

Basic science for the clinician

Nitric oxide synthases: regulation and function

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Nitric oxide (NO), the smallest signalling molecule known, is produced by three isoforms of NO synthase (NOS; EC 1.14.13.39). They all utilize L-arginine and molecular oxygen as substrates and require the cofactors reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydrobiopterin (BH₄). All NOS bind calmodulin and contain haem. Neuronal NOS (nNOS, NOS I) is constitutively expressed in central and peripheral neurons and some other cell types. Its functions include synaptic plasticity in the central nervous system (CNS), central regulation of blood pressure, smooth muscle relaxation, and vasodilatation via peripheral nitrenergic nerves. Nitrenergic nerves are of particular importance in the relaxation of corpus cavernosum and penile erection. Phosphodiesterase 5 inhibitors (sildenafil, vardenafil, and tadalafil) require at least a residual nNOS activity for their action. Inducible NOS (NOS II) can be expressed in many cell types in response to lipopolysaccharide, cytokines, or other agents. Inducible NOS generates large amounts of NO that have cytostatic effects on parasitic target cells. Inducible NOS contributes to the pathophysiology of inflammatory diseases and septic shock. Endothelial NOS (eNOS, NOS III) is mostly expressed in endothelial cells. It keeps blood vessels dilated, controls blood pressure, and has numerous other vasoprotective and anti-atherosclerotic effects. Many cardiovascular risk factors lead to oxidative stress, eNOS uncoupling, and endothelial dysfunction in the vasculature. Pharmacologically, vascular oxidative stress can be reduced and eNOS functionality restored with renin- and angiotensin-converting enzyme-inhibitors, with angiotensin receptor blockers, and with statins.

Keywords

(6R-)5,6,7,8-Tetrahydrobiopterin • L-Arginine • Asymmetric dimethyl-L-arginine • NADPH oxidase • Peroxynitrite

Introduction

Nitric oxide (NO) is an unorthodox messenger molecule, which has numerous molecular targets. NO controls servoregulatory functions such as neurotransmission^{1,2} or vascular tone^{3,4} (by stimulating NO-sensitive guanylyl cyclase), regulates gene transcription^{5,6} and mRNA translation (e.g. by binding to iron-responsive elements),^{7,8} and produces post-translational modifications of proteins (e.g. by ADP ribosylation).^{9,10} An important mode of inactivation of NO is its reaction with superoxide anion (O₂^{-•}) to form the potent oxidant peroxynitrite (ONOO⁻). This compound can cause oxidative damage, nitration, and S-nitrosylation of biomolecules including proteins, lipids, and DNA.^{11,12} Nitrosative stress by ONOO⁻ has been implicated in DNA single-strand breakage, followed by poly-ADP-ribose polymerase (PARP) activation.¹³

In mammals, NO can be generated by three different isoforms of the enzyme NO synthase (NOS; L-arginine, NADPH: oxygen oxidoreductases, NO forming; EC 1.14.13.39). The isozymes are referred

to as neuronal 'n'NOS (or NOS I), inducible 'i'NOS (or NOS II), and endothelial 'e'NOS (or NOS III) (Figure 1). This review will cover all three isoforms. However, a focus of the article will be on eNOS and functions of NO in the cardiovascular system.

Mechanisms of nitric oxide synthesis

All isoforms of NOS utilize L-arginine as the substrate, and molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH₄) are cofactors of all isozymes. All NOS proteins are homodimers (Figure 2). A functional NOS transfers electrons from NADPH, via the flavins FAD and FMN in the carboxy-terminal reductase domain, to the haem in the amino-terminal oxygenase domain. The oxygenase domain also binds the essential

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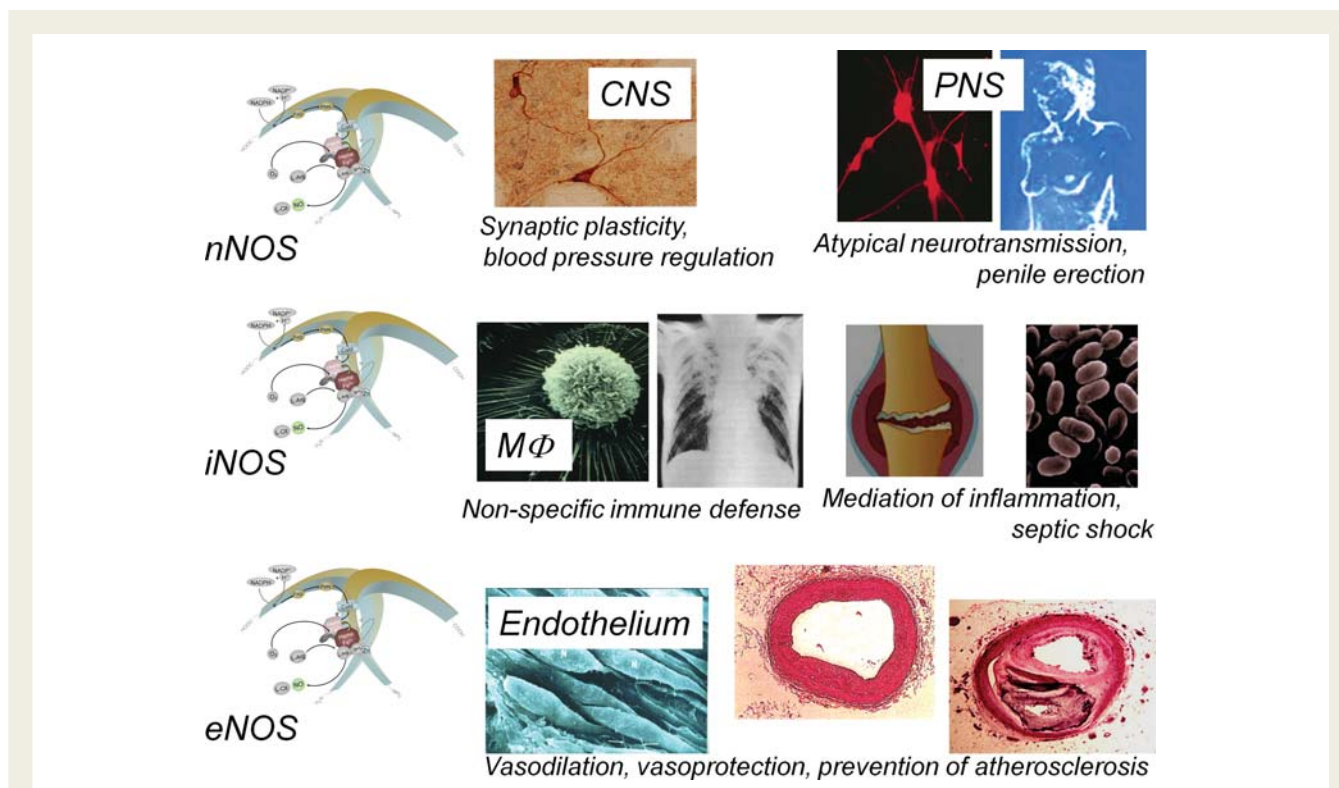


Figure 1 Important functions of the different NOS isoforms. (Top panel) Neuronal NOS is expressed in specific neurons of the central nervous system (CNS). It has been implicated in synaptic plasticity (i.e. phenomena such as long-term potentiation and long-term inhibition). These phenomena are involved in learning and memory formation. Neuronal NOS-derived NO also participates in central control of blood pressure. In the peripheral nervous system (PNS), neuronal NOS-derived NO acts as an atypical neurotransmitter, which mediates relaxing components of gut peristalsis, vasodilation, and penile erection. At least a minimal stimulation of soluble guanylyl cyclase in corpus cavernosum by nNOS-derived NO, and the subsequent formation of small amounts of cyclic GMP, is a prerequisite for the pro-erectile effects of the phosphodiesterase 5 inhibitors sildenafil (Viagra[®]), vardenafil (Levitra[®]), and tadalafil (Cialis[®]). (Middle panel) Inducible NOS expression can be induced by cytokines and other agents in almost any cell type. This had initially been shown for macrophages (MΦ). The induction of inducible NOS in MΦ is essential for the control of intracellular bacteria such as *Mycobacterium tuberculosis*^{156,157} or the parasite *Leishmania*.^{158,159} However, inducible NOS is also up-regulated in various types of inflammatory disease, and the NO generated by the enzyme mediates various symptoms of inflammation.^{160,161} Finally, inducible NOS-derived NO is the predominant mediator of vasodilation and fall in blood pressure seen in septic shock.¹⁶¹ In fact, mice with a disrupted inducible NOS gene are protected from many symptoms of septic shock.¹⁵⁹ (Bottom panel) Endothelial NOS-derived NO is a physiological vasodilator, but can also convey vasoprotection in several ways. NO released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall. Besides protection from thrombosis, this also prevents the release of platelet-derived growth factors that stimulate smooth muscle proliferation and its production of matrix molecules. Endothelial NO also controls the expression of genes involved in atherogenesis. NO decreases the expression of chemoattractant protein MCP-1 and of a number of surface adhesion molecules, thereby preventing leucocyte adhesion to vascular endothelium and leucocyte migration into the vascular wall. This offers protection against early phases of atherogenesis. Also the decreased endothelial permeability, the reduced influx of lipoproteins into the vascular wall and the inhibition of low-density lipoprotein oxidation may contribute to the anti-atherogenic properties of endothelial NOS-derived NO. Finally, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells as well as smooth muscle cell migration, thereby protecting against a later phase of atherogenesis. Based on the combination of those effects, NO produced in endothelial cells can be considered an anti-atherosclerotic principle (for review, see Li and Förstermann¹⁶²).

cofactor BH₄, molecular oxygen, and the substrate L-arginine^{14,15} (Figure 2). At the haem site, the electrons are used to reduce and activate O₂ and to oxidize L-arginine to L-citrulline and NO (Figure 2). Sequences located near the cysteine ligand of the haem are also apparently involved in L-arginine and BH₄ binding.¹⁶ In order to synthesize NO, the NOS enzyme goes through two steps. In a first step, NOS hydroxylates L-arginine to N^ω-hydroxy-L-arginine (which remains largely bound to the

enzyme). In a second step, NOS oxidizes N^ω-hydroxy-L-arginine to L-citrulline and NO.^{17,18} All isoforms of NOS bind calmodulin (Figure 2). In nNOS and eNOS, calmodulin binding is brought about by an increase in intracellular Ca²⁺ (half-maximal activity between 200 and 400 nM). When calmodulin affinity to NOS increases, it facilitates the flow of electrons from NADPH in the reductase domain to the haem in the oxygenase domain. In inducible NOS (iNOS), calmodulin already binds at extremely low

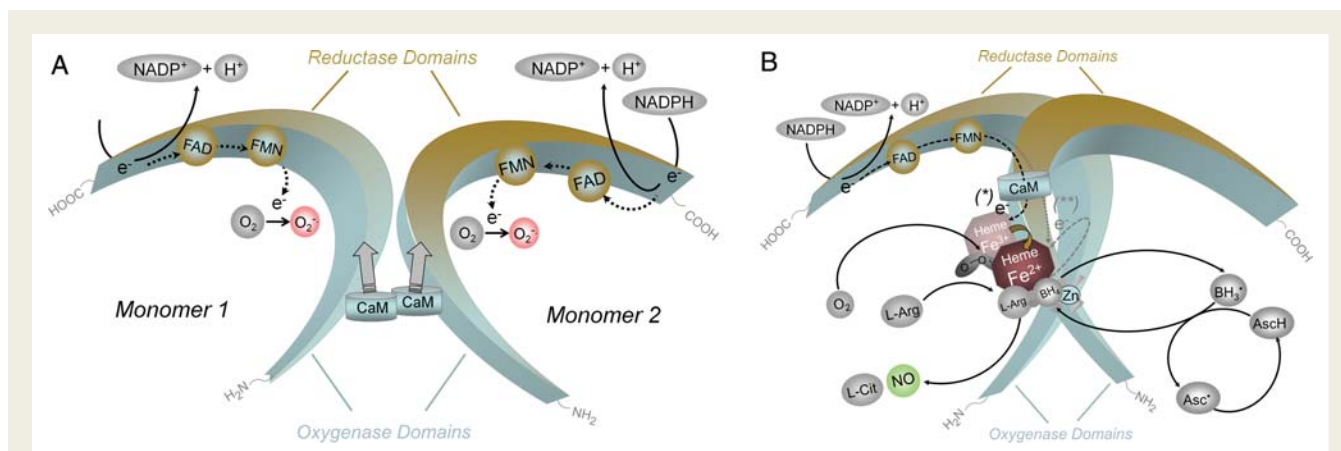


Figure 2 Structure and catalytic mechanisms of functional NOS. (A) NOS monomers are capable of transferring electrons from reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), to flavin-adenine-dinucleotide (FAD) and flavin-mononucleotide (FMN) and have a limited capacity to reduce molecular oxygen to superoxide ($O_2^{\cdot -}$).^{18,163,164} Monomers and isolated reductase domains can bind calmodulin (CaM), which enhances electron transfer within the reductase domain.¹⁶⁵ NOS monomers are unable to bind the cofactor (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) or the substrate L-arginine and cannot catalyze NO production.^{163,166} (B) In the presence of haem, NOS can form a functional dimer.^{163,166} Haem is essential for the interdomain electron transfer from the flavins to the haem of the opposite monomer.^{165,167} Due to differences in the calmodulin-binding domain, elevated Ca^{2+} is required for calmodulin binding (and thus catalytic activity) in nNOS and eNOS, whereas calmodulin binds to inducible NOS with high affinity even in the absence of Ca^{2+} . When sufficient substrate L-arginine (L-Arg) and cofactor BH_4 are present, intact NOS dimers couple their haem and O_2 reduction to the synthesis of NO (fully functional NOS). L-Citrulline (L-Cit) is formed as the byproduct. For clarity, the flow of electrons is only shown from the reductase domain of one monomer to the oxygenase domain of the other monomer. NOS enzymes perform two separate oxidation steps, one to form *N*^ω-hydroxy-L-arginine and a second to convert this intermediate to NO.¹⁸ All NOS isoforms contain a zinc ion (Zn) coordinated in a tetrahedral conformation with pairs of CXXXXC motifs at the dimer interface. This site is of major importance for the binding of BH_4 and L-arginine. Electron transfer from the reductase domain (*) enables NOS ferric (Fe^{3+}) haem to bind O_2 and form a ferrous (Fe^{2+})-dioxy species. This species may receive a second electron (***) preferentially from BH_4 or from the reductase domain. The nature of the resulting oxidized BH_4 has been identified as the trihydrobiopterin radical (BH_3^{\cdot}) or the trihydrobiopterin radical cation protonated at N5 ($BH_3^{\cdot}H^+$). The BH_3^{\cdot} radical (or radical cation) can be recycled to BH_4 by the NOS itself (using an electron supplied by the flavins). Alternatively, there is evidence that reducing agents such as ascorbic acid (AscH, which is present in cells in millimolar concentrations) can reduce the BH_3^{\cdot} radical back to BH_4 ¹⁰³ (Asc[•], ascorbate radical).

intracellular Ca^{2+} concentrations (below 40 nM) due to a different amino acid structure of the calmodulin-binding site.^{19,20} All NOS proteins contain a zinc–thiolate cluster formed by a zinc ion that is tetrahedrally coordinated to two CysXXXXCys motifs (one contributed by each monomer) at the NOS dimer interface.^{21–23} Zinc in NOS has a structural rather than a catalytic function.²¹

The NO formed by NOS can act on a number of target enzymes and proteins. The most important physiological signalling pathway stimulated by NO is the activation of soluble guanylyl cyclase and the generation of cyclic GMP.^{3,4,24–26}

Neuronal nitric oxide synthase

Neuronal NOS is constitutively expressed in specific neurons of the brain (Figure 1). Enzyme activity is regulated by Ca^{2+} and calmodulin. Brain nNOS is found in particulate and soluble forms in cells and the differential subcellular localization of nNOS may contribute to its diverse functions. Neuronal NOS contains a PDZ domain and can interact directly with the PDZ domains of other proteins. These interactions determine the subcellular distribution and the activity of the enzyme.²⁷ In addition to brain tissue, nNOS has been identified by immunohistochemistry in the spinal cord, in

the sympathetic ganglia and adrenal glands, in peripheral nitroergic nerves, in epithelial cells of various organs, in kidney macula densa cells, in pancreatic islet cells, and in the vascular smooth muscle.²⁸ In mammals, the largest source of nNOS in terms of tissue mass is the skeletal muscle.^{28,29}

Physiological functions of neuronal nitric oxide synthase

In the past years, an increasing number of reports have confirmed the significance of nNOS in a variety of synaptic signalling events. Neuronal NOS has been implicated in modulating physiological functions such as learning, memory, and neurogenesis.²⁷ In the central nervous system (CNS), nNOS mediates long-term regulation of synaptic transmission (long-term potentiation, long-term inhibition),^{1,2,30,31} whereas there is no evidence for an involvement of nNOS-derived NO in acute neurotransmission (Figure 1). Retrograde communication across synaptic junctions is presumed to be involved in memory formation, and there is evidence that inhibitors of NOS impair learning and produce amnesia in animal models.^{32,33} There is also evidence that NO formed in the CNS by nNOS is involved in the central regulation of blood pressure^{34–36}

(Figure 1). Blockade of nNOS activity in the medulla and hypothalamus causes systemic hypertension.³⁷

In the periphery, many smooth muscle tissues are innervated by *nitrergic nerves*, i.e. nerves that contain nNOS and generate and release NO (Figure 1). Nitric oxide produced by nNOS in nitrergic nerves can be viewed as an unusual neurotransmitter that stimulates NO-sensitive guanylyl cyclase in its effector cells, thereby decreasing the tone of various types of smooth muscle including blood vessels.^{28,38} The conventional notion that eNOS is mostly responsible for the regulation of vascular tone in the periphery (see later in this article) has been challenged by a human study with *S*-methyl-L-thiocitrulline (SMTC), a selective inhibitor of nNOS. *S*-Methyl-L-thiocitrulline reduces basal blood flow in the human forearm and in the coronary circulation. This effect can be reversed by L-arginine. Interestingly, SMTC does not affect classical eNOS-mediated vasodilatation in response to acetylcholine, substance P, or fluid shear stress. These data are coherent with the notion that nNOS plays an important role in the regulation of vascular tone, independent of effects of nNOS in the CNS. Thus, eNOS and nNOS may have distinct roles in the physiological regulation of human microvascular tone *in vivo*.³⁹ Interestingly, vascular smooth muscle cells also express low levels of nNOS, which have been shown to maintain some degree of vasodilation, when the predominant eNOS becomes dysfunctional.⁴⁰

By mediating the relaxation of the corpus cavernosum smooth muscle, nNOS-containing nitrergic nerves are responsible for penile erection^{41,42} (Figure 1). Also in the corpus cavernosum, NO-induced smooth muscle relaxation is mediated by cyclic GMP.⁴² Cyclic GMP is degraded by phosphodiesterases. The predominant isoform in the corpus cavernosum is isoform 5.⁴³ Thus, a residual nNOS activity is essential for the proerectile effect of selective phosphodiesterase 5 inhibitors such as sildenafil (Viagra[®]), vardenafil (Levitra[®]), and tadalafil (Cialis[®]).^{43,44} Interestingly, because phosphodiesterase 5 is also significantly expressed in pulmonary arteries, sildenafil (under the trade name Revatio[®]) and tadalafil (under the trade name Adcirca[®]) have also been approved for the treatment of pulmonary arterial hypertension.

Role of neuronal nitric oxide synthase in pathophysiology

Abnormal NO signalling is likely to contribute to a variety of neurodegenerative pathologies such as excitotoxicity following stroke, multiple sclerosis, Alzheimer's, and Parkinson's diseases.⁴⁵ Hyperactive nNOS, stimulated by massive Ca²⁺ influx into neuronal cells, has been implicated in *N*-methyl-D-aspartate receptor-mediated neuronal death in cerebrovascular stroke.⁴⁶ Under those conditions, NO can contribute to excitotoxicity, probably via peroxynitrite activation of PARP and/or mitochondrial permeability transition. High levels of NO can also produce energy depletion, due to inhibition of mitochondrial respiration and inhibition of glycolysis.⁴⁷

Some disturbances of smooth muscle tone within the gastrointestinal tract (e.g. gastro-oesophageal reflux disease) may also be related to an overproduction of NO by nNOS in peripheral nitrergic nerves.^{48,49}

Inducible nitric oxide synthase

Inducible NOS is not usually expressed in cells, but its expression can be induced by bacterial lipopolysaccharide, cytokines, and other agents. Although primarily identified in macrophages (Figure 1), expression of the enzyme can be stimulated in virtually any cell or tissue, provided that the appropriate inducing agents have been identified.^{28,38} Once expressed, iNOS is constantly active and not regulated by intracellular Ca²⁺ concentrations.

Physiological functions of inducible nitric oxide synthase

Inducible NOS, when induced in macrophages, produces large amounts of NO, which represent a major cytotoxic principle of those cells.⁵⁰ Due to its affinity to protein-bound iron, NO can inhibit key enzymes that contain iron in their catalytic centres. These include iron–sulfur cluster-dependent enzymes (complexes I and II) involved in mitochondrial electron transport, ribonucleotide reductase (the rate-limiting enzyme in DNA replication), and *cis*-aconitase (a key enzyme in the citric acid cycle).⁵⁰ In addition, higher concentrations of NO, as produced by induced macrophages, can directly interfere with the DNA of target cells and cause strand breaks and fragmentation.^{51,52} A combination of these effects is likely to form the basis of the cytostatic and cytotoxic effects of NO on parasitic microorganisms and certain tumour cells (Figure 1). Interestingly, non-immune cells can also be induced with cytokines to release amounts of NO large enough to affect the neighbouring cells. Cytokine-activated endothelial cells, for example, have been shown to lyse tumour cells,⁵³ and induced hepatocytes can use NO to kill malaria sporozoites.⁵⁴ Inducible NOS activity is likely to be responsible for all of these effects.

Role of inducible nitric oxide synthase in pathophysiology

The high levels of NO produced by activated macrophages (and probably neutrophils and other cells) may not only be toxic to undesired microbes, parasites, or tumour cells, but—when released at the wrong site—may also harm healthy cells. *In vivo*, cell and tissue damage can be related to the NO radical itself or an interaction of NO with O₂^{•-} leading to the formation of peroxynitrite (ONOO⁻). The large majority of inflammatory and autoimmune lesions are characterized by an abundance of activated macrophages and neutrophils. Significant amounts of NO can be secreted by those cells, leading to damage of the surrounding tissue.^{52,55} (Figure 1). Inducible NOS-derived NO is also likely to be involved in non-specific allograft rejection.⁵⁶

Inflammatory neurodegeneration contributes to a number of brain pathologies. Mechanisms by which activated microglia and astrocytes kill neurons have been identified in cell culture. These mechanisms include the activation of the phagocyte NADPH oxidase in microglia and expression of iNOS in glia. This combination produces apoptosis via ONOO⁻ production. Inducible NOS-derived NO also synergizes with hypoxia to induce neuronal

death because NO inhibits cytochrome oxidase. This can result in glutamate release and excitotoxicity^{57,58} (see the Role of neuronal nitric oxide synthase in pathophysiology section on nNOS and excitotoxicity).

Lastly, excessive NO production by iNOS plays a crucial role in septic shock (Figure 1). This condition is characterized by massive arteriolar vasodilatation, hypotension, and microvascular damage. Bacterial endotoxins usually initiate the symptoms. A number of mediators such as platelet-activating factor, thromboxane A₂, prostanooids, and cytokines such as interleukin-1, tumour necrosis factor- α , and interferon- γ are elevated in septic shock and have been implicated in its pathophysiology. However, the fall in blood pressure is predominantly due to excess NO production by iNOS induced in the vascular wall.^{59,60}

Endothelial nitric oxide synthase

Endothelial NOS is mostly expressed in endothelial cells (Figure 1). However, the isozyme has also been detected in cardiac myocytes, platelets, certain neurons of the brain, in syncytiotrophoblasts of the human placenta and in LLC-PK₁ kidney tubular epithelial cells.^{28,38}

Similar to nNOS, Ca²⁺-activated calmodulin is important for the regulation of eNOS activity. Endothelial NOS synthesizes NO in a pulsatile manner with eNOS activity markedly increasing when intracellular Ca²⁺ rises. Ca²⁺ induces the binding of calmodulin to the enzyme.²⁰ However, several other proteins also interact with eNOS and regulate its activity. For example, heat shock protein 90 (hsp90) has been found associated with eNOS and serves as an allosteric modulator activating the enzyme⁶¹ and promoting eNOS (re)coupling^{62,63} (see later in the text). The fraction of eNOS that is localized in caveolae⁶⁴ can interact with the caveolae coat protein, caveolin-1. Caveolin-1 is a tonic inhibitor of eNOS activity. This concept has been proven genetically because blood vessels from caveolin-1-deficient mice show enhanced endothelium-dependent relaxations.⁶⁵ Mechanistically, the recruitment of calmodulin and hsp90 to eNOS can displace caveolin-1 from the enzyme thereby leading to enzyme activation.⁶⁶

However, eNOS can also be activated by stimuli that do not produce sustained increases in intracellular Ca²⁺, but still induce a long-lasting release of NO. The best established such stimulus is fluid shear stress. This activation is mediated by phosphorylation of the enzyme.^{67,68} The eNOS protein can be phosphorylated on several serine (Ser), threonine (Thr), and tyrosine (Tyr) residues. Phosphorylation of Ser1177 stimulates the flux of electrons within the reductase domain, increases the Ca²⁺ sensitivity of the enzyme, and represents an additional and independent mechanism of eNOS activation.^{68,69} Oestrogen and vascular endothelial growth factor (VEGF) phosphorylate eNOS mainly via the Ser/Thr kinase Akt, insulin probably activates both Akt and the AMP-activated protein kinase (AMPK), the bradykinin-induced phosphorylation of Ser1177 is mediated by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), and shear stress elicits phosphorylation mainly by activating protein kinase A (PKA) (Figure 3). Recent evidence using Akt1-deficient mice carrying knock-in mutations of the critical Akt1 phosphorylation site on eNOS has proven that kinase Akt1 is a critical regulator of eNOS function

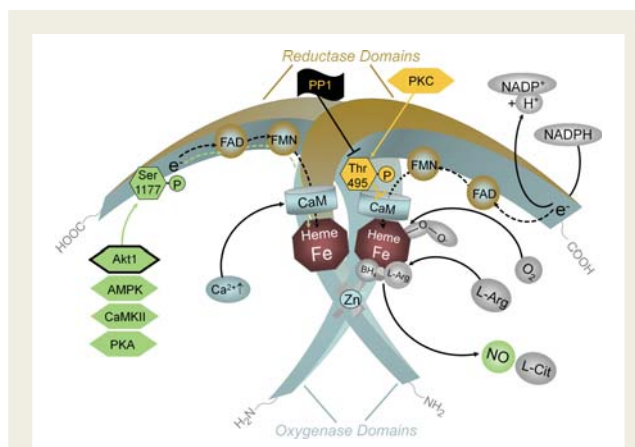


Figure 3 Regulation of endothelial NOS activity by intracellular Ca²⁺ and phosphorylation. An increase in intracellular Ca²⁺ leads to an enhanced binding of calmodulin (CaM) to the enzyme, which in turn displaces an auto-inhibitory loop and facilitates the flow of electrons from NADPH in the reductase domain to the haem in the oxygenase domain. Established functionally important phosphorylation sites in human endothelial NOS are Ser1177 and Thr495. In resting endothelial cells, Ser1177 is usually not phosphorylated. Phosphorylation is induced when the cells are exposed to oestrogens, vascular endothelial growth factor (VEGF), insulin, bradykinin or fluid shear stress. The kinases responsible for phosphorylation (green hexagons) depend on the primary stimulus. Oestrogen and vascular endothelial growth factor elicit phosphorylation of Ser1177 by activating serine/threonine kinase Akt. So far, Akt1 is the only kinase proven to regulate endothelial NOS function *in vivo* (framed green hexagon). Insulin probably activates both Akt and the AMP-activated protein kinase (AMPK), the bradykinin-induced phosphorylation of Ser1177 is mediated by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), and shear stress leads to phosphorylation of endothelial NOS mainly via protein kinase A (PKA). Phosphorylation of the Ser1177 residue increases the flux of electrons through the reductase domain and thus enzyme activity. The Thr495 residue of human endothelial NOS tends to be constitutively phosphorylated in endothelial cells. Thr495 is a negative regulatory site, and its phosphorylation is associated with a decreased electron flux and enzyme activity. The constitutively active kinase that phosphorylates endothelial NOS Thr495 is most probably protein kinase C (PKC, yellow hexagon). Phosphorylation of Thr495 reduces endothelial NOS activity (yellow block arrow). The phosphatase that dephosphorylates Thr495 appears to be protein phosphatase1 (PP1, black flag with black block arrow).

also *in vivo*.⁷⁰ Ser1176 is the Akt1 phosphorylation site in the mouse that corresponds to Ser1177 in the human species. The phosphomimetic mutation Ser1176Asp rendered the enzyme constitutively active, whereas the mutation Ser1176Ala reduced enzyme activity.⁷⁰ Thus, although all the kinases mentioned can regulate eNOS Ser1177 *in vitro*, Akt1 is the only kinase proven to regulate eNOS function *in vivo*.

Thr495 tends to be phosphorylated under non-stimulated conditions (most probably by protein kinase C). Phosphorylation of Thr495 is likely to interfere with the binding of calmodulin to

the calmodulin-binding domain. In fact, dephosphorylation of Thr495 is associated with stimuli that elevate intracellular Ca^{2+} concentrations and increase eNOS activity. Substantially more calmodulin binds to eNOS when Thr495 is dephosphorylated.⁶⁸ However, dephosphorylation of Thr495 has also been shown to favour eNOS uncoupling (see below).⁷¹

Other phosphorylation sites of human eNOS include Ser114, Ser633, Tyr81, and Tyr657 residues. Phosphorylation of these residues is an intensively studied area and may have important consequences for enzyme activity as recently reviewed.⁷²

Physiological functions of endothelial nitric oxide synthase

Vasodilation and inhibition of platelet aggregation and adhesion

Endothelial NOS appears to be a homeostatic regulator of numerous essential cardiovascular functions. Endothelial NOS-derived NO dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells.^{3,4,73} Deletion of the eNOS gene leads to elevated blood pressure.^{74,75} Nitric oxide released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall.^{76–78} Besides protection from thrombosis, this also prevents the release of platelet-derived growth factors that stimulate smooth muscle proliferation and its production of matrix molecules. Endothelial NOS is also critical for adaptive vascular remodelling to chronic changes in flow.⁷⁹

Inhibition of leucocyte adhesion and vascular inflammation

Endothelial NO controls the expression of genes involved in atherogenesis. Nitric oxide decreases the expression of chemoattractant protein MCP-1.⁸⁰ Nitric oxide can also inhibit leucocyte adhesion to the vessel wall by either interfering with the ability of the leucocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface or by suppressing CD11/CD18 expression on leucocytes.^{81,82} Leucocyte adherence is an early event in the development of atherosclerosis, and therefore, NO may protect against the onset of atherogenesis.

A disturbed integrity of the endothelial monolayer barrier can initiate proinflammatory events. Endothelium-derived NO prevents endothelial cell apoptosis induced by proinflammatory cytokines and proatherosclerotic factors including reactive oxygen species (ROS) and angiotensin II (AT). The suppression of apoptosis may also contribute to the antiinflammatory and anti-atherosclerotic effects of endothelium-derived NO.⁸³

Control of vascular smooth muscle proliferation

Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells.^{84–87} These antiproliferative effects are likely to be mediated by cyclic GMP.^{84,85,88} The inhibition of platelet aggregation and adhesion protects smooth muscle from exposure to platelet-derived

growth factor(s). Therefore, NO also prevents a later step in atherogenesis, fibrous plaque formation. Based on the combination of those effects, NO produced in endothelial cells can be considered an anti-atherosclerotic principle⁸⁹ (Figure 1).

Stimulation of angiogenesis by endothelial nitric oxide synthase-derived NO

Endothelial NOS-derived NO plays a critical role in post-natal angiogenesis, mediating signals downstream of angiogenic factors. Recent findings in eNOS-deficient mice point to a novel and previously unrecognized role of NO in foetal lung development and lung morphogenesis. The lung phenotype of eNOS-deficient mice closely resembles alveolar capillary dysplasia in humans, a form of malignant pulmonary hypertension of the newborn that presents with defective lung vascular development and respiratory distress.⁹⁰ Similarly, eNOS had been found to be critical for collateral formation and angiogenesis post-ischaemia.⁹¹ Furthermore, the positive effects of NO on endothelial cell survival are likely to also contribute to the pro-angiogenic effects of NO.⁸³

Activation of endothelial progenitor cells by endothelial nitric oxide synthase-derived nitric oxide

Mice with a deleted eNOS gene show an impaired neovascularization. This was related to a defect in progenitor cell mobilization. Mobilization of endothelial progenitor cells by VEGF is reduced in eNOS-deficient mice. In a model of hind-limb ischaemia in these mice, intravenous infusion of wild-type progenitor cells, but not bone marrow transplantation, can rescue the defective neovascularization. This suggests that mobilization of progenitor cells from the bone marrow is impaired in eNOS-deficient mice. Indeed, matrix metalloproteinase-9, which is required for stem cell mobilization, was reduced in the bone marrow of eNOS-deficient mice. Thus, eNOS expressed by bone marrow stromal cells influences recruitment of stem and progenitor cells. Reduced systemic NO bioactivity seen in ischaemic heart disease may therefore contribute to impaired neovascularization.⁹²

Also in patients with ischaemic cardiomyopathy, bone marrow mononuclear cells show a reduced neovascularization capacity *in vivo*. As mentioned above, NO plays an important role in neovascularization and NO bioavailability is typically reduced in patients with ischaemic heart disease. Pre-treatment of bone marrow cells from these patients with the enhancer of eNOS expression and activity 4-fluoro-*N*-indan-2-yl-benzamide (AVE9488)⁹³ significantly increased eNOS expression and activity.⁹⁴ This is associated with an enhanced migratory capacity of the bone marrow cells *in vitro* and improved neovascularization capacity of these cells in a mouse ischaemic hind-limb model *in vivo*. This improved limb perfusion by AVE9488-treated bone marrow cells was NO mediated because it was abrogated by pre-treatment of the cells with the eNOS inhibitor N^{G} -nitro-L-arginine methyl ester. Also, compound AVE9488 showed no effect on the impaired migratory capacity of bone marrow cells from eNOS-deficient mice. Thus, pharmacological enhancement of eNOS expression and activity at least partially reverses the impaired functional activity of bone marrow

cells from patients with ischaemic cardiomyopathy.⁹⁴ Similarly, the eNOS stimulator simvastatin (see below) enhanced the number of functionally active endothelial progenitor cells in patients with myocardial infarction.⁹⁵

Gene therapy with nitric oxide synthase

Gene therapy refers to the transfer of a specific gene to the host tissue to intervene in a disease process, with resultant alleviation of the symptoms. Nitric oxide synthase gene therapy has been the focus of numerous studies as dysfunction of this enzyme has been implicated in several types of cardiovascular diseases. Research has concentrated on effects of gene delivery of NOS isoforms (eNOS, iNOS, or nNOS) in animal models of vascular tone, ischaemia–reperfusion injury, intimal hyperplasia, and restenosis. In many pre-clinical models of cardiovascular disease, vascular gene delivery proved to be therapeutically beneficial. Endothelial NOS appears particularly promising as it inhibits intimal hyperplasia and enhances reendothelialization in injured blood vessels. The obvious long-term goal is to translate the benefits of NOS gene therapy seen in animal models into clinical practice. However, further work is required along this way to improve delivery systems and to minimize negative side effects.^{96,97}

Role of endothelial nitric oxide synthase in pathophysiology

Patients with cardiovascular risk factors (such as hypertension, hypercholesterolaemia, diabetes mellitus, cigarette smoking, etc.) and patients with vascular disease show endothelial dysfunction, i.e. the inability of the endothelium to generate adequate amounts of bioactive NO (and to produce NO-mediated vasodilation). Cardiovascular risk factors and vascular disease are also associated with an increased production of ROS. There are several enzyme systems that can potentially produce ROS in the vessel wall. These include the NADPH oxidases, xanthine oxidase, enzymes of the mitochondrial respiratory chain, and uncoupled eNOS (see below).⁹⁸ Of these, NADPH oxidases are of primary importance for ROS generation (Figure 4A). Several isoforms of $O_2^{\cdot-}$ -producing NADPH oxidase exist in the vascular wall. They are expressed in endothelial and smooth muscle cells, as well as in the adventitia.

Molecular basis of endothelial dysfunction in vascular disease: inactivation of bioactive nitric oxide and endothelial nitric oxide synthase uncoupling

Due to the enhanced oxidative stress seen in vascular disease, an increased degradation of NO by its reaction with $O_2^{\cdot-}$ will occur. However, oxidative stress has also been shown to convert eNOS from an NO-producing enzyme to an enzyme that generates $O_2^{\cdot-}$.

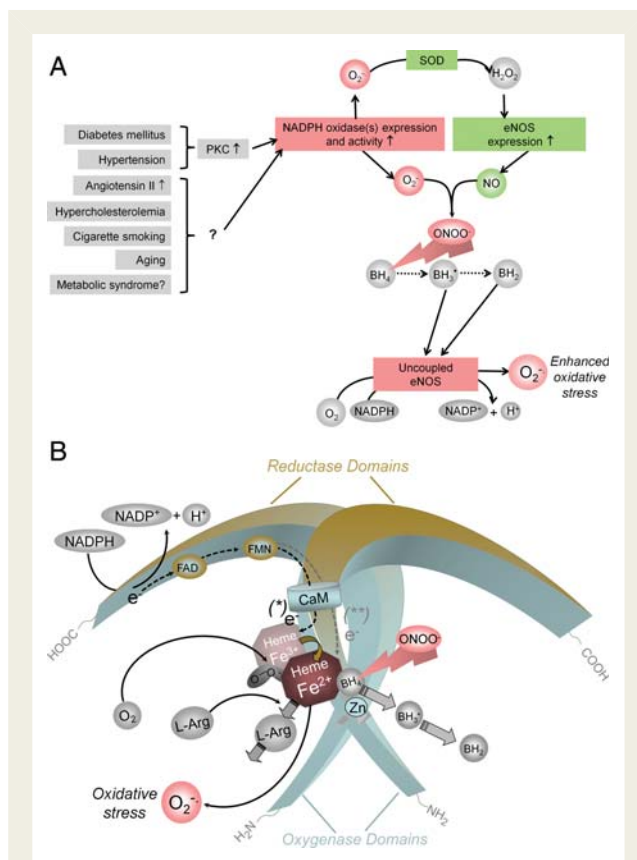


Figure 4 Potential mechanisms by which cardiovascular risk factors lead to oxidative stress and endothelial NOS uncoupling. (A) In many types of vascular disease, NADPH oxidases are up-regulated in the vascular wall and generate superoxide ($O_2^{\cdot-}$). In experimental diabetes mellitus and angiotensin II-induced hypertension, this has been shown to be mediated by protein kinase C (PKC).^{168,169} Expression of endothelial NOS is also increased in vascular disease. H_2O_2 , the dismutation product of $O_2^{\cdot-}$ can increase endothelial NOS expression via transcriptional and post-transcriptional mechanisms (SOD, superoxide dismutase).¹⁷⁰ In addition, also protein kinase C activation can enhance endothelial NOS expression,¹⁷¹ and protein kinase C inhibitors reduce endothelial NOS expression levels in vascular disease.¹⁶⁹ The products of NADPH oxidases and endothelial NOS, $O_2^{\cdot-}$ and NO, rapidly recombine to form peroxynitrite ($ONOO^-$). This can oxidize the essential cofactor of endothelial NOS (6R-)5,6,7,8-tetrahydrobiopterin (BH_4) to trihydrobiopterin radical (BH_3^{\cdot}).^{172,173} BH_3^{\cdot} can disproportionate to the quinonoid 6,7-[8H]- H_2 -biopterin (BH_2). As a consequence, oxygen reduction and O_2 reduction by endothelial NOS are uncoupled from NO formation, and a functional NOS is converted into a dysfunctional $O_2^{\cdot-}$ -generating enzyme that contributes to vascular oxidative stress. The enhanced endothelial NOS expression (see above) aggravates the situation. (B) Oxidation of BH_4 to biologically inactive products such as the BH_3^{\cdot} radical or BH_2 also reduces the affinity of the substrate L-arginine (L-Arg) to NO, and NOS catalyzes the uncoupled reduction in O_2 , leading to the production of $O_2^{\cdot-}$ (and possibly also H_2O_2).

This process has been referred to as NOS uncoupling (Figure 4B). Mechanisms implicated in eNOS uncoupling include oxidation of the critical NOS cofactor BH_4 , depletion of L-arginine, and

accumulation of endogenous methylarginines. More recently, S-glutathionylation of eNOS has been proposed as yet another mechanism leading to eNOS uncoupling.

The (6R)-5,6,7,8-tetrahydrobiopterin hypothesis

As detailed above, a functional eNOS transfers electrons from NADPH, via the flavins FAD and FMN to the haem, where the substrate L-arginine is oxidized to L-citrulline and NO.⁹⁹ The reaction product of NO and O_2^- , ONOO⁻, can uncouple oxygen reduction from NO generation in NOS. Oxidation or removal of the essential cofactor BH₄^{99–101} or oxidative damage of the zinc–thiolate cluster involved in BH₄ and L-arginine binding¹⁰² may be the cause of eNOS uncoupling in this situation (Figure 4A and B). ONOO⁻ can oxidize BH₄ to the biologically inactive BH₃[•] radical that can disproportionate to the quinonoid 6,7-[8H]-H₂-biopterin.^{103,104} It has been shown that NO production by eNOS correlates closely with the intracellular concentration of BH₄,^{105,106} and BH₄ levels have been found decreased in many models of cardiovascular disease^{107–109,101} and in patients with endothelial dysfunction.^{110–112}

L-Arginine supply of endothelial nitric oxide synthase and endothelial nitric oxide synthase uncoupling

In animal and human pathophysiology (hypercholesterolaemia and hypertension), L-arginine supplementation can improve endothelial dysfunction.^{113–116} Normal L-arginine plasma concentrations are ~100 μmol/L. Even in pathophysiology, they hardly fall below 60 μmol/L, and there is an up to 10-fold accumulation of L-arginine within cells.¹¹⁷ On the other hand, the *K_m* of eNOS for L-arginine is only ~3 μmol/L.¹¹⁸ Also, human endothelial cells are not even dependent on L-arginine uptake from the extracellular space; they can effectively recycle L-citrulline to L-arginine and can also obtain L-arginine from proteolysis.^{119,120}

However, endothelial cells express arginases that can compete with eNOS for substrate and, if highly expressed, ‘starve’ eNOS.^{121–124} A relative L-arginine deficiency in the vicinity of eNOS caused by excessive arginase activity is conceivable and could explain part of the beneficial effects of L-arginine supplementation. Also non-substrate effects of L-arginine can contribute to these effects. These include potential direct radical-scavenging properties of the guanidino nitrogen group, the cooperativity between L-arginine and BH₄-binding sites of NOS,^{125,126} or the competition of L-arginine with asymmetric dimethyl-L-arginine (ADMA).¹²⁷

Asymmetrical dimethyl-L-arginine and endothelial nitric oxide synthase uncoupling

Asymmetric dimethyl-L-arginine is considered a risk factor for all-cause cardiovascular mortality.¹²⁸ Asymmetric dimethyl-L-arginine is an endogenous inhibitor of eNOS, but elevated ADMA has also been associated with eNOS uncoupling.¹²⁹ The activities (not the expression) of the key enzyme for ADMA production, protein arginine N-methyltransferase (PRMT, type I),¹³⁰ and of

the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH)¹³¹ are redox-sensitive. In various models, oxidative stress has been shown to increase the activity of PRMT(s) and decrease that of DDAH, thereby leading to increased ADMA concentrations.^{127,130,131} Thus, an increased production of ROS could trigger increased ADMA levels.

S-Glutathionylation of endothelial nitric oxide synthase: yet another mechanism leading to endothelial nitric oxide synthase uncoupling

In several disease conditions associated with oxidative stress (see above), BH₄ supplementation only partly restores eNOS functionality. Cysteine residues are important for eNOS function. Protein thiols can be subject to S-glutathionylation, a protein modification involved in cell signalling. Conditions of oxidative stress promote S-glutathionylation of proteins. S-Glutathionylation of eNOS reversibly decreases NO production and increases O_2^- generation primarily from the reductase domain. Two highly conserved cysteine residues in the reductase domain have been identified as sites of S-glutathionylation.¹³² Endothelial NOS S-glutathionylation in endothelial cells goes along with an impaired endothelium-dependent vasodilation. In blood vessels from hypertensive animals, eNOS S-glutathionylation is increased and endothelium-mediated vasodilation is reduced. That condition is reversed by thiol-specific reducing agents, which reverse S-glutathionylation. Thus, S-glutathionylation of eNOS is likely to represent an additional mechanism involved in eNOS uncoupling.^{132,133}

Pleiotropic actions of conventional cardiovascular drugs that improve endothelial function

Drugs interfering with the renin–angiotensin–aldosterone system and statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are able to prevent endothelial dysfunction and eNOS uncoupling.

Drugs interfering with the renin–angiotensin–aldosterone system

Several components of the renin–angiotensin–aldosterone system are up-regulated in atherosclerotic vessels. Angiotensin II and aldosterone both promote endothelial dysfunction.¹³⁴ Angiotensin II activates NADPH oxidases via AT₁ stimulation.¹³⁵ In addition, the AT₁ receptor is up-regulated *in vitro* by low-density lipoprotein.¹³⁶

In Watanabe heritable hyperlipidaemic rabbits, the renin inhibitor aliskiren increases eNOS expression, enhances eNOS phosphorylation at Ser1177 (thereby increasing activity), decreases NADPH oxidase expression, augments vascular BH₄ levels, and restores eNOS uncoupling.¹³⁷

Angiotensin-converting enzyme-inhibitors and AT₁ receptor blockers have indirect antioxidant effects by preventing the activation of NADPH oxidase.^{138–141} In addition, they can also increase the activity of extracellular superoxide dismutase

(SOD3).¹⁴² Angiotensin-converting enzyme-inhibitors significantly reduce cardiovascular events in patients with established coronary artery disease or at high risk for the disease.¹⁴³ The AT₁ receptor blocker losartan restores glomerular NO production by increasing protein expression of GTP cyclohydrolase1 (the rate-limiting enzyme for BH₄ synthesis) and elevating BH₄ levels in diabetic rats.¹⁴⁴

The selective aldosterone antagonist eplerenone and enalapril reduce NADPH oxidase activity, elevate vascular BH₄ levels, and enhance eNOS expression and NO bioavailability. Eplerenone also increases eNOS phosphorylation at Ser1177. Both drugs decrease atherosclerotic plaque formation.¹³⁴

These pleiotropic effects of compounds interfering with the renin–angiotensin–aldosterone system may contribute significantly to the therapeutic benefit of such drugs.

Statins

The cholesterol-lowering statins have additional cholesterol-independent or pleiotropic effects in cardiovascular disease.¹⁴⁵ These properties include the improvement of endothelial function, stabilization of atherosclerotic plaques, inhibition of oxidative stress and inflammation, and reduction in thrombotic responses.¹⁴⁶ These effects of statins are, in part, mediated by an effect on eNOS, because they can be inhibited by eNOS inhibitors¹⁴⁷ and are absent in eNOS-deficient mice.⁹⁵

Statins increase the expression of eNOS,¹⁴⁸ but also enhance eNOS activity by decreasing caveolin abundance¹⁴⁹ and by activation of the phosphatidylinositol 3-kinase/Akt pathway.¹⁵⁰

Several statins inhibit endothelial O₂^{-•} formation by reducing the expression and/or activity of NADPH oxidase and by preventing the isoprenylation of p21 Rac, which is critical for a functional NADPH oxidase.¹⁵¹ In addition, SOD3 activity is more than doubled by simvastatin.

Statins have also been shown to increase GTP cyclohydrolase1 mRNA expression in endothelial cells and to elevate intracellular BH₄ levels.¹⁵² Atorvastatin has been shown to normalize endothelial function and reduces oxidative stress by inhibiting vascular NADPH oxidases and preventing eNOS uncoupling by an up-regulation of GTP cyclohydrolase1.¹⁵³ Together, these effects may contribute to the anti-atherogenic action of statins.^{154,155}

Conclusions

All three NOS isozymes have regulatory functions in the cardiovascular system. Neuronal NOS is involved in central regulation of blood pressure, and nNOS-containing (nitrenergic) nerves can dilate certain vascular beds. Nitrenergic nerves are of particular importance in the relaxation of corpus cavernosum and penile erection. Phosphodiesterase 5 inhibitors require at least a residual nNOS activity for their action. Inducible NOS is found expressed in atherosclerotic plaque and is an important mediator of the fall in blood pressure in septic shock. The most important isoform is eNOS, which keeps blood vessels dilated, controls blood pressure, and has numerous other vasoprotective and anti-atherosclerotic effects. Although there is no evidence that eNOS is a ‘disease gene’, many cardiovascular risk factors lead to oxidative stress, eNOS uncoupling, and endothelial dysfunction in the vasculature.

Drugs interfering with the renin–angiotensin–aldosterone system as well as statins are useful in preventing endothelial dysfunction. Further elucidation of how these therapeutic agents promote eNOS coupling, in face of elevated oxidative stress, may yield insights into other potential avenues leading to the beneficial actions of NO in the cardiovascular system.

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