

# Tetrahydrobiopterin and Cardiovascular Disease

An L. Moens, David A. Kass

**Abstract**—Tetrahydrobiopterin (BH<sub>4</sub>) 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 BH<sub>4</sub> supplementation. Recent evidence supports potential cardiovascular benefits from BH<sub>4</sub> replacement for the treatment of hypertension, ischemia-reperfusion injury, and cardiac hypertrophy with chamber remodeling. Such disorders exhibit BH<sub>4</sub> 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 BH<sub>4</sub> suggest its novel potential as a therapeutic agent. This review discusses the biochemistry, physiology, and evolving therapeutic potential of BH<sub>4</sub> 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 (BH<sub>4</sub>).<sup>1</sup> BH<sub>4</sub> was subsequently found to be an essential cofactor for several other aromatic amino acid hydroxylases (tyrosine<sup>2</sup> and tryptophane<sup>3</sup>) involved with neurotransmitter biosynthesis, glyceryl-ether mono-oxygenase, and nitric oxide synthase (NOS). To be functional, BH<sub>4</sub> must be in its fully reduced form, and depletion and/or BH<sub>4</sub> oxidation to BH<sub>3</sub> and BH<sub>2</sub> reduces its activity. For the cardiovascular system, the role of BH<sub>4</sub> in NOS activity is particularly relevant. Reduced BH<sub>4</sub> 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 BH<sub>4</sub> 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 BH<sub>4</sub>.

## BH<sub>4</sub> Biosynthesis

BH<sub>4</sub> is formed by either a de novo or salvage pathway (Figure 2). De novo synthesis starts with guanine triphosphate cyclohydrolase (GTPCH) in a magnesium, zinc, and NADPH-dependent reaction, and continues through 2 intermediates (7,8-dihydroneopterin triphosphate and 6-pyruvoyl-5,6,7,8-tetrahydropterin) mediated by 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase.<sup>4</sup> GTPCH is the rate limiting enzyme and is under negative feedback regulation by GTPCH feedback regulatory protein (GFRP) and BH<sub>4</sub> itself, and positive

feedback by phenylalanine.<sup>5</sup> GTPCH is also regulated at the expression level, being increased by calcium<sup>6</sup> and 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibition,<sup>7</sup> and by cytokines such as interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , and interleukin-1 $\beta$ . Cytokine activation may involve coordinated activation of NF- $\kappa$ B and the Jak2/Stat pathway,<sup>8</sup> and can increase BH<sub>4</sub> levels by increasing GTPCH-1 expression,<sup>9–12</sup> reducing GFRP expression,<sup>5</sup> and increasing PTPS expression.<sup>12</sup> BH<sub>4</sub> synthesis is also stimulated by insulin via a phosphatidylinositol-3-kinase-dependent activation of GTPCH-1,<sup>13</sup> whereas insulin-resistant states impair this mechanism.<sup>14–17</sup> Suppressors of GTPCH-1 activity include glucocorticoids<sup>18,19</sup> and cyclic GMP, the latter generated by short-term treatment with NO donors or sodium nitroprusside<sup>20</sup> and high levels of 7,8-BH<sub>2</sub>.<sup>21</sup> These and other factors are summarized in the Table.

The salvage pathway generates BH<sub>4</sub> from oxidized forms via sepiapterin and sepiapterin reductase<sup>22</sup> but cannot compensate for defects in biosynthesis or recycling.<sup>22–25</sup> Two other enzymes are also involved with regenerating reduced BH<sub>4</sub> from oxidized forms, dihydrofolate reductase and dihydropterine reductase. Dihydrofolate reductase is mainly involved in folate metabolism and converts inactive 7,8-BH<sub>2</sub> back to BH<sub>4</sub>, and plays an important role in the metabolism of exogenously administered BH<sub>4</sub>. Recently, Chalupsky et al<sup>26</sup> demonstrated the role of dihydrofolate reductase in the regulation of BH<sub>4</sub> and NO bioavailability in the endothelium. Endothelial NAD(P)H oxidase-derived H<sub>2</sub>O<sub>2</sub> downregulated dihydrofolate reductase expression in response to angiotensin II, resulting in BH<sub>4</sub> deficiency and uncoupling of eNOS.

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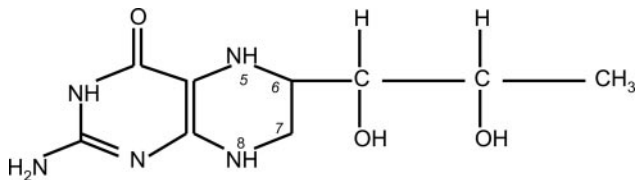
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**Figure 1.** Biochemical structure of 5,6,7,8-tetrahydrobiopterin.

Dihydropteridine reductase catalyzes  $\text{BH}_4$  regeneration from qBH2 formed under oxidative stress.

### $\text{BH}_4$ and NOS Function

$\text{BH}_4$  is an essential cofactor for all 3 NOS isoforms,<sup>21,27,28</sup> and basal enzyme activity correlates with the amount of  $\text{BH}_4$  bound tightly to the protein. NOS is a homodimeric oxidoreductase containing iron protoporphyrin IX (heme), flavin adenine dinucleotide, flavin mononucleotide, and  $\text{BH}_4$ .<sup>29,30</sup> The flavin-containing reductase domain and a heme-containing oxygenase domain are connected by a regulatory calmodulin-binding domain. Binding of  $\text{Ca}^{2+}$ /calmodulin orients the other domains to allow NADPH-derived electrons generated in the reductase domain to flow to the oxygenase domain,<sup>31</sup> ultimately resulting in the conversion of L-arginine to NO and L-citrulline. This occurs if  $\text{BH}_4$  is bound<sup>32,33</sup> 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  $\text{BH}_4$  on NOS occurs at several levels.  $\text{BH}_4$  can shift the NOS heme iron to a high spin state, increasing arginine binding and stabilizing the active dimeric form.<sup>34–36</sup> NOS-bound  $\text{BH}_4$  may act as a redox-active cofactor via an unknown mechanism.<sup>34</sup>  $\text{BH}_4$  increases substrate affinity of NOS<sup>21,35,37</sup> and participates in the electron transfer process, being converted to  $\text{BH}_3\cdot$  radical during the NOS catalytic cycle and then restored to  $\text{BH}_4$ . The best-characterized structural effect of  $\text{BH}_4$  is its stabilization of NOS dimers, particularly striking for

### Influencing Factors of GTPCH

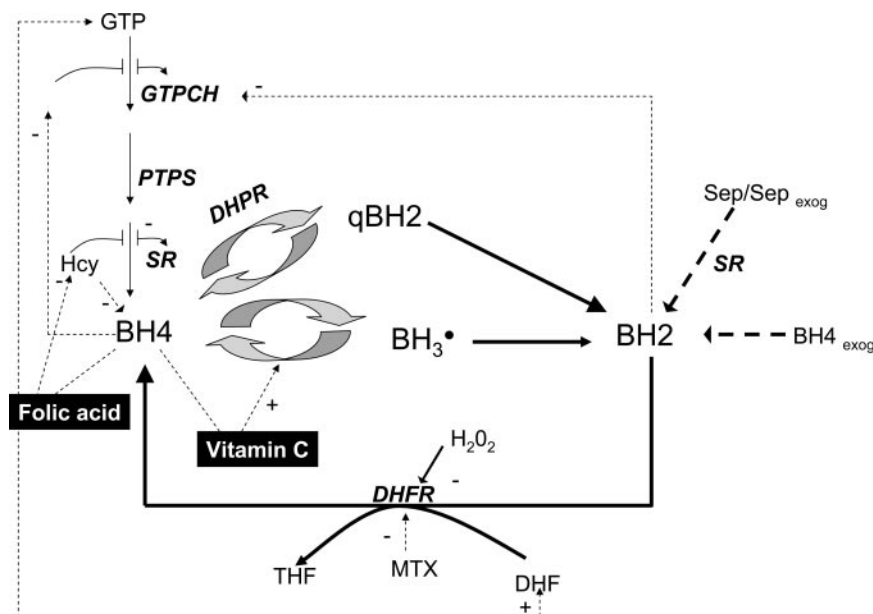
#### Inhibiting factors

NO donors/cyclic GMP  
IL-4/IL-10/TGF- $\beta$   
Melatonin  
Glucocorticoids  
2,4-diamino-6-hydroxypyrimidine

#### Stimulating factors

TNF- $\alpha$ /Interferon- $\gamma$ /IL-1 $\beta$   
Insulin  
Statins  
Ca influx  
Follicle stimulating hormone  
Epidermal growth factor  
Nerve growth factor  
Platelet-derived growth factor  
Vasoactive intestinal peptide

inducible NOS (iNOS).<sup>38</sup> Under certain conditions iNOS dimerization strictly depends on  $\text{BH}_4$ . However, dimeric forms of all 3 isoforms can be obtained in the absence of  $\text{BH}_4$ .<sup>39,40</sup> 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,  $\text{BH}_4$  donates an electron to form an iron-oxy species (FeII-O) that in turn participates in arginine hydroxylation and NO generation.  $\text{BH}_4$  also critical effects on the heme including the shift of the ferric iron spin state equilibrium toward a high spin state,<sup>41–43</sup> altering the stability of the Fe(II)O<sub>2</sub> complex<sup>44</sup> and stabilizing 6-coordinate forms of NOS-ferrous-CO and ferrous-NO complexes.<sup>40,45</sup> Lastly,  $\text{BH}_4$  has some modest anti-



**Figure 2.**  $\text{BH}_4$  biosynthesis and metabolism of  $\text{BH}_4$ .  $\text{BH}_4$  can be formed by both a de novo pathway and a salvage pathway. The de novo pathway starts from guanine triphosphate (GTP) and is regulated by the enzymes GTP cyclohydrolase (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). The salvage pathway starts from sepiapterin (Sep) and is mediated by the enzymes SR and dihydrofolate reductase (DHFR).

oxidant effects and can scavenge NOS derived reactive nitrogen and oxygen species.<sup>37,46</sup>

When BH<sub>4</sub> bioavailability declines, NOS undergoes multiple changes. The dimer architecture is altered possibly because of malrotation of the oxidase domains to yield “molecular” uncoupling,<sup>47,48</sup> 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 BH<sub>4</sub> levels and NOS activity have been elegantly explored both in vitro and in vivo. Cai et al<sup>49</sup> showed in endothelial cells that GTPCH gene transfer increases BH<sub>4</sub> >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 overexpression targeted to endothelial cells under control of the mouse *Tie2* promoter.<sup>48</sup> These mice demonstrated a 3-fold increase in vascular BH<sub>4</sub>, 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 BH<sub>4</sub> and NOS enzyme levels using combined GTPCH-transgenic and NOS3 knockout models.<sup>50</sup> A hph-1 mouse<sup>51</sup> has decreased hepatic GTPCH activity and defective BH<sub>4</sub> biosynthesis. These mice display pulmonary hypertension with right heart hypertrophy, and enhanced sensitivity to chronic hypoxia.<sup>52</sup>

### BH<sub>4</sub> Bioavailability: Role of Oxidant Stress

BH<sub>4</sub> bioavailability is potentially influenced by oxidative stress, by decreasing expression of GTPCH,<sup>53</sup> depleting NADPH, which is required for de novo synthesis<sup>54</sup> and is involved with BH<sub>4</sub> recycling,<sup>55</sup> and by oxidation to inactive BH<sub>2</sub>.<sup>56,57</sup> Oxidized BH<sub>4</sub> further augments superoxide anion synthesis from NOS3, increasing the synthesis of peroxynitrite (ONOO<sup>-</sup>), which is a potent oxidizer of BH<sub>4</sub>.<sup>58,59</sup> Angiotensin II reduces BH<sub>4</sub> by endothelial NAD(P)H oxidase-derived H<sub>2</sub>O<sub>2</sub>-dependent downregulation of DHFR,<sup>26</sup> an enzyme involved with reduction of BH<sub>2</sub> back to BH<sub>4</sub>. This response is associated with a significant increase in endothelial O<sub>2</sub><sup>-</sup> production<sup>60,61</sup> and impaired endothelial function and homeostasis. BH<sub>4</sub> oxidation is observed in a number of vascular diseases,<sup>48,62</sup> and although it cannot act as an NO cofactor, it can exacerbate BH<sub>4</sub> availability by competitive binding to NOS.<sup>63</sup>

### BH<sub>4</sub> Bioavailability and Inflammation/Atherosclerosis

Unlike hypertension, hypertrophy, and oxidant stress stimulation, other stimuli such as inflammatory cytokines have been found to increase BH<sub>4</sub> biosynthesis, and this may play a role in atherosclerosis. For example, d'Uscio et al<sup>64</sup> detected elevated BH<sub>4</sub> in atherosclerotic aortas of apolipoprotein

E-deficient mice caused by increased expression and enzyme activity of GTPCH. Upregulation of GTPCH and BH<sub>4</sub> synthesis has been linked to stimulation by certain inflammatory cytokines<sup>8,10,65–68</sup> such as tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , and IL-1 $\beta$ , and may in this setting serve as a counter response to enhance NO production.<sup>64</sup> In atherosclerotic vessels, total NOS activity is three times higher than in control arteries,<sup>69</sup> caused mostly by increased expression and activity of iNOS.<sup>68</sup> Additional support for upregulated BH<sub>4</sub> synthesis in the setting of inflammation comes from studies showing increased neopterin, a side-product of GTPCH-1 activity.<sup>70,71</sup> Intrinsic upregulation of BH<sub>4</sub> biosynthesis per se still does not rule out potential utility of exogenous BH<sub>4</sub> supplementation, because uncoupling is often still observed.<sup>71</sup>

### BH<sub>4</sub> Bioavailability: Role of Homocysteine, Folate, and Ascorbate

Increased vascular homocysteine is a potent risk factor for atherosclerosis and endothelial dysfunction, and some of this effect maybe mediated by its influence on BH<sub>4</sub>. Homocysteine reduces intracellular BH<sub>4</sub> accompanied by apparent inhibition of de novo synthesis<sup>72</sup> likely by blunting sepiapterin reductase. BH<sub>4</sub> administration has beneficial effects on homocysteine-induced impairment of endothelial function, increased superoxide production, and impaired agonist-stimulated NO release.<sup>73</sup>

Folic acid (folate) enhances the binding-affinity of BH<sub>4</sub> 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 BH<sub>4</sub> from the NOS reductase domain to the heme.<sup>74</sup> Folate also enhances regeneration of BH<sub>4</sub> from inactive BH<sub>2</sub><sup>75</sup> by stimulating DHFR, and it chemically stabilizes BH<sub>4</sub>.

Ascorbic acid (Vitamin C) assists in BH<sub>4</sub> stabilization primarily through antioxidant and other effects.<sup>76,77</sup> Vitamin C also prevents formation of BH<sub>2</sub> from the BH<sub>3</sub><sup>-</sup> radical by facilitating the recycling to BH<sub>4</sub>.<sup>76</sup> This may explain some of the benefits of ascorbate on endothelial function independent of superoxide scavenging.<sup>78</sup>

### BH<sub>4</sub> Supplementation: Vascular Effects

Clinical data supporting vascular benefits of exogenous BH<sub>4</sub> are largely based on acute or subacute studies examining endothelium-dependent vasodilation by agonists or flow stimuli. BH<sub>4</sub> improves endothelial function in those who smoke,<sup>79</sup> diabetic subjects,<sup>80</sup> hypertensive subjects,<sup>81</sup> patients with hypercholesterolemia,<sup>82</sup> and those with coronary artery disease.<sup>83,84</sup> More recently, Setoguchi et al<sup>85</sup> showed BH<sub>4</sub> improves endothelial function in patients with systolic heart failure. Intracoronary administration of BH<sub>4</sub> to patients with cardiovascular risks but without flow-limiting coronary artery stenoses (<75%), enhanced endothelial-dependent vasodilation to acetylcholine.<sup>83</sup> Some studies contrasting acute BH<sub>4</sub> infusion versus more chronic treatment found beneficial effects on endothelial function only with the latter.<sup>86</sup> This supports changes in NOS3 coupling rather than a less specific antioxidant effect likely explain the response. Preliminary results of chronic treatment with BH<sub>4</sub> (400 mg twice daily, 4 weeks; Schircks Laboratories, Zurich, Switzerland) revealed

benefits on endothelial dysfunction measured by acetylcholine response in forearm venous occlusion plethysmography in subjects with hypercholesterolemia.<sup>87</sup>

### BH<sub>4</sub> and the Heart

Reduced BH<sub>4</sub> likely represents an important cellular defect involved with both endothelial and myocyte dysfunction in hearts exposed to ischemia/reperfusion. BH<sub>4</sub> prevents ischemia/reperfusion cardiac dysfunction in vitro,<sup>88</sup> 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 BH<sub>4</sub>.<sup>89</sup> Takimoto et al<sup>47</sup> recently revealed the importance of BH<sub>4</sub> 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 oxidant 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. BH<sub>4</sub> tissue levels declined >50%, and BH<sub>4</sub> replacement therapy was able to reduce oxidative stress and inhibit cardiac dilation and depressed function in nonmutant controls. These data support potential benefits of BH<sub>4</sub> to the heart under conditions of stress, such as postinfarction remodeling, dilated myopathic remodeling, and hypertrophy.

### Clinical Pharmacology

Exogenous BH<sub>4</sub> or its precursor sepiapterin first increases systemic BH<sub>2</sub> (Figure 2) that is subsequently reduced to BH<sub>4</sub><sup>90,91</sup> by DHFR. Oral sapropterin hydrochloride, the synthetic form of 6R-BH<sub>4</sub>, at 2 mg/kg causes a 3-fold increase in BH<sub>4</sub> after 3 hours, returning to baseline at 24 hours.<sup>92</sup> Intracoronary infusion of 1 mg/min results in a rapid increase within 2 minutes raising coronary sinus BH<sub>4</sub> levels nearly 100-fold.<sup>93</sup> These doses are high and unlikely to be used as chronic therapy. They may also have amplified nonspecific antioxidants effects<sup>94</sup> of BH<sub>4</sub> independent of its role to NOS coupling and NO synthesis. Unfortunately, measurement of systemic (plasma) BH<sub>4</sub> 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.<sup>95</sup> Shinozaki et al<sup>96</sup> demonstrated that patients with insulin resistance have lower ratios of plasma BH<sub>4</sub>:BH<sub>2</sub> and plasma BH<sub>4</sub>:total biopterin, whereas BH<sub>4</sub> levels remained unchanged in patients with insulin resistance versus controls.

A potential disadvantage of BH<sub>4</sub> 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 BH<sub>4</sub> induced by IL-2 treatment in cancer patients,<sup>97</sup> although studies in PKU patients receiving BH<sub>4</sub> have not reported this effect.

To date, the major factor limiting clinical BH<sub>4</sub> use has been its pharmacological preparation. BH<sub>4</sub> tablets have been large with an acidic taste and unstable as BH<sub>4</sub> is hygroscopic and easily oxidized. Thus, the medication had to be maintained frozen at -20°C to maintain long-term stability. However, BH<sub>4</sub> 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

BH<sub>4</sub> 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 BH<sub>4</sub> 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 BH<sub>4</sub> treatment of hypertension, vascular dysfunction, and cardiac remodeling in the relatively near future.

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### Disclosures

None.

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