylations at Cys15 and Cys26 facilitate the hydrophobic interactions of eNOS with the caveolae, where, as mentioned above, it comes in close contact with regulatory molecules including G-protein-coupled receptors and Akt. Hence, thiopalmitoylation (which occurs mainly in the Golgi apparatus after eNOS synthesis and after its depalmitoylation and translocation to the cytosol [repalmitoylation]) contributes to the proper localization of the enzyme and permits its integration with upstream signaling initiated by neurohumoral (agonists and hormones) and physical (shear stress) stimuli.^{25,27,28,50,51} This process is facilitated by the availability of palmitoyl coenzyme A (CoA), formed by fatty acid synthase (the expression of which is induced by insulin, possibly contributing to the vasoprotective role of the hormone) by increased levels of plasma glucose and fatty acids as occurs in obesity and during hypertension (see Reduced Protein Activity section of this article). Depalmitoylation, mediated by palmitoyl protein thioesterase and acyl-protein thioesterase 1 and facilitated by the Ca²⁺-calmodulin complex, occurs after

prolonged agonist stimulation, resulting in the translocation of the enzyme to the cytosol and distancing it from exogenous stimuli^{28,50,51}; (2) S-nitrosylation (by NO produced by eNOS itself), which occurs when the enzyme is localized in the cell membrane/caveolae because the local hydrophobic environment favors the accumulation of NO and hence the formation of S-nitrosothiols. Among several cysteine residues that can be nitrosylated, only those at Cys94 and Cys99 (the cysteine residues that are located at the interface for dimer-formation) affect eNOS function causing its inhibition. Denitrosylation takes place when eNOS is translocated, on activation, to the cytosol, which contains a high concentration of reduced thiols thus facilitating reduction of S-nitrosothiols on eNOS, and permitting for maximal activity; this process is impaired under conditions of oxidative stress (eg, aging, hypertension, and diabetes mellitus) or by excessive intracellular levels of NO (eg, after iNOS induction during inflammation; see Reduced Protein Dimerization and Reduced NO Bioavailability sections of this article)^{28,50,51}; (3) both acetylation of eNOS at Lys610 by lysine acetyltransferases (depending on the availability of acetyl-CoA [major metabolite of the catabolism of sugars, fatty acids, and proteins], the breakdown of which is reduced by AMPK [inhibiting acetyl-CoA carboxylase to prevent metabolism of acetyl-CoA to melonyl-CoA] and enhanced by PP2A [dephosphorylating and hence activating acetyl-CoA carboxylase]) and its deacetylation at Lys497 and Lys507 (sites that are acetylated constitutively and prevent calmodulin binding) by silent mating-type information regulation 2 homologue 1 (SIRT1, the activity of which is reduced when the thiol group of its cysteine residues is oxidized and when NAD⁺ is depleted by oxidative stress, as with aging and hyperglycemia; see Reduced NO Bioavailability section of this article), leading to increased enzyme activity^{28,51}; (4) glycosylation (attachment of N-acetylglucosamine by O-linked N-acetylglucosamine transferase) at Ser1177, a post-translational modification (characteristic of hyperglycemic conditions) that prevents phosphorylation and hence decreases eNOS activity; and (5) S-glutathionylation (addition of glutathione) at Cys689 and Cys908, determined by the cellular levels of oxidized versus reduced glutathione and prevented by thiol-reducing agents and enzymes (eg, thioredoxin reductase and glutathione reductase); such S-glutathionylation favors the uncoupling of eNOS (by promoting the reaction of the enzyme with oxygen and preventing the binding of the cofactors flavin adenine dinucleotide [FAD] and flavin mononucleotide [FMN] to it for NO synthesis; Figure 1) and hence its inactivation (see Reduced Protein Activity section of this article).^{25,28,51}

Other Endothelial Sources of NO

Nitrite and nitrate not only are products of the metabolism of NO but also act as a reservoir for the endothelial mediator (Figure 2, left). Indeed, several enzymes (hemoglobin, myoglobin, xanthine oxidoreductase, mitochondrial complex IV [cytochrome *c* oxidase], aldehyde dehydrogenase 2, cytochrome P450 reductase, and cytochrome P450) can catalyze the reduction of nitrite or nitrate to generate NO.²⁹ In particular, in the anoxic vascular wall, production of NO from nitrate and nitrite by xanthine oxidoreductase and mitochondrial cytochrome *c* oxidase can contribute to the

redistribution of blood flow to ischemic tissues.²⁹ By contrast, both cytosolic and mitochondrial aldehyde dehydrogenase 2 convert organic nitrate compounds (eg, nitroglycerin) to NO under normoxic conditions. In addition, cytochrome P450 reductase and cytochrome P450 reduce nitrate and nitrite, respectively, to yield NO. For example, cytochrome P450 reductase is an eNOS-independent endothelial generator of NO, at least in arteries of the spontaneously hypertensive rat, which may be a compensatory mechanism to restore NO bioavailability when release from its canonical source (eNOS) is deficient.^{29,53–55}

S-nitrosothiols can also act as a source of NO. Endothelial cells can form such S-nitrosothiols from either exogenous donors (eg, S-nitrosoglutathione) or NO produced by eNOS; they are considered to be intermediates in the storage and transport of NO. S-nitrosothiols are stable under physiological conditions; however, they liberate NO when exposed to trace amounts of redox-sensitive transition metal ions (eg, Cu⁺ or Fe²⁺) in the presence of thiol-reducing agents (eg, reduced glutathione, L-cysteine and hydrogen sulfide), the levels of which are reduced by oxidative stress.^{29,53,54,56–58}

Disposition of NO

To play a moment-to-moment role as local regulator of vascular tone, NO has to disappear soon after its release and its action has to be short lasting. This is the case under most physiological conditions because NO has an unpaired electron in the highest occupied orbital, making it highly reactive with other molecules that also contain an unpaired electron in that orbital, as is the case for free radicals and heme iron. NO is thus inactivated into not only peroxynitrite by superoxide anions but also nitrite and nitrate by oxygen, which contains 2 such unpaired electrons and is present in large amounts in oxygenated tissues.^{59,60} These processes within seconds nearly completely remove the endothelium-derived mediator.^{8,10,15,25,29,59,60} In addition, as mentioned above, NO reacts with protein thiol groups yielding S-nitrosothiols that act as a further sink for the mediator.²⁹

Normal Responses to Endothelium-Derived NO

Endothelium-derived NO not only affects the tone of the underlying vascular smooth muscle but also inhibits platelet aggregation, the expression of adhesion molecules at the surface of the endothelial cells, and hence the adhesion and penetration of white blood cells (Figure 2, left).^{5,8,13–15,61} It also plays an important role in the regulation of the biogenesis of mitochondria in vascular cells and in adipocytes and monocytes. By initiating downstream cGMP/PKG signaling, NO stimulates peroxisome proliferator-activated receptor γ coactivator 1 α , which in turn activates nuclear peroxisome proliferator-activated receptor- γ receptors enhancing the synthesis of mitochondrial DNA and proteins (eg, complexes I–IV of the electron transport chain), thereby contributing to the maintenance of the mitochondrial mass and its function (including regulation of the cellular production of reactive oxygen species [ROS] and energy metabolism).^{62–64} Overall, in terms of local vasomotor control, with few exceptions (see Vasoconstrictor Effect section of this article), NO is a powerful vasodilator that prevents or reduces vasoconstrictions.

Soluble guanylyl cyclase (sGC) is the major target (receptor) for NO in vascular smooth muscle cells, and this enzyme catalyzes the formation of cGMP. The latter in turn activates PKG, which inhibits both the release of Ca²⁺ from the SR (by phosphorylating IP₃ receptor-associated PKG substrate, and thus inhibiting calcium release caused by IP₃ receptor activation in vascular smooth muscle cells) and its influx from the extracellular space (by opening large-conductance calcium-activated potassium channels resulting in hyperpolarization, which in turn prevents the opening of voltage-dependent L-type channels and hence calcium influx).^{3,10,15,29,61} PKG also promotes the uptake of Ca²⁺ to the SR by phosphorylating the integral SR-membrane protein, phospholamban, which increases the activity of SR calcium-ATPase.^{29,61,65–67} At the vascular smooth muscle cell membrane, PKG similarly stimulates Ca²⁺-ATPase accelerating calcium-extrusion.^{29,61,66} These actions concur to reduce the intracellular concentration of the activator ion to the point that the calcium-dependent myosin light-chain kinase can no longer phosphorylate myosin and thus that the contractile process is prevented or interrupted; hence, the vascular smooth muscle cells relax.²⁹ PKG also phosphorylates the regulatory subunit of myosin light-chain phosphatase, activating the enzyme dephosphorylating myosin and hence facilitating relaxation. In addition, NO can bypass the sGC–cGMP–PKG pathway by S-glutathionylation and direct stimulation of the SR calcium-ATPase further reducing the intracellular calcium concentration and facilitating relaxation.^{29,61,65,66} Furthermore, in vascular smooth muscle cells, NO also reacts with cysteine thiols to form S-nitrosylated proteins (S-nitrosylation), which affect the expression and function of certain G-protein-coupled receptors contributing to the regulation of contractile tone. For example, S-nitrosothiols modulate the activity of G-protein-coupled receptor kinase 2 (GRK2), which reduces G-protein signaling by phosphorylating β -adrenoceptors, thus inducing their internalization and desensitization. Because activation of vascular β -adrenoceptors causes vasodilatation, an increased production of NO with greater S-nitrosylation of GRK2 prevents the loss of β -adrenergic signaling and improves organ perfusion.^{29,58}

Besides its direct vasodilator effect, endothelium-derived NO exerts a gatekeeper function in that it modulates EDH-mediated hyperpolarizations^{15,68–70} and prevents the production and action of both endothelium-derived contracting factors^{15,19,20,68,71} and endothelin-1.^{15,21,72}

The Signals for Moment-to-Moment Changes in Endothelial NO Production and Their Targets

To judge mainly from ex vivo/in vitro work and appropriate pharmacological analysis, endothelial cells are endowed with many ion channels (eg, Ca_v3.1 and Ca_v3.2 T-type calcium channels, and TRPV1 and TRPV4 channels; Figure 2; see Calcium-Dependent Activation of eNOS section of this article) and cell membrane receptors (eg, acetylcholine M₃, adiponectin, bradykinin B₂, and serotonin 5HT_{1D} receptors; Figure 3) which, when opened or activated, respectively, are coupled to intracellular events leading to instantaneous increases in eNOS activity and hence endothelium-dependent,

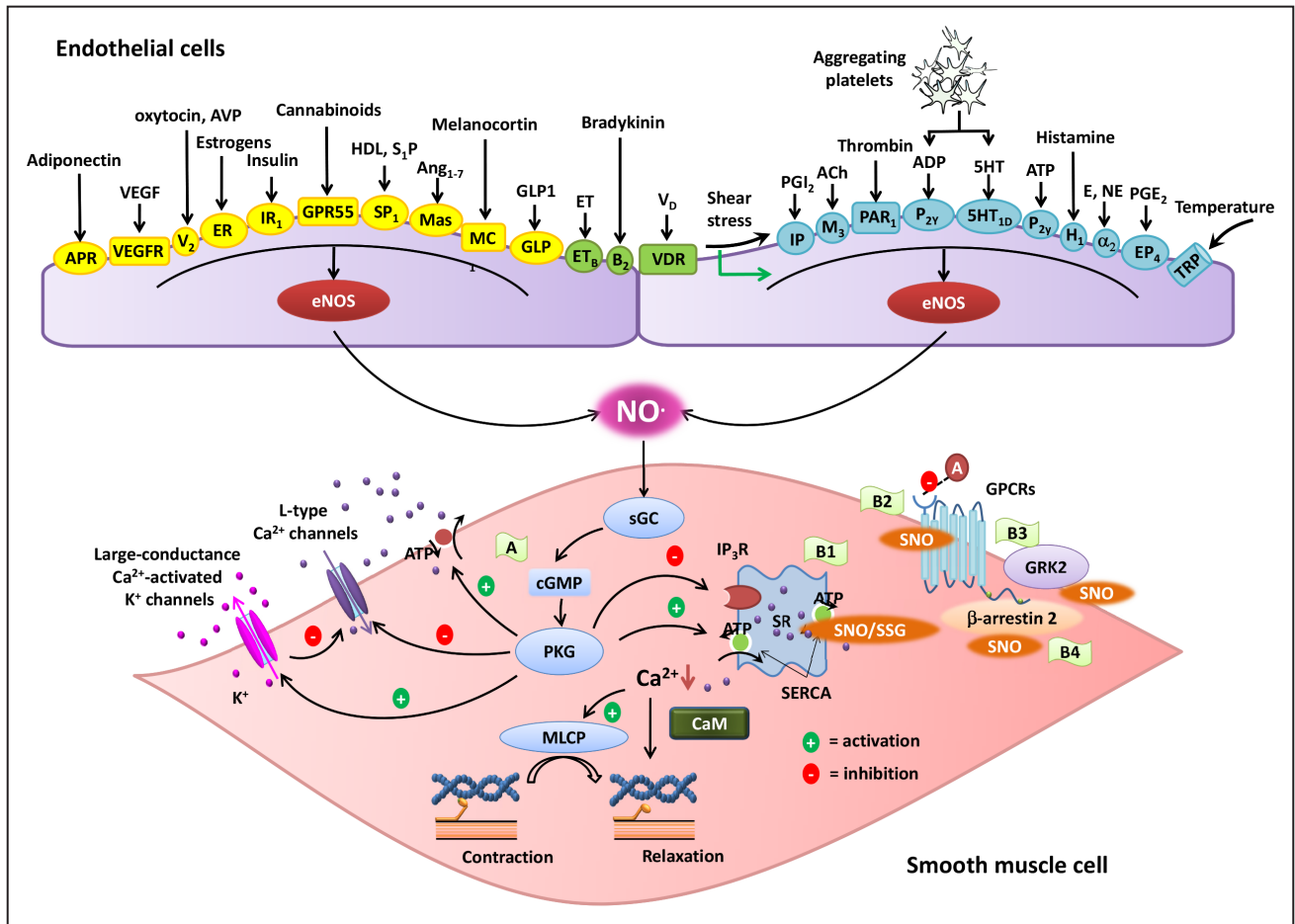


Figure 3. Regulation of vascular tone by endothelium-derived nitric oxide (NO). Top, Physical factors (increases in shear stress and temperature lowering) and neurohumoral mediators (through activation of specific endothelial cell membrane receptors) cause instantaneous increases in the release of endothelium-derived NO by endothelial nitric oxide synthase (eNOS). Color code: Signals leading to activation of eNOS dependent mainly on increases in Ca²⁺ concentration, mainly on post-translational modifications or on both are indicated by blue, yellow, and green, respectively. Image derived from Vanhoutte et al¹⁴ and modified from Zhao et al²⁹ (Copyright ©2015, The Authors). Bottom, The contraction of vascular smooth muscle cells is initiated by increases in the intracellular calcium (Ca²⁺) concentration either from internal stores (sarcoplasmic reticulum [SR]) or by influx into the cell after opening of calcium channels in the cell membrane. The intracellular free calcium ions bind to calmodulin (CaM) and the calcium-calmodulin complex activates myosin light-chain kinase; when activated the latter phosphorylates myosin light chain, which leads to cross-bridge formation between the myosin heads and the actin filaments. Cross-bridge formation results in contraction of the smooth muscle cell. NO modulates the contraction process in different ways: (A) NO stimulates soluble guanylyl cyclase (sGC) in the vascular smooth muscle cells which produces cGMP. Cyclic GMP activates protein kinase G (PKG), preventing calcium influx through voltage-dependent calcium channels and calcium release mediated by inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃R). In addition, PKG not only activates the calcium-ATPase on the cell membrane and the sarco/endoplasmic reticulum calcium-ATPase (SERCA) accelerating the efflux of cytosolic calcium outside the cell and its reuptake into the sarcoplasmic reticulum (SR), respectively, but also opens large-conductance calcium-activated potassium channels resulting in hyperpolarization, which in turn prevents the opening of voltage-dependent L-type channels and hence calcium influx. The resulting decrease in free intracellular calcium ion concentration inactivates calmodulin and thus myosin light-chain kinase. Calcium depletion also augments the activity of myosin light-chain phosphatase (MLCP). The actin-myosin cross-bridges are interrupted and the vascular smooth muscle cell relaxes and (B) NO also S-nitrosylates (SNO) intracellular proteins: (1) SERCA with resulting accelerated calcium depletion; NO also facilitate the S-glutathionylation (SSG) of SERCA; (2) G-protein-coupled receptors (GPCR) with reduced binding of ligands (A) or blunted G-protein coupling; (3) G-protein-coupled receptor kinase 2 (GRK2) preventing desensitization and internalization of β-adrenoceptors; and (4) β-arrestin 2 increasing receptor-internalization. Modified from Zhao et al²⁹ (Copyright ©2015, The Authors). α indicates α-adrenergic receptor; 5HT, serotonin (5-hydroxytryptamine); 5HT_{1D}, serotoninergic (5 hydroxytryptamine) receptor 1D subtypes; ACh, acetylcholine; Ang₁₋₇, angiotensin₁₋₇; AdipoR, adiponectin receptor; AVP, arginine vasopressin; B, kinin receptor; E, epinephrine; EP₄, prostaglandin E₂-receptor 4; ER, nongenomic estrogen receptor; ET, endothelin-1; ET_B, endothelin-receptor B subtypes; GLP, glucagon-like peptide-1, glucagon-like peptide receptor; GPR55, G-protein-coupled receptor 55; HDL, high-density lipoproteins; IP, prostacyclin receptor; IR₁, insulin receptor; M, muscarinic receptor; Mas, Mas receptor; MC, melanocortin receptor; NE, norepinephrine; P, purinergic receptor; PAR, protease activated receptor; PGE₂, prostaglandin E₂; PGI₂, prostacyclin; SP₁, sphingosine 1-phosphate; TRP, transient potential receptor V4; V, vasopressin receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF-receptor; VD, vitamin D; and VDR, vitamin D receptor.

NO-mediated relaxations/dilatations. This increased activity subsides when the signal disappears, insuring moment-to-moment local control of the tone of the underlying vascular smooth muscle cells (Figure 3; Table 1).

Blood-Borne Signals

Many hormones present in the blood cause endothelium-dependent, NO-mediated relaxations of isolated blood vessels. This is the case for adiponectin, catecholamines, estrogens (acting

Table 1. Major Endogenous Signals, With Origin and Underlying Mechanisms, Which Acutely or Chronically Cause or Facilitate Endothelium-Dependent, NO-Mediated Vasodilatation

Signal	Likely Origin	Mechanism	eNOS Presence	eNOS Activation		Examples of Particular Heterogeneous Prominence*/Absence†	Physiological Relevance
				CaDA	PTM		
Acetylcholine	Endothelial cells	M ₃	...	+	...	Coronaries (human, pig) ^{15†}	Thermoregulatory adjustments?
	Cholinergic nerves						Sympathetic cholinergic vasodilatation?
Adiponectin	Adipocytes	AdipoR	+	...	Metabolic modulation of blood supply and angiogenesis
	PVAT						
ADP, ATP	Aggregating platelets	P _{2y} (G _q)	...	+	Prevention of vasospasm
							Inhibition of platelet aggregation
Angiotensin ₁₋₇	Vascular wall	Mas	+	...	Flow-dependent vasodilatation?
							Feedback inhibition of AT II action
Bradykinin	Vascular wall	B ₂ (G _q)	...	+	+	Coronaries (human, pig)*	Flow-dependent vasodilatation
						Rodent arteries ^{15†}	
Catecholamines	Adrenal glands	α ₂	...	+	...	Coronaries	Preservation of BF during sympathetic activation
						Arteries feeding muscles* ¹⁵	
CGRP	Sensorimotor nerves	...	+	...	+	...	Vasodilatation during sensory nerve stimulation
Endothelin-1	Endothelial cells	ET _B	...	+	+	Pulmonary veins* ⁷³	Feedback inhibition of the production/action of ET-1
Estrogens	Ovaries	ER	+	...	+	Human uterine arteries*/placental arteries ^{†74}	Vascular protective role, underlying premenopausal gender differences
GLP-1	Gastrointestinal tract	GLP	+	...	+	...	Sustaining splanchnic blood flow during digestion
Normal HDL	Plasma	S ₁ P	+	...	Vascular protective role
Histamine	Mastocytes	H ₁	...	+	Vasodilatation during allergic reactions
Insulin	β-cells of pancreas	IR ₁ (G _i)	+	Mouse mesenteric*/tibial arteries†	Sustaining BF in metabolically active tissues
						Canine coronary conduit arteries*/microvessels†	
						Porcine large coronaries†	
						Human mammary arteries and saphenous veins† ⁷⁵	
Irisin	Myocytes	...	+	...	+	...	Mediating beneficial effect of exercise and PPAR _α agonists
MSH	Pituitary gland	MC ₁	+	...	+	...	Reduction of arterial stiffness
Oxytocin	Pituitary gland	V ₂	+	Canine cerebral*/femoral arteries ^{†76}	Sustaining uterine BF and preservation of cerebral BF during delivery?
Prostacyclin	Endothelial cells	IP	...	+	Feedback inhibition of the production of vasoconstrictor prostanoids
							Inhibition of platelet aggregation
Serotonin	Aggregating platelets	5HT _{1D} (G _i)	...	+	...	Coronary and cerebral arteries*	Prevention of vasospasm
							Inhibition of platelet aggregation

(Continued)

Table 1. Continued

Signal	Likely Origin	Mechanism	eNOS Presence	eNOS Activation		Examples of Particular Heterogeneous Prominence*/Absence†	Physiological Relevance
				CaDA	PTM		
Shear stress	Flowing blood	Cell membrane adjustments	...	+	+	...	Flow-dependent vasodilatation
Thrombin	Coagulating blood	PAR ₁ (G _i)	...	+	...	Canine arteries*/veins† ⁷⁷	Prevention of vasospasm Inhibition of platelet aggregation
Vasopressin	Pituitary gland	V ₂	...	+	...	Canine cerebral and coronary*/femoral arteries† ⁷⁸	Preservation of cerebral BF during hemorrhage
VEGF	Vascular wall	VEGFR	+	...	+	...	Angiogenesis, maintenance of ED dilatations
Vitamin D	Plasma	VDR	+	+	+	...	Vascular protective role

The endothelial cell membrane receptors mentioned in the third column have been identified in the legend of Figure 3; the indication between brackets refer to the G-protein family coupling the cell membrane receptors to activation of eNOS, when identified. The seventh column shows selected examples of heterogeneity between vascular beds or species, showing absence of response in †, and its prominence in *; the latter may reflect mainly the type of arteries in which the mentioned response has received the most attention. The physiological relevance column reflects the authors' perception of the possibly most important role of the individual responses listed. 5HT_{1D} indicates serotonergic (5 hydroxytryptamine) receptor 1D subtype; AdipoR, adiponectin receptor; AT II, angiotensin II; BF, blood flow; CaDA, calcium-dependent activation of eNOS; CGRP, α -calcitonin-gene-related peptide; eNOS, endothelial NOS; ED, endothelium dependent, NO mediated; ER, estrogen receptor; ET-1, endothelin-1; GLP-1, glucagon-like peptide-1; H1, histamine receptor 1 subtype; HDL, high-density lipoprotein; IP, inositol phosphate; IR₁, insulin receptor; MSH, α -melanocyte-stimulating hormone; PAR₁, protease activated receptor 1; PPAR α , peroxisome proliferator-activated receptor α ; PTM, post-translational activation of eNOS; PVAT, perivascular adipose tissue; S1P, sphingosine 1-phosphate; VDR, vitamin D receptor; and VEGFR, vascular endothelial growth factor receptor.

in a nongenomic manner on G-protein-coupled receptors), glucagon-like peptide-1, high-density lipoproteins (HDL; associating with sphingosine₁-phosphate), insulin (by activating the PI3K/Akt pathway), irisin⁵² (an autacoid/hormone secreted by myocytes), melanocortin (α -melanocyte-stimulating hormone), oxytocin, secretoneurin,⁷⁹ and vasopressin.¹⁵ Likewise, aggregating platelets (Figure 2) release adenine nucleotides (in particular, ADP) and 5-hydroxytryptamine (serotonin), which induce endothelium-dependent relaxations.^{5,15,36} Finally, if the coagulation cascade is set in motion, the first response of healthy endothelial cells to thrombin will be to augment the release of NO.^{5,8,14,15,36}

Vascular Signals

The cells composing the vascular wall can produce several autacoids that activate endothelial cells in an autocrine/paracrine manner and augment the production of NO. These include acetylcholine (produced by endothelial cells; see Physical Signals section of this article), adiponectin (produced by perivascular adipose tissues; see Other Hormones and Circulating Mediators section of this article), angiotensin₁₋₇ (formed from angiotensin II by angiotensin-converting enzyme 2), bradykinin (produced by vascular kallikrein), endothelin-1, histamine (produced by mast cells), prostacyclin (produced by endothelial cells), prostaglandin E₂ (produced presumably by endothelial cells), and vascular endothelial growth factor (produced in response to hypoxia).^{5,14,15,80}

Physical Signals

In isolated arteries, increases in flow, and thus in shear stress, immediately augment the release of endothelium-derived relaxing factors, which has been attributed mainly to increased activity of eNOS resulting in a greater production

of NO although EDH-type responses can contribute.^{15,81-85} Shear stress is signaled through the glycocalyx (a network of membrane-bound proteoglycans and glycoproteins, including syndecans and glypicans, at the luminal surface of the endothelium), membrane-associated proteins localized to the intercellular junctions (integrins and platelet endothelial cell adhesion molecules [CD31]), and proteins (G-protein-coupled receptors, ion channels, and PKs) of the plasma membrane and the caveolae.^{81-83,85} Mechanisms responsible for the initial phase (lasting from seconds to \approx 30 minutes) of eNOS activation by increases in shear stress, which are associated with an augmented intracellular calcium concentration include (1) opening of TRPV1 and TRPV4 channels for Ca²⁺ influx and of calcium-activated potassium (KCa_{2,3} and KCa_{3,1} subtypes) channels causing hyperpolarization and increasing the electrochemical driving force for calcium entry^{40,82,85}; (2) increased interaction between the oligosaccharide components of the glycocalyx and lectinic moiety of G-protein-coupled receptors leading to the sensitization of the latter; (3) release of ATP activating purinergic P2Y₂ receptors that are coupled by G_q proteins to activation of the phospholipase C/IP₃ pathway^{45,86}; (4) activation of the local production of bradykinin that stimulates the release of NO through a G_q-dependent mechanism; and (5) increased production of angiotensin₁₋₇.^{15,80,81,84} Shear stress also causes conformational change of caveolae promoting dissociation of eNOS from caveolin-1 and association of the enzyme with calmodulin. The most important post-translational modifications of eNOS responsible for maintaining eNOS activation throughout the exposure to increased shear stress is its phosphorylation at Ser1177, the reasons for which include (1) activation of PKC- α by syndecans; (2) tyrosine phosphorylation of platelet endothelial cell adhesion molecule allowing

it to serve as a scaffold for the formation of a multiprotein complex including the catalytic subunit of PKA and eNOS; (3) activation of integrins followed by that of focal adhesion kinase (a member of the tyrosine kinase family) resulting in the stimulation, in succession, of vascular endothelial growth factor receptor-2 kinase, PI3K, and Akt; and (4) increased membrane fluidity activating the α subunit of G_i proteins coupled to tyrosine kinase, which in turn stimulates the small GTPase Ras and subsequently ERK1/2.^{15,25,80–82,84} The immediate effect of an increase in shear stress on the release of NO explains flow-mediated dilatation, a phenomenon often used to estimate the functional state of the endothelium in humans although there are several limitations when equating flow-mediated dilatation with the release of NO, particularly in humans.¹⁵

Moderate decreases in temperature stimulate cold-sensitive TRP-channels (TRP ankyrin type 1 and TRPV4 subtypes) leading to increased endothelial synthesis of acetylcholine and autocrine initiation of endothelium-dependent, eNOS-dependent relaxations.⁸⁷ The physiological role, if any, played by this phenomenon in thermoregulatory vascular adaptations is unknown.

Chronic Upregulation of Endothelial NO Production

For each blood vessel, the actual response in terms of endothelial release of NO is determined by many chronic modulators, in particular, hormonal impregnation and chronic/repeated changes in shear stress.

Hormonal Modulation

Sex and Estrogens

The consensus is that endothelium-dependent relaxations are more pronounced in arteries from young female animals and humans than in those of males of the same age, and that this difference subsides after menopause. In young female animals, ovariectomy causes blunting of such responses, whereas reintroduction of physiological levels of estrogens potentiates them, which permits to conclude that these female hormones, in addition to their nongenomic direct effect (see Blood-Borne Signals section of this article), favor eNOS-dependent responses.^{15,88} Several mechanisms have been invoked to explain the chronic facilitating effect of estrogens on NO-mediated, endothelium-dependent relaxations: (1) sensitization of G_i -coupled receptors on the endothelial cells; (2) prevention of BH_4 deficiency (see Reduced Cofactor Availability section of this article); (3) reduced production of the endogenous inhibitor of eNOS, asymmetrical dimethyl arginine (ADMA; see Increased Presence of Endogenous Inhibitors section of this article); (4) recruitment of SIRT1, which activates eNOS by deacetylating the enzyme directly and by causing its further phosphorylation at Ser1177 through recruitment of Akt (see Post-Translational Modulation of eNOS Activity section of this article); (5) stimulation of calcium/calmodulin-dependent PK kinase β , activating AMPK, which phosphorylates eNOS at Ser1177; (6) improving the association of eNOS with heat shock protein 90 (see NO Synthase section of this article); (7) reduction in oxidative stress⁸⁹ and thus increased bioavailability of endothelium-derived NO (see Reduced NO

Bioavailability section of this article); and (8) increased responsiveness of the vascular smooth muscle cells to NO by increasing cGMP production and PKG sensitivity.^{15,88,89}

Other Hormones and Circulating Mediators

Insulin enhances the expression of eNOS in endothelial cells by upregulating the PI3K/Akt pathway and stimulating transcription factor activator protein-1.¹⁵ Adiponectin facilitates endothelial function by recruiting the adaptor protein containing a pleckstrin homology domain, a phosphotyrosine-binding domain, and a leucine zipper motif to its receptors; the adaptor protein then promotes the translocation of liver kinase B1 from the nucleus to the cytosol for the activation of AMPK, thereby enhancing the coupling and activity of eNOS. In addition, adiponectin stimulates adenylyl cyclase elevating the intracellular level of cAMP, which in turn activates PKA to increase the expressions of mitochondrial complex I of the electron transport chain (nicotinamide adenine dinucleotide:ubiquinone oxidoreductase) and heme oxygenase-1 (HO-1) thereby reducing the production of superoxide anions and increasing NO bioavailability (see Reduced NO Bioavailability section of this article). The adipokine also suppresses endothelial inflammatory activation by inhibiting the expression of proinflammatory cytokines and chemokines.^{15,90–92} Glucagon-like peptide-1 potentiates endothelium-dependent, NO-mediated relaxations by upregulating the protein presence and activity of eNOS.¹⁵ Melanocortin increases the expression of eNOS and facilitates NO-dependent relaxations.¹⁵ Prolonged incubation with secretoneurin potentiates endothelium-dependent relaxations in isolated arteries.⁷⁹ Thyroid hormone, acting on thyroid hormone receptors (TR α_1) also upregulates eNOS, augments the endothelial production of NO, and facilitates endothelium-dependent relaxations.¹⁵

Chronic Changes in Shear Stress

Both acute and chronic exercise improve endothelium-dependent relaxations in the coronary and skeletal muscle circulations. This beneficial impact of exercise on endothelial vasomotor regulation, resulting from repeated augmentations in blood flow, is explained by several long-term effects of increases in shear stress that include: (1) upregulation of tyrosine kinase c-Src increasing the expression and activation (by phosphorylation) of eNOS; (2) increased production of transforming growth factor- β and subsequent activation of Krüppel-like factor-2, which promotes eNOS gene transcription; (3) upregulation of longevity-associated bactericidal permeability increasing fold-containing-family-B-member-4, the activation of which promotes the phosphorylation of eNOS at Ser1177; (4) production of endogenous hydrogen peroxide, which activates calcium/calmodulin-dependent PK II leading to activation of, on the one hand, janus kinase II upregulating eNOS expression/presence (see Reduced Protein Presence section of this article) and, on the other hand, of kinases (including Akt, AMPK, and ERK1/2), which increase phosphorylation at Ser1177 and hence activate eNOS; (5) increased production of irisin⁵²; and (6) improved mitochondrial biogenesis/function thereby permitting greater metabolic capacity (including glycolysis and oxidative phosphorylation) and preventing mitochondrial ROS formation.¹⁵

Dysfunction of Endothelial NO Production

With aging, with dietary unbalance (eg, high salt intake, hypercholesterolemia, and hypovitaminosis D), with exposure to deleterious environmental factors (active and passive smoking, chronic exposure to air pollution) and under pathological conditions such as obesity (with exaggerated release of deleterious adipokines [eg, chemerin, leptin,⁹³ lipocalin-2, and resistin] from inflamed fat, in particular, in perivascular adipose tissues, and reduced secretion of irisin from myocytes),⁵² diabetes mellitus (with insulin resistance [initiating inflammatory endothelial responses] and hyperglycemia [favoring glycosylation of eNOS]), hypertension (with reduced expression of antioxidant enzymes and increased ROS production [in particular when the levels of angiotensin II are elevated]),^{94,95} endocrinologic disorders (eg, increased circulating levels of aldosterone,⁹³ cortisol, progesterone, or testosterone), atherosclerosis, endothelial regeneration after injury (eg, angioplasty and heart transplantation), immunodeficiency and certain inflammatory situations (eg, rheumatoid arthritis), the eNOS-NO system becomes dysfunctional.^{12,14,15} When comparing NO-mediated responses between preparations of control, aged, and diseased animals or humans, examining only responses to strong stimuli for NO production (eg, acetylcholine, bradykinin or shear stress) is not necessarily indicative of endothelial normality because the parallel use of less efficacious endothelium-dependent dilators (α_2 -adrenergic agonists, insulin, or serotonin) may reveal an obvious endothelial dysfunction. Reduced NO-dependent relaxations/dilatations can be because of both decreased activity of eNOS and accelerated disposition (reduced bioavailability) of NO. Finally, the absence of response to endothelium-dependent vasodilator

signals can always reflect an abnormal responsiveness of the vascular smooth muscle cells to NO (Table 2).

Dysfunction of eNOS

Abnormal Coupling of Endothelial Cell Membrane Receptors

After in vivo regeneration following endothelial denudation, or after cardiac transplantation, NO-mediated, G_i-protein-dependent relaxations are impaired selectively in isolated porcine coronary arteries, whereas responses that depend on G_q proteins are preserved initially (possibly because of the protective role exerted by endogenous histamine⁹⁶), demonstrating that the functionality of eNOS is intact; this selective dysfunction is exacerbated by chronic hypercholesterolemia and has been attributed to the overexpression of adipocyte-fatty acid binding protein resulting in the increased production of ROS (generated by endothelial nicotinamide adenine dinucleotide phosphate oxidase [NOX]) and oxidized-LDLs that perturb G_i-dependent coupling between cell membrane receptors and the activation of eNOS.^{15,36} (Figure 2, right; Table 2).

Reduced Substrate Availability

L-arginine, the precursor of NO, is an essential amino acid in young mammals, but in healthy adults, can be synthesized de novo from L-citrulline by the enzymes argininosuccinate synthase and argininosuccinate lyase and thus its supply is rarely a limiting factor for the endothelial production of NO under physiological conditions. However, decreased availability of L-citrulline and L-arginine, in particular when combined, can contribute to NO deficiency and acute supplementation with the latter can curtail endothelial dysfunction although such beneficial effect is not always seen chronically.^{15,29,97-99} Insufficient availability of the precursor for NO production

Table 2. Summary of the Multiple and Diverse Impacts of Aging and Major Pathological Situations on the Different Steps of the eNOS-NO-sGC Pathway Responsible for Endothelium-Dependent, NO-Mediated Vasodilatations

Condition	Receptor Coupling	Arginase Activity	BH ₄ Levels	ADMA Levels	eNOS Expression* and Presence	eNOS Dimerization	eNOS Coupling	eNOS Activity	NO Disposition†	NO Vasodilator Effect
Aging	...	↑	↓	↓	↓	↓	↑	↓
Postmenopause	↓	↑	↓	↓	↑	↓
Hyperlipidemia	↓	↓
Obesity	↓	↓	↑	...
Diabetes mellitus (type II)	...	↑	↓	↑	↓	...	↓	↓	↑	↓
Hypertension‡	...	↑	↓	↑	↓	...	↓	↓	↑	↓
Heart failure	↓	↓	↑	↓
Ischemia/reperfusion, angioplasty	↓	↓	↓	↑	...
Chronic inflammation	...	↑	↓	↑	↓	↓	↑	...

For description of the molecular events underlying the changes shown see text and Vanhoutte et al.¹⁵ ↓ indicates reduction; ↑, acceleration; ADMA, asymmetrical dimethyl arginine; BH₄, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; and NO, nitric oxide.

*Due mainly to induction of nuclear factor κ B.

†Due mainly to increased reactive oxygen species production.

‡In particular, if accompanied by increased levels of angiotensin II and/or aldosterone.

can occur when the L-arginine transporter (cationic amino acid transporter 1) is deficient in the endothelial cell membrane or when the breakdown of L-arginine by arginase is accelerated.²⁹ Indeed, L-arginine is also a substrate for arginases (arginase I and II), enzymes of the urea cycle present in the blood vessel wall, which catalyze the hydrolysis of L-arginine into urea and ornithine.¹⁰⁰ When the activity of the endothelial arginases increases (eg, with aging, diabetes mellitus, or hypertension), they compete with eNOS for the common substrate, at the expense of the endothelial production of NO.^{29,101–105} Major causes of L-arginine steal in the blood vessel wall include increased ROS production and activation of Rho-kinase that stimulates both mitochondrial processing peptidase (promoting translocation of arginase from mitochondria to the cytosol and hence increasing its activity) and p38 mitogen-activated PK (phosphorylating transcription factors [activating transcription factor-2 and c-Jun] upregulating the expression and activity of the enzyme); conversely, inhibition of arginases improves endothelium-dependent, NO-mediated dilatations/relaxations when they are impaired because of upregulation of these enzymes.^{100–109} Another sink for L-arginine can be the induction of iNOS, which is minimally present under physiological conditions, but when expressed/present during infection, chronic inflammation, and in tumors, continuously produces large amounts of NO, thus competing with eNOS for the common substrate and accelerating S-nitrosylation of the enzyme (Figure 5).²⁹

Reduced cofactor availability

The biosynthesis of BH₄, from sepiapterin, is catalyzed, in sequence, by GTP-cyclohydrolase I (the rate-limiting step), 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. Hence, mutation or deletion of these enzymes leads to deficient production of this essential cofactor for eNOS, resulting in reduced NO-mediated, endothelium-dependent relaxations, whereas the overexpression of GTP-cyclohydrolase I has beneficial effects.^{29,110,111} A similar endothelial dysfunction is observed when the circulating BH₄ levels are low or if the bioavailability of the cofactor is reduced by exaggerated oxidation to BH₂, as can be the case in postmenopausal females, in diabetes mellitus, on prolonged exposure to aldosterone or cortisol, in hypertension (in particular, if caused by angiotensin II, which promotes ROS production) and during inflammation (accompanied by induction of iNOS); under those circumstances, supplementation with exogenous BH₄ improves endothelial function, as does the administration of erythropoietin,¹¹² which increases BH₄ levels by increasing the expressions of endogenous antioxidant enzymes (superoxide dismutases [SOD], catalase, and HO-1) and reducing that of NOX (see Reduced NO Bioavailability section of this article).^{25,29,33,110–113} Major causes of dysregulation in the BH₄-balance include glucocorticoid receptor activation (decreasing GTP-cyclohydrolase I mRNA expression),¹¹⁴ mineralocorticoid receptor activation (upregulating the expression of NOX) or increased oxidative stress (accelerating the degradation of BH₄ to BH₂), stimulation of the Rho-kinase pathway¹¹³ (reducing the reconversion of BH₂ to BH₄ by dihydrofolate reductase or the synthesis of BH₄ by GTP-cyclohydrolase I), and impairment of eNOS function (see Reduced Protein Activity

section of this article; reducing the amount of NO available for S-nitrosylation of dihydrofolate reductase that protects the latter from degradation; hence, reducing S-nitrosylation slows down the reconversion of BH₂ to BH₄; Figure 4).^{25,29,33,110–114}

Increased Presence of Endogenous Inhibitors

Increased levels of endogenous inhibitors of eNOS (in particular, ADMA [metabolic by-product created during protein methylation in the cytoplasm]), which as preferential substrates displace the physiological precursor L-arginine from the enzyme, curtail the production of NO and hence blunt endothelium-dependent relaxations/vasodilatations.^{115–120} The production of ADMA can be accelerated by increased oxidative stress, by augmented levels of oxidized and carbamylated LDL, and by proton pump or cyclooxygenase-2 inhibitors.¹²¹ Such acceleration also contributes to the endothelial dysfunction in smokers and diabetic subjects; conversely, the production of ADMA can be decreased by induction of SIRT1 (eg, by resveratrol and physical exercise) or by administration of antioxidants (Figure 4).^{15,29,98,115–121}

Reduced Protein Presence

Experiments with genetically modified mice have demonstrated beyond doubt that the reduced gene expression and the resulting decreased protein presence of eNOS is associated with diminished endothelium-dependent relaxations although the shortage of NO production can be compensated for by the emergence of EDH-mediated responses.¹⁵ With aging, diabetes mellitus or hypertension, mechanisms leading to reduced expression/presence of eNOS include (1) activation of tumor necrosis factor- α (eg, by lipopolysaccharides) causing binding of polypyrimidine tract-binding protein-1 and of translation elongation factor 1- α to eNOS mRNA thereby destabilizing it; (2) increased presence of micro-RNA-155 (as a result of dysfunctional insulin receptor substrate-1, impairment of PI3K signaling, and activation of nuclear factor- κ B [NF κ B; by, eg, tumor necrosis factor- α , oxidative stress, and reduced SIRT1 activity] and Rho-kinase), which reduces the stability of eNOS mRNA; (3) increased levels of the adipokine resistin activating p38 mitogen-activated PK and c-Jun NH₂-terminal mitogen-activated PK leading to diminished endogenous antioxidant enzyme (SOD and catalase) activity and augmented ROS production that inhibits activator protein-1 and thus reduces eNOS gene transcription; and (4) increased levels of oxidized-LDL recruiting myocardin-related transcription factor-A to the nucleus in turn repressing eNOS gene transcription. Decreased expression/presence of eNOS also can be caused genetically by deletion of α -calcitonin gene-related peptide, heterozygous deletion of *Alk1* (coding for endothelium-specific receptors for transforming growth factor- β , which increases the transcription and hence expression of eNOS), or by histone demethylase deficiency resulting in reduced transcription of the eNOS genes (Figure 5).¹⁵

Reduced Protein Dimerization

The dimeric structure of eNOS is essential for its function (see NO Synthase section of this article).^{15,51,122} Increased monomerization of eNOS is observed with aging, diabetes mellitus, hypoxia, hyperlipidemia, and increased oxidative stress. S-nitrosylation at Cys94 and Cys99 (see

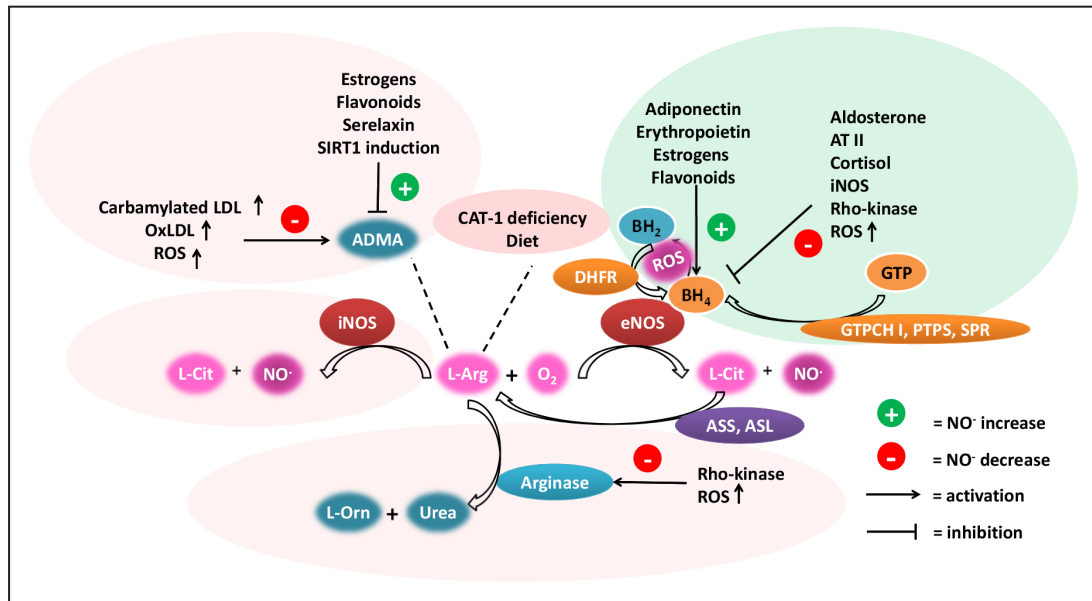


Figure 4. Dysfunction of endothelial nitric oxide synthase (eNOS): reduction in supply and access of substrate and cofactors. Examples of factors that affect the supply of the substrate for eNOS, L-arginine (L-Arg), the availability of the essential cofactor tetrahydrobiopterin (BH₄) and the presence of the endogenous inhibitor dimethyl arginine (ADMA). The green and red color surrounding the + or - signs indicate facilitation or inhibition of NO-dependent dilatations, respectively. ASS indicates argininosuccinate synthase; ASL, argininosuccinate lyase; AT II, angiotensin II; BH₂, dihydrobiopterin; CAT-1, cationic amino acid transporter 1; DHFR, dihydrofolate reductase; GTPCH I, GTP-cyclohydrolase I; iNOS, inducible nitric oxide synthase; L-Cit, L-citrulline; LDL, low-density lipoproteins; L-Orn, L-ornithine; Ox-LDL, oxidized low-density lipoproteins; PTPS, 6-pyruvoyl-tetrahydropterin synthase; ROS, reactive oxygen species; SIRT1, silent mating-type information regulation 2 homologue 1; and SPR, sepiapterin reductase.

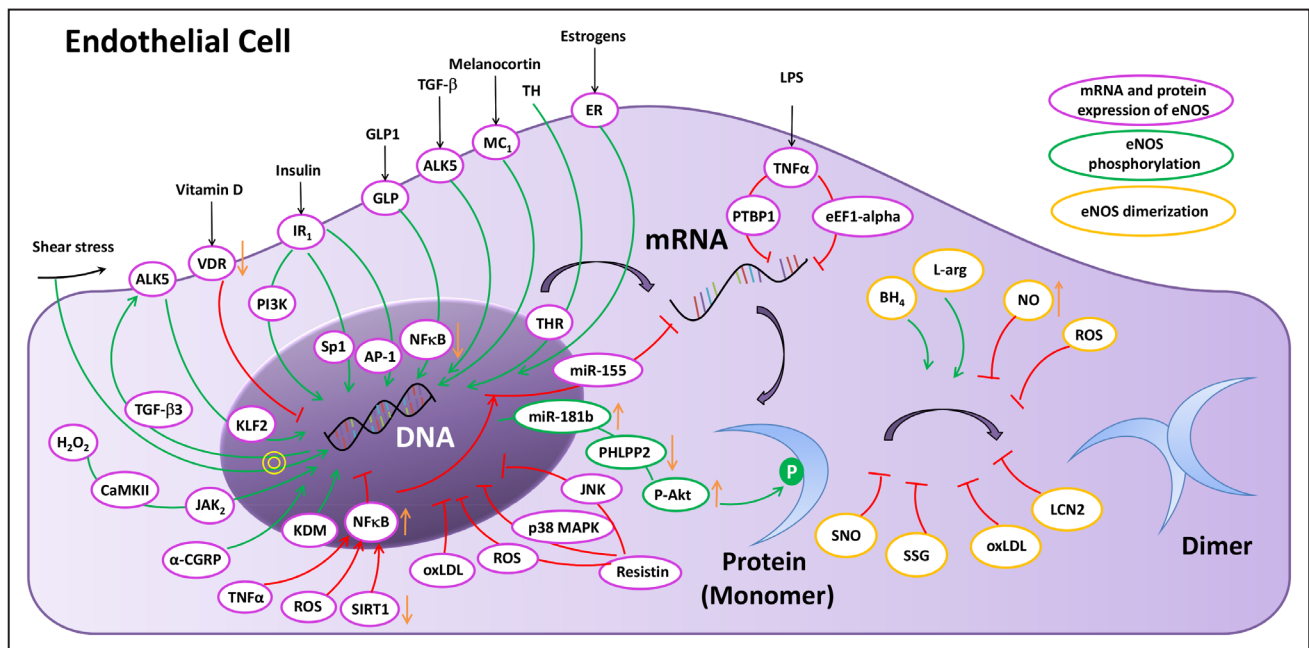


Figure 5. Dysfunction of endothelial nitric oxide synthase (eNOS): protein changes. Examples of factors increasing or reducing the mRNA expression, the protein presence and the degree of dimerization of endothelial NO synthase (eNOS). The green and red arrows indicate facilitation or inhibition of NO-dependent dilatations, respectively. ALK1 indicates transforming growth factor-β receptor; ALK5, transforming growth factor-β receptor; α-CGRP, α-calcitonin gene-related peptide; AP-1, activator protein-1; BH₄, tetrahydrobiopterin; CaMKII, calcium/calmodulin-dependent protein kinases II; eEF1-α, eukaryotic translation elongation factor 1 α; ER, estrogen receptor; GLP, glucagon-like peptide; H₂O₂, hydrogen peroxide; IR₁, insulin receptor 1; JAK₂, janus kinase II; KLF2, Krüppel-like factor-2; KDM, histone demethylase; LCN2, lipocalin-2; JNK, c-Jun NH₂-terminal mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; L-arg, L-arginine; MC₁, melanocortin 1 receptor; miR, microribonucleic acid; NFκB, nuclear factor κB; ox-LDL, oxidized low-density lipoproteins; P-Akt, phosphor-protein kinase B; PI3K, phosphoinositide 3-kinase; PHLPP2, phosphotyrosine-binding domain and leucine-rich repeat protein phosphatase 2; PTBP-1, polypyrimidine tract-binding protein 1; R, receptor; ROS, reactive oxygen species; SIRT1, silent mating-type information regulation 2 homologue 1; SNO, S-nitrosylation; Sp1, specificity protein 1; SSG, S-glutathionylation; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TH, thyroid hormone; THR, thyroid hormone receptor; and VDR, vitamin D receptor.

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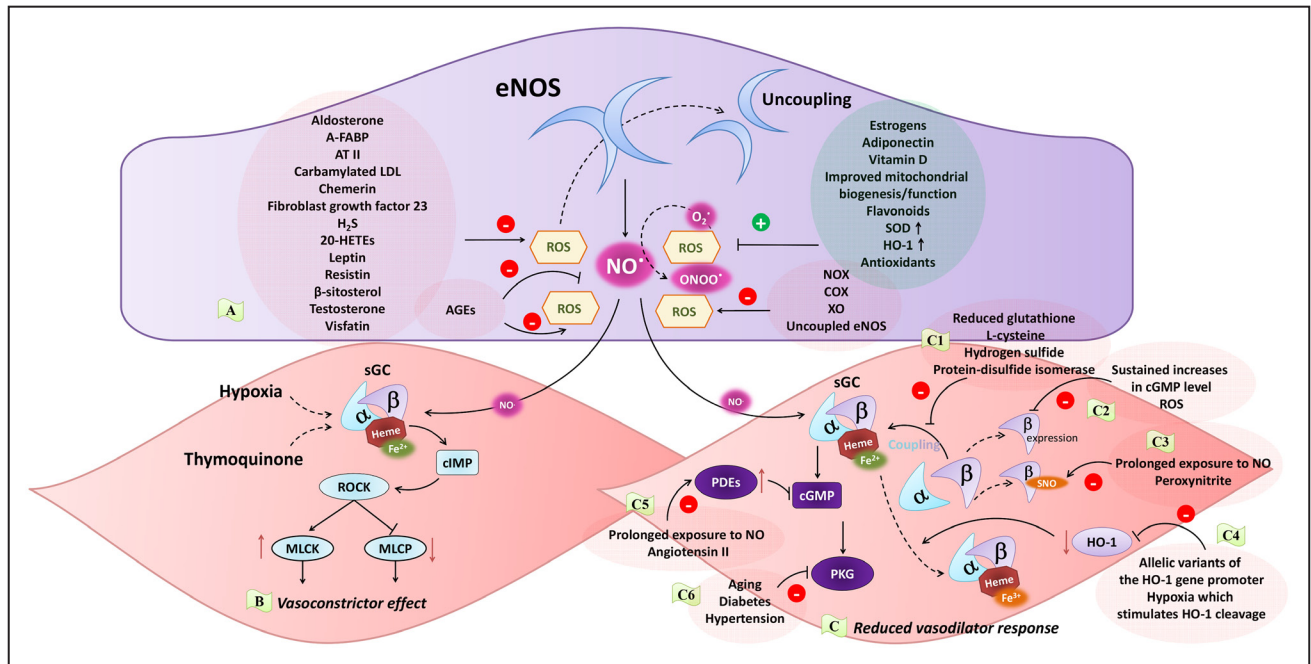


Figure 6. Apparent endothelial dysfunction. **A, Top**, Examples of factors that reduce or enhance the production of reactive oxygen species (ROS; in particular, superoxide dismutase [SOD] anions) and thus favor or inhibit nitric oxide (NO)-mediated, endothelium-dependent relaxations, indicated by green (+) and red (−) symbols, respectively. The scavenging of NO by superoxide anions forming peroxynitrite can occur in the endothelial cells, the intercellular space or the vascular smooth muscle cells. In addition to scavenging NO, ROS also uncouple eNOS. **Bottom**, Examples of factors altering the responsiveness of soluble guanylyl cyclase (sGC) in vascular smooth muscle to endothelium-derived NO. **B**, Under hypoxic conditions (or exposure to thymoquinone), the biased activity of sGC produces 3′-5′-cyclic inosine monophosphate (cIMP) instead of cGMP, which activates Rho-associated protein kinase (ROCK) and inhibits myosin light-chain phosphatase (MLCP), facilitating contraction. **C**, Reduced activity of sGC can be because of (1) reduced dimerization by thiol-reducing agents; (2) reduced expression of the β-subunit; (3) desensitization by S-nitrosylation at Cyst122 of the β-subunit; and (4), reduced expression/activity of heme oxygenase (HO-1) favoring oxidation of the sGC-heme (containing Fe³⁺ instead of Fe²⁺). Reduced vascular responses to endothelium-derived NO can be because of (5) increased expression/presence of phosphodiesterases (PDEs) that reduce cGMP or (6) reduced expression/activity of protein kinase G (PKG). ↓, − indicates inhibition; ↑, +, stimulation; α, β, subunits of sGC; 20-HETE, 20-hydroxyeicosatetraenoic acid; AGE, advanced glycation end-products; AT II, angiotensin II; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; H₂S, hydrogen sulfide; LDL, low-density lipoproteins; MLCK, myosin light-chain kinase; NOX, nicotinamide adenine dinucleotide phosphate oxidase; SNO, S-nitrosylation; and XO, xanthine oxidase.

Post-Translational Modulation of eNOS Activity section of this article; sites that are located at the interface for dimerization) disrupts the dimeric structure; this process is promoted by elevated levels of NO (during maximal enzymatic activity of eNOS [possibly as a negative-feedback inhibition of enzyme activity] or when iNOS is induced during inflammation) and ROS. Other factors that destabilize the dimeric structure include reduced levels of BH₄ and L-arginine and increased presence of the inflammatory adipokine lipocalin-2 (Figure 5).^{15,51,122}

Reduced Protein Activity

In arteries of young, healthy animals and humans, the operation of the eNOS-NO system seems to be optimal and potentiation of endothelium-dependent, NO-mediated relaxations can only be achieved when augmenting the degree of eNOS activation by increased stimulation of the enzyme, by introducing a synergistic signal, or by prolonging the activity of the tested agonist. For example, as regards the latter possibility, inhibitors of angiotensin-converting enzyme 1 potentiate endothelium-dependent relaxations to endogenously released (eg, by increases in shear stress) and exogenously added bradykinin because the enzyme is the major

degradation route for the kinin.^{15,80} However, many molecular mechanisms have been identified which with aging or under pathological conditions (obesity, diabetes mellitus, and systemic or pulmonary hypertension) impair the production of NO by eNOS and precipitate endothelial dysfunction. Major causes include increased production of ROS (causing S-glutathionylation of eNOS and uncoupling/inactivating the enzyme),^{123,124} augmented presence of oxidized-LDL (causing reduced turnover, uncoupling, and dephosphorylation of eNOS) or carbamylated-LDL,¹²⁵ activation of toll-like receptors (TLR; key components of innate immunity responses) of type 4 (TLR4; stimulated by circulating lipopolysaccharides) and the downstream NFκB pathway,¹²⁶ insulin resistance (favoring proinflammatory signaling of wingless-type family member 5a activating c-Jun NH₂-terminal mitogen-activated PK),¹²⁷ increased expression of peptidyl prolyl *cis-trans* isomerase (in diabetic patients; causing isomerization of the inhibitory Ser116 of eNOS), or increased levels of plasma fatty acids (during hypertension and in obesity; promoting repalmitoylation for caveolae docking [see Post-Translational Modulation of eNOS Activity section of this article], thus favoring the inhibitory interaction of eNOS with caveolin-1).^{15,98,117} Other chronic deleterious influences

on eNOS activity are prolonged exposure of the endothelial cells to increased levels of circulating aldosterone (leading to intracellular sodium accumulation thereby interrupting endothelial calcium-dependent signaling), cortisol (down-regulating estrogen production), glucose (diverting glucose metabolism to the hexosamine pathway and thus increasing endothelial levels of N-acetylglucosamine which glycosylates eNOS), abnormal HDL (in patients with coronary artery disease),^{128–130} carbamylated-HDL^{125,131} (elevated in patients with end-stage renal disease and characterized by a reduced activity of the HDL-associated antioxidant enzyme paraoxonase I, and downregulating endothelial PI3K expression), high concentrations of dietary nitrate¹³² (increasing eNOS phosphorylation at Thr495 and reducing that at Ser1177), the proinflammatory adipokine lipocalin-2 (in patients with obesity), the inflammatory mediator pentraxin 3¹³³ (activating the NFκB pathway), or progesterone (preventing the beneficial effect of estrogens).¹⁵ Likewise, eNOS dysfunction can be favored by the relative absence of facilitating mediators such as adiponectin, angiotensin_{1–7} (to judge from angiotensin-converting enzyme 2 deletion experiments), or apelin.¹⁵ Abnormal intracellular calcium handling (eg, because of downregulation of SR-transmembrane protein stromal interaction molecule 1 [see Calcium-Dependent Activation of eNOS section of this article]) by the endothelial cells and modification of the phosphorylation of eNOS (eg, by inhibition of agonist-induced phosphorylation of Ser1177 or phosphorylation of Thr495 by Rho-kinase) can also reduce eNOS activity.¹⁵ Other intracellular mechanisms favoring eNOS dysfunction include disruption of the interaction of the enzyme with caveolae, NFκB activation, suppression by transcription factor forkhead box O-1 of Krüppel-like factor-2 and increased endoplasmic reticulum stress.^{15,126} In addition, eNOS dysfunction can be exacerbated by many genetic manipulations that include (1) deletion of bone morphogenetic protein type II receptors [receptors that activate PKA to cause eNOS phosphorylation at Ser1177; their mutation is associated with the development of pulmonary arterial hypertension]; (2) deletion of α -calcitonin gene-related peptide [neuropeptide that induces the expression of eNOS and causes its phosphorylation by activating PKA and AMPK]; (3) deletion of small GTPase Ras-related protein 1¹³⁴ impairing activation of integrins (eg, by shear stress) and the subsequent stimulation of the focal adhesion kinase/PI3K/Akt pathway; (4) endothelium-specific deletion of liver kinase B1 impairing AMPK activation; (5) reduction in the level of S100A1, enhancing PKC activity with increased eNOS phosphorylation at Thr495 and reducing that of the PI3K/Akt and the ERK1/2 pathways with decreased eNOS phosphorylation at Ser1177; and (6) expression of myristoylation-deficient eNOS preventing the membrane targeting and thiopalmitoylation of the enzyme (Figure 6).^{15,51}

Experimentally, the occurrence of dysfunction of the NO pathway can be prevented/alleviated by (1) elevation of the circulating levels of adiponectin; (2) activation of angiotensin-converting enzyme 2 or administration of angiotensin_{1–7}; (3) stimulation of the production or long-term administration of erythropoietin¹¹² enhancing the activation of Akt; (4) administration of stable melanocortin analogs, cannabidiol,¹³⁵

and dietary capsaicin, which activate TRPV1 channels of the endothelial cell membrane leading to calcium influx and calcium-dependent stimulation of calcium/calmodulin-dependent PK II phosphorylating eNOS at Ser1177; (5) administration of AMPK-activators such as AICAR; and (6) upregulation of micro-RNA-181b¹³⁶ (present in endothelial cells of adipose tissue after high-fat diet; which suppresses the expression of the leucine-rich repeat PP-2 that dephosphorylates and inactivates Akt).¹⁵ In addition, the following molecular maneuvers improve endothelium-dependent, NO-mediated responses: (1) overexpression of SR-transmembrane protein stromal interaction molecule 1, which increases capacitive calcium entry; (2) deletion of caveolin-1 thereby facilitating calcium-calmodulin binding to eNOS; (3) activation of NAD(P)H:quinone oxidoreductase with increased production of cyclic ADP ribose; (4) deletion, reduction, or inhibition of TLR2 and TLR4 reducing the activation of the NFκB pathway; (5) Krüppel-like factor-2 gene therapy increasing eNOS gene transcription; (6) knockin of eNOS with a single amino acid mutation replacing serine by aspartate at the Ser1176 phosphorylation site (the murine site corresponding to Ser1177 in humans) to produce a phosphomimetic hyperactive form of eNOS; (7) heterogeneous deletion of GRK2, which in endothelial cells interact with Akt inhibiting its activity; (8) heterozygous knockout of Rho-kinase isoforms (ROCK 1 and ROCK 2) reducing eNOS phosphorylation at Thr495 and increasing the availability of both the substrate L-arginine (see Reduced Substrate Availability section of this article) and the cofactor BH₄ (see Reduced Cofactor Availability section of this article) for NO production; (9) gene transfer of a variant form (with enhanced activity) of longevity-associated bactericidal permeability increasing fold-containing-family-B member-4 increasing eNOS phosphorylation at Ser1177; (10) endothelium-selective expression of a constitutively active AMPK; and (11) genetic deletion of soluble epoxide hydrolase, the phosphatase domain of which mediates the dephosphorylation of eNOS at Ser1177 and is activated (eg, upon administration of statins) by c-Src tyrosine kinase.¹⁵ Pharmacotherapeutically, besides obviously treatment of underlying disorders (eg, obesity, diabetes mellitus, and hypertension), certain available therapeutic agents can improve dysfunctional NO-mediated vasodilatation by exerting a so-called pleiotropic direct effect on NO bioavailability; they include antidiabetic drugs (eg, metformin, peroxisome proliferator-activated receptor- γ activators, and saxagliptin),^{137–139} antihypertensive drugs (eg, nebivolol and telmisartan),^{140–142} lipid-lowering drugs (eg, statins and peroxisome proliferator-activated receptor- α activators),^{113,143–145} mineralocorticoid receptor antagonists (eg, epleronone), and Rho-kinase inhibitors.^{146,147} Such protective pleiotropic effect involves not only augmented production and bioavailability of NO (as a result of prevention of eNOS uncoupling by increasing BH₄ levels [see Reduced Cofactor Availability section of this article],^{113,138} reduction in ROS production,^{140,141,145} and promotion of calcium-independent phosphorylation of eNOS [eg, by Akt and AMPK; see Post-Translational Modulation of eNOS Activity section of this article])^{137,139} but also augmented expression of eNOS protein by increasing its transcription^{141,142} and by stabilizing its mRNA.¹⁴⁴ In addition, the shortage of endogenous NO can

obviously be bypassed by exogenous NO donors such as nitrates, by activators/stimulators of sGC or by phosphodiesterase inhibitors.¹⁵

Reduced NO Bioavailability

Increased presence of superoxide anions with exaggerated formation of peroxynitrite is the major factor accelerating abnormally the disposition of endothelium-derived NO; in addition to reducing the amount of NO available to relax the vascular smooth muscle cells, peroxynitrite per se has deleterious effects on cell function.^{32,148,149} (Figure 6; Table 2) Peroxynitrite is a potent oxidant that avidly reacts with certain molecules including carbon dioxide (to form toxic carbonate radicals and nitrogen dioxide), proteins containing a heme group (eg, eNOS, hemoglobin, myoglobin, or cytochrome *c*) or a thiol moiety (eg, mitochondrial complexes I, II [succinate dehydrogenase], III [cytochrome *c* reductase], and V [ATP synthase]), lipids (eg, LDL, myelin, and membrane lipids) and nucleic acids (eg, guanine and deoxyribose), leading to their dysfunction. Nitrogen dioxide causes the nitration (addition of a nitro-group adjacent to the hydroxyl group on the aromatic ring of tyrosine residues) of proteins (eg, mitochondrial SOD, prostacyclin synthase, cardiomyocyte structural proteins, neuronal presynaptic protein α -synuclein, and cytoskeletal proteins) and the nitrotyrosine formed renders these proteins dysfunctional, resulting in cardiovascular (eg, endothelial dysfunction, vascular aging, and heart failure) and other disorders. The combination of oxidative modification and nitrotyrosine formation of cellular proteins by peroxynitrite eventually can result in apoptotic cell death.^{32,148–151}

In addition, intermediates of the formation of advanced glycation end-products (formed by the reaction of reducing sugars [eg, glucose] with amino groups of proteins, and prominent in diabetic patients) contain a reactive oxidizing moiety that can reduce NO bioavailability.¹⁵

The endothelial enzymes that can produce superoxide anions in amounts sufficient to reduce NO bioavailability include NOX, xanthine oxidase, cyclooxygenases, members (complexes I, II, and III) of the electron transport chain of the mitochondria when dysfunctional, and eNOS itself if uncoupled.^{15,148,152,153} In particular, the expressions of NOX and cyclooxygenase-2 are upregulated after the activation of transforming growth factor- β receptors¹⁵⁴ (eg, during high salt intake), receptors for advanced glycation end-products (leading to activation of the tumor necrosis factor- α /NF κ B pathway) and TLR9¹⁵⁵ (eg, by damage-associated molecular pattern molecule formed from the release of mitochondrial DNA, as observed in hypertensive animals).¹⁵ Increased ROS production reducing NO bioavailability contributes to the endothelial dysfunction caused by androgens, perturbed flow patterns, hyperglycemia (causing increased presence of advanced glycation end-products), smoking, intoxications (arsenic or mercury), and exposure to air pollution.¹⁵ It contributes importantly to the blunting of NO-mediated responses observed in obesity, diabetes mellitus, and hypertension.¹⁵ Endogenous substances, when present in pathophysiological amounts, that can increase oxidative stress to the extent of reducing endothelium-dependent, NO-mediated relaxations/vasodilatations include adipocyte-fatty acid binding protein, aldosterone, angiotensin

II (activating NOX), carbamylated-LDL, chemerin, fibroblast growth factor-23, hydrogen sulfide, 20-hydroxyeicosatetraenoic acid, leptin, resistin, β -sitosterol, testosterone, and visfatin.^{15,98,117} Under normal conditions, the endothelial ROS levels are kept under control by cellular and extracellular antioxidant enzymes, in particular, SOD, catalase, and HO-1. Reduced expression of these antioxidant enzymes subsequent to increased degradation of the transcription factor nuclear factor-erythroid 2-related factor-2 that stimulates HO-1 gene transcription, resulting, for example, from oscillatory disturbed shear forces as seen in hypertension, can lead to endothelial dysfunction. The same is true for selective deletion of endothelial SOD, which leads to increased oxidative stress and reduced endothelium-dependent, NO-mediated relaxations. The exaggerated reduction in NO bioavailability caused by ROS can be alleviated experimentally by (1) inhibiting the activation of endothelial cell membrane transforming growth factor- β ¹⁵⁴ and endoplasmic reticulum membrane TLR9 receptors¹⁵⁵ thereby reducing the expression of NOX and cyclooxygenase-2; (2) augmenting (eg, with the administration of adiponectin, capsaicin, or glucagon-like peptide-1; see Other Hormones and Circulating Mediators section of this article) the intracellular concentration of cAMP to activate PKA that increases uncoupling protein 2 in the mitochondria and cAMP-responsive element-binding protein in the nucleus, boosting the expression of the antioxidant enzymes mitochondrial complex I and HO-1, respectively; (3) stimulating AMPK (eg, by augmenting the production of adiponectin) to activate nuclear factor-erythroid 2-related factor-2 and thus stimulates HO-1 gene transcription; (4) reducing ROS production and DNA release in the mitochondria (by activating telomerase and increasing the presence of its catalytic subunit¹⁵⁶); and (5) by the exogenous administration of epigallocatechins,¹⁵⁷ erythropoietin,¹¹² ghrelin,¹⁵⁸ estrogens,⁸⁹ or other antioxidants.^{15,92,120} Part of the improvement of endothelium-dependent relaxations caused by HO-1 induction or exogenous antioxidants can be attributed to restoration of the gatekeeper role of NO in preventing endothelium-dependent contractions.¹⁵

Abnormal Responsiveness of Vascular Smooth Muscle to NO

Reduced Vasodilator Response

When faced with reduced endothelium-dependent, NO-mediated vasodilatations/relaxations, one should always consider the possibility that reductions in responses to shear stress or endothelium-dependent vasodilator agents can be because of a reduced responsiveness of sGC in the vascular smooth muscle cells to the endothelial mediator.^{15,67,159} Such reduced responses have been attributed to (1) reduced expression of the β -subunit of sGC (because of increased degradation after sustained increases in cGMP level [by C-type natriuretic peptide activating particulate guanylyl cyclase or by upregulation of phosphodiesterases] or increased oxidative stress [with aging, hypertension, and diabetes mellitus]), which contains the heme-binding domain responding to NO^{159–161}; (2) reduced dimerization by thiol-reducing agents (eg, reduced glutathione, L-cysteine, and hydrogen sulfide) or by interaction with protein-disulfide isomerase (eg, hypoxia in the pulmonary circulation), preventing the formation of disulphide bonds between

the cysteine residues of the α - and β -subunits of the enzyme, which are essential for its activity^{162–164}; (3) desensitization by oxidation or S-nitrosylation at Cys122 of the β -subunit caused by prolonged exposure to NO or increased oxidative stress (with the resultant formation of peroxynitrite)^{165,166}; and (4) reduced expression/activity of HO-1 (caused by allelic variants of the HO-1 gene promoter with reduced transcriptional activity or to hypoxia that stimulates its cleavage) favoring oxidation of the sGC-heme (containing Fe³⁺ instead of Fe²⁺) because the reduced form is necessary for the activation by NO and the oxidized form promotes enzyme degradation.¹⁶⁷ Alternatively, reduced vascular responses to endothelium-derived NO can be because of increased expression/activity of phosphodiesterases (in particular, phosphodiesterase-1 and phosphodiesterases-5, after prolonged exposure to NO or angiotensin II)^{160,168} accelerating the breakdown of cGMP, reduced expression/activity of PKG (eg, in aging and hypertension),^{169,170} or reduced expression/activity of large-conductance calcium-activated potassium channels, one of the downstream signaling targets of PKG (see Normal Responses to Endothelium-Derived NO section of this article), resulting in reduced hyperpolarization of the vascular smooth muscle cells (eg, in aging, diabetes mellitus, hypertension, and obesity; Figure 6).^{171–173}

Vasoconstrictor Effect

By contrast to its vasodilator effects under most circumstances, in isolated coronary arteries suddenly exposed to hypoxia endothelium-derived NO causes augmentations of contractions, which require the activation of sGC but are not mediated by the canonical product of the enzyme, cGMP (Figure 6). Thus, in such precontracted preparations, acute hypoxia induces further increases in tension (augmentation), which are endothelium-dependent, are abrogated by inhibitors of either eNOS or sGC, but restored by NO donors.¹⁷⁴ However, the cGMP levels do not increase under these conditions and incubation with an exogenous cell permeable analog of this cyclic nucleotide does not restore the hypoxic augmentation in preparations treated with sGC inhibitors.¹⁷⁴ Furthermore, inhibition of PKG (a main target of cGMP) does not prevent the hypoxic augmentation. By contrast to the lack of involvement of cGMP, the levels of 3'-5'-cyclic inosine monophosphate (cIMP)¹⁷⁵ augment during the hypoxic contractions and exogenous cIMP restores the response in preparations treated with sGC inhibitors.¹⁷⁴ A similar biased activity of sGC can be evoked by the natural compound thymoquinone.¹⁷⁶ These findings demonstrate the existence of a novel, cIMP-mediated signaling mechanism for NO distinct from the classical sGC–cGMP–PKG pathway, which favors the occurrence of vasospasms.^{174,177}

Conclusion

The genesis of NO-mediated relaxations/dilatations is a complex process involving, in sequence, activation of endothelial cell membrane receptors and opening of ion channels, interacting with intracellular mechanisms allowing stimulation of eNOS, provided that the enzyme is coupled, its cofactors are available, and its substrate (L-arginine) is plentiful. Once formed, the gaseous mediator diffuses to the vascular smooth muscle cells where it initiates the relaxation process, mainly by activating sGC (yielding increased levels of its canonical

product, cGMP). To insure proper physiological control of local vasomotor tone by the endothelial cells, NO is rapidly inactivated. Under normal circumstances, in arteries of young and healthy subjects, the system functions optimally. However, with aging and under pathological conditions (in particular obesity, diabetes mellitus, and hypertension), NO-mediated relaxations/dilatations become curtailed, a hallmark of endothelial dysfunction. The review of the literature indicates that such dysfunction can be multifaceted and can be explained not only by reduced presence or activity of eNOS but also by shortcomings in the supply of substrate and cofactors for the enzyme or by accelerated destruction of NO, not to mention abnormal responsiveness of the vascular smooth muscle cells (eg, by the generation by sGC of the noncanonical product cIMP). When searching for the culprits causing endothelial dysfunction, irrespective of the underlying pathological process and the resulting molecular malfunction(s), repeated offenders seem to be augmented presence of the endogenous eNOS inhibitor ADMA, stimulation of mineralocorticoid receptors, activation of endothelial Rho-kinase, stimulation of the NF κ B pathway, and exaggerated oxidative stress, which not only uncouples eNOS but also accelerates the inactivation of NO. Hence, all pharmacological and genetic manipulations that favor these villains result in reduced NO-mediated relaxations/vasodilatations and improper local control of vasomotor tone by the endothelial cells. Conversely, when reviewing earlier work, it becomes apparent that all pharmacological or genetic maneuvers that protect the supply of substrate and cofactors for eNOS, preserve the presence and activity of the enzyme, and reduce the generation of oxygen-derived free radicals improve NO-mediated relaxations reduced by aging or disease. Across the border, the more common achievers of such improvement seem to be maintained levels of the female hormones estrogens, increased production of adiponectin, and induction of SIRT1. As stated in the first paragraph, endothelium-dependent relaxations are only one of the beneficial actions mediated by NO in the vascular wall. Hence, it is not surprising that reduced endothelium-dependent, NO-mediated responses, caused by an imbalance between the villain and the protective impacts in favor of the former on the eNOS–NO–sGC–cGMP pathway, remain such a strong predictor of cardiovascular complications, in particular, the occurrence of atherosclerosis.^{13–15}

Although the past 30 years have increased considerably our knowledge of the importance of NO bioavailability in the control of vascular tone, many questions remain to be answered before we will totally comprehend the role of this major endothelium-derived vasodilator. The most important challenges for the future, at least for the authors of this review, include (1) comprehending why such a multiplicity of signals exist, and often cohabit, which lead to the activation of eNOS, the physiological relevance of which can be perceived (Table 1) but is not always ascertained; (2) understanding what underlies the striking diversity (heterogeneity) of the responses to endothelium-dependent vasodilators because their absence or presence and their amplitude differ not only between arteries and veins but also between individual arteries of the same animal/human, not to mention the variability between similar preparations of different species (Table 1) or

evolving responses of a given blood vessel during the maturation process.^{15,178} The molecular basis for this diversity is ill-defined, but may involve either absence of receptor proteins or their improper coupling to intracellular downstream signaling. The latter interpretation is comforted by the observation that endothelium-dependent relaxations in response to acetylcholine require the presence of NO synthase traffic inducer¹⁷⁹ and that those to serotonin and α_2 -adrenergic agonists require proper coupling to G_i proteins (see Abnormal Coupling of Endothelial Cell Membrane Receptors section of this article). In any case, the mere diversity in endothelium-dependent responsiveness precludes extrapolation from experiments on 1 blood vessel to endothelial function (or dysfunction) in general; (3) understanding the relative importance/relevance of, and the interactions between the different molecular mechanisms that modulate the presence and the activity of eNOS, beyond merely acknowledging that almost every genetic manipulation in the mouse affects this key endothelial enzyme; (4) understanding better the exact role of calcium-dynamics and mitochondrial oscillatory calcium signals in the regulation of eNOS activity under physiological and pathological conditions; (5) resolving the controversies on the ultimate impact of certain regulatory proteins (eg, ERK1/2 and PKC subtypes) on the post-translational activation of eNOS. In this review, we have selected to favor the most commonly accepted view although we realize that alternative situations have been aired in the literature; (6) understanding better the modulatory influences of micro-RNAs, the importance of which as modulators of NO-dependent vasodilatations is just emerging; (7) understanding the exact physiological role of oxygen-derived free radicals. Indeed, although there is consensus that exaggerated oxidative stress is a major villain in causing endothelial dysfunction, one cannot ignore the fact that physiological amounts of ROS can activate eNOS, thus increasing NO production and evoking/potentiating endothelium-dependent relaxations.^{180–185} Even when accepting that ROS are villains at least in rats and mice, the therapeutic relevance of experimental animal findings appears maybe questionable because chronic treatment with antioxidants usually fails to improve endothelial function in humans^{15,94,186–188}; this lack of efficacy may be explained by the largely ignored observation of large amounts of extracellular SOD contained in the human vascular wall¹⁸⁹; (8) understanding why in resistance arteries the relative lack of NO production is brought about, permitting the emergence of EDH-mediated dilatations; (9) understanding how at the molecular level NO tempers the occurrence of endothelium-dependent vasoconstrictions; (10) understanding the link between overexpression/presence of adipocyte-fatty acid binding protein and the accelerated production of oxidized-LDL leading to selective loss of G_i-mediated endothelium-dependent relaxations; and (11) understanding why under hypoxic conditions (or during exposure to thymoquinone) NO stimulates the biased activity of sGC leading to the production of the vasoconstrictor cyclic nucleotide cIMP. Hypoxic vasospasm has been demonstrated in vivo in coronary arteries of the pig previously exposed to ischemia-reperfusion injury; if they were to occur in humans, they may help to understand the greater cardiovascular risk associated with sleep apnea¹⁵

and raise concerns on the use of sGC activators/stimulators in coronary patients with a history of ischemia-reperfusion.

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