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Tetrahydrobiopterin, Superoxide and Vascular Dysfunction

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Abstract

(6R)-5,6,7,8-Tetrahydrobiopterin (BH₄) is an endogenously produced pterin that is found widely distributed in mammalian tissues. BH₄ works as a cofactor of aromatic amino acid hydroxylases and nitric oxide synthases. In the vasculature a deficit of BH₄ is implicated in the mechanisms of several diseases including atherosclerosis, hypertension, diabetic vascular disease, and vascular complications from cigarette smoking and environmental pollution. These ill-effects are connected to the ability of BH₄ to regulate reactive oxygen species levels in the endothelium. The possibility of using BH₄ as a therapeutic agent in cardiovascular medicine is becoming more compelling and many biochemical and physiological aspects involved in this application are currently under investigation. This review summarizes our current understanding of BH₄ reactivity and some aspects of cellular production and regulation.

Keywords

GTP cyclohydrolase I; dihydrobiopterin; neopterin; endothelial nitric oxide synthase

1. Introduction

It is clear that tetrahydrobiopterin is vital to human health in many ways and that research to elucidate its exact role is even more essential with new therapies arising. This year the first BH₄ formulation (Sapropterin, *Kuvan*) received FDA approval for use in patients afflicted with phenylketonuria (PKU) [1], a genetic condition causing accumulation of phenylalanine in the blood [2]. While this treatment will not be positive for all PKU patients, certainly it represents a tremendous benefit for those that until now only relied on a strict diet, of questionable taste, to control their disease. In a broader sense, this application opens the door to the use of BH₄ in the treatment of other human conditions where BH₄ supply is limiting. This step certainly requires a more clear understanding of the mechanisms regulating BH₄ in different organs and tissues, its pharmacology and its physiological effects.

Upon the demonstration that nitric oxide (NO) production from nitric oxide synthases is dependent on optimal BH₄ levels, we have witnessed a renewed interest in understanding BH₄ sources and reactivity. It is now recognized that tissue availability and distribution in different redox forms may explain its biological activity. Although these variables have been shown to affect iron-dependent enzymes such as phenylalanine and tyrosine hydroxylase, they only recently have been connected with NOS and NO synthesis. Some confounding

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characteristics that have delayed a full understanding of the role of BH₄ in the regulation of eNOS activity include a high turnover rate, low redox potential and reactivity with antioxidant molecules such as thiols and ascorbate [4–7]. Also, in the endothelium the differential activity of the enzymes assisting in the recycling of oxidized products of BH₄ (i.e. dihydrofolate reductase, dihydropteridine reductase) has limited our understanding as to what extent BH₄ may be considered a limiting pathophysiological factor in vascular disease.

There is now a large body of literature implicating BH₄ and its oxidized metabolites in the regulation of superoxide anion radical (O₂^{•−}) release in the endothelium. There is also strong evidence showing that this activity is linked to loss of NO. The shift in endothelial O₂^{•−} and NO levels is thought to significantly change endothelial redox state and create a chronic state of BH₄ deficiency. This way BH₄ could cause a sizeable state of oxidant stress in the endothelium conducive to cellular changes and loss of function. Within this context, this review focuses on the BH₄ biochemistry, and its role in reactive oxygen species formation and current evidence on the potential implications of this agent in vascular medicine.

2. Basic Redox Biochemistry of BH₄

A significant feature of the biochemical systems where BH₄ is found to play a role is the existence of redox active metals and oxygen. Formation of reactive oxygen species (superoxide radical anion and hydrogen peroxide) in those systems is generally seen as an avoidable product of BH₄ reactions. Available evidence indicates that this may not be always the case however. Studies on the redox chemistry of BH₄ have been undertaken by many research groups over the years. This section presents accumulated evidence and current discussion relative to the involvement of BH₄ in the production of reactive oxygen and reactive nitrogen species under physiological conditions.

2.1. Redox chemistry of BH₄

Initial data on the oxidation mechanisms of BH₄ were obtained through electrochemical studies with the BH₄-analog, tetrahydropterin (Fig. 1). Those studies showed that under anaerobic conditions at physiological pH, tetrahydropterin undergoes a reversible two electron oxidation via a stepwise one-electron transfer mechanism [3,4]. This mechanism was consistent with electron paramagnetic resonance (EPR) data showing the intermediacy of a pterin a cation radical in the oxidation of BH₄. The quinonoid dihydropteridine (qPH₂; Fig. 1) was identified as the final product of oxidation. In protic solvents and under anaerobic conditions, the qPH₂ was shown to rearrange in a general base catalyzed reaction to 7,8-dihydropteridine (7,8-PH₂) [5]. This indicated that BH₄ oxidation reaction in the polarographic system even in anaerobic conditions may not be fully reversible. It also indicated that the quantification of the quinonoid species would be only possible in conditions that fully inhibit this rearrangement. Most biological buffers (pK_a 6.2–9.4) and reaction temperature [6] influence the 7,8-dihydro (bio)pterin yield, however (Fig.1).

In biochemical systems, BH₄ oxidation is caused by a variety of oxidants including horseradish peroxidase/H₂O₂ [7], heme proteins such as ferricytochrome c [8], and non heme iron (III) [9]. In all these systems and under anaerobic conditions, the formation of qBH₂ has been implicated and the 7,8-BH₂ product detected (Fig.2). The products of the reaction of BH₄ with oxygen have been more difficult to define. This is in part due to the fact that chemical oxidation of BH₄ in oxygenated buffers follows a more uncharacteristic product distribution than is anticipated from a two-electron mechanism to generate qBH₂ and H₂O₂ as main products (eq. 1)

Therefore, a one-electron transfer mechanism has been considered to better explain product yield. This mechanism predicts that the intermediacy of a BH_4 radical species influences product yield (eq.2 and eq.3)

(eq. 2)

(eq. 3)

A reaction influencing BH_4 radical formation is the dismutation reaction between oxidized and reduced BH_4 . Actually, direct evidence for the formation of a nitrogen centered BH_4 radical ($\cdot\text{BH}_4$) was obtained by EPR by mixing equimolar amounts of $q\text{BH}_2$ and BH_4 under acidic conditions [10] (eq.2).

2.2 BH_4 reactions in oxygenated solutions

The reaction of BH_4 with oxygen generates a variety of products depending on the experimental conditions [6,10,11]. At neutral pH, BH_4 autoxidation is slow and yields $q\text{BH}_2$, 7,8- BH_2 and 7,8-dihydropterin (upon loss of the side chain in the form of lactoyl aldehyde) (Fig.2) [5,6] and non-stoichiometric amounts of H_2O_2 [10,11]. The calculated rate constant for the reaction between oxygen and BH_4 is $0.60 \pm 0.03 \text{ M}^{-1}\text{s}^{-1}$ [11] and an even slower rate constant of $0.005 \text{ M}^{-1}\text{s}^{-1}$ has been reported for the synthetic analog 6,6,7,7-methyltetrahydropterin [10].

The mechanism of reaction between BH_4 and oxygen is controversial especially when considering that the reaction of BH_4 with the triplet oxygen molecule is slow and most likely the rate limiting step of the reaction. The intermediacy of a BH_4 radical has been proposed to alleviate the singlet-triplet spin barrier that limits the reactivity of reduced pterin ring with oxygen. Based on electron density calculations, an electron-rich carbon centered radical ($\cdot\text{BH}_4$), carbon-4 of the pterin structure (Fig. 1), is anticipated to be the reactive species. Upon the rapid combination of this radical with oxygen, the BH_4 peroxy radical ($\text{BH}_4\text{OO}\cdot$) is generated. The $\text{BH}_4\text{OO}\cdot$ is anticipated to propagate the oxidation reaction by abstracting a hydrogen atom from another BH_4 molecule that subsequently will react with another oxygen molecule. The product of the reaction, BH_4 hydroperoxide (BH_4OOH), rearranges to generate $q\text{BH}_2$ and H_2O_2 . Thus, the yield of BH_4 hydroperoxide and H_2O_2 will be mostly controlled by fluxes of BH_4 radical reacting with oxygen versus the amount of BH_4 radical decaying via dismutation. Whether the $\cdot\text{BH}_4$ (carbon-centered radical) and/or the $\cdot\text{BH}_4$ (nitrogen-5 cation radical) is the reactive species in the dismutation reaction is not yet definitively established.

Another pathway by which BH_4 could react with oxygen influencing reactive oxygen species formation is via an electron transfer mechanism with oxygen producing $\text{O}_2^{\cdot-}$ and BH_4 radical species [10]. This reaction is very slow yet it is a thermodynamically feasible reaction. It has been argued, however, that the recombination reactions between the radicals $\text{O}_2^{\cdot-}$ and BH_4 radical will likely take place almost instantaneously ($k \sim 10^{10} \text{ M}^{-1}\text{s}^{-1}$) to generate $\text{BH}_4\text{OO}(\text{H})$. This predicts that BH_4 autoxidation through this mechanism would be an unlikely source of superoxide. By the arrangement of BH_4OOH , however some accumulation of H_2O_2 occurs.

This mechanism though does not fully explain the inhibitory effect of superoxide dismutase (SOD) on oxygen consumption by BH_4 solutions [11,13]. Clearly the effect of SOD indicates that some $\text{O}_2^{\bullet-}$ likely escapes the reaction cage, and rapidly reacts with BH_4 to propagate oxidation reaction. In agreement, when $\text{O}_2^{\bullet-}$ is scavenged by SOD the oxidation of BH_4 slows down. We have shown that the reaction between $\text{O}_2^{\bullet-}$ and BH_4 is feasible [14] and calculated a reaction constant of $\sim 10^5 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4 and in the presence of the metal iron chelator, DTPA [14]. Also formation of the $^+\bullet\text{BH}_4$ was demonstrated in the reaction of BH_4 with a bolus amount of potassium superoxide in acidic conditions [14]. Thus $\text{O}_2^{\bullet-}$ regulates BH_4 oxidation which implies that any reaction interfering with $\text{O}_2^{\bullet-}$ will slow down BH_4 oxidation rates. This effect likely explains the lack of convincing EPR evidence for $\text{O}_2^{\bullet-}$ generation from BH_4 solutions at neutral pH since superoxide spin-trapping will compete with the propagation steps in the oxidation reaction of BH_4 [11,15].

Therefore the most likely fates of $\text{O}_2^{\bullet-}$ in BH_4 reactions are: (1) oxidation to generate oxygen or (2) radical recombination to BH_4 radical. These reactions may better explain the low yield of H_2O_2 generated by BH_4 oxidation and negates a further reaction between BH_4 and H_2O_2 which occurs at very slow rates in the presence of iron chelators, if at all [14]. Overall these studies show that autooxidation of BH_4 is a slow process that generates low amounts of free reactive oxygen species and a variety of BH_4 intermediates. Thus a role for BH_4 in endothelial oxidative stress is unlikely mediated by the direct generation of reactive oxygen species. Other reactions are likely to better explain this activity.

2.3 Antioxidant activity of BH_4

Another theory involving BH_4 in the regulation of reactive oxygen species in cells is that BH_4 could act as a direct ROS scavenger. This is inferred from kinetic studies on the reaction of BH_4 with free radicals showing that BH_4 , at neutral pH, very rapidly neutralizes reactive species. It has been calculated that the reaction rate constant for the reaction with hydroxyl, carbonate or thiyl radical is $\sim 10^9 \text{ M}^{-1}\text{s}^{-1}$ indicating the superior reactivity of BH_4 with respect to other biological reducing agents such as ascorbate [16]. The further reactions of the BH_4 radical, the product of this reaction, remain however less defined.

In view of the reactivity of $^+\bullet\text{BH}_4$ or $^-\bullet\text{BH}_4$, their decay is as important as BH_4 reactions when considering an antioxidant role for BH_4 in biological systems. There is the proposal that in combination with other antioxidants such as ascorbate, the reducing power of BH_4 is preserved via rapid conversion of the BH_4 radical to its fully reduced form. It has been estimated that the reaction constant for BH_4 radical (not yet identified) and ascorbate is $\sim 10^5 \text{ M}^{-1}\text{s}^{-1}$ [16]. While this evidence favors the idea that BH_4 recycling is coupled to ascorbate, these studies alone cannot distinguish whether the reducing capacity of BH_4 is more relevant than that of ascorbate in a cellular context. Ascorbate can reach much higher concentrations than BH_4 and reacts with several oxidants including $\text{O}_2^{\bullet-}$ and peroxynitrite at comparable rates. Thus ascorbate appears to be a better antioxidant in its own. Another issue is that these studies did not examine whether the reaction of ascorbate is the same with all the different BH_4 radical species ($^-\bullet\text{BH}_4$ or $^+\bullet\text{BH}_4$ or the peroxy radical $\text{BH}_4\text{OO}^\bullet$) and if a differential reactivity could explain its biological effect. This appears an important aspect in assessing the significance of the antioxidant activity of the BH_4 :ascorbate couple.

Both ascorbate and thiol reagents such as glutathione and 1,4-dithioerythritol efficiently slow down BH_4 oxidation when occurring under certain mild oxidant conditions like in BH_4 autooxidation. This is explained by the fact that BH_4 radical species and qBH_2 are generated in a low flow and can be reduced back to BH_4 by these reducing agents. When the rate of BH_4 oxidation increases, however, the conversion of BH_4 to products other than qBH_2 such as 7,8-PH₂ and dihydroxanthopterin is more rapid [7]. These products cannot be converted back to BH_4 . In the reaction of BH_4 with peroxynitrite, non-stoichiometric amounts of 7,8-BH₂, 7,8-

PH₂ and dihydroxanthopterin are generated [17] (Fig.2). Thus, in some cases the reaction of BH₄ with oxidants may actually lead to a net loss of the cofactor since not all of these products can be recycled back to BH₄ by chemical or enzymatic means.

In summary, the evidence supporting an antioxidant role for BH₄ is weak. Clearly, to better value this possibility the product(s) of reaction need to be characterized. This entails to understand the efficiency of the recycling of the BH₄ oxidized products generated in the reaction with superoxide and oxidants in different conditions and cellular milieu. It is possible that increased accumulation of BH₄ oxidized products may actually increase ROS production by eNOS uncoupling.

3. BH₄ metabolism

Deficient BH₄ production caused by genetic defects in biosynthetic enzymes is associated with a variety of health conditions including increased cardiovascular risk. In endothelial cells, BH₄ turnover is high. This is inferred from the rapid depletion of cellular BH₄ upon inhibition of synthetic enzymes [18,19]. Therefore endothelial cells rely on a constant *de novo* synthesis of the cofactor to maintain a critical BH₄ pool. The first committed step in the synthesis of the cofactor is catalyzed by the enzyme GTP cyclohydrolase I. This enzyme is also considered a critical regulator of BH₄ levels in endothelial cells.

3.1. GTPCH and synthesis of BH₄

The first enzyme of the biosynthetic pathway of BH₄, GTP cyclohydrolase I (GTPCH, EC 3.5.4.16) (Fig.3), is a guanosine-5'-triphosphate (GTP) 7,8-8,9-dihydrolase that produces 7,8-dihydroneopterin triphosphate, formate and water. This enzyme is constitutively expressed in many cells and tissues but with wide variation in activity with the liver showing the highest activity [20,21]. Pharmacological inhibition of GTPCH causes BH₄ deficiency in many cell types which indicates both the fast cellular BH₄ turnover and the key role of GTPCH in the regulation of cellular BH₄ levels.

Defects in the GTPCH gene causes defective production of BH₄ that manifests itself in dissimilar phenotypes depending on whether it occurs in autosomal recessive (AR) or dominant (AD) form. Carriers of AR form present high blood phenylalanine levels reaching 300–1200 µmol/L [22,23], neurological dysfunction and low neurotransmitters and pterin in cerebral spinal fluid (CSF). Patients presenting AD form are affected by the dopa-responsive dystonia syndrome (DRD) [24,25], a defect characterized by low BH₄ and neopterin in cerebrospinal fluid and also deficient neurotransmitters production in the brain. Differential diagnosis of AR and AD can be assisted by the fact that DRD patients do not develop hyperphenylalaninemia.

More recently, GTPCH variants have been associated with increased cardiovascular risk across different populations including those with high blood pressure and coronary artery disease. The discovery of frequent GTPCH mRNA variants in the 3'-untranslated regions (3'-UTR) C +273T (rs841) was recently reported [26]. Phenotypic characterization of twin pairs showed that both decreased renal and urinary NO and some autonomic traits such as pulse variability and baroreceptor coupling were linked to GTPCH polymorphisms in this region. Furthermore, increased systolic and diastolic blood pressure in outpatients was shown associated with these characteristics. The 3'-UTR variant, however, did not predict neopterin or catecholamine secretion [26]. In this study, however, additional GTPCH single nucleotide polymorphisms (SNP) were identified in the promoter region whose influence in phenotypic changes are not fully understood. Also the possibility that those specific polymorphic sites regulate functions in different tissues warrants investigation. Another study screened a population composed of coronary artery disease patients for three SNPs in the putative promoter region (rs8007267G>A), intron 1 region (rs3783641A>T) and 3'UTR (rs10483639C>G) that were

used to define a characteristic haplotype X (ATG) presenting low GTPCH activity in stimulated leukocytes [27]. The functional consequences of this haplotype were described as reduced GTPCH mRNA expression, low plasma BH₄:total biopterin ratio and altered vasorelaxation to acetylcholine, an index of NO release, among other characteristics [27]. Clearly, these studies are essential in demonstrating the association between vascular disease and GTPCH-BH₄ pathway and disturbances in NO production. They also underscore the complexity of population GTPCH gene variants in the different aspects determining vascular phenotypic characteristics.

3.2. Regulation of GTPCH activity

The GTPCH activity is increased at the transcriptional level by upregulating protein expression levels [28,29]. Several cytokines (TNF- α , IFN- γ , IL-1) and lipopolysaccharide (LPS) alone or in combination with cytokines are known to induce GTPCH expression several fold in many cell types. Macrophages and T cells, upon activation as a consequence of infectious and non-infectious conditions, show a strong GTPCH upregulation associated with increased formation of GTP-breakdown products dihydroneopterin triphosphate and neopterin (Fig.2) [30]. Since neopterin is found in human fluids such as plasma and cerebrospinal fluid, its application in the diagnosis and prognosis of diseases linked to immune cell activation has prompted active research in HIV and inflammatory diseases [31–33]. Typically, GTPCH in non-inflammatory cell types such as neonatal cardiomyocytes, smooth muscle cells and endothelial cells also responds to cytokine stimulation with increased protein levels [34–36].

In endothelial cells, GTPCH expression is regulated by a variety of signals. Cytokines such as TNF α , IFN γ and IL-1 increase *de novo* expression of GTPCH protein. This stimulation, however, is more complex than other cells types in that it requires the concerted activation of transcription factor signaling pathways involving NF- κ B and STAT1, but not STAT3, for an effective several fold upregulation of the enzyme [37]. Human umbilical vein endothelial cells treated with TNF α and IFN γ for 18–24 h increase GTPCH protein levels and activity, and BH₄ by >150-fold [34,37]. The effects of TNF α are mediated by NF- κ B while IFN γ acts via Jak2/STAT1. The NF- κ B pathway is regulated by ubiquitin/proteasome pathway and its contribution to increasing GTPCH is indicated by the lack of upregulation in I κ B mutants resistant to degradation or cells treated with proteasome inhibitor. The synergism between NF- κ B and Jak2/STAT1 in the regulation of endothelial GTPCH provided an understanding for the observation that LPS failed to increase GTPCH protein expression in endothelial cells [38]. We found that constitutive GTPCH protein levels in human aortic endothelial cells are not changed by LPS or by the protein synthesis inhibitor cycloheximide [38]. These findings are important in showing that constitutive GTPCH in human endothelial cells presents a low enzyme activity and low protein turnover rate and that LPS signaling via TLR4/TRAFF6/NF- κ B pathway is insufficient to stimulate *de novo* protein expression.

Variable increases in GTPCH mRNA levels are found in endothelial cells stimulated with insulin, HMG-CoA reductase or 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors and H₂O₂ [39–41]. Concurrent increase in BH₄ levels are reported for all those treatments suggesting that GTPCH protein is also increased. The transcriptional control of GTPCH activity, however, is not the only mechanism increasing enzyme activity in endothelial cells. Shear stress (oscillatory and laminar) stimulates GTPCH activity by mechanisms that are not fully characterized. It is currently proposed that shear stress increases either *de novo* protein synthesis and/or protein phosphorylation [42,43].

Regarding the latter, human endothelial cell casein kinase 2 (CK2) was shown to drive GTPCH phosphorylation at serine 81 causing a ~28-fold increase in enzyme activity [43]. Transient increases in GTPCH activity following protein phosphorylation is also a known mechanism in other cell types [44]. In mast cells, both CK2 and protein kinase C (PKC) were shown to

phosphorylate GTPCH protein. The predicted sites for casein kinase II activity are at serine positions 14, 51, 82, 103, 131 and 231 while for PKC only one potential site at serine position 167 is found. Hesslinger et al. [44] found that the extent of GTPCH activation directly correlates with the level of hyperphosphorylation. In endothelial cells, however, S81-modification is the only modification detected after several hours of shear stress. Whether phosphorylation of serine 81 prevents enzyme hyperphosphorylation in endothelial cells is an interesting possibility that warrants further investigation.

3.3. GTPCH protein interactions

Another mechanism regulating GTPCH activity is via interaction with the partner protein GTP cyclohydrolase feedback regulatory protein (GFRP) [45]. While this mechanism appears critical in tuning GTPCH activity to the constant changes in metabolic demand in the liver, its role in regulating GTPCH in endothelial cells is less clear [37,46]. Werner et al. [47] showed that GFRP downregulation increased GTPCH activity in endothelial cells stimulated with LPS. This response elucidated the mechanism for increasing BH₄ in endothelial cells which do not increase GTPCH protein levels in response to LPS, as previously discussed. Increased GFRP mRNA levels were found in H₂O₂ treated cells, suggesting that oxidative stress decreases endothelial BH₄ levels by upregulating GFRP expression [38]. Furthermore, Shimizu et al. [48] explained that H₂O₂ treatment actually increased BH₄ and GTPCH activity by lowering GFRP in an analogous way to LPS mechanisms. At this point the reasons behind the different responses of endothelial cell BH₄ to H₂O₂ challenge remain unclear and may be due to dissimilar H₂O₂ fluxes to which endothelial cells were exposed. The important aspect of this data is that GTPCH activity in the endothelium may be influenced by GFRP availability. The activity of GTPCH in protein complex with GFRP (1GTPCH:2GFRP) is influenced by BH₄ (BH₂) levels where high concentrations inhibits activity and low concentrations increases it. However, in endothelial cells GTPCH activity and BH₄ levels are low [38,47] raising questions about the role of a GTPCH:GFRP mechanism in these cells. Nonetheless a direct control by modulating protein association may be a possibility. There are some additional evidences however that negate this role. It is known that GFRP mediates pharmacological inhibition of GTPCH by 2,4-diamino-6-hydroxypyrimidine (DAHP) in decreasing the concentrations required for optimal inhibition [49]. But again, DAHP concentrations needed to inhibit GTPCH activity in endothelial cells are much higher than necessary to inhibit GTPCH activity in other cell types suggesting that GTPCH:GFRP complex is scarce. Furthermore we could not detect GFRP protein in human endothelial cells by traditional Western blot analysis using polyclonal rabbit antibodies, although the protein was detected in liver homogenates and when GFRP was transfected in endothelial cells [38]. Together this data suggests that low content of GFRP in endothelial cells may explain the refractoriness of GTPCH to regulation by known allosteric mechanisms.

3.4. GTPCH and oxidant damage

We and others found that under oxidative stress conditions GTPCH activity can be regulated via the ubiquitin-proteasome system [50,51]. We found that 4-hydroxynonenal mediates an irreversible step in the signaling of GTPCH degradation by the 26S proteasome. The proteasome inhibitor, lactacystin, was shown to increase the level of ubiquitinated proteins and prevent loss of GTPCH [50]. Similar findings are described in endothelial cells cultured with high concentrations of glucose [51]. The involvement of ubiquitin-proteasome activity in the regulation of GTPCH activity is new and may have important implications in terms of modulation of GTPCH-BH₄ in different cell types under oxidative stress. Removal of damaged proteins is a normal process in cells and modification of GTPCH makes it a prime target of proteasome activity. Since GTPCH is a low turnover protein in ECs, its degradation will likely lead to irreversible loss of BH₄. The exact steps and signals involved in the degradation of the

enzyme and when the enzyme is irreversibly marked for degradation are currently under investigation.

3.5 Genetic models of increased GTPCH activity

Overexpression of GTPCH by adenoviral gene transfer techniques in human endothelial cells and rabbit carotid artery was shown to efficiently increase intracellular BH₄ levels several fold [52,53]. This finding further indicated that GTPCH is a primary determinant in the control of endothelial BH₄ levels. It also indicated that downstream enzymes (6-pyruvoyltetrahydropterin reductase, sepiapterin reductase; Fig.3) in human and rabbit endothelial cells do not appear to be a major factor in regulating biopterin levels. In addition, it has been considered that GTPCH gene transfer circumvents the potential contribution of GFRP by limiting BH₄ production via feedback inhibition mechanism [45]. But again this mechanism has proven to be open to discussion.

Paradoxically other studies testing gene transfer in normal human endothelial cells reported increases in 7,8-BH₂ which accumulates inside the cells at much faster rates than BH₄ itself (96.1 pmol BH₂/mg protein versus 31.7 pmol BH₄/mg protein, electrochemical detection) [52]. Others reported an insignificant effect of GTPCH gene transfer in rabbit carotid artery relaxations [53]. These seemingly contradictory responses in those two independent studies may be related to GTPCH increases to non-physiological levels that enhance 7,8-BH₂ accumulation. One exception to these observations is that GTPCH gene transfer into aortic rings from spontaneously BB diabetic (BBd) and Zucker diabetic fatty (ZDF) rats increased BH₄ several fold and concomitantly improved vascular relaxation. In this model the values of 7,8-BH₂, however, were not reported [54].

Transgenic mice with endothelium specific overexpression of GTPCH have about a 3–6 fold higher basal aortic BH₄ levels (5.0± 2.5 pmol/mg protein) and improved vascular relaxation compared to wild type C57BL6 controls [55,56]. Improved vascular relaxation is also reported in animal models of diabetes, arteriosclerosis and hypertension with increased expression of GTPCH. In all these cases, an increase in aortic BH₄ is associated with improved vascular function which has been taken as indication of increased NO bioavailability [54–59]. While these findings highlight the beneficial effects of BH₄ in preserving vascular relaxations, they also show the rather narrow range of concentrations in which BH₄ is beneficial. As mentioned above, the arbitrary increase in GTPCH and BH₄ levels may actually worsen endothelial function by increasing 7,8-BH₂ accumulation in endothelial cells. Clearly, this aspect brings additional challenges to the design of therapeutic interventions to correct BH₄ deficiency in the endothelium.

3.6. Auxiliary systems in the regulation of BH₄

As shown in Fig.3, GTPCH catalyzes the first step of the *de novo* biosynthesis of BH₄. The following downstream biochemical reactions are regulated by several other enzymes including 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). The PTPS is constitutively expressed and responds to the same cytokine signals that upregulate GTPCH synthesis in the endothelium [60–62]. While this mechanism is not yet fully established, PTPS is thought not to be a limiting factor in BH₄ synthesis in endothelial cells when stimulated by cytokines. As mentioned above, endothelial targeting of GTPCH is known to increase BH₄ synthesis. In the lung and aorta of transgenic animals neopterin levels were unchanged implying that intermediates in BH₄ pathway do not accumulate to any significant extent and that PTPS and SR must be active.

The activity of PTPS in human macrophages however is limiting in producing BH₄ in response to cytokine stimulation while releasing high amounts of neopterin and 7,8-dihydroneopterin

(Fig.3) [62]. In these cells, defective activity appears linked to skipping of exon 3, and transduction with intact PTPS mRNA restores enzyme activity. Post transcriptional mechanisms regulating PTPS activity have been reported [63]. There is evidence that phosphorylation of PTPS serine 19 in human PTPS ensures maximal enzyme activity under in vivo conditions. Whether this modification is important in controlling BH₄ synthesis in the endothelium warrants more investigation.

Another key enzyme in BH₄ biosynthetic pathways is dihydrofolate reductase (DHFR). This enzyme was considered part of the de novo pathway for many years until studies by Milstien and Kaufman [64] unequivocally showed that DHFR was better explained by the existence of a BH₄ salvage pathway (Fig.3). The DHFR is also found constitutively expressed in the endothelium and its inhibition is associated with accumulation of 7,8-BH₂ from BH₄ oxidation [65]. The importance of DHFR in the regulation of physiological endothelial BH₄ levels is still not clear. Since the inhibition of GTPCH with the pharmacological inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) causes the rapid depletion of BH₄ levels, it is plausible to infer that DHFR has little or no influence in maintaining cellular BH₄ pool. It is recognized however that DHFR is a key enzyme in the successful treatment of endothelial cells with BH₄ and BH₄ analogs [66,67]. Thus it is likely that DHFR's contribution becomes important in a therapeutic setting rather than as an active regulator of BH₄ pools.

The alternative role for carbonyl and aldose reductase in BH₄ synthesis has also been indicated [68] (Fig.3). The role of these enzymes appears to be tissue specific, but limited information about their role in the regulation of endothelial BH₄ in health and disease is available.

4. Nitric oxide synthase

Tetrahydrobiopterin is a key component of NOSs [69,70]. Not only is BH₄ critical for the production of optimal amounts of NO but suboptimal BH₄ availability promotes superoxide release from eNOS and nNOS. This finding has fostered extensive work in characterizing the exact role of the cofactor in enzyme catalysis and its potential influence in vascular functions.

4.1. Biochemical characteristics

The catalytic domain of the three isoforms of NOS, the endothelial, neuronal and inducible, is composed of a reductase and oxygenase domain. The N-terminal reductase domain binds the flavin cofactors FAD and FMN that transfer electrons from NADPH to the heme group and BH₄ in the C-terminal oxygenase domain. This electron transfer is dependent upon activation of the enzyme with calcium/calmodulin complex [70,71]. The oxygenase domain contains binding sites for L-arginine and BH₄ that are located in close proximity to the heme-iron group. The active NOS arranges in a homodimeric conformation in which the reductase domain of one subunit interacts with the oxygenase-heme in the other subunit [72]. This arrangement increases efficiency of iron reduction and enzyme turnover.

All NOS isoforms catalyze the reaction of 1.5 mol NADPH + 1 mol L-arginine → 1 mol citrulline + 1 mol NO + 1.5 NADP⁺ which is fully dependent on BH₄. For many years the exact role of the BH₄ cofactor in catalysis was unknown. So far, studies have established the following key observations about the many roles of BH₄:

- a. Both reduced and oxidized BH₄ analogs bind to NOS but only the reduced and *R*-isomer is able to sustain optimal levels of NO production. The C6-chain substituent and stereochemical conformation greatly influences the *K*_{on} of binding;
- b. BH₄ binding to the nNOS increases high-spin heme-iron, heme-iron reduction and turnover rates;

- c. BH₄ binding to NOS subunits is anticooperative [73] albeit NOS has higher affinity for BH₄ than other BH₄-dependent enzymes like tyrosine and phenylalanine hydroxylase. This may explain why NOS isolated from BH₄-containing cellular systems is only partially saturated with the cofactor (<0.5BH₄/heme). Reconstitution of the enzyme with BH₄ in vitro restores V_{max} activity of the enzyme;
- d. BH₄ binding to NOS stabilizes “loose” dimer conformation and to a certain extent promotes the formation of homodimeric quaternary structure in iNOS [74]. The same role in maintenance of eNOS homodimeric conformation in solution and in cells remains debatable [75–78];
- e. BH₄ binding to NOS enhances arginine binding and alters the stability of the catalytic intermediate heme-iron dioxygen complex;
- f. BH₄ binding to eNOS and nNOS inhibits superoxide release from the heme-oxygenase domain of the enzyme [79–81].

All these activities have been critical in delineating a role for BH₄ in the NOS catalytic mechanism and regulation of product formation. It is now recognized that upon activation of the enzyme, the heme-iron(III) is reduced by FMNH₂ to heme-iron(II) which binds molecular oxygen to generate unstable heme-iron dioxygen complex that rearranges to heme-iron(III) and caged superoxide radical pair (heme-[Fe(III)-O₂^{•-}]). In the absence of BH₄ this complex dissociates to generate heme-iron(III) resting enzyme and superoxide. In the presence of BH₄, however, the heme-dioxygen complex is reduced to generate heme-iron-peroxo [heme-Fe-OO⁻] species and BH₄ is oxidized to ^{•+}BH₄. New advances in understanding the mechanism and kinetic constraints in the recycling of the pterin radical species are defining new aspects of enzyme activity regulation for the three isoforms of NOS [82].

4.2 Superoxide release from eNOS

The unequivocal demonstration that eNOS generates catalytic amounts of superoxide was aided by two important advances. The first was the expression of recombinant bovine eNOS protein in a dimeric BH₄-free form [83]. The purified enzyme showed a specific activity >120 nmoles citrulline/min/mg protein that was fully dependent on calcium/calmodulin, NADPH and BH₄ but not FAD or FMN. The protein showed a denatured molecular mass of 135 kDa in SDS-PAGE conditions. The second was the synthesis of improved spin traps like 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) [84] and 5-ethoxycarbonyl-5-methyl-pyrroline *N*-oxide (EMPO) [85] that produce more stable superoxide spin adducts and do not generate hydroxyl radical adducts as decay products.

Quantitative EPR experiments showed that BH₄-free eNOS produces superoxide with a specific activity of ~100 nmoles/min/mg protein (Fig.4). This compares to ~69% of the NO producing V_{max} activity of eNOS (~145 nmoles/min/mg protein). Superoxide formation showed full dependence on the heme-iron. This finding was supported by the demonstration that heme-iron ligands (e.g., cyanide, imidazole and phenyl-radical) significantly diminished superoxide yields. In addition superoxide release was inhibited upon supplementation of the enzyme with BH₄ indicating that BH₄ inhibited superoxide release from the heme oxygenase domain (Fig.5). In the presence of L-arginine, BH₄ stimulates NO production at V_{max} as previously shown by several groups. We proposed that the role of BH₄ is to couple the NADPH to L-arginine oxidation preventing superoxide release from the heme-iron at the oxygenase domain of the enzyme. These observations were also valid for neuronal NOS, except that more BH₄ was necessary to inhibit superoxide formation from eNOS than from nNOS [79–80]. For nNOS, however, L-arginine inhibited both superoxide release and NADPH consumption, an effect not seen with eNOS. L-arginine does not inhibit superoxide from BH₄-free eNOS but actually stimulates superoxide release. Other substrate analogs such as L-nitroarginine methyl

ester showed little effect suggesting that occupation of L-arginine binding site is not inhibitory for this activity. This is also indicated in recent studies with other L-arginine analogs such as asymmetrical asymmetric dimethylarginine (ADMA) and N^(G)-monomethyl- L-arginine which do not inhibit superoxide from BH₄-eNOS [86].

The formation of a blue-shifted intermediate from an oxyferrous cytochrome P450-like intermediate of NOS in the presence of BH₄ provided a solid clue on its role in enzyme catalysis [87]. This intermediate was attributed to a reductive product of the oxygenated heme-iron complex, which indicated that BH₄ provided the electrons and therefore that BH₄ redox state is key to this activity. This was in agreement with the observation that oxidized analogs, 7,8-BH₂ and sepiapterin were inefficient at inhibiting superoxide release from eNOS and that BH₄ was more efficient than 6-methyl- and 5-methyl-BH₄ at inhibiting this activity [88]. These observations gave the first insight into the possibility that superoxide release from eNOS could actually be regulated by the ratio of BH₄:7,8-BH₂ in the endothelium [88]. Following both NO and superoxide formation we showed that 7,8-BH₂ inhibited NO formation with a relatively high efficiency (BH₄:BH₂ 1:1), which was followed by increased superoxide release at higher BH₄:7,8-BH₂ ratios (1:7) [88]. Thus, 7,8-BH₂ can effectively compete for eNOS BH₄ binding site disrupting catalytic cycle. The possibility that eNOS uncoupling by 7,8-BH₂ explains low NO production and increased O₂^{•−} production in the endothelium is an active area of research. Our understanding of cellular control of BH₄ production, oxidation mechanisms and recycling is not complete and further studies will be needed.

Caveolin-1 scaffolding peptide inhibits both NO and superoxide generation from eNOS [89–90]. This inhibition is reversed by calcium/calmodulin, which is consistent with the proposal that caveolin blocks heme iron reduction [91]. Endothelial cell activation with vascular endothelial growth factor (VEGF) and bradykinin (BK) favors protein trafficking and increased interaction between eNOS and hsp90 [92]. The eNOS:hsp90 interaction is implicated in several mechanisms increasing eNOS activity including protein phosphorylation, improved calcium responses and stabilization of eNOS homodimer [92]. But hsp90 does not alter BH₄'s affinity for eNOS nor does it inhibit superoxide release from BH₄-free enzyme [77]. It was found however, that BH₄ increases eNOS-hsp90 interaction in endothelial cells [77] and stimulates eNOS activity which may offer another mechanism of control. Furthermore phosphorylation of BH₄-free eNOS was shown to increase superoxide production [93] indicating that hsp90 may contribute to this activity. In combination these results substantiate that the key determinant of superoxide formation from eNOS is BH₄ itself, while hsp90 and protein phosphorylation may potentiate this activity.

5. BH₄ and vascular dysfunction

Deficient BH₄ availability in the endothelium is associated with low NO bioavailability, impaired cGMP and vasoconstriction. This connection is quite significant since NO has many important vascular functions including local regulation of vasomotor tone, anti-thrombotic characteristic of the endothelium, and endothelial permeability. A worsening in NO bioavailability is anticipated if superoxide release is increased from uncoupled eNOS. To a certain degree the outcome of these changes are modulated by the cell's ability to cope with oxidative damage. That low BH₄ tips the endothelial cell redox balance towards a more oxidative state has been suggested by high levels of aorta lipid peroxide contents and plasma 8-F₂-isoprostanes in high fructose fed rats and hypercholesterolemic patients, respectively [94–95].

Early studies showed that *in vitro* supplementation of ECs with BH₄ analogs (6-methyl-BH₄ and sepiapterin) increased NO production indicating that BH₄ is limiting and that cellular BH₄ concentrations influence eNOS activity. More recently the effects of supplementation

have been connected to the re-coupling of eNOS by binding of BH₄ to BH₄-deficient eNOS and increasing NO production, and simultaneous inhibition of superoxide release from eNOS. The results of BH₄ supplementation in human patients, however, are still controversial. An underlying problem in several of these studies is that the effective BH₄ increase in ECs is difficult to demonstrate. This directly relates to one of the most critical aspects yet to be resolved: is BH₄ depletion a cause of disease? and how does BH₄ become limiting in the endothelium?

5.1. Inhibition of GTPCH activity

A primary cause of shortage in normal BH₄ supply can be explained by the loss of GTPCH protein and/or protein activity. Analysis of GTPCH protein by western blot indicated a significant down regulation of the protein levels in spontaneously diabetic (type 1) BioBreeding (BB) but not BB-normal rats [96]. Concordantly very low BH₄ (0.17 pmol/10⁶ cells) was found. The reasons explaining the lack of GTPCH is unclear; however, this deficiency in the *de novo* synthesis pathway appears specific since these cells retain the ability to produce BH₄ from sepiapterin. As discussed in the preceding sections, glucose-mediated oxidant production in endothelial cells may explain increased proteasome degradation of GTPCH. Whether loss of GTPCH explains BH₄ deficiency in diabetes is not yet firmly established. In type 1 diabetes mellitus induced by streptozotocin increased GTPCH activity was found [97]. There was, however, a concomitant loss of BH₄ that was explained by its accelerated oxidative degradation [97]. In contrast, another study showed that streptozotocin treatment caused loss of GTPCH protein which was also attributed to oxidative stress [98]. Thus oxidative stress has been thought to modulate GTPCH levels, but the exact mechanisms are yet controversial. However, oxidative stress overrides the effects of GTPCH increase by accelerating BH₄ degradation.

Glucocorticoid like dexamethasone has been shown to block GTPCH induction by cytokines in microvascular endothelial cells [99]. As dexamethasone decreases endothelial-dependent vasorelaxation, a decrease in GTPCH has been implicated in high blood pressure in glucocorticoid hypertensive rats [100]. A decrease in GTPCH mRNA, has been tested by PCR although how exactly glucocorticoid alters expression of GTPCH is not explained. Opposite effects have been described for dexamethasone treatment on rat lungs which increase GTPCH mRNA [101]. Whether these differences are explained by cell specific responses needs to be resolved considering that this may be an important side effect of current applied therapies in human.

5.2. BH₄ oxidation

An increased oxidation of BH₄ is often considered as a mechanism explaining BH₄ deficiency in several vascular conditions. A particular reaction that may increase BH₄ consumption is that with iron which is found in low molecular weight iron pools and in iron-containing proteins such as cytochrome *c* [102]. It is recognized that enhanced iron mobilization and storage is associated with endothelial dysfunction and progression of vascular diseases [103]. Thus there is a possibility that this reaction is significant in cells under increased oxidative stress. In support of this, we showed that the iron chelator, desferroxamine, inhibits BH₄ depletion in endothelial cells submitted to a flux of H₂O₂ by glucose/glucose oxidase indicating that iron maybe involved [38]. Through this pathway it is anticipated that H₂O₂ may have a real impact in accelerating the decay of BH₄ via oxidative pathways since the direct reaction of BH₄ with H₂O₂ is very slow. In a more indirect way, depletion of glutathione by H₂O₂ may increase oxidative degradation since less oxidized BH₄ may be regenerated [104]. Oxidation of BH₄ by superoxide is feasible and this pathway maybe favored in cells with depleted levels of antioxidants like ascorbate. The latter condition may also be a requirement for peroxynitrite mediated BH₄ depletion since antioxidants may outcompete BH₄ in the reaction with

peroxynitrite considering that their concentrations are an order of magnitude higher than those normally found for BH₄ in the endothelium.

Whether increased oxidation of BH₄ to 7,8-BH₂ fully explains BH₄ deficiency is debatable since 7,8-BH₂ is known to be reduced to BH₄ via DHFR. A caveat to this argument is that DHFR might not remain fully active under oxidative stress. Angiotensin II causes DHFR inhibition in endothelial cells at levels commonly associated with oxidative stress [65]. Thus increased oxidation of BH₄ and impaired reduction back to BH₄ may explain alterations in the BH₄:7,8-BH₂ ratios in cells. In this case, it is anticipated that decreasing oxidative stress by supplementation with ascorbate and/or glutathione should correct the loss of BH₄. This possibility was tested in chronic smokers that present impaired blood flow, an index of endothelial dysfunction, and low NO bioavailability. It was found that supplementation with ascorbate by acute infusion did not improve blood flow while acute BH₄ infusion did. Since tetrahydroneopterin (NH₄), a BH₄ analog without NOS cofactor activity, had no effect it was concluded that BH₄ infusion corrects endothelial BH₄ deficiency to stimulate NO formation and blood flow [105]. Ascorbate, however, has been shown to improve vascular relaxations in ApoE knockout (ApoE^{-/-}) mice fed a western diet. In this study BH₄ deficiency was shown not to be the cause of impaired vasorelaxation, but high 7,8-BH₂ was found. In agreement long-term treatment with ascorbate decreased 7,8-BH₂ and improved vascular relaxations [106]. Together these results indicate that DHFR is likely inhibited in ApoE^{-/-} mice but not in chronic smokers. To this date there is no data on DHFR activity in any of these conditions. This information clearly shows the need for further studies establishing better correlations between endothelial BH₄, 7,8-BH₂ and redox changes before a clear treatment including timing of intervention, dose and potential combinational therapies is proposed.

5.3. eNOS uncoupling and superoxide release

Deficient BH₄ levels and/or low BH₄:7,8-BH₂ ratios in several in vitro and in vivo disease models correlate with low NO and cGMP production in the vascular wall [56–58,94,98,105–106]. Thus endothelial dysfunction is explained at least in part by alteration in the normal BH₄ supply possibly via the mechanisms discussed above that result in deficient NO production from eNOS. A more difficult task however has been to demonstrate that low BH₄ or enhanced 7,8-BH₂ stimulates superoxide release from eNOS uncoupling. While most investigators have used L-NAME inhibitable superoxide formation as an index of uncoupled activity, this result is not firmly supported by studies with purified enzyme. Contradictory EPR data showing little effects [80,88] or full inhibition [98] of superoxide release from eNOS has been presented. If the differences reflect specific susceptibility of bovine or human eNOS to L-arginine analogs is a possibility with important consequences. This aspect of eNOS biochemistry remains a work-in-progress.

6. Concluding remarks

There is a sizeable amount of evidence supporting a role for BH₄ in the alterations of vascular reactivity in atherosclerosis, diabetes, cigarette smoking and hypertension. Recent studies have also implicated BH₄ in the salutary effects of exercise, shear stress, statins and insulin. It appears that BH₄ therapy is the next logical step in the treatment of endothelial dysfunction associated with vascular diseases. A phase 2 clinical trial for the effects of BH₄ on blood pressure in subjects with poorly controlled systemic hypertension, however, was recently terminated by the lack of evidence in support of significant beneficial effect [Biomarin News, 2007]. Positive results in phase 2A clinical studies of endothelial dysfunction with sickle cell patient were recently reported [Biomarin News, 2008]. The dissimilar results in these clinical trials highlights the importance of fully addressing basic questions about the mechanism of endothelial BH₄ regulation that will be critical in the design of BH₄-based therapies including

dose and length of intervention. The most compelling issues appear to be (1) to define whether BH₄ deficiency causes oxidative stress; (2) to define how oxidative stress causes BH₄ deficiency and (3) the existence or not of a genetically- enhanced susceptibility for BH₄ deficiency that may explain eNOS uncoupling.

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List of Abbreviations

ApoE ^{-/-}	Apolipoprotein E knockout mice
BH ₄	(6R)-5,6,7,8-Tetrahydrobiopterin
•BH ₄	BH ₄ carbon centered radical
⁺ •BH ₄	BH ₄ nitrogen centered radical
BH ₄ OO•	BH ₄ peroxy radical
7,8-BH ₂	7,8-dihydrobiopterin
qBH ₂	5,6-dihydrobiopterin
DHFR	Dihydrofolate reductase
EPR	Electron paramagnetic resonance
GTPCH	GTP Cyclohydrolase I
GFRP	GTP Cyclohydrolase I Feedback Regulatory Protein
H ₂ O ₂	Hydrogen peroxide
7,8-PH ₂	7,8-dihydropteridine
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O ₂ ^{•-}	Superoxide anion radical
PKU	Phenylketonuria
PTPS	6-Pyruvoyl Tetrahydropterin Synthase
SR	Sepiapterin Reductase

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Figure 1. BH_4 autoxidation reactions

(A) The deprotonated carbon-centered radical ($\cdot BH_4$) reacts with oxygen to generate a BH_4 peroxy radical ($BH_4OO\cdot$). This radical species reacts with another BH_4 in the propagation reaction to generate the corresponding peroxide (BH_4OOH), which decomposes into qBH_2 and hydrogen peroxide. (B) General base-catalyzed rearrangement of qBH_2 to generate 7,8- BH_2 in neutral solution. (C) NON-enzymatic conversion of qBH_2 to 7,8-dihydropterin (7,8- PH_2) and lactoyl aldehyde (RCHO).

Figure 2. BH₄ degradative pathways

(Upper left) L-Biopterin is produced from BH₄ oxidative metabolism. Only the pterin ring is oxidized, the side chain hydroxyl groups remain in *cis*-conformation as predicted to be the favorable conformation in solution. (Upper right) D-neopterin is a byproduct in BH₄ synthesis that also presents hydroxyl groups in *cis*-position. Both biopterin and neopterin are found in biological fluids and their concentration ratios are used as markers in the diagnosis of BH₄ metabolic disorders. (Lower left) Oxidative degradation of BH₄ by biological oxidants (peroxidase/H₂O₂, peroxynitrite, heme proteins) s convert BH₄ into 7,8-dihydro-L-biopterin (7,8-BH₂), 7,8-dihydropterin (7,8-PH₂) and dihydroxanthopterin. Dihydrofolate reductase (DHFR) can reduce 7,8-BH₂ back to BH₄. (Lower right) BH₄ cofactor activity in the hydroxylation reactions catalyzed by tyrosine and phenylalanine hydroxylase (TH, PAH respectively). Upon binding to enzymes BH₄ side chain hydroxyl groups re-orient in a *trans*-like conformation presumably to increase binding forces. The BH₄ oxidation product, pterin-4a-carbinolamine, generates quinonoid BH₂ (*q* BH₂) by spontaneous or catalyzed dehydration by pterin carbinolamine dehydratase (PCD) also known as dimerization cofactor

of hepatocyte nuclear factor 1 alpha (DCoH). The qBH_2 is either reduced back to BH_4 by NADH-dependent enzyme dihydropterin reductase (DHPR) or rearranges to form 7,8- BH_2 .

Figure 3. Biosynthetic pathway of BH₄

(Solid arrow) *de novo* synthesis pathway involving GTPCH, GTP cyclohydrolase 1, PTPS, 6-pyruvoyl tetrahydropterin synthase, AR, aldose reductase, SR, sepiapterin reductase, CR, carbonyl reductase. (Broken arrow) Salvage pathway of tetrahydrobiopterin via SR, sepiapterin reductase and DHFR, dihydrofolate reductase.

Figure 4. Superoxide release from BH_4 -free eNOS

Superoxide production from BH_4 free-eNOS was detected with EMPO spin trap upon activation of the enzyme with calcium-calmodulin. Incubation of the enzyme with L-arginine prior to activation increases superoxide yield, while L-NAME at 10-fold higher concentration than L-arginine only decreases superoxide yield by ~25%. BH_4 dose dependently inhibits superoxide release. Both BH_4 and L-arginine decrease superoxide to stimulate NO production as confirmed by L-citrulline production.

Figure 5. Superoxide release from heme-oxygenase domain of eNOS is inhibited by BH_4
 Superoxide release occurs in the absence of BH_4 and is increased by L-arginine (L-Arg) upon activation of the resting state enzyme by calcium/calmodulin. In the absence or presence of L-Arg, BH_4 inhibits superoxide release. Together with L-arg BH_4 dose-dependently increases NO and citrulline production.