Tetrahydrobiopterin and Cardiovascular Disease

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Abstract—Tetrahydrobiopterin (BH₄) is an essential cofactor for the aromatic amino acid hydroxylases, which are essential in the formation of neurotransmitters, and for nitric oxide synthase. It is presently used clinically to treat some forms of phenylketonuria (PKU) that can be ameliorated by BH₄ supplementation. Recent evidence supports potential cardiovascular benefits from BH₄ replacement for the treatment of hypertension, ischemia-reperfusion injury, and cardiac hypertrophy with chamber remodeling. Such disorders exhibit BH₄ depletion because of its oxidation and/or reduced synthesis, which can result in functional uncoupling of nitric oxide synthase (NOS). Uncoupled NOS generates more oxygen free radicals and less nitric oxide, shifting the nitroso–redox balance and having adverse consequences on the cardiovascular system. While previously difficult to use as a treatment because of chemical instability and cost, newer methods to synthesize stable BH₄ suggest its novel potential as a therapeutic agent. This review discusses the biochemistry, physiology, and evolving therapeutic potential of BH₄ for cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2006;26:2439-2444.)

Key Words: tetrahydrobiopterin ▪ nitric oxide synthase ▪ atherosclerosis ▪ inflammation

In 1963, a naturally occurring coenzyme for phenylalanine hydroxylase (PAH) was discovered to be the unconjugated pterin 5,6,7,8-tetrahydrobiopterin (BH₄).¹ BH₄ was subsequently found to be an essential cofactor for several other aromatic amino acid hydroxylases (tyrosine² and tryptophane³) involved with neurotransmitter biosynthesis, glyceryl-ether mono-oxygenase, and nitric oxide synthase (NOS). To be functional, BH₄ must be in its fully reduced form, and depletion and/or BH₄ oxidation to BH₂ and BH₃ reduces its activity. For the cardiovascular system, the role of BH₃ in NOS activity is particularly relevant. Reduced BH₃ was first shown to contribute to vascular pathophysiology and hypertension, whereas more recent studies have found important roles in cardiac hypertrophy and remodeling, and ischemia/reperfusion physiology. Development of genetic mouse models that modulate BH₄ synthesis have greatly advanced understanding of its role to normal NOS and vascular function. Here we briefly review the pharmacology, physiology, and therapeutic potential of BH₄.

BH₄ Biosynthesis

BH₄ is formed by either a de novo or salvage pathway (Figure 2). De novo synthesis starts with guanidine triphosphate cyclohydrolase (GTPCH) in a magnesium, zinc, and NADPH-dependent reaction, and continues through 2 intermediates (7,8-dihydronicotinamide riboside and 6-pyruvolyt-5,6,7,8-tetrahydropterin) mediated by 6-pyruvolyt-tetrahydropterin synthase and sepiapterin reductase.⁴ GTPCH is the rate limiting enzyme and is under negative feedback regulation by GTPCH feedback regulatory protein (GFRP) and BH₄ itself, and positive feedback by phenylalanine.⁵ GTPCH is also regulated at the expression level, being increased by calcium⁶ and 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibition,⁷ and by cytokines such as interferon-γ, tumor necrosis factor-α, and interleukin-1β. Cytokine activation may involve coordinated activation of NF-κB and the Jak2/Stat pathway,⁸ and can increase BH₄ levels by increasing GTPCH-1 expression,⁹–¹² reducing GFRP expression,⁵ and increasing PTPS expression.¹² BH₄ synthesis is also stimulated by insulin via a phosphatidylinositol-3-kinase–dependent activation of GTPCH-1,¹³ whereas insulin-resistant states impair this mechanism.¹⁴–¹⁷ Suppressors of GTPCH-1 activity include glucocorticoids¹⁸,¹⁹ and cyclic GMP, the latter generated by short-term treatment with NO donors or sodium nitroprusside²⁰ and high levels of 7,8 BH₃.²¹ These and other factors are summarized in the Table.

The salvage pathway generates BH₄ from oxidized forms via sepiapterin and sepiapterin reductase²² but cannot compensate for defects in biosynthesis or recycling.²²–²⁵ Two other enzymes are also involved with regenerating reduced BH₄ from oxidized forms, dihydrofolate reductase and dihydopterine reductase. Dihydrofolate reductase is mainly involved in folate metabolism and converts inactive 7,8-BH₂ back to BH₄, and plays an important role in the metabolism of exogenously administered BH₄. Recently, Chalupsky et al²⁶ demonstrated the role of dihydrofolate reductase in the regulation of BH₄ and NO bioavailability in the endothelium. Endothelial NAD(P)H oxidase-derived H₂O₂ downregulated dihydrofolate reductase expression in response to angiotensin II, resulting in BH₄ deficiency and uncoupling of eNOS.

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Dihydropteridine reductase catalyzes BH4 regeneration from qBH2 formed under oxidative stress.

**BH4 and NOS Function**

BH4 is an essential cofactor for all 3 NOS isoforms, and basal enzyme activity correlates with the amount of BH4 bound tightly to the protein. NOS is a homodimeric oxidoreductase containing iron protoporphyrin IX (heme), flavin adenine dinucleotide, flavin mononucleotide, and BH4. The flavin-containing reductase domain and a heme-containing oxygenase domain are connected by a regulatory calmodulin-binding domain. Binding of Ca2+ or calmodulin orients the other domains to allow NADPH-derived electrons generated in the reductase domain to flow to the oxygenase domain, ultimately resulting in the conversion of L-arginine to NO and L-citrulline. This occurs if BH4 is bound in the dimer interface, where it interacts with amino acid residues from both monomers to stabilize NOS dimerization and participate in arginine oxidation through the N-hydroxyl-L-arginine intermediate and the subsequent generation of NO.

The functional influence of BH4 on NOS occurs at several levels. BH4 can shift the NOS heme iron to a high spin state, increasing arginine binding and stabilizing the active dimeric form. NOS-bound BH4 may act as a redox-active cofactor via an unknown mechanism. BH4 increases substrate affinity of NOS and participates in the electron transfer process, being converted to BH3 radical during the NOS catalytic cycle and then restored to BH4. The best-characterized structural effect of BH4 is its stabilization of NOS dimers, particularly striking for inducible NOS (iNOS). Under certain conditions iNOS dimerization strictly depends on BH4. However, dimeric forms of all 3 isoforms can be obtained in the absence of BH4. Functional dimerization is thought to be a general requirement for normal NOS activity by biophysical alignment of the 2 oxidase domains linked to the opposing monomer reductase domain, thus this influence is thought to impact on enzyme function. Reduction of the ferric iron of endothelial NOS (eNOS) results in formation of an FeII-dioxygen complex, which would yield superoxide. However, BH4 donates an electron to form an iron-oxy species (FeII-O) that in turn participates in arginine hydroxylation and NO generation. BH4 also critical effects on the heme including the shift of the ferric iron spin state equilibrium toward a high spin state, altering the stability of the Fe(II)O2 complex and stabilizing 6-coordinate forms of NOS-ferrous-CO and ferrous-NO complexes. Lastly, BH4 has some modest anti-

**Figure 1.** Biochemical structure of 5,6,7,8-tetrahydrobiopterin.

**Figure 2.** BH4 biosynthesis and metabolism of BH4. BH4 can be formed by both a de novo pathway and a salvage pathway. The de novo pathway starts from guanidine triphosphate (GTP) and is regulated by the enzymes GTP cyclohydrolase (GTPCH), 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). The salvage pathway starts from sepiapterin (Sep) and is mediated by the enzymes SR and dihydrofolate reductase (DHFR).
When BH4 bioavailability declines, NOS undergoes multiple changes. The dimer architecture is altered possibly because of malrotation of the oxidase domains to yield “molecular” uncoupling, and the catalytic activity becomes “functionally” uncoupled. In the latter situation, the stoichiometric coupling between the reductase domain and L-arginine at the active site is lost, resulting in formation of superoxide and/or hydrogen peroxide. While increased generation of superoxide by uncoupled eNOS has become general accepted, it should be noted that these findings are all based on in vitro measurements and that this remains to be confirmed by in vivo real-time measurements.

The importance of GTPCH to BH4 levels and NOS activity have been elegantly explored both in vitro and in vivo. Cai et al. showed in endothelial cells that GTPCH gene transfer increases BH4 >10-fold over baseline, accompanied by a 25% increase in NOS3-dependent NO production. In the control cells, NOS3 was principally monomeric, whereas GTPCH gene transfer induced a 3-fold increase of NOS3 dimerization. Alp et al. reported on a transgenic mouse with human GTPCH over-expression targeted to endothelial cells under control of the mouse Tie2 promoter. Theses mice demonstrated a 3-fold increase in vascular BH4, reduced endothelial superoxide production, and preserved NO bioavailability comp with wild-type littermates in a streptozotocin model of diabetic vascular disease. These investigators also revealed enhanced NOS activity by gene transfer of GTPCH, and evidence of tight stoichiometry between BH4 and NOS enzyme levels using combined GTPCH-transfer of GTPCH, and evidence of tight stoichiometry between BH4 recycling, and by oxidation to inactive BH2. Oxy-

BH4 Bioavailability: Role of Oxidant Stress
BH4 bioavailability is potently influenced by oxidative stress, by decreasing expression of GTPCH, depleting NADPH, which is required for de novo synthesis, and is involved with BH4 recycling, and by oxidation to inactive BH2. Oxidized BH4 further augments superoxide anion synthesis from NOS3, increasing the synthesis of peroxynitrite (ONOO-), which is a potent oxidizer of BH4. Angiotensin II reduces BH4 by endothelial NAD(P)H oxidase-derived H2O2-dependent downregulation of DHFR, and an enzyme involved with reduction of BH4 back to BH2. This response is associated with a significant increase in endothelial O2- production and impaired endothelial function and homeostasis. BH4 oxidation is observed in a number of vascular diseases, and although it cannot act as an NO cofactor, it can exacerbate BH4 availability by competitive binding to NO.

BH4 Bioavailability and Inflammation/Atherosclerosis
Unlike hypertension, hypertrophy, and oxidant stress stimulation, other stimuli such as inflammatory cytokines have been found to increase BH4 biosynthesis, and this may play a role in atherosclerosis. For example, d’Uscio et al. detected elevated BH4 in atherosclerotic aortas of apolipoprotein E-deficient mice caused by increased expression and enzyme activity of GTPCH. Upregulation of GTPCH and BH4 synthesis has been linked to stimulation by certain inflammatory cytokines such as tumor necrosis factor-α, interferon-γ, and IL-1β, and may in this setting serve as a counter response to enhance NO production. In atherosclerotic vessels, total NOS activity is three times higher than in control arteries, caused mostly by increased expression and activity of iNOS. Additional support for upregulated BH4 synthesis in the setting of inflammation comes from studies showing increased neopterin, a side-product of GTPCH-1 activity. Intrinsically upregulated BH4 biosynthesis per se still does not rule out potential utility of exogenous BH4 supplementation, because uncoupling is often still observed.

BH4 Bioavailability: Role of Homocysteine, Folate, and Ascorbate
Increased vascular homocysteine is a potent risk factor for atherosclerosis and endothelial dysfunction, and of some of this effect maybe mediated by its influence on BH4. Homocysteine reduces intracellular BH4 accompanied by apparent inhibition of de novo synthesis, likely by blunting sepiapterin reductase. BH4 administration has beneficial effects on homocysteine-induced impairment of endothelial function, increased superoxide production, and impaired agonist-stimulated NO release.

Folic acid (folate) enhances the binding-affinity of BH4 to NOS by a pteridine-binding domain serving as a locus through which the active form 5-methyl tetrahydrofolate (5MTHF) facilitates the electron transfer by BH4 from the NOS reductase domain to the heme. Folate also enhances regeneration of BH4 from inactive BH2 by stimulating DHFR, and it chemically stabilizes BH4.

Ascorbic acid (Vitamin C) assists in BH4 stabilization primarily through antioxidant and other effects. Vitamin C also prevents formation of BH3 from the BH4 radical by facilitating the recycling to BH4. This may explain some of the benefits of ascorbate on endothelial function independent of superoxide scavenging.

BH4 Supplementation: Vascular Effects
Clinical data supporting vascular benefits of exogenous BH4 are largely based on acute or subacute studies examining endothelium-dependent vasodilation by agonists or flow stimuli. BH4 improves endothelial function in those who smoke, diabetic subjects, hypertensive subjects, patients with hypercholesterolemia, and those with coronary artery disease. More recently, Setoguchi et al. showed BH4 improves endothelial function in patients with systolic heart failure. Intracoronary administration of BH4 to patients with cardiovascular risks but without flow-limiting coronary artery stenoses (≤75%), enhanced endothelium-dependent vasodilation to acetylcholine. Some studies contrasting acute BH4 infusion versus more chronic treatment found beneficial effects on endothelial function only with the latter. This supports changes in NO synthase coupling rather than a less specific antioxidant effect likely explain the response. Preliminary results of chronic treatment with BH4 (400 mg twice daily, 4 weeks; Schirks Laboratories, Zurich, Switzerland) revealed...
benefits on endothelial dysfunction measured by acetylcholine response in forearm venous occlusion plethysmography in subjects with hypercholesterolemia.\textsuperscript{87}

**BH₄ and the Heart**

Reduced BH₄ likely represents an important cellular defect involved with both endothelial and myocyte dysfunction in hearts exposed to ischemia/reperfusion. BH₄ prevents ischemia/reperfusion cardiac dysfunction in vitro,\textsuperscript{88} attenuating the normally observed rise in malondialdehyde levels, a marker of lipid peroxidation, and improving endothelial-dependent vasorelaxation. These changes appear independent of the intrinsic radical scavenging action of BH₄.\textsuperscript{89} Takimoto et al\textsuperscript{97} recently revealed the importance of BH₄ depletion and consequent NOS3 uncoupling in mice subjected to sustained pressure overload. In this model, myocardial and myocyte hypertrophy, interstitial fibrosis, and eventual cardiac dilation and dysfunction were linked to increased oxidative stress generated by uncoupled NOS3. Mice lacking NOS3 and exposed to the same pressure load developed more compensated concentric hypertrophy with preserved function, whereas control animals displayed marked dilation and dysfunction after 9 weeks of pressure stress. BH₄ tissue levels declined >50\%, and BH₄ replacement therapy was able to reduce oxidative stress and inhibit cardiac dilation and depressed function in nonmutant controls. These data support potential benefits of BH₄ to the heart under conditions of stress, such as postinfarction remodeling, dilated myopathic remodeling, and hypertrophy.

**Clinical Pharmacology**

Exogenous BH₄ or its precursor sepiapterin first increases systemic BH₄ (Figure 2) that is subsequently reduced to BH₄⁰⁻⁰⁻² by DHFR. Oral sepiapterin hydrochloride, the synthetic form of 6R-BH₄, at 2 mg/kg causes a 3-fold increase in BH₄ after 3 hours, returning to baseline at 24 hours.\textsuperscript{92} Intracoronary infusion of 1 mg/min results in a rapid increase within 2 minutes raising coronary sinus BH₄ levels nearly 100-fold.\textsuperscript{93} These doses are high and unlikely to be used as chronic therapy. They may also have amplified nonspecific antioxidants effects\textsuperscript{94} of BH₄ independent of its role to NOS coupling and NO synthesis. Unfortunately, measurement of systemic (plasma) BH₄ has not been particularly useful for assessing local tissue levels and abnormal bioavailability. This has been shown to be true for coronary artery disease in which no significant differences were demonstrated compared with control population.\textsuperscript{95} Shinozaki et al\textsuperscript{96} demonstrated that patients with insulin resistance have lower ratios of plasma BH₄+/BH₄ and plasma BH₄/total bipterin, whereas BH₄ levels remained unchanged in patients with insulin resistance versus controls.

A potential disadvantage of BH₄ is that it might stimulate neuronal and inducible NOS activity, leading to excessive NO production and toxicity, particularly in inflammatory disorders. This remains conjectural. There are also some reports of elevated catecholamines with BH₄ induced by IL-2 treatment in cancer patients.\textsuperscript{97} although studies in PKU patients receiving BH₄ have not reported this effect.

To date, the major factor limiting clinical BH₄ use has been its pharmacological preparation. BH₄ tablets have been large with an acidic taste and unstable as BH₄ is hygroscopic and easily oxidized. Thus, the medication had to be maintained frozen at −20°C to maintain long-term stability. However, BH₄ has recently been developed in the form of a thermostable and photostable tablet, with stability at room temperature of nearly 2 years (BioMarin, San Francisco, Calif). This development has opened up broader potential use for cardiovascular indications.

**Conclusion**

BH₄ plays a central role to normal NOS3 activity, yet remarkably it appears vulnerable to depletion, thereby providing a key mechanism underlying a number of cardiovascular disorders. This also opens up intriguing potential for replacement therapy, and new developments in BH₄ pharmaceutical preparation should facilitate larger scale testing of such efficacy. Such studies are being initiated now and we can anticipate new information regarding the therapeutic potential for BH₄ treatment of hypertension, vascular dysfunction, and cardiac remodeling in the relatively near future.

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**Disclosures**

None.

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