
Tetrahydrobiopterin

Regulator of Endothelial Nitric Oxide Synthase in Vascular Disease

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In vascular disease states such as atherosclerosis and diabetes, endothelial nitric oxide (NO) bioactivity is reduced and oxidative stress is increased, resulting in endothelial dysfunction. Recent studies suggest that changes in the activity and regulation of endothelial NO synthase by its cofactor tetrahydrobiopterin (BH4) is an important contributor to endothelial dysfunction. Pharmacologic studies and more recent insights from genetically modified mouse models have improved the understanding of the mechanistic role and importance of BH4 in vascular disease pathogenesis. Targeting BH4 may provide new therapeutic strategies in vascular disease. (Trends Cardiovasc Med 2004;14:323–327) © 2004, Elsevier Inc.

Nitric oxide (NO) is a key regulator of multiple aspects of cardiovascular homeostasis, including blood pressure and flow, smooth muscle contraction, inflammation, and platelet activation. In the vascular wall, loss of normal NO production from the endothelium is a cardinal feature of endothelial dysfunction that characterizes diverse vascular disease states such as diabetes, hypertension, and atherosclerosis. In addition to providing a biologic marker of vascular disease, endothelial dysfunction contributes to vascular disease pathogenesis and progression through loss of the atheroprotective actions of NO. Indeed,

recent prospective studies indicate that measures of endothelial function provide independent predictors of cardiovascular risk.

Although the importance of endothelial NO in vascular pathophysiology is well established, the cellular and biochemical mechanisms underlying the loss of endothelial NO bioavailability in endothelial dysfunction states has remained unclear. In general, diseased blood vessels with impaired NO-mediated endothelial function still have an anatomically intact endothelium. Furthermore, endothelial NO synthase (eNOS) protein remains present in the dysfunctional endothelium at normal or even, in some cases, increased levels. Accordingly, attention has focused on mechanisms that could reduce NO bioavailability either by reduced NO production or increased NO loss. In particular, increased production of reactive oxygen species (ROS) is a feature of vascular disease states that directly parallels the deficit in NO-mediated endothelial function. Increased superoxide production by oxidase enzymes in the vascular wall may have direct effects on NO bioavailability, due to the rapid interaction between the NO and superoxide radicals, leading to

loss of NO bioavailability and the production of peroxynitrite, another ROS with potentially toxic effects. The principal enzymatic sources of vascular superoxide production include the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidase. However, more recent studies have found evidence that abnormal eNOS regulation in vascular disease states is accompanied not only by reduced NO production but also by a direct contribution to vascular ROS production by eNOS. Some of the potential mechanisms contributing to these observations, discussed below, provide a mechanistic link between endothelial dysfunction and increased vascular ROS production through changes in eNOS regulation and activity.

• Regulation of Endothelial NOS

In common with all three NOS enzymes, endothelial NOS (NOS3) is a homodimeric oxidoreductase that catalyzes the production of NO from the guanidino nitrogen of L arginine, using molecular oxygen. The reductase domain of eNOS shares a close homology with the cytochrome P450 enzymes, generating electron flow from NADPH through the flavins FAD and FMN that are transferred to the oxidase domain of the other monomer where L-arginine oxidation occurs at the heme group in the active site (Figure 1A). In endothelial cells, eNOS activity is regulated through multiple integrated pathways including activation by calcium—calmodulin, membrane localization in caveolae through lipid modifications, protein—protein interactions with caveolin and hsp90, phosphorylation at key serine and threonine residues, and subcellular trafficking between caveolae and cytosol (Fleming and Busse 2003).

A critical aspect of NOS function is the requirement for the cofactor tetrahydrobiopterin (BH4). BH4 binds close to the heme active site at the interface between the two monomers, interacting with residues from both (Raman et al. 1998). Maintenance and stabilization of NOS dimers is dependent on BH4, and BH4 also plays a direct role in the multistep oxidation of arginine through the N-hydroxy-L-arginine intermediate and the subsequent generation of NO.

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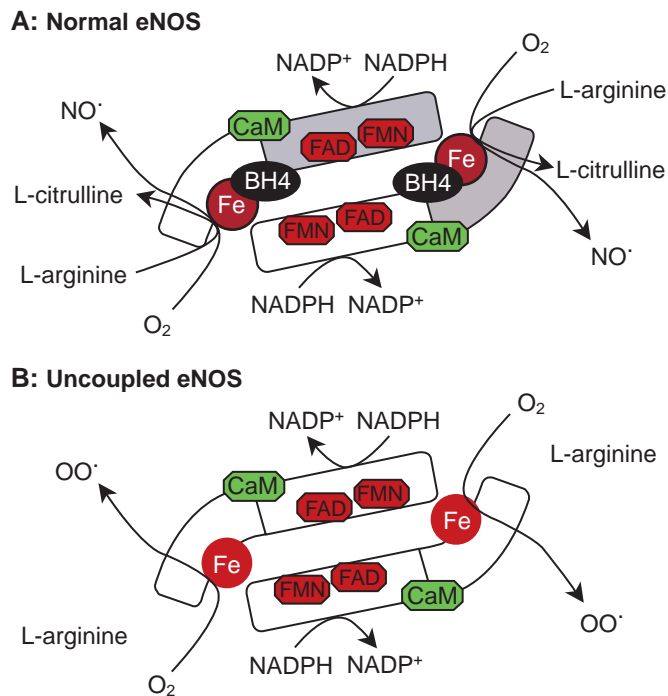


Figure 1. Mechanisms of endothelial nitric oxide synthase (eNOS) regulation by tetrahydrobiopterin (BH4). Under optimal conditions (A), the eNOS enzyme is a homodimer whereby electron flow generated from NADPH is transferred through the flavins FAD and FMN to the oxygenase domain of the other monomer (*gray shading*). BH4 is bound close to the heme group (Fe) in the active site and also interacts with residues in both monomers, contributing to eNOS dimer formation. NO is generated by the oxidation of the guanidino nitrogen of L arginine using molecular oxygen. In conditions of BH4 deficiency (B), electron flow from flavins to L arginine becomes “uncoupled” from L-arginine oxidation, resulting in formation of superoxide from molecular oxygen.

In contrast to the aromatic amino acid hydroxylases for which BH4 is also a cofactor, BH4 is not oxidized to the dihydrobiopterin derivative (BH2) but rather forms a BH3⁺ radical during the NOS catalytic cycle from which BH4 is regenerated directly. Intracellular BH4 levels are regulated through de novo BH4 synthesis from guanosine triphosphate (GTP) (Figure 2); the rate-limiting enzyme is GTP-cyclohydrolase I (GTP-CH) (Thony et al. 2000). In inflammatory cells, GTP-CH is regulated by transcriptional induction in response to stimuli such as cytokines, and studies in cultured endothelial cells showed that GTP-CH and other enzymes in the biosynthetic pathways can also be induced by cytokines (Hattori et al. 1997). However, inflammatory stimuli do not appear to greatly upregulate vascular GTP-CH expression in vivo (Alp et al. 2003). Posttranslational modification of GTP-CH activity by phosphorylation and by feedback inhibition through protein–protein interactions may be important additional regulators of BH4 synthesis

(Thony et al. 2000). GTP-CH feedback regulatory protein expression in endothelium is reduced in response to cytokines, resulting in increased BH4 synthesis independent of GTP-CH expression (Werner et al. 2002).

When BH4 is limiting or absent, eNOS biochemistry is fundamentally altered in a number of ways, not merely by a loss of enzymatic activity. First, eNOS dimerization is destabilized, leading to a reduction in the relative proportion of eNOS dimers versus monomers present in the cell. Second, eNOS catalytic activity becomes “uncoupled.” In this situation, the stoichiometric coupling between the reductase domain and L-arginine oxidation at the active site is lost. However, electron transfer from NADPH through the flavins to molecular oxygen is not inhibited, but results in a formation of superoxide and/or hydrogen peroxide. NOS uncoupling is regulated not just by BH4 but also by other mechanisms, including oxidation status of the Zn²⁺-thiolate center (Zou et al. 2002) and by

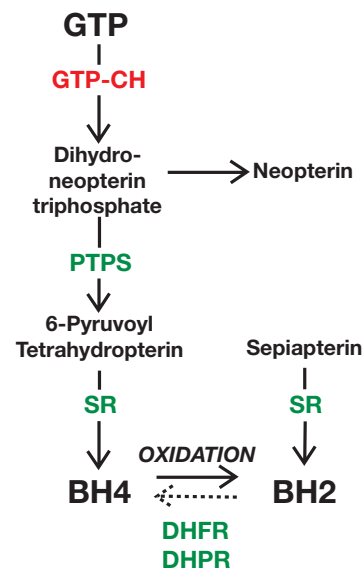


Figure 2. Tetrahydrobiopterin (BH4) biosynthesis and metabolism. BH4 is synthesized de novo from guanosine triphosphate (GTP) by the enzymes GTP-cyclohydrolase I (GTP-CH), 6-pyruvoyl-tetrahydrobiopterin synthase (PTPS), and sepiapterin reductase (SR). BH4 is susceptible to oxidation by reactive oxygen species such as peroxynitrite, forming dihydrobiopterin (BH2) and ultimately biopterin. Synthesis of BH4 is also possible through the “salvage pathway” from the synthetic pterin, sepiapterin, which is metabolized to dihydrobiopterin by SR and thence to BH4 by dihydrofolate reductase (DHFR) when BH2 levels are supraphysiologic. Regeneration of BH4 from BH2 is also facilitated by dihydropteridine reductase (DHPR), in relation to BH4’s function as a cofactor for the aromatic amino acid hydroxylases, but this pathway does not appear to play a role in vascular BH4 regulation. The stable metabolite neopterin is formed from the product of GTP-CH, 7,8-dihydroneopterin triphosphate.

availability of the substrate L arginine. The relative contributions of BH4 versus L-arginine availability appear to vary in different cell types under different conditions and between different NOS isoforms. Nevertheless, BH4 appears necessary, even if not always sufficient to prevent NOS uncoupling.

• Vascular Disease: A Tetrahydrobiopterin Deficiency State?

The ability of BH4 to modulate both NO production and superoxide production in the endothelium has received considerable attention as a potential mechanism underlying endothelial dysfunction in vascular disease. Numerous studies have found that pharmacologic supple-

mentation of BH4 augments NO-mediated effects in either cell culture or in vitro vessel rings or in animal models or patients with vascular disease risk factors (Alp and Channon 2004, Katusic 2001). However, more direct mechanistic evidence showing that relative BH4 deficiency is a significant contributor to endothelial dysfunction that can be specifically rescued by restoration of BH4 in relation to eNOS function remains the focus of investigation. The pharmacologic approach of BH4 supplementation, either by administration of BH4 or sepiapterin, is potentially confounded by the administration of very high supraphysiologic concentrations that may exert nonspecific effects on NO bioavailability through scavenging of superoxide and other ROS. In the case of supplementation with high doses of sepiapterin, which is converted to BH4 in cells through the pterin salvage pathway, nonspecific effects may be marked and unpredictable (Vasquez-Vivar et al. 2002a). However, some investigators have attempted to control for nonspecific effects. For example, administration of BH4 improved forearm blood flow in smokers, whereas the chemically similar tetrahydropterin, which has antioxidant properties but cannot act as a NOS cofactor, had no effect (Heitzer et al. 2000).

Important evidence suggesting that BH4 may be limiting in vascular disease states has come recently from direct measurements of BH4 levels in vascular tissues and cultured endothelial cells obtained from animal models of vascular disease; for example, models of diabetes in the rat and mouse (Meininger et al. 2000, Shinozaki et al. 1999). In addition to absolute BH4 levels, the ratio between the fully reduced BH4 and the partially oxidized dihydrobiopterin (BH2) may regulate eNOS activity and uncoupling. BH2 is unable to act as a NOS cofactor, but may effectively reduce BH4 availability by competitive binding to NOS. Certainly, loss of BH4 to BH2 by oxidation is a feature of the increased oxidative stress that is characteristic of vascular disease states (Alp et al. 2003, Landmesser et al. 2003). However, some data suggest that the BH4/BH2 ratio can directly modulate superoxide release from eNOS, independent of absolute BH4 levels (Vasquez-Vivar et al. 2002b). In apoE

KO mouse liver, the BH4/BH2 ratio was reduced compared with wild-type controls, whereas absolute BH4 levels were unchanged (d'Uscio et al. 2003). In apoE KO mouse aorta, both the BH4/BH2 ratio and absolute BH4 levels were reduced (Alp et al. 2004). Other studies suggest that the absolute level of BH4, rather than the BH4/BH2 ratio, is the principal determinant of eNOS activity in vivo (Alp et al. 2003). Further studies are required to specifically investigate whether BH4/BH2 ratio, independent of BH4 levels, is a key determinant of eNOS activity and coupling.

• Endothelial NOS Uncoupling in Vascular Disease: Role of BH4

Several recent studies have sought to investigate the mechanisms relating BH4 availability to eNOS uncoupling in vascular disease using approaches other than pharmacologic BH4 supplementation.

In streptozotocin (STZ) diabetic rats, hyperglycemia resulted in endothelial dysfunction and increased vascular superoxide production. Increased vascular superoxide production was mediated by upregulation of NAD(P)H oxidases through a pathway in part dependent on protein kinase C (Hink et al. 2001). However, eNOS expression and protein levels were increased, whereas NO production was decreased, suggesting a stoichiometric discordance in eNOS activity. These findings were supported by studies in human blood vessels obtained from patients undergoing coronary artery bypass surgery (Guzik et al. 2002). Vessels from patients with diabetes had reduced NO-mediated endothelial function and increased endothelial superoxide production. Uncoupling of eNOS in diabetic endothelium was revealed by NOS inhibition, which reduced total superoxide production, whereas NOS inhibition in nondiabetic vessels increased net superoxide release by reducing superoxide scavenging by NO. Furthermore, the specific increase in endothelial superoxide release in diabetes was inhibited by BH4.

Evidence of endothelial dysfunction and eNOS uncoupling was also observed in atherosclerotic apoE knockout (KO) mice (Laursen et al. 2001). In these animals, eNOS-dependent superoxide production was increased, resulting in the formation of peroxynitrite, which can

oxidize BH4 to BH2. Restoration of BH4 levels or reduction in peroxynitrite formation improved endothelial function.

Further important observations implicating eNOS regulation in vascular disease pathogenesis have come from studies in eNOS KO and in endothelial-targeted eNOS transgenic mice. In eNOS KO mice, both atherosclerotic plaque formation and the proliferative response to vascular injury were exaggerated (Knowles et al. 2000, Rudic et al. 1998), confirming that eNOS plays a vascular protective role. The corollary would be that increased eNOS levels might be predicted to reduce atherosclerosis. However, in transgenic mice with endothelial-targeted eNOS overexpression, atherosclerotic plaque in the apoE KO model was paradoxically increased (Ozaki et al. 2002). Although eNOS overexpression modestly increased NO production, there was a much larger increase in endothelial superoxide release. These features could be rescued by BH4 supplementation, directly implicating BH4-mediated eNOS regulation in vascular disease pathogenesis. More importantly, this study provided clear proof that eNOS activity and regulation, not eNOS levels, are the critical determinants of NO-mediated endothelial function in vascular disease states.

More mechanistic insights into the role of BH4 and eNOS uncoupling in endothelial function came from studies in genetically modified mice rendered hypertensive by deoxycorticosterone acetate-salt (DOCA-salt) feeding (Landmesser et al. 2003). These animals developed endothelial dysfunction and features of eNOS uncoupling, including loss of BH4 by oxidation. However, eNOS KO mice were protected from the adverse vascular effects of DOCA-salt feeding, providing direct evidence for the importance of eNOS in mediating endothelial dysfunction. Furthermore, animals with targeted deletion of the gene encoding p47phox, one of the subunits of the NAD(P)H oxidase, were also protected from the effects of DOCA-salt on BH4 oxidation and eNOS uncoupling. From these studies implicating the NAD(P)H oxidases in vascular disease comes the paradigm that increased oxidative stress in vascular disease, mediated by NAD(P)H oxidases, leads to BH4 depletion by oxidation, resulting in eNOS uncoupling. Loss of

NO and increased eNOS-dependent superoxide production further contribute to vascular oxidative stress and endothelial dysfunction.

Several recent studies have sought to investigate the importance of BH4 in eNOS regulation by targeting intracellular BH4 biosynthesis as a means of regulating BH4 levels, both in cell culture and in vivo, without recourse to pharmacologic BH4 supplementation. GTP-CH gene transfer in endothelial cells greatly increased BH4 levels without significantly altering the BH4/BH2 ratio, confirming that other enzymes in the pathway do not become significantly rate limiting, even when GTP-CH is overexpressed (Cai et al. 2002). Furthermore, increasing BH4 biosynthesis in cultured endothelial cells, which are relatively BH4-deficient, restored eNOS activity and increased the proportion of eNOS protein present as the homodimeric form. Gene transfer of GTP-CH in carotid arteries of DOCA-salt hypertensive rats restored BH4 levels and improved endothelial function (Zheng et al. 2003). When GTP-CH was constitutively overexpressed in endothelial cells in transgenic mice, tissue BH4 levels were increased and eNOS activity was increased (Alp et al. 2003), whereas plasma BH4 levels were unchanged. In GTP-CH transgenic mice rendered diabetic with STZ, the loss of vascular BH4 was prevented, leading to reduced features of eNOS uncoupling and restored endothelial function. When GTP-CH transgenic mice were crossed with apoE KO mice, endothelial function was improved and atherosclerotic plaque progression was reduced (Alp et al. 2004). These studies show that increased endothelial BH4 synthesis is sufficient to rescue endothelial dysfunction in vascular disease, and demonstrate that BH4-mediated improvement in endothelial function can directly influence vascular disease pathogenesis.

Whether eNOS uncoupling due to BH4 deficiency is a necessary step in vascular disease pathogenesis remains less clear. KO animals with targeted disruption of BH4 biosynthetic enzymes have severely altered systemic phenotypes, which limits their utility in studies of vascular disease. However, the hph-1 mouse, generated by ethinyl-nitrosourea mutagenesis (McDonald et al. 1988), has greatly reduced GTP-CH expression and

moderate BH4 deficiency. Initial studies in this mouse model found evidence that eNOS-derived hydrogen peroxide, rather than NO, mediated vasorelaxation (Cosentino et al. 2001), but the effects of constitutive BH4 deficiency on susceptibility to vascular diseases remain to be determined.

• Tetrahydrobiopterin As a Therapeutic Target

The importance of BH4 as a critical regulator of eNOS function suggests that BH4 may be a rational therapeutic target in vascular disease states. Indeed, several studies have already explored the effect of BH4 administration, either intravascular or oral, on endothelial functions. However, these studies have been limited to acute or short-term administration, used very high doses, and only determined the effects on endothelial-dependent relaxation rather than other variables related to vascular disease progression or risk. Indeed, whether pharmacologic administration of BH4 in humans changes intracellular BH4 levels in the endothelium and can modify eNOS activity and/or uncoupling remains unexplored. In animal models, oral BH4 administration can increase tissue levels, but there are unpredictable dose effects on oxidative stress in atherosclerosis (Vasquez-Vivar et al. 2002a).

A more promising alternative strategy may be to normalize BH4 levels in the endothelium by reducing oxidative loss, using antioxidants such as ascorbic acid. In cultured endothelial cells, ascorbic acid maintains eNOS activity by stabilizing BH4 against oxidation to BH2 (Kuzkaya et al. 2003, Patel et al. 2002). However, the reaction kinetics between reactive oxygen species, ascorbate and BH4 favor oxidation of BH4 rather than ascorbate. More detailed studies have shown that peroxynitrite is highly efficient in oxidizing BH4 to BH2 via the BH3⁺ radical; the effect of ascorbate is to maintain BH4 availability by recycling BH3⁺ back to BH4 (Kuzkaya et al. 2003). In apoE KO mice with atherosclerosis, chronic supplementation with ascorbic acid increased the BH4/BH2 ratio in the aorta, restored eNOS activity, and improved endothelial function (d'Uscio et al. 2003). However, the effects of low-dose oral antioxidants such as ascorbic acid appear to have no significant clinical

benefit in patients with vascular disease, and may be insufficiently effective and specific in modifying redox signalling at the level of the vessel wall. New agents that modulate oxidant stress and BH4 availability are required to test the true potential of BH4 as a therapeutic target. Agents such as folic acid may exert some of their effects on endothelial function through BH4 (Stroes et al. 2000). Other existing drugs, such as statins, may also have beneficial effects through modulation of GTP-CH expression and BH4 levels (Hattori et al. 2003).

Taken together, these studies showing the salutary effects of increased vascular BH4 levels clearly establish proof of principle that BH4 is a potential therapeutic target in vascular disease. The challenge for the future is to identify new strategies that target BH4 supplementation, synthesis, and/or stabilization with sufficient potency and specificity for use in long-term clinical studies.

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