Endothelial Nitric Oxide Synthase in Vascular Disease

From Marvel to Menace

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Abstract—Nitric oxide (NO·) is an important protective molecule in the vasculature, and endothelial NO· synthase (eNOS) is responsible for most of the vascular NO· produced. A functional eNOS oxidizes its substrate L-arginine to L-citrulline and NO·. This normal function of eNOS requires dimerization of the enzyme, the presence of the substrate L-arginine, and the essential cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄), one of the most potent naturally occurring reducing agents. Cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, or chronic smoking stimulate the production of reactive oxygen species in the vascular wall. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases represent major sources of this reactive oxygen species and have been found upregulated and activated in animal models of hypertension, diabetes, and sedentary lifestyle and in patients with cardiovascular risk factors. Superoxide (O₂⁻) reacts avidly with vascular NO· to form peroxynitrite (ONOO⁻). The cofactor BH₄ is highly sensitive to oxidation by ONOO⁻. Diminished levels of BH₄ promote O₂⁻ production by eNOS (referred to as eNOS uncoupling). This transformation of eNOS from a protective enzyme to a contributor to oxidative stress has been observed in several in vitro models, in animal models of cardiovascular diseases, and in patients with cardiovascular risk factors. In many cases, supplementation with BH₄ has been shown to correct eNOS dysfunction in animal models and patients. In addition, folic acid and infusions of vitamin C are able to restore eNOS functionality, most probably by enhancing BH₄ levels as well. (Circulation. 2006;113:1708-1714.)

Key Words: endothelium ■ arteriosclerosis ■ endothelium-derived factors ■ nitric oxide synthase ■ risk factors

The roughly 10¹⁴ endothelial cells of our vasculature protect us against atherosclerosis and thrombosis. A major weapon of endothelial cells to fight vascular disease is endothelial nitric oxide synthase (eNOS), an enzyme that generates the vasoprotective molecule nitric oxide (NO·). However, many of us unintentionally mistreat our endothelial cells. We expose them to risk factors such as cigarette smoke, high blood pressure, high glucose, or high lipids. Despite this abuse, our endothelium bears with us for some time, tries to maintain NO· production, and preserves vascular protection. However, the risk factors lead to excess production of superoxide (O₂⁻; ie, they produce oxidative stress). O₂⁻ reacts with NO· to form peroxynitrite, and vascular protection slowly vanishes. But that is only the beginning of the calamity. Our eNOS now enters into a vicious biochemical cycle. It changes its enzymology, starts making peroxynitrite (ONOO⁻) itself, and eventually becomes an enzyme that generates only O₂⁻. This brief review discusses how and when this happens and how it may be prevented.

Vascular Protection by eNOS-Derived NO·

eNOS, the predominant NOS isoform in the vasculature, is responsible for most of the NO· produced in this tissue.¹ Vascular NO· dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in smooth muscle cells.¹ NO· released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion. NO· also can inhibit leukocyte adhesion to the vessel wall either by interfering with the ability of the leukocyte adhesion molecule CD11/CD18 to form an adhesive bond with the endothelial cell surface or by suppressing CD11/CD18 expression on leukocytes. White cell adherence is an early event in the development of atherosclerosis; therefore, NO· may protect against the onset of atherogenesis. Furthermore, NO· has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells. 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, endothelial NO· probably represents the most important antiatherogenic defense principle in the vasculature.¹

NOS Structure and Structure-Related Enzymology

All NOS isoforms are modular enzymes. In intact NOS, a C-terminal reductase domain (which binds nicotinamide adenine dinucleotide phosphate [NADPH], flavin mononucleo-
tide [FMN], and flavin adenine dinucleotide [FAD]) is linked to the N-terminal oxygenase domain of the other monomer (Figure 1). As shown in Figure 1C, the oxygenase domain carries a prosthetic heme group. The oxygenase domain also binds (6R)-5,6,7,8-tetrahydrobiopterin (BH4), molecular oxygen, and the substrate l-arginine.1 Sequences located near the cysteine ligand of the heme are apparently also involved in l-arginine and BH4 binding (Figure 2A). All 3 NOS isoforms possess a zinc-thiolate cluster formed by a zinc ion that is tetrahedrally coordinated to 2 CXXXXC motifs (1 contributed by each monomer) at the NOS dimer interface2 (Figure 2A). Chemical removal of zinc from NOS or the possibility of expressing a zinc-deficient NOS that remained catalytically active demonstrated that the zinc in NOS is structural rather than catalytic. All NOS isozymes catalyze flavin-mediated electron transfer from the C-terminally bound NADPH to the heme on the N terminus. Calmodulin (on calcium-induced binding) increases the rate of electron transfer from NADPH via the reductase domain flavins to the heme center (Figures 1B and 1C). At the heme, the electrons are used to reduce and activate O2. To synthesize NO, the enzyme needs to cycle twice. In a first step, NOS hydroxylates l-arginine to N^-hydroxy-l-arginine (which remains largely bound to the enzyme). In a second step, NOS oxidizes N^-hydroxy-l-arginine to l-citrulline and NO (Figure 1C).3 In human eNOS, Cys99, which is part of the zinc-thiolate cluster, is thought to represent (or largely contribute to) the binding site for BH4; zinc itself does not contribute to BH4 binding. Mutation of the homologous Cys331 in nNOS to alanine (C331A) led to an enzyme that lost its binding affinity for BH4 and became catalytically incompetent.4

\[ \text{O}_2^{-} \text{Generation by eNOS and Enzyme Dimerization} \]

The flow of electrons within NOS is tightly regulated. If disturbed, the ferrous-dioxygen complex dissociates, and \( \text{O}_2^{-} \) is generated from the oxygenase domain instead of \( \text{NO} \). (Figures 2B through 2D). This is referred to as NOS uncoupling.

In the recent literature, NOS-catalyzed reduction of molecular oxygen to \( \text{O}_2^{-} \) has been attributed to the failure of the enzyme to form dimers. Indeed, it has been shown that monomers of NOS and even isolated reductase domains are sufficient for \( \text{O}_2^{-} \) production (Figure 1A). However, the NADPH oxidase activity of such enzyme fragments is limited; the dimeric form has much higher enzymatic activity (Figure 1B). Studies with inhibitors of dimerization on inducible NOS have suggested that once a dimer is formed, there is little or no significant return to the monomer. Most probably, this also applies to eNOS. Thus, uncoupling of oxygen reduction from NO formation is unlikely to go along with significant monomerization of the enzyme in vivo.

**Cardiovascular Risk Factors Cause Endothelial Dysfunction: Potential Mechanisms Involved**

In the presence of cardiovascular risk factors, endothelial dysfunction frequently is encountered. Several molecular defects could account for reductions in endothelium-dependent vascular relaxation.
Endothelial dysfunction could be due to decreased eNOS expression. However, several studies have shown that cardiovascular risk factors are associated with an increase rather than a decrease in eNOS expression. The increased expression of eNOS in vascular disease is likely to be a consequence of an excess production of H$_2$O$_2$, the dismutation product of O$_2^-$/H$_2$O$_2$ can increase eNOS expression through transcriptional and posttranscriptional mechanisms.

On the other hand, an accelerated degradation of NO$^-$ (by its reaction with O$_2^-$) is likely to occur in vascular disease.

Cardiovascular Risk Factors and Vascular Disease Are Associated With Increased Levels of Reactive Oxygen Species

Cardiovascular risk factors increase the expression and/or activity of NADPH oxidases (NOX) in the vascular wall, thereby enhancing the production of reactive oxygen species.
Evidence for an activation of NOX has been provided in animal models of hypertension such as angiotensin II infusion\(^8\) or spontaneously hypertensive rats (SHRs)\(^9\) and models of diabetes mellitus.\(^10\) In addition, experimental hypercholesterolemia is associated with an activation of NOX.\(^11\) In atherosclerotic arteries, increased expression of \(\text{gp91phox (Nox2) and Nox4 has been observed}\(^12\) (Figure 3). The stimulating effects of angiotensin II on the activity of these enzymes suggests that an activated (local or systemic) renin-angiotensin system can cause vascular dysfunction.\(^13\) In addition, in hypercholesterolemia, local renin-angiotensin systems may be activated.\(^14\) In vessels from hypercholesterolemic animals\(^15\) and in platelets from hypercholesterolemic patients,\(^16\) the \(\text{AT}1\) receptor has been found to be upregulated.

Xanthine oxidase is another potential source of ROS in vascular disease. The enzyme readily donates electrons to molecular oxygen, thereby producing \(\text{O}_2^{\text{-}}\) and \(\text{H}_2\text{O}_2\). Oxypurinol, an inhibitor of xanthine oxidase, has been shown to reduce \(\text{O}_2^{\text{-}}\) production and improve endothelium-dependent vascular relaxations to acetylcholine in blood vessels from hyperlipidemic animals.\(^17\) This suggests a contribution of xanthine oxidase to endothelial dysfunction in early hypercholesterolemia. Unlike NOX, however, the general importance of xanthine oxidase for endothelial dysfunction is uncertain. Whereas some investigators reported an improvement in endothelial dysfunction in hypercholesterolemic and diabetic patients with xanthine oxidase inhibitors,\(^18\) other failed to show an effect with allopurinol.\(^19\)

**Uncoupled eNOS Contributes to Endothelial Dysfunction**

Evidence for uncoupling of eNOS has been obtained in endothelial cells treated with low-density lipoprotein (LDL),\(^20\) in ONOO\(^-\)-treated rat aorta,\(^21\) and in isolated blood vessels from animals with pathophysiological conditions such as SHRs,\(^22\) stroke-prone SHRs,\(^23\) angiotensin II–induced hypertension,\(^24\) hypertension induced with the mineralocorticoid deoxycorticosterone acetate (DOCA),\(^25\) streptozotocin-induced diabetes,\(^10\) or nitroglycerin tolerance.\(^26\)

Importantly, NOS uncoupling has also been seen in patients with endothelial dysfunction resulting from hypercholesterolemia,\(^27\) diabetes mellitus,\(^28\) or essential hypertension\(^29;\) in chronic smokers\(^30;\) and in nitroglycerin-treated patients.\(^31\)

This raises questions about the pathophysiological mechanism(s) leading to eNOS uncoupling in vascular disease. There is a growing body of evidence that vascular NOX plays a crucial role in the phenomenon of eNOS uncoupling in humans. The important hint came from experiments with NOX (p47phox)-knockout animals.\(^25\) DOCA-salt–treated hypertensive mice showed an increased production of vascular ROS. This was significantly reduced by the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), demonstrating a marked contribution of uncoupled eNOS to oxidative stress in vascular tissue. p47phox-knockout animals showed much less oxidative stress on DOCA-salt treatment, and levels of ROS could no longer be reduced with L-NAME.\(^25\)

**Potential Role of L-Arginine in eNOS Uncoupling**

Beneficial effects of L-arginine supplementation have been documented in both animal studies and humans under pathophysiological conditions such as hypercholesterolemia and hypertension.\(^32–34\) This raises the question as to whether L-arginine concentrations can become critical as a substrate in vivo (Figure 2B). At first glance, this appears unlikely. The \(K_d\) of eNOS for L-arginine is \(\approx 3 \, \text{mmol/L}\); normal L-arginine plasma concentrations are \(\approx 100 \, \text{mmol/L}\) (even in pathophysiology, they hardly fall below 60 mmol/L); and there is up to a 10-fold accumulation of L-arginine within cells.\(^36\) In addition, human endothelial cells can effectively recycle L-citrulline to L-arginine and can obtain L-arginine from protein breakdown.\(^37\)

On the other hand, endothelial cells express arginases that can compete with eNOS for substrate and, if highly expressed, “starve” eNOS. Arginase exists in 2 isoforms; in human endothelial cells, arginase II seems to be the predominant isozyme.\(^38,39\) Upregulated expression and activity of arginase II have been found in corpus cavernosum of diabetic
individuals\textsuperscript{40} and in endothelium from the lung of pulmonary hypertensive patients.\textsuperscript{41} Evidence for a role of increased enzymatic activity of arginine in endothelial dysfunction also has been provided in animal models of cardiovascular disease such as aging,\textsuperscript{42} atherosclerosis,\textsuperscript{38} endothelial dysfunction after ischemia-reperfusion,\textsuperscript{43} and hypertension induced by aortic coarctation or high salt.\textsuperscript{44,45} In apolipoprotein

BH4,\textsuperscript{49} and supplementation with BH4 is capable of correcting inhibitor asymmetric dimethyl-L-arginine (ADMA)\textsuperscript{46} (see below). Thus, 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. Effects of supplemental l-arginine also could be due to local competition with the endogenous eNOS scavenging properties of the guanidino nitrogen group or the cooperativeness between the l-arginine and BH4 binding sites.\textsuperscript{46} (see below).

However, also nonsubstrate effects of l-arginine can contribute to these effects. These include potential direct radical scavenging properties of the guanidino nitrogen group or the cooperativeness between the l-arginine and BH4 binding sites on NOS\textsuperscript{4} (Figure 2A).

**Potential Role of ADMA in eNOS Uncoupling**

ADMA represents a novel independent predictor for all-cause cardiovascular mortality. The activities (not the expression) of both protein arginine N-methyltransferase (PRMT, type I)\textsuperscript{47} and the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH)\textsuperscript{48} are redox sensitive. In cultured endothelial cells, rat models, and humans, oxidative stress has been shown to increase the activity of PRMT(s) and decrease that of DDAH, thereby leading to increased ADMA concentrations.\textsuperscript{46–48} Thus, an increased production of ROS could be the reason for increased ADMA levels. Elevated ADMA may inhibit NO\textsuperscript{-} synthesis by eNOS or could even uncouple the enzyme, which would enhance oxidative stress.\textsuperscript{46} However, it remains to be established whether ADMA concentrations reached in vivo (even in pathophysiology) are sufficient to effectively interact with eNOS.

**Role of BH4 in eNOS Uncoupling**

NO\textsuperscript{-} and l-citrulline production by eNOS in endothelial cells correlates closely with the intracellular concentration of BH4.\textsuperscript{49} and supplementation with BH4 is capable of correcting eNOS dysfunction in several types of pathophysiology. In isolated aortas from prehypertensive SHRs, BH4 supplementation diminished the NOS-dependent generation of O2\textsuperscript{-}.\textsuperscript{22} Administration of BH4 restored endothelial function in animal models of diabetes\textsuperscript{50} and insulin resistance,\textsuperscript{51} as well as in patients with hypercholesterolemia,\textsuperscript{57} diabetes mellitus,\textsuperscript{28} and essential hypertension\textsuperscript{59} and in chronic smokers.\textsuperscript{50}

Intracellular BH4 levels depend on the balance of its de novo synthesis and its oxidation/degradation. BH4 is one of the most potent naturally occurring reducing agents. It is therefore reasonable to hypothesize that oxidative stress may lead to excessive oxidation and depletion of BH4\textsuperscript{21,52} (Figure 2C). Thus, oxidation of BH4 may be the common cause of eNOS dysfunction in vascular pathophysiology. In agreement with this concept, BH4 levels have been found to be decreased in the aorta of insulin-resistant rats,\textsuperscript{53} in plasma of SHRs compared with age-matched Wistar-Kyoto rats,\textsuperscript{54} in aorta of hypercholesterolemic apolipoprotein E–knockout mice,\textsuperscript{23} and in DOCA-salt–treated hypertensive rats.\textsuperscript{24}

It is important to note that particularly ONOO\textsuperscript{-}, the direct reaction product of NO\textsuperscript{-} and O2\textsuperscript{-}, is able to oxidize BH4. Recently published studies revealed that ONOO\textsuperscript{-} oxidizes BH4 to the BH2 radical, which can be re-reduced to BH4 by NO\textsuperscript{-} or by appropriate chemical reducing agents such as ascorbic acid (vitamin C)\textsuperscript{55,56} (Figure 2A). Thus, the improvement in endothelial function seen with infusions of vitamin C\textsuperscript{57–59} may involve mechanisms beyond mere protection of NO from inactivation by free oxygen radicals. Because of an enhanced regeneration of BH4,\textsuperscript{55,56} ascorbic acid can “recouple” eNOS and enhance its enzymatic activity.

**Improvement in Endothelial Dysfunction by Folic Acid**

Folic acid has proved effective in reversing endothelial dysfunction in animal models of cardiovascular disease and in patients with cardiovascular risk factors.\textsuperscript{60–62} Recent studies have indicated that folates possess stabilizing effects on the heme-containing oxygenase domain of eNOS.\textsuperscript{63} First, folates may rescue or stabilize BH4 by stimulating the endogenous regeneration of quinoid BH2 to BH4. This can recouple the eNOS enzyme, thereby increasing NO production. Second, folates as reduced pteridines, have potent antioxidant properties per se and can directly scavenge the O2\textsuperscript{-}. produced by an uncoupled eNOS. Third, folates may interact with the pteridine-binding site in NOS. This can enhance the binding of BH4, leading to a facilitated electron transfer from the reductase domain or BH4 itself to the catalytic heme center.

**Oxidation of the Zinc-Thiolate Cluster in eNOS May Lead to Enzyme Uncoupling**

Zou et al\textsuperscript{64} have put forth an alternative concept potentially explaining eNOS uncoupling. They showed that the exposure of the isolated enzyme to ONOO\textsuperscript{-} leads to a disruption of the zinc-thiolate cluster, resulting in an uncoupling of the enzyme (Figure 2D). BH4 was oxidized at concentrations 10- to 100-fold higher than those needed to disrupt the zinc-thiolate complex. From these findings, the authors suggested that the principal mechanism of uncoupling is the oxidation of the zinc-thiolate center rather than BH4 oxidation.\textsuperscript{64} However, it should be kept in mind that Cys99 in the thiolate center of eNOS is also essential for BH4 binding (Figure 2A); its oxidation would damage the BH4 binding site (Figure 2D) with similar consequences for the enzyme as oxidation of the cofactor itself. In addition, it is not clear whether a loss of zinc from eNOS ever occurs in intact cells in vivo.

**Potential Clinical Interventions to Restore Normal eNOS Function**

On the basis of the pathophysiology mentioned above, there are several possible approaches to restore eNOS functionality
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(ie, recouple eNOS) in the clinical situation. These include the intra-arterial infusion of the eNOS cofactor BH₄ as demonstrated by studies in chronic smokers, diabetics, hypercholesterolemic patients, and hypertensive individuals.

Folic acid increases intracellular BH₄ levels and has been used successfully to restore endothelial function in patients with hypercholesterolemia, diabetes mellitus, or hyperhomocysteinemia. Folic acid also prevented or reversed eNOS dysfunction in nitroglycerin-treated patients and in healthy volunteers with postprandial endothelial dysfunction. In addition, infusions of high doses of vitamin C have been found to improve endothelial function acutely. The exact mechanism of action of ascorbic acid is unknown, but as detailed above, vitamin C also is likely to recouple eNOS

Conclusions
Oxidative stress and endothelial dysfunction in the coronary and peripheral circulation have important prognostic implications for subsequent cardiovascular events. As detailed here, an increased production of ROS by uncoupled eNOS contributes markedly to this pathophysiology.

Disclosures
None.

References


