T he roughly 10^{14} 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 (O_{2}^{-}); i.e., they produce oxidative stress. O_{2}^{-} 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 O_{2}^{−}. 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. Vascular NO· dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in smooth muscle cells. 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.
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. 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 interface (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^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 (Figure 1C). 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.

O₂⁻ Generation by eNOS and Enzyme Dimerization
The flow of electrons within NOS is tightly regulated. If disturbed, the ferrous-dioxygen complex dissociates, and O₂⁻ is generated from the oxygenase domain instead of NO (Figures 2B through 2D). This is referred to as NOS uncoupling.

In the recent literature, NOS-catalyzed reduction of molecular oxygen to O₂⁻ 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 O₂⁻ 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.

**Figure 1.** A, Basic structure of eNOS and scheme of NOS catalysis. All NOS enzymes are synthesized as monomers. Each subunit consists of a reductase domain and an oxygenase domain. Monomers and even isolated reductase domains are able to transfer electrons from NADPH to the flavins FAD and FMN and have a limited capacity to reduce molecular oxygen to O₂⁻. Monomers and isolated reductase domains can bind calmodulin (CaM), which stimulates the electron transfer within the reductase domain. However, monomers are unable to bind the cofactor BH₄ or the substrate L-arginine and cannot catalyze NO production. B, The presence of heme allows NOS dimerization; in fact, heme is the only cofactor that is absolutely required for the formation of active NOS dimers. Heme also is essential for the interaction between reductase and oxygenase domains and for the interdomain electron transfer from the flavins to the heme of the opposite monomer. NADPH oxidation rates are significantly enhanced in heme-containing substrate-free NOS dimers compared with monomers, consistent with a more effective O₂⁻ production. C, When sufficient substrate L-arginine and cofactor BH₄ are present, intact NOS dimers couple their heme and O₂ reduction to the synthesis of NO: L-citrulline is formed as the byproduct; N^ω-hydroxy-L-arginine is an intermediate in the reaction.
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 $\text{H}_2\text{O}_2$. $\text{H}_2\text{O}_2$, the dismutation product of $\text{O}_2^-/\text{H}_2\text{O}_2$, can increase eNOS expression through transcriptional and posttranscriptional mechanisms.

On the other hand, an accelerated degradation of NO· (by its reaction with $\text{O}_2^-$) 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 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 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 \(O_2^-\) and \(H_2O_2\). Oxypurinol, an inhibitor of xanthine oxidase, has been shown to reduce \(O_2^-\) 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 \(K_m\) of eNOS for l-arginine is \(\approx 3 \mu\text{mol}/L\); normal l-arginine plasma concentrations are \(\approx 100 \mu\text{mol}/L\) (even in pathophysiology, they hardly fall below 60 \(\mu\text{mol}/L\)); 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 arginase 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 E–knockout mice, the expression of arginase II was unchanged compared with wild-type mice, but the activity of the enzyme was markedly increased.\textsuperscript{38} Similarly, in human umbilical vein endothelial cells, arginase II enzymatic activity was enhanced after an 18- to 24-hour exposure to thrombin\textsuperscript{38} or a 24-hour stimulation with inflammatory cytokines.\textsuperscript{39}

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 inhibitor asymmetric dimethyl-L-arginine (ADMA)\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 BH\textsubscript{4} 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 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 BH\textsubscript{4} in eNOS Uncoupling

NO- and L-citrulline production by eNOS in endothelial cells correlates closely with the intracellular concentration of BH\textsubscript{4}\textsuperscript{49} and supplementation with BH\textsubscript{4} is capable of correcting eNOS dysfunction in several types of pathophysiology. In isolated aortas from hypertensive SHRs, BH\textsubscript{4} supplementation diminished the NOS-dependent generation of O\textsubscript{2}•.\textsuperscript{22} Administration of BH\textsubscript{4} 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 BH\textsubscript{4} levels depend on the balance of its de novo synthesis and its oxidation/degradation. BH\textsubscript{4} 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 BH\textsubscript{4}\textsuperscript{21,52} (Figure 2C). Thus, oxidation of BH\textsubscript{4} may be the common cause of eNOS dysfunction in vascular pathophysiology. In agreement with this concept, BH\textsubscript{4} levels have been found to be decreased in the aorta of insulin-resistant rats,\textsuperscript{53} in plasma of SHR compared with age-matched Wistar-Kyoto rats,\textsuperscript{54} in aorta of hypercholesterolemic apolipoprotein E–knockout mice,\textsuperscript{22} and in DOCA-salt–treated hypertensive rats.\textsuperscript{23}

It is important to note that particularly NOO\textsuperscript{−}, the direct reaction product of NO- and O\textsubscript{2}•−, is able to oxidize BH\textsubscript{4}. Recently published studies revealed that NOO\textsuperscript{−} oxidizes BH\textsubscript{4} to the BH\textsubscript{2} radical, which can be re-reduced to BH\textsubscript{4} by NO\textsubscript{•−} 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 BH\textsubscript{4},\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. First, folates may rescue or stabilize BH\textsubscript{4} by stimulating the endogenous regeneration of quinoid BH\textsubscript{4} to BH\textsubscript{4}. 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 O\textsubscript{2}•− produced by an uncoupled eNOS. Third, folates may interact with the pteridine-binding site in NO. This can enhance the binding of BH\textsubscript{4}, leading to a facilitated electron transfer from the reductase domain or BH\textsubscript{4} 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 NOO\textsuperscript{−} leads to a disruption of the zinc-thiolate cluster, resulting in an uncoupling of the enzyme (Figure 2D). BH\textsubscript{4} 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 BH\textsubscript{4} oxidation.\textsuperscript{64} However, it should be kept in mind that Cys99 in the thiolate center of eNOS is also essential for BH\textsubscript{4} binding (Figure 2A); its oxidation would damage the BH\textsubscript{4} 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, diabetic, 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 (Figure 2A).

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 (Figure 3).

Disclosures

None.

References


Endothelial Nitric Oxide Synthase in Vascular Disease: From Marvel to Menace
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