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Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease

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Abstract

Nitric oxide, generated by the nitric oxide synthase (NOS) enzymes, plays pivotal roles in cardiovascular homeostasis and in the pathogenesis of cardiovascular disease. The NOS cofactor, tetrahydrobiopterin (BH4), is an important regulator of NOS function, since BH4 is required to maintain enzymatic coupling of L-arginine oxidation, to produce NO. Loss or oxidation of BH4 to 7,8-dihydrobiopterin (BH2) is associated with NOS uncoupling, resulting in the production of superoxide rather than NO. In addition to key roles in folate metabolism, dihydrofolate reductase (DHFR) can ‘recycle’ BH2, and thus regenerate BH4 [1,2]. It is therefore likely that net BH4 cellular bioavailability reflects the balance between *de novo* BH4 synthesis, loss of BH4 by oxidation to BH2, and the regeneration of BH4 by DHFR. Recent studies have implicated BH4 recycling in the direct regulation of eNOS uncoupling, showing that inhibition of BH4 recycling using DHFR-specific siRNA and methotrexate treatment leads to eNOS uncoupling in endothelial cells and the hph-1 mouse model of BH4 deficiency, even in the absence of oxidative stress. These studies indicate that not only BH4 level, but the recycling pathways regulating BH4 bioavailability represent potential therapeutic targets and will be discussed in this review.

Keywords

Tetrahydrobiopterin recycling; Nitric oxide; eNOS uncoupling; Dihydrofolate reductase; Superoxide

Tetrahydrobiopterin

(6R) 5,6,7,8-tetrahydrobiopterin (BH4) is a pteridine, defined by its unique heterocyclic ring structure (Fig. 1). The biological synthesis of pteridines was first discovered in 1889, when Sir Fredrick Gowland Hopkins isolated the yellow pigments from the wings of English butterflies [3]. The identity of these compounds was not established until the 1940s, when three compounds were isolated and shown to share a novel pyrimidine ring system. The name pterin was given to these compounds after the Greek name *Ptera*, meaning wing, for the source from which these molecules were isolated.

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Biopterin is defined as the pteridine analogue in which the heterocyclic ring is substituted with amino, carbonyl oxygen and 1,2-dihydroxypropyl at the 2, 4 and 6 positions, respectively. The redox state of this substituted ring has profound effects on chemical, spectral and biological activity, existing as fully oxidized (biopterin), partially reduced (BH2), or fully reduced (BH4) forms.

Kaufman and coworkers were the first to demonstrate a cofactor function for BH4 in mammalian biology. BH4 was shown to be an essential cofactor for the metabolism of phenylalanine to tyrosine by phenylalanine hydroxylase in the liver [4]. BH4 was later found to also play a similar cofactor role for the two other mammalian aromatic amino acid hydroxylases (AAAH), tyrosine hydroxylase and tryptophan hydroxylase [5,6]. These findings implicated BH4 in the biosynthesis of epinephrine, norepinephrine, dopamine and 5-HT. The role of BH4 in AAAH catalysis is well established to include redox-based activation of molecular oxygen and allosteric stabilization of the enzyme. The enzymatic reactions of the AAAHs also have a strict requirement for oxygen and iron. During catalysis, BH4 is oxidized to the intermediates, BH4-4a-carbinolamine [7] and quinonoid-BH2 (q-BH2) [8]. In AAAHs, one mole of BH4 is able to support a single catalytic turnover; continued catalysis is dependent upon the NADPH-mediated regeneration of the BH4 cofactor by the enzyme dihydropteridine reductase (DHPR).

The observation that BH4 synthesis is dramatically induced in mammalian cells by cytokines puzzled researchers for many years, as there was no known enzyme that was both inducible and BH4-dependent. This led to the discovery that BH4 is an essential cofactor for iNOS activity [9,10]. It was naturally assumed that the function of BH4 in NOSs was identical to that shown for the AAAHs. However, it is now appreciated that while BH4 is essential for activity of all NOS isoforms, it is not utilized in the same way as for the AAAHs.

In addition to serving as a cofactor for AAAH and NOS catalysis, BH4 has been reported to have growth factor and proliferative actions on hematopoietic cells [11,12]. Another function may be scavenging O_2^- as shown in experiments with both xanthine/xanthine oxidase and rat macrophage/phorbol myristate acetate O_2^- generating systems [13]. Indirect support for this role came from studies where endothelial cell death induced by the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) could be prevented by increasing intracellular BH4 levels [14].

***De novo* synthesis by GTP cyclohydrolase I**

BH4 is formed *de novo* from GTP, via a sequence of enzymatic steps carried out by GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) [15]. In the first, and rate-limiting step, guanosine 5' triphosphate (GTP) is reduced to 7,8-dihydroneopterin 3' triphosphate (DNTP) by GTPCH [16]. GTPCH-catalyzed formation of DNTP is a common initial step in the biosynthesis of unconjugated pterins, folates and riboflavin, but not molybdopterin (a cofactor of sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase in man). GTPCH is the rate-limiting enzyme in BH4

biosynthesis and its activity is regulated at the transcriptional, translational and post-translational levels (Fig. 2) [17].

GTPCH protein levels have been shown to be induced by H₂O₂ in oxidative stress [18] and bacterial LPS elicits a 2–3-fold increase in GTPCH activity in a variety of rat tissues that constitutively express GTPCH, including cerebellum, liver, spleen and the adrenal gland. Macrophages, dermal fibroblasts and tumor cell lines all demonstrate a profound increase in GTPCH activity after treatment with IFN γ and TNF α ; LPS and IFN γ has also been observed to increase activity and act in a synergistic manner [19,20]. BH₄ synthesis by GTPCH is subject to feedback inhibition by BH₄ and other reduced pterins via a mechanism that requires a regulatory protein known as GTPCH feedback regulatory protein (GFRP) [21]. This inhibition of GTPCH by BH₄/GFRP can be reversed by high levels of phenylalanine [21,22].

The conversion of DNTP to 6-pyruvoyl tetrahydropterin (PTP) is catalyzed by the zinc-dependent metalloprotein, PTPS. Although GTPCH is rate-limiting to BH₄ synthesis in most cells, PTPS has been suggested to be rate-limiting in some, most notably human hepatocytes. PTPS may become rate-limiting in other tissues and cells, following stimulation with cytokines and other immunological stimuli that induce BH₄ synthesis by upregulation of GTPCH expression [12]. The final steps in the biosynthesis of BH₄ are two successive propyl side-chain reduction reactions, catalyzed by SR [12].

Nitric oxide synthase

The role of BH₄ in NOS catalysis

Following the discovery of BH₄ as an essential cofactor in NOS catalysis, it became clear that BH₄ adopted a different role in NOS catalysis when it was discovered that NOS contained a cytochrome P450-type heme, a moiety able to support the activation of oxygen without the need for a pterin cofactor. The main evidence against a direct redox role for BH₄ came from the observation that citrulline formation is not stoichiometric with BH₄ consumption and the fact that BH₄ had little effect on the initial rate of NOS catalysis [23]. Comparison of eNOS [24,25], iNOS [26] and nNOS [27] oxygenase domains with crystal structures of the AAHs has revealed fundamental structural differences between the BH₄ binding sites. In NOSs, BH₄ functions in part as an allosteric modulator of arginine binding. Binding of BH₄ to NOS elicits a conformational change that increases the affinity for binding of arginine-based ligands [28,29]. Support for an allosteric role of BH₄ was confirmed by spectrophotometric and electron paramagnetic resonance (EPR) studies, which show that BH₄ binding converts the heme iron from a low-spin to a high-spin state [30]. Another possible role of BH₄ was thought to be in dimer assembly [31]. Although proven not to be essential for dimer assembly [32], BH₄-binding does play a role in dimer stabilization [33].

The above mentioned allosteric effects do not fully explain the essential role of BH₄ in NO synthesis and electron donation from BH₄ to the heme iron remains almost certain. Indeed, only fully reduced pterins such as tetrahydrobiopterin have ever been shown to support catalysis [34–36]. Moreover, tetrahydrobiopterins with modifications that would cause them

to be redox silent are unable to catalyze NO synthesis [37,38]. Spectral and EPR studies have revealed the presence of a trihydrobiopterin ($\cdot\text{BH}_3$) radical intermediate, which strongly supports a redox role for BH4 in NOS catalysis and may explain the ability of eNOS-bound BH4 to limit the release of oxygen as O_2^- [39].

Tetrahydrobiopterin bioavailability and eNOS uncoupling

Many studies have focused on the potential role of BH4 oxidation to BH2 and other oxidized biopterin species in reducing BH4 bioavailability for eNOS (Fig. 3). Although superoxide can indeed react directly with BH4, the rate constant of this reaction ($3.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) [64] is many orders of magnitude lower than that for NO with superoxide ($6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) [65]. A more likely mechanism for BH4 oxidation is the interaction with peroxynitrite (generated from the interaction between NO and superoxide). Experiments *in vitro* [66] and *ex vivo* [67] indicate that peroxynitrite can oxidize BH4 within minutes at physiologically relevant concentrations. EPR (electron paramagnetic resonance) spectroscopy experiments have demonstrated that peroxynitrite oxidizes BH4 to the (non-protonated) BH3 (trihydrobiopterin) radical, and then to BH2, with a rate constant estimated to be $6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, several-fold higher than reactions between peroxynitrite and ascorbate, glutathione or thiol groups [68]. 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 [40].

Depletion of BH4 in oxidatively stressed endothelial cells can result in eNOS 'uncoupling', where electron transfer from NOS flavins becomes 'uncoupled' from L-arginine oxidation, the ferrous-dioxygen complex dissociates, and superoxide is produced from the oxygenase domain [27,41–43]. Indeed, it is now believed that the intracellular BH4:BH2 ratio, rather than absolute concentrations of BH4, is the key determinant of eNOS uncoupling [27,40,44,45].

Superoxide generation by eNOS has been implicated in a variety of experimental and clinical vascular disease states, including diabetes [46,47], cigarette smoking [48], hypertension [49] and atherosclerosis [50]. In the apolipoprotein E knock-out mouse model of hypercholesterolemia, eNOS mRNA and protein levels remain unchanged or may even be increased compared with wild type litter mate controls. In these animals transgenic overexpression of eNOS paradoxically increases vascular superoxide production because of enzymatic uncoupling, which is reversed when mice are crossed with animals overexpressing GTPCH [51,52].

Tetrahydrobiopterin recycling

Depending on its cofactor function for the aromatic amino acid hydroxylases versus the NOS enzymes, BH4 turnover products and regeneration reactions differ. In the aromatic amino acid hydroxylase reactions, BH4 is oxidized to tetrahydrobiopterin-4a-carbinolamine; BH4 is subsequently regenerated in a two-step process. Actions of pterin-4a-carbinolamine dehydratase (EC 4.2.1.96; PCD) lead to the production of the quinonoid dihydrobiopterin intermediate, which is subsequently reduced by dihydropteridine reductase (EC 1.6.99.7; DHPR). Both enzymes are expressed in mammalian liver [53], kidney, and brain [54], and

mutations in the PCD and DHPR genes are associated with clinical systemic BH4 deficiency and hyperphenylalaninemia.

As a cofactor for NOS, BH4 is not oxidized to tetrahydrobiopterin-4a-carbinolamine but, during the transfer of electrons to the ferrous–dioxygen complex in the NOS active site, forms the protonated trihydrobiopterin cation radical (BH3.H⁺), which is subsequently reduced in the next catalytic cycle by electron transfer from eNOS flavins [55,56]. Since the BH3.H⁺ radical returns to the BH4 state after each electron transfer step, continuous BH4 regeneration by PCD and DHPR is not a requirement for eNOS activity in endothelial cells.

Dihydrofolate reductase

In addition to key roles in folate metabolism, dihydrofolate reductase (DHFR; E.C. 1.5.1.3) can reduce BH2, regenerating BH4 [1,2]. Thus, it is likely that net BH4 cellular bioavailability reflects the balance between *de novo* BH4 synthesis, loss of BH4 by oxidation to BH2, and the regeneration of BH4 by DHFR. In human liver extracts, DHFR has been shown to reduce BH2 to BH4 as part of the salvage pathway for biopterin synthesis [57]. A BH2 reductase activity of DHFR was first observed in cell and tissue extracts – in a Chinese hamster ovary cell mutant lacking dihydrofolate reductase (DUKX-BII), endogenous formation of BH4 proceeds normally, but unlike the parent cells that express DHFR, extracts do not convert sepiapterin or BH2–BH4 [58]. These studies implicate the biopterin recycling pathway in the regulation of steady-state BH4 levels. Recent data from our group and others suggest that this BH2 reductase activity of DHFR is crucial in determining cellular BH4 homeostasis, NO bioavailability, and ultimately eNOS coupling. Moreover, recent studies have investigated the recycling function of DHFR in cultured endothelial cells. Exposure to angiotensin II down-regulated DHFR expression, decreased BH4 levels, and increased eNOS uncoupling, that was restored by overexpression of DHFR [59]. Pharmacological inhibition of DHFR activity by methotrexate, or genetic knockdown of DHFR by RNA interference, reduced intracellular BH4 and increased BH2 levels resulting in enzymatic uncoupling of eNOS in endothelial cells. In cells expressing eNOS with low biopterin levels, DHFR inhibition or knockdown further diminished the BH4:BH2 ratio and exacerbated eNOS uncoupling [60,61].

Despite these insights from studies in cultured endothelial cells, the extent to which DHFR regulates intracellular BH4 levels and eNOS uncoupling *in vivo* remains unknown. We previously demonstrated that DHFR activity is critical in regulating BH4:BH2 ratio and hence eNOS coupling *in vitro*, particularly at low biopterin levels. Interestingly, some previous studies have shown that DHFR levels or activity are diminished in experimental models of cardiovascular disease states, suggesting that insufficient recycling of BH2–BH4 by DHFR is at least in part responsible for the reduced BH4 levels and the accumulation of BH2, leading to eNOS uncoupling. For example, DHFR protein levels are significantly decreased in streptozotocin-induced diabetic mice and diabetes-induced impairment of cardiac myocyte function is exacerbated following treatment of the mice with the DHFR inhibitor, MTX [62]. Furthermore, reduced DHFR activity in adult cardiac myocytes underlies their limited capacity to synthesize BH4 after cytokine stimulation following treatment of rat cardiac allograft recipients with sepiapterin [63]. In support of these

findings, upregulation of BH4 recycling enzymes is sufficient to restore BH4 levels, and effectively 'recouple' eNOS within the aorta of STZ-induced diabetic mice [64]. Insufficient DHFR activity might also explain impaired vasorelaxation in atherosclerotic vessels from hypercholesterolemic rabbits, despite exposure to sepiapterin, that increases biopterin levels through BH2, requiring DHFR to increase BH4 [65].

Furthermore, we compared the effect of DHFR inhibition by MTX treatment on BH4 levels and eNOS coupling *in vivo*, using mice with either BH4-deficiency (hph-1 mice) or elevated BH4 levels due to GTPCH-1 overexpression (GCH-Tg mice), in comparison with wild type mice. We report that DHFR activity is required to maintain eNOS coupling *in vivo* and that this effect is more prominent in conditions of overall BH4 deficiency, whereas BH4 augmentation protects against these deleterious effects of DHFR inhibition by MTX. Moreover, we have demonstrated that MTX treatment in patients with inflammatory disease leads to a striking accumulation of BH2 in plasma, further illustrating the requirement for DHFR activity in the maintenance of BH4 homeostasis in humans *in vivo* [66].

Sepiapterin reductase

In contrast to other BH4 metabolic enzymes, sepiapterin reductase (SR) is involved in both the synthetic and salvage pathways of BH4 synthesis. SR was first discovered in chicken and rat liver by Matsubara et al. and purification of SR from rat erythrocytes showed it to consist of two 28 kDa sub units [67]. Exogenous sepiapterin can be reduced in all cells by sepiapterin reductase to 7,8-dihydrobiopterin (BH2) and further by DHFR to form BH4, known as the 'Salvage Pathway' that has been exploited by many investigators as an approach to increase BH4 levels by pharmacological supplementation of sepiapterin. The designation of this alternate route as a 'salvage pathway' is by analogy with the conservation of intact purines by their conversion to nucleotides by a route separate from *de novo* synthesis. This salvage pathway is mediated by both SR and DHFR and is therefore sensitive to methotrexate. During the hydroxylation of tyrosine, tryptophan, and phenylalanine, BH4 is converted to Q-BH2 which can be reduced to BH4 by DHPR. Q-BH2 is rearranged rapidly to the more stable 7,8-BH2 isomer which does not serve as a substrate for DHPR [58]. Little is known about the expression and regulation of mammalian SR. The only evidence for a regulatory role of SR in NO synthesis comes from a study by Gao and co-workers where knockdown of SR in endothelial cells was associated with dramatic decreases in both BH4 content and NO levels [68]. Evidence for the sepiapterin synthesis pathway in humans comes from a recent study of rare patients with sepiapterin reductase deficiency, in which sepiapterin levels were elevated in cerebrospinal fluid, which suggests endogenous production of sepiapterin [69].

Dihydropteridine reductase

DHPR, along with PCD is critical for the regeneration of BH4 which is required for activity of the AAHs. DHPR concentrations compared with those of the AAHs, is relatively high. The enzyme is almost ubiquitously expressed and its presence in tissues lacking enzymes of the AAHs suggests that DHPR may be involved in other metabolic processes. DHPR may preserve tetrahydrofolate levels in brain where the concentration of DHFR is comparatively

low [70]. The presence of DHPR in lung and liver may be protective in BH4 homeostasis, catalysing the reduction of q-BH2 to BH4 to maintain NO production by NOS. Deficiency in DHPR is an autosomal recessive condition and has been shown to cause hyperphenylalaninemia due to BH4 deficiency.

Is biopterin transport dependent on BH4 recycling?

The high concentrations of pharmacological BH4 that are required to generate a biological effect in many studies suggest that biopterins do not enter cells merely by passive diffusion. The precise mechanisms for the transport of BH4 into and out of cells have not yet been fully elucidated but recent studies suggest a recycling-dependent regulation for BH4 transport.

Hasegawa and coworkers compared the uptake of 6R-BH4 and sepiapterin RBL2H3 cells (rat basophilic leukemia cells). Sepiapterin was found to elevate the intracellular concentration of BH4 much more effectively than exposure to 6R-BH4 [71]. In this experimental setting, BH4 taken up by cells from the medium was immediately oxidized to BH2 and released without being incorporated into the intracellular BH4 pool. These data suggest that regulation of BH4 bioavailability, at least in RBL2H3 cells, requires BH4 oxidation to BH2 and then controlled reduction back to BH4 in the cell [72–74]. The small increase in cellular BH4 observed after BH4 administration in cell cultures was demonstrated to be methotrexate-sensitive, suggesting that intracellular BH4 accumulation following BH4 exposure was dependent on DHFR-mediated reduction of BH2 [75].

Intracellular BH4 accumulation following sepiapterin administration is thought to be accomplished by at least three functional steps: (1) sepiapterin entry into cells through the plasma membrane; (2) a forward-driven in-out equilibrium of sepiapterin favoring BH4 accumulation by two successive and reversible enzyme reactions catalyzed by sepiapterin reductase and dihydrofolate reductase; and (3) BH4 deposition inside the cell due to the relatively low permeability of the cell membrane towards BH4 (with an uptake half-time of approximately 120 min) [76–78].

In vivo experiments where mice were treated with BH4, BH2 or sepiapterin (10 mg/kg), either orally or intraperitoneally, revealed that sepiapterin was able to increase tissue BH4 levels most efficiently. A comparable but smaller increase in tissue biopterin levels was observed after oral administration of equivalent doses of BH4 and BH2 [79,80]. The increased tissue biopterin levels were predominantly comprised of BH4, but analogous to the *in vitro* experiments, the increase in tissue BH4 was markedly inhibited by pretreatment with 10 mg/kg methotrexate before administration of either sepiapterin, BH2 or BH4. Indeed, in these conditions, the increase in tissue biopterin was almost exclusively comprised of BH2.

Supporting the hypothesis that the salvage pathway has a major function in regulating intracellular BH4 levels, the same investigators observed that despite administering the unnatural diastereomer 6*S*-BH4 to mice, a large proportion of the deposited BH4 (more than 95% was in the liver) was in the form of the 6*R*-diastereomer [81,33]. This particular finding provided very strong evidence that accumulating BH4 required the DHFR pathway in which

achiral 7,8-dihydrobiopterin formed from chiral 6S-BH4 was converted to chiral 6R-BH4 [82,83].

However, there may be multiple cell-specific mechanisms for BH4 uptake, since erythrocytes and the epithelial cell line CaCo-2 are reported to readily import BH4 directly from the extracellular compartment in a methotrexate-independent fashion [84]. This may be relevant for transport of orally-administered BH4 from the GI tract, and for the renal excretion of BH4.

Tetrahydrobiopterin recycling 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 and are outlined in Table 1. In clinical studies, pharmacological supplementation of BH4 improves endothelium-dependent relaxations and augments NO-mediated effects on forearm blood flow in smokers and those with diabetes and elevated cholesterol [85–88]. However, this may be due to nonspecific scavenging of superoxide by high dose BH4 treatment. 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, numerous studies have found that pharmacologic supplementation 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 [89,90]. Specifically, increasing BH4 biosynthesis in cultured endothelial cells, which are relatively BH4-deficient, restores eNOS activity and increases the proportion of eNOS protein present as the homodimeric form. Gene transfer of GTPCH in carotid arteries of DOCA-salt hypertensive rats restores BH4 levels and improves endothelial function [91] and when GTPCH is constitutively overexpressed specifically within endothelial cells in transgenic mice, tissue BH4 levels were increased and eNOS activity was restored [92]. In GTPCH transgenic mice rendered diabetic with streptozotocin, the loss of vascular BH4 was prevented, leading to reduced evidence of eNOS uncoupling and restored endothelial function. Finally, when GTPCH transgenic mice were crossed with ApoE KO mice, endothelial function was improved and atherosclerotic plaque progression was reduced [93]. Together, many 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.

Conclusions

Current research indicates that the maintenance of adequate BH4 levels within the endothelium is likely to be critical in regulating eNOS coupling and hence, the balance between the production of eNOS-derived NO and superoxide. As well as BH4 supplementation, a promising strategy may be to normalize BH4 levels in the endothelium by either reducing oxidative stress with pharmacological agents such as ascorbic acid, or as suggested by more recent investigations, to protect against the accumulation of oxidized forms of tetrahydrobiopterin, such as BH2 and biopterin by activation of the recycling

pathways of biopterin synthesis. Further understanding of pharmacological approaches to target BH₄ biosynthesis and regeneration may provide exciting new therapeutic opportunities in vascular diseases.

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Fig. 1.
Chemical structures of the essential NOS cofactor BH4 and increasingly more oxidized non-cofactor derivatives: q-BH2, 7,8-BH2 and biopterin.

Fig. 2.

BH₄ 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. The following steps are catalysed by the enzymes PTPS and SR. An alternative pathway for BH₄ synthesis has been documented whereby 6-pyruvoyl-5,6,7,8-tetrahydrobiopterin is converted into sepiapterin by an enzyme termed 'sepiapterin synthase'. Exogenous sepiapterin can be reduced in all cells by SR to BH₂, and further by DHFR to form BH₄, the so-called 'salvage pathway'. The principle oxidant species leading to BH₄ oxidation to BH₂

is peroxynitrite. As a cofactor for the aromatic amino acid hydroxylases in the liver and neurons, but not as a cofactor for NOS, BH₄ is converted into tetrahydrobiopterin-4a-carbinolamine, which is recycled to BH₄ by the actions of PCD and DHPR.

Fig. 3.

Schematic representation of the BH₄ recycling pathway and eNOS coupling. BH₄ is synthesised *de novo* from GTP via a series of reactions involving GTPCH, 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase (---). DHFR can regenerate BH₄ from BH₂ as part of the recycling pathway. Both BH₄ and BH₂ bind eNOS with equal affinity, altering the 'balance' between NO and superoxide production by eNOS: BH₄ bound eNOS produces NO while BH₂ bound eNOS promotes uncoupling and eNOS-derived superoxide rather than NO.

Table 1

Overview of current translational studies that demonstrate the potential of BH4 therapy in vascular disease.

Setting	Effect	Reference
Hypercholesterolemia		[88]
Hypercholesterolemia		[85]
Coronary artery disease		[50]
Chronic Smokers		[86]
Long-term smokers		[94]
Coronary risk factors	Prevents endothelial dysfunction	[95]
Angina		[96]
Chronic heart failure		[97]
Glucose challenge		[98]
Aging		[99]
Ischemia reperfusion		[100]
Aging	Improves flow mediated dilatation	[101]
Erectile dysfunction	Prevents erectile dysfunction	[102]
Atherosclerosis	Does not improve endothelial function	[103]
Hypertension	Augments endothelium-dependent vasodilatation	[104]
Hypertension	Prevents endothelial dysfunction and decreases arterial blood pressure	[105]
Hypercholesterolemia	Prevents endothelial dysfunction and decreases oxidative stress	[106]
Hypercholesterolemia	Improves dysfunction of coronary microcirculation	[107]
Type 2 diabetes	Increases insulin sensitivity but does not improve endothelial function	[108]
Type 2 diabetes	Prevents endothelial dysfunction	[87]