

# Regulation of Endothelial Nitric Oxide Synthase by Tetrahydrobiopterin in Vascular Disease

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**Abstract**—Nitric oxide (NO), produced by endothelial nitric oxide synthase (eNOS), is a key signaling molecule in vascular homeostasis. Loss of NO bioavailability due to reduced synthesis and increased scavenging by reactive oxygen species is a cardinal feature of endothelial dysfunction in vascular disease states. The pteridine cofactor tetrahydrobiopterin (BH4) has emerged as a critical determinant of eNOS activity: when BH4 availability is limiting, eNOS no longer produces NO but instead generates superoxide. In vascular disease states, there is oxidative degradation of BH4 by reactive oxygen species. However, augmentation of BH4 concentrations in vascular disease by pharmacological supplementation, by enhancement of its rate of de novo biosynthesis or by measures to reduce its oxidation, has been shown in experimental studies to enhance NO bioavailability. Thus, BH4 represents a potential therapeutic target in the regulation of eNOS function in vascular disease. (*Arterioscler Thromb Vasc Biol.* 2004;24:413-420.)

**Key Words:** tetrahydrobiopterin ■ nitric oxide synthase ■ superoxide

Nitric oxide (NO), produced by endothelial NO synthase (eNOS), is a key signaling molecule in vascular homeostasis.<sup>1</sup> Originally identified as endothelium-derived relaxing factor,<sup>2,3</sup> NO is an important regulator of vascular tone and blood pressure. In addition, NO has multiple antiatherogenic roles, including anti-inflammatory, antithrombotic, antiproliferative, and antioxidant effects (reviewed in Ignarro<sup>1</sup>). Loss of NO bioavailability is a cardinal feature of endothelial dysfunction<sup>4</sup> that precedes the development of overt atherosclerosis and is an independent predictor of adverse cardiovascular risk.<sup>5,6</sup> Several factors contribute to loss of NO bioavailability in endothelial dysfunction states, including both reduced NO synthesis and NO scavenging by reactive oxygen species.<sup>7</sup> The regulation of NO production by eNOS is complex, but the pteridine cofactor tetrahydrobiopterin (BH4) has emerged as a critical determinant of NO synthesis. Indeed, endothelial BH4 availability appears to be a key requirement for maintaining normal endothelial function. In this article, we review the current knowledge on the regulation of eNOS by BH4 and discuss the potential importance of mechanisms linking BH4 availability with endothelial function in vascular disease.

## Tetrahydrobiopterin

Tetrahydrobiopterin (BH4) is an essential cofactor for all 3 NO synthase (NOS) isoforms and for the aromatic amino acid hydroxylases (phenylalanine hydroxylase, tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase). BH4 biosynthesis proceeds from GTP via 2 intermediates, 7,8-dihydroneopterin triphosphate and 6-pyruvoyl-5,6,7,8-

tetrahydropterin (reviewed in Thony et al<sup>8</sup>; Figure 1). In endothelial cells (ECs), the first and rate-limiting step in this pathway is GTP cyclohydrolase I (EC 3.5.4.16; GTPCH). The subsequent steps are catalyzed by the enzymes 6-pyruvoyl tetrahydropterin synthase (EC 4.6.1.10; PTPS) and sepiapterin reductase (EC 1.1.1.153; SR). In some cell types (for example, macrophages), inflammatory cytokines upregulate GTPCH expression,<sup>9</sup> but in these conditions, PTPS is not upregulated to the same extent and becomes rate limiting.<sup>10</sup> As a result, the intermediate 7,8-dihydroneopterin triphosphate accumulates and is oxidized to the stable metabolite neopterin, which can be detected in plasma and serves as an indirect marker of inflammation, for example, in acute coronary syndromes.<sup>11</sup>

An alternative pathway for BH4 synthesis has been documented in bacteria<sup>12</sup> and in *Drosophila*,<sup>13</sup> whereby 6-pyruvoyl-5,6,7,8-tetrahydropterin is converted to sepiapterin by a poorly defined enzyme termed “sepiapterin synthase.” The only evidence for a sepiapterin synthesis pathway in mammals comes from a recent study of rare patients with sepiapterin reductase deficiency, in whom sepiapterin levels were elevated in cerebrospinal fluid, which suggests endogenous production of sepiapterin.<sup>14</sup> Certainly, exogenous sepiapterin can be reduced in all cells by sepiapterin reductase to 7,8-dihydrobiopterin (BH2) and further by dihydrofolate reductase (EC 1.5.1.3; DHFR) to form BH4, the so-called salvage pathway<sup>15</sup> that has been exploited by many investigators as an approach to increase BH4 levels by pharmacological supplementation of sepiapterin (Figure 1).

As a cofactor for the aromatic amino acid hydroxylases, BH4 is oxidized to tetrahydrobiopterin-4a-carbinolamine.

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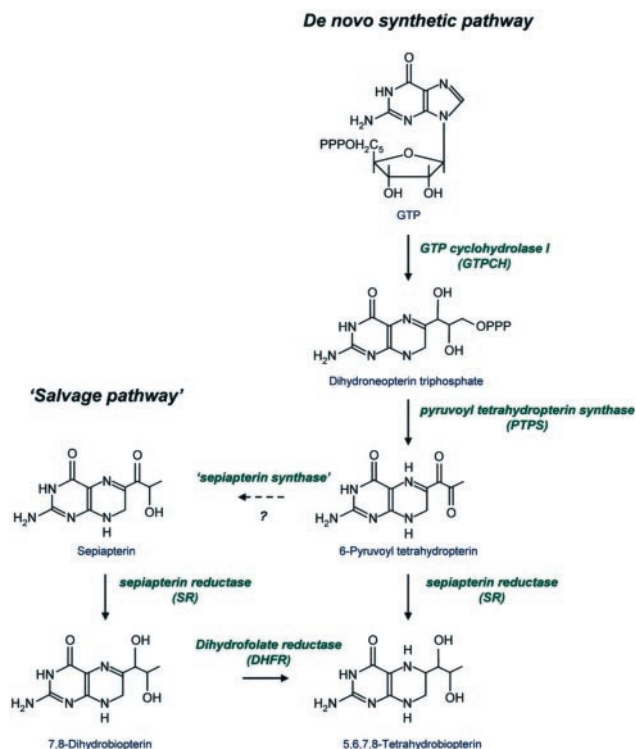
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**Figure 1.** Pathways of BH<sub>4</sub> biosynthesis. BH<sub>4</sub> biosynthesis proceeds from GTP via 7,8-dihydroneopterin triphosphate and 6-pyruvoyl-5,6,7,8-tetrahydropterin. The first and rate-limiting step in the pathway is GTPCH. Subsequent steps are catalyzed by the enzymes 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase. An alternative pathway for BH<sub>4</sub> synthesis has been documented in bacteria<sup>12</sup> and in *Drosophila*,<sup>13</sup> whereby 6-pyruvoyl-5,6,7,8-tetrahydropterin is converted to sepiapterin by an enzyme termed “sepiapterin synthase.” There is limited evidence for a sepiapterin synthesis pathway in mammals. However, exogenous sepiapterin can be reduced in all cells by sepiapterin reductase to BH<sub>2</sub>, and further by dihydrofolate reductase to form BH<sub>4</sub> (the so-called salvage pathway).

BH<sub>4</sub> is regenerated by the actions first of pterin-4a-carbinolamine dehydratase (EC 4.2.1.96; PCD), forming the quinonoid dihydrobiopterin intermediate, which is then reduced by dihydropteridine reductase (EC 1.6.99.7; DHPR). These 2 enzymes are expressed in mammalian liver,<sup>16</sup> kidney, and brain,<sup>17</sup> and mutations in the PCD and DHPR genes are associated with clinical systemic BH<sub>4</sub> deficiency and hyperphenylalaninemia. However, in its action as a cofactor for NOS, BH<sub>4</sub> is not oxidized to tetrahydrobiopterin-4a-carbinolamine. Instead, it appears that BH<sub>4</sub> forms the protonated trihydrobiopterin cation radical (BH<sub>3</sub>.H<sup>+</sup>) in the transfer of electrons to the ferrous-dioxygen complex, coupling oxygen reduction to L-arginine oxidation.<sup>18</sup> After each electron transfer step, the BH<sub>3</sub>.H<sup>+</sup> radical species returns to the BH<sub>4</sub> state. Thus, continuous BH<sub>4</sub> regeneration does not appear to be a requirement for eNOS activity in ECs.

Clinical BH<sub>4</sub> deficiency is associated with a rare variant of hyperphenylalaninemia that is unresponsive to a low-phenylalanine diet.<sup>19</sup> The syndrome is characterized by a deficit of the neurotransmitters dopamine and serotonin and generally presents in childhood with progressive neurological symptoms.<sup>20</sup> The variable presentation reflects a range of

mutations in genes encoding the enzymes involved in BH<sub>4</sub> biosynthesis<sup>21</sup> or regeneration.<sup>22</sup> A form of BH<sub>4</sub> deficiency without hyperphenylalaninemia is also recognized, known as dopa-responsive dystonia,<sup>23</sup> and this is associated with mutations in the gene encoding GTPCH, transmitted in an autosomal-dominant fashion.<sup>24</sup> Study of these syndromes has helped elucidate the role of BH<sub>4</sub> in neurotransmitter synthesis and phenylalanine metabolism, but there are as yet no published data regarding vascular function in these patients.

### GTP Cyclohydrolase I

The human *GCH1* gene, which encodes GTPCH, is located on chromosome 14q22.1-q22.2 and consists of 6 exons spanning ≈30 kb (reviewed in Thony et al<sup>8</sup>). This gene encodes a subunit protein of 221 amino acid residues (27.9 kDa), and the subunits assemble to form a toroidal homododecamer of 2 pentamers.<sup>25</sup> Each active site is located at the interface of 3 subunits, 2 from 1 pentamer and 1 from the other; there are thus 10 equivalent active sites per functional unit.<sup>26</sup>

As the committing and rate-limiting enzyme for BH<sub>4</sub> synthesis, GTPCH is subject to direct regulation at transcriptional and posttranscriptional levels. Cytokines including interferon- $\gamma$  and tumor necrosis factor- $\alpha$  and inflammatory mediators such as lipopolysaccharide are reported to increase GTPCH transcription,<sup>27,28</sup> enzyme activity,<sup>29</sup> and BH<sub>4</sub> levels in human ECs. Insulin may also upregulate GTPCH expression in ECs via a phosphatidylinositol-3-kinase-dependent pathway.<sup>30</sup>

GTPCH is also subject to posttranslational modification by phosphorylation. In rat mesangial cells, angiotensin II and platelet-derived growth factor are reported to increase GTPCH activity by phosphorylation via a protein kinase C-dependent mechanism.<sup>31</sup> However, the role of GTPCH phosphorylation in ECs has not been determined.

Finally, GTPCH activity is governed by interactions with GTPCH feedback regulatory protein (GFRP), which has been cloned and purified<sup>32</sup> and is a homopentamer of 52-kDa subunits.<sup>33,34</sup> Recently, the nature of the physical interaction between GFRP and GTPCH has been demonstrated with x-ray crystallography.<sup>35,36</sup> Through GFRP, phenylalanine exerts positive regulation of GTPCH activity, and BH<sub>4</sub> exhibits negative feedback regulation.<sup>37</sup> GFRP expression has been documented in various cell types and is highly expressed in hepatocytes,<sup>32</sup> but whether GFRP has a biologically important role in regulating GTPCH activity in ECs remains to be determined.

### Regulation of eNOS Activity in Vascular Disease

In ECs, eNOS is subject to rapid changes in activity by regulation through signaling molecules that are integrated in caveolae, for example, by receptor-mediated agonist stimulation that leads to rapid enzyme activation by the binding of calcium-calmodulin, dephosphorylation, displacement of caveolin-1, and release of eNOS from caveolae.<sup>38,39</sup> Changes in eNOS activity are also mediated through phosphorylation of key residues. Akt-dependent phosphorylation at Ser 1177 activates eNOS in response to stimuli such as fluid shear

stress, estrogen, and insulin,<sup>40</sup> whereas phosphorylation at Thr 459 (eg, by protein kinase C) reduces activation. Dephosphorylation of Thr 459 by protein phosphatase 1 contributes to eNOS activation in response to bradykinin.<sup>41</sup>

Although eNOS is constitutively expressed in ECs, eNOS gene transcription may be modulated by a variety of factors. Laminar shear stress is an important stimulus in maintaining or upregulating eNOS expression.<sup>42</sup> Although eNOS protein levels may be reduced in ECs overlying advanced atherosclerotic plaques,<sup>43</sup> eNOS protein may remain normal or even increase, despite marked endothelial dysfunction, in preatherosclerotic states such as diabetes,<sup>44</sup> experimental heart failure,<sup>45</sup> and hypertension.<sup>46</sup> Overexpression of eNOS by gene transfer can increase NO bioactivity in the vessel wall, but constitutive endothelial overexpression of eNOS paradoxically accelerated atherosclerosis in apoE knockout mice, in association with increased oxidative stress.<sup>47</sup> Thus, reductions in NO bioactivity in vascular disease are not explained simply by loss of eNOS protein, and indeed, plentiful eNOS protein is no guarantee of adequate NO bioavailability. These findings have led to the concept of “eNOS dysfunction” or “eNOS uncoupling,” characterized by a stoichiometric discordance between eNOS protein levels and NO production.

### eNOS Uncoupling: Role of BH4 in eNOS Regulation

Increased reactive oxygen species generation is associated with many vascular disease states. Several oxidase systems contribute to increased oxidative stress, notably the NADPH oxidases.<sup>48</sup> However, increasing evidence suggests that eNOS itself can generate superoxide under certain pathophysiological conditions. Electron transfer within the active site becomes “uncoupled” from L-arginine oxidation; instead, molecular oxygen is reduced to form superoxide.<sup>49,50</sup> Superoxide generation by eNOS has been implicated in a variety of experimental and clinical vascular disease states, including diabetes,<sup>44,51</sup> cigarette smoking,<sup>52</sup> hypertension,<sup>53</sup> chronic nitrate tolerance,<sup>54</sup> and overt atherosclerosis.<sup>55</sup> However, demonstration of eNOS-dependent superoxide production has relied on currently available ex vivo methods, all of which have potential limitations and shortcomings.<sup>56</sup> The effect of eNOS uncoupling on NO bioavailability in vascular disease may be profound, because NO production is reduced and superoxide production is increased, which leads to further reductions in NO bioactivity by scavenging and increasing oxidative stress within ECs by formation of peroxynitrite.

A number of molecular mechanisms likely contribute to eNOS uncoupling. Limited availability of the substrate L-arginine may reduce NO synthesis, and there is some evidence that superoxide production may be increased.<sup>57</sup> The interaction of heat shock protein 90 with eNOS appears to modulate the relative production of NO and superoxide<sup>58</sup>; this interaction is inhibited in hypercholesterolemia, associated with eNOS uncoupling.<sup>59</sup> However, BH4 appears to have a particularly important role in regulating NO and superoxide production by eNOS.

The importance of BH4 in the catalytic process of L-arginine oxidation and NO synthesis by eNOS is well established. There is 1 BH4 binding site in the oxygenase

domain of each monomer, so 2 molecules of BH4 are incorporated into each functional eNOS dimer. In vitro experiments with electron paramagnetic resonance spectroscopy have demonstrated that BH4 both stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain, as the initiating step of L-arginine oxidation.<sup>60–62</sup> In this reaction, BH4 forms the protonated trihydrobiopterin cation radical BH<sub>3</sub>H<sup>+</sup>, which is subsequently reduced by electron transfer from eNOS flavins.<sup>61</sup> When BH4 is limiting, electron transfer from eNOS flavins becomes uncoupled from L-arginine oxidation, the ferrous-dioxygen complex dissociates, and superoxide is produced from the oxygenase domain.<sup>49,63</sup>

It is likely that NOS must assemble in its dimeric form for catalytic activity, whether producing NO or superoxide. However, the role of BH4 in stabilizing the dimeric conformation of NOS isoforms is not fully resolved. In vitro enzyme reconstitution experiments suggest that the addition of BH4 has only a minimal effect on eNOS dimerization.<sup>64</sup> However, other evidence indicates a possible role and mechanism for BH4 in stabilizing eNOS dimers. A zinc ion is tetrahedrally coordinated to pairs of cysteine residues at the dimer interface, 1 pair from each subunit, close to the BH4 binding site.<sup>65</sup> Site-directed mutagenesis studies indicate that the cysteine residues are critical for locating BH4 in its binding site and maintaining NO production by eNOS.<sup>66</sup> Recent evidence suggests that disruption of the zinc-thiolate complex by peroxynitrite leads to loss of BH4 from the active site, enzymatic uncoupling, and destabilization of eNOS dimers.<sup>67</sup> Results from recombinant enzyme expression experiments<sup>68–70</sup> and from endothelial cell culture studies<sup>71</sup> also indicate that BH4 indeed promotes eNOS dimerization, protein stability, and NO synthesis. Thus, BH4 may have an important role in the physical stabilization of eNOS in its active dimeric form, in addition to its catalytic functions.

A variety of experimental approaches have been used to investigate BH4 availability as a modulator of eNOS function in vascular disease states, including diabetes, hypertension, and hypercholesterolemia, in both animal models and clinical studies.

### Animal Models

Several recent studies have investigated the relationships between BH4 levels and eNOS function in diabetes. ECs isolated from the BB rat (a strain genetically predisposed to diabetes) have reduced BH4 levels compared with cells from control strains, which results in reduced NO production despite normal eNOS protein levels.<sup>72</sup> This deficit is restored by supplementation with sepiapterin. Insulin-resistant fructose-fed rats demonstrate increased vascular superoxide production, oxidative degradation of BH4 in the aorta, reduced eNOS activity, and impaired endothelium-dependent vascular relaxations compared with controls.<sup>73,74</sup> These abnormalities can be prevented by coadministration of sepiapterin (a synthetic precursor of BH4) in the diet<sup>75</sup> or by incubation of aortic rings in BH4 solution.<sup>73</sup> Similarly, streptozotocin-induced diabetes in rats<sup>76</sup> and hyperglycemia in spontaneously diabetic (*db/db*  $-/-$ ) mice<sup>77</sup> lead to impaired endothelium-dependent relaxations of vascular rings,

which can be restored acutely by incubation with high doses of BH4.

Pharmacological BH4 supplementation also improves endothelial dysfunction in spontaneously hypertensive rats.<sup>78,79</sup> In a deoxycorticosterone acetate-salt (DOCA-salt) model of hypertension in mice, there is increased aortic superoxide production, BH4 oxidation, and eNOS uncoupling, which can be prevented by oral BH4 supplementation.<sup>53</sup> The hyperphenylalaninemic (Hph-1) mouse, generated by *N*-ethyl-*N*-nitrosourea mutagenesis of the *GCH1* locus, has systemically reduced levels of GTPCH expression and reduced BH4 synthesis. Initial studies of the vascular phenotype in the Hph-1 mouse suggest that BH4 deficiency leads to hypertension, increased vascular oxidative stress, and reduced eNOS activity,<sup>80</sup> which demonstrates that reduced BH4 levels appear sufficient to produce eNOS uncoupling in the absence of coexisting vascular disease.

In hypercholesterolemic apoE-knockout mice, endothelium-dependent vascular relaxations are impaired and vascular superoxide production is increased; both can be normalized by *in vitro* incubation of vessels with sepiapterin.<sup>81</sup> Transgenic endothelial overexpression of eNOS in apoE-knockout mice paradoxically increases vascular superoxide production because of enzymatic uncoupling of the increased eNOS protein levels, which leads to accelerated atherosclerosis.<sup>47</sup> BH4 levels are reduced in the aortas of these apoE-knockout mice compared with wild-type controls, but dietary BH4 supplementation with sapropterin appears to reduce superoxide production and restore NO synthesis. These results indicate that increased eNOS protein alone is insufficient to maintain NO synthesis in hypercholesterolemia and that adequate BH4 levels are essential to prevent enzymatic uncoupling.

Thus, in these different animal models, multiple risk factors for endothelial dysfunction appear to act via a common pathway that involves reduced bioavailability of BH4, leading to uncoupling of eNOS, reduced NO synthesis, and increased superoxide production.

### Clinical Studies

A number of clinical studies have sought to investigate the effects of BH4 on eNOS function by exploring the effects of pharmacological supplementation of BH4 on NO-mediated endothelial function. Using venous occlusion plethysmography as a measure of endothelial function, acute administration of BH4 improves endothelial function of patients with hypercholesterolemia<sup>82</sup> or type 2 diabetes.<sup>83</sup> Heitzer and colleagues<sup>84</sup> showed that the beneficial effect of acute BH4 administration in chronic smokers was likely via a direct effect on eNOS coupling rather than simply an antioxidant effect, because a control infusion of tetrahydrobiopterin (NH4) had no effect on endothelial function despite a similar ability to scavenge superoxide anion *in vitro*. Direct intracoronary infusion of BH4 during cardiac catheterization also improves endothelial function, measured by quantitative coronary angiography and Doppler flow studies, in the coronary arteries of patients with hypercholesterolemia<sup>85</sup> or overt coronary atherosclerosis.<sup>55</sup>

However, many of these experimental and clinical studies are limited by the high doses of sepiapterin or BH4 used (often >100-fold in excess of physiological concentrations), which may increase NO bioactivity via nonspecific antioxidant effects. Conversely, the experimental effects of high-dose BH4 added to vessel rings in organ bath chambers may be confounded by auto-oxidation of BH4, generating superoxide.<sup>86</sup> Where sepiapterin has been used to augment BH4 concentrations in a rabbit model of hypercholesterolemia, unexpected uncoupling of eNOS was observed, possibly as a result of competition with BH4 at the active site of the enzyme.<sup>87</sup> In addition, all the experimental interventions have been short term, and there were no studies evaluating the effects of chronic low-dose BH4 augmentation on NOS function in vascular disease. Some of these issues have been addressed by the recent generation of a transgenic mouse model of increased vascular BH4 synthesis, in which human GTPCH expression is targeted to vascular endothelium under the control of the mouse *Tie2* promoter. These mice demonstrate a persistent modest (3-fold) increase in vascular BH4 levels and have reduced endothelial superoxide production and preserved NO bioavailability compared with wild-type littermates in a streptozotocin model of diabetic vascular disease.<sup>88</sup>

## Factors Affecting BH4 Bioavailability in Vascular Disease

### Reduced BH4 Synthesis

Some evidence suggests that GTPCH expression may be downregulated, and thereby BH4 synthesis reduced, in certain vascular pathological states. This hypothesis has conflicted with the observation that inflammatory cytokines are reported to upregulate GTPCH expression in ECs.<sup>27,28</sup> In a glucocorticoid-induced rat model of hypertension, GTPCH mRNA levels were reduced, and impaired endothelium-dependent relaxations could be restored by incubating vessels in sepiapterin, which suggests reduced BH4 bioavailability as a cause of eNOS uncoupling.<sup>89</sup> However, it was not determined whether these abnormalities were a direct effect of glucocorticoid treatment or secondary to the experimental hypertension, and the relevance of these findings to human vascular disease remains uncertain. More recent studies in the DOCA-salt hypertensive mouse also found that decreased BH4 levels were related to reduced GTPCH activity; supplementation of BH4 or increased BH4 synthesis by adenoviral gene delivery of GTPCH restored BH4 levels and normalized eNOS function.<sup>90</sup> Other mechanisms regulating GTPCH activity, such as phosphorylation and GFRP interactions, have not yet been explored in the endothelium, so the importance of altered BH4 synthesis in determining BH4 bioavailability in vascular disease remains uncertain.

### BH4 Oxidation

Many studies have focused on the potential role of BH4 oxidation to BH2 and other oxidized biopterin species in reducing BH4 bioavailability for eNOS. Many preatherosclerotic disease states are associated with increased vascular oxidative stress, particularly superoxide production. Al-

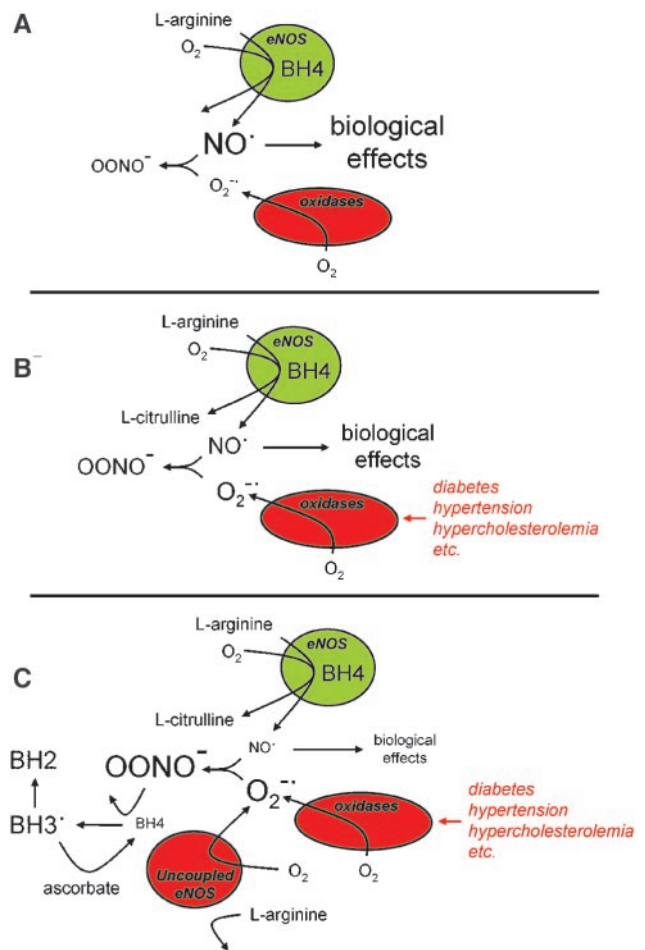
though superoxide can indeed react directly with BH4, the rate constant of this reaction ( $3.9 \times 10^5 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ )<sup>18</sup> is many orders of magnitude lower than that for NO with superoxide ( $6.7 \times 10^9 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ ),<sup>91</sup> which indicates that BH4 is unlikely to be acting merely as an antioxidant at physiological (as opposed to pharmacological) concentrations.

A more likely mechanism for BH4 oxidation is the interaction with peroxynitrite (generated from the interaction between NO and superoxide). Experiments *in vitro*<sup>92</sup> and *in vivo*<sup>81</sup> indicate that peroxynitrite can oxidize BH4 within minutes, at physiologically relevant concentrations, and lead directly to eNOS uncoupling and endothelial dysfunction. Recent electron paramagnetic resonance experiments have demonstrated that peroxynitrite oxidizes BH4 to the (nonprotonated) BH3 radical, and thence to BH2, with a rate constant estimated to be  $6 \times 10^3 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ , several-fold higher than reactions between peroxynitrite and ascorbate, glutathione, or thiol groups.<sup>93</sup> However, the importance of these observations *in vivo* remains to be confirmed. In mice with streptozotocin-induced diabetes, endothelial dysfunction is associated with reduced BH4 levels but no change in total biopterins, which suggests that BH4 is oxidized to BH2 or biopterin. Increased endothelial BH4 biosynthesis by transgenic GTPCH overexpression partially corrects eNOS uncoupling, but the ratio of reduced BH4 in relation to oxidized biopterins in diabetes remains largely unaffected.<sup>88</sup> More direct evidence showing that oxidative loss of BH4 is sufficient to produce eNOS uncoupling comes from studies of DOCA-salt hypertension in mice.<sup>53</sup> DOCA-salt hypertension in eNOS-knockout mice did not increase vascular superoxide production to the same extent as in wild-type mice, which clearly implicates eNOS as an important source of vascular superoxide. Furthermore, in DOCA-salt hypertension, p47phox knockout mice were relatively protected from BH4 oxidation and eNOS uncoupling, which suggests that NADPH oxidase-mediated superoxide production is an important contributor to BH4 oxidative loss and eNOS uncoupling. Other interventions that reduce NADPH oxidase activation in vascular disease states, such as angiotensin II receptor blockade<sup>94</sup> or inhibition of protein kinase C,<sup>95</sup> have also been shown to reduce eNOS uncoupling and enhance NO bioavailability. These observations have contributed to a paradigm whereby superoxide in the endothelium (principally from NADPH oxidase) is thought to generate peroxynitrite (by reacting with NO), which oxidizes BH4, leading to eNOS uncoupling, thus perpetuating a cycle of vascular oxidative stress<sup>7</sup> (Figure 2).

Oxidation not only directly reduces BH4 bioavailability, but the oxidation products themselves (such as BH2), which have no cofactor activity, may compete with BH4 for binding to eNOS.<sup>60</sup> However, it is uncertain whether the ratio of BH4 to oxidized biopterins is as important as the absolute BH4 concentration in determining eNOS activity *in vivo*.

### Tetrahydrobiopterin as a Therapeutic Target in Vascular Disease

What are the prospects for vascular disease therapies that target BH4-dependent eNOS function? High-dose pharmacological supplementation of BH4, given acutely, may not be specific for eNOS cofactor function and is not practical as a



**Figure 2.** Schematic illustrating the role of BH4 in regulating eNOS activity in vascular disease. A, In healthy vascular endothelium, BH4 availability is not limiting. eNOS production of NO is appropriate for the regulation of multiple antiatherogenic biological effects. Superoxide production by various oxidases acts predominantly in a signaling capacity. Peroxynitrite formed by interaction between NO and superoxide is minimal. B, In vascular disease states such as diabetes, hypertension, or hypercholesterolemia, superoxide production by oxidases is markedly increased. NO production may remain unaffected initially, but NO bioavailability is reduced because of scavenging interactions with superoxide, forming increased peroxynitrite. C, Peroxynitrite and other reactive oxygen species oxidize BH4, via the BH3 radical to BH2 and biopterin, which reduces the bioavailability of BH4 and promotes eNOS uncoupling. eNOS now generates superoxide rather than NO, which contributes to vascular oxidative stress and further reduces NO bioavailability. Ascorbate may protect against oxidative degradation of BH4 by directly reducing the BH3 radical to BH4.

therapy for chronic vascular disease states. However, several studies have shown that ascorbate (vitamin C) may be important in maintaining BH4 levels in the setting of vascular oxidative stress. Acute or chronic ascorbate supplementation improves endothelial dysfunction in smokers,<sup>96</sup> in subjects with hypercholesterolemia,<sup>97</sup> and in patients with diabetes mellitus<sup>98,99</sup> or overt coronary artery disease.<sup>100,101</sup> Similarly, chronic dietary supplementation with ascorbate increased the BH4:BH2 ratio and NO bioavailability in the aortas of apoE-knockout mice,<sup>102</sup> and there is a dose-dependent effect of ascorbate on BH4 levels and NO synthesis in cultured

ECs.<sup>103,104</sup> The mechanism for this effect is not fully resolved. Initial reports suggested that ascorbate chemically protects BH4 from oxidation by peroxynitrite,<sup>105</sup> but recent data indicate that the rate constant for the reaction between peroxynitrite and ascorbate is 10-fold lower than that for peroxynitrite and BH4 (generating the BH3 radical).<sup>93</sup> Instead, ascorbate may act by reducing the BH3 radical to regenerate BH4, a reaction with a kinetically favorable rate constant of  $1.7 \times 10^5 \text{ mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$ .<sup>106</sup>

### Conclusions

Current research indicates that maintenance of adequate BH4 levels in the endothelium is likely to be critical in regulating the balance of NO and superoxide synthesis by eNOS in vascular disease. Strategies to maintain BH4 availability may include measures to increase BH4 biosynthesis or to reduce oxidative degradation. Despite significant recent insights into the molecular interactions between BH4 and eNOS that regulate endothelial biology, a number of important questions remain unanswered. The high concentrations of exogenous BH4 that are required to generate a biological effect in many studies suggest that biopterins do not enter cells merely by passive diffusion. However, the nature of a possible biopterin receptor/transporter is currently unknown. A better understanding of cellular biopterin transport would open new therapeutic approaches for endothelial dysfunction in vascular disease. An additional question concerns the role of the BH4 regeneration pathway (mediated by PCD and DHPR) in maintaining BH4 concentrations; this is a critical pathway in hepatocytes, but its importance in the vascular endothelium is unclear.

It is likely that the development of new mouse models with tissue-specific overexpression or deficiency of enzymes relevant to biopterin metabolism will provide important additional information on the role of BH4 in regulating eNOS function in vascular disease. Similarly, a better understanding of pharmacological approaches to target BH4 synthesis, oxidation, or regeneration in the endothelium may provide new therapeutic opportunities in vascular diseases.

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