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Review

The Role of Tetrahydrobiopterin in Superoxide Generation from eNOS: Enzymology and Physiological Implications

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Tetrahydrobiopterin (BH_4) is a ubiquitous pteridine metabolite that serves as a NOS cofactor. Recently, we showed that BH_4 efficiently inhibits superoxide generation from the heme group at the oxygenase domain of eNOS. This role indicates that BH_4 acts as a redox switch in the catalytic mechanism of the enzyme, which may have important consequences in the physiology of the endothelium. Here the mechanism by which BH_4 inhibits superoxide release from eNOS and the "uncoupling" effects of oxidized BH_4 metabolites are presented. The implications of the disparate actions of fully reduced and oxidized BH_4 metabolites in the control of eNOS biochemistry are discussed in the light of clinical data indicating that BH_4 levels are important in the regulation of superoxide levels and of endothelial reactivity.

Keywords: Tetrahydrobiopterin; Endothelium; Electron paramagnetic resonance; Spin trapping; Superoxide

INTRODUCTION

Nitric oxide synthase is a heme and flavin-containing enzyme that uses tetrahydrobiopterin (BH_4) as a cofactor and L-arginine, NADPH and molecular oxygen as substrates.[1,2] Three isoforms of the enzyme have been cloned and characterized. They are known as neuronal (nNOS or NOS-I, 165 KDa), inducible (iNOS or NOS-II, 130 KDa) and endothelial (eNOS or NOS-III, 133 KDa). These isoforms have been extensively studied in regard to catalysis mechanism and cellular control of enzyme activity. At the physiological level, eNOS has been shown to play an important role in regulating vascular function. It is known, for instance, that knockout mice lacking eNOS develop hypertension,[3] insulin resistance,[4] hyperlipidemia[4] and show augmented ischemia–reperfusion damage.[5] Similar damage is associated with increased generation of superoxide. Since superoxide and $^{\bullet}NO$ combine at diffusion limited-rates, increased superoxide is associated with diminish $^{\bullet}NO$ levels[6,7] and consequently, increased superoxide is associated with decreased endothelium-dependent relaxation. This phenomenon is known as endothelial dysfunction. The current debate in the vascular literature is focused on mechanisms that control the balance between $^{\bullet}NO$ and superoxide formation as contributory factors in the pathophysiology of vascular disease.

The pteridine cofactor BH_4 has been shown to critically control eNOS activity.[8–10] It has been postulated that in disease states such as diabetes, hypertension and atherosclerosis endothelial levels of BH_4 are reduced which correlates with diminished $^{\bullet}NO$ production.[11–13] Recently, we have shown that activation of eNOS under limited availability of BH_4 not only results in low rates of $^{\bullet}NO$ formation but also increases superoxide formation.[14] This indicates that BH_4 by controlling superoxide formation from eNOS maybe an important metabolite in vascular physiology. In this work, we discuss the

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role of BH_4 in the catalytic mechanism of eNOS and its implications in endothelial functioning.

NOS STRUCTURE AND COMPOSITION

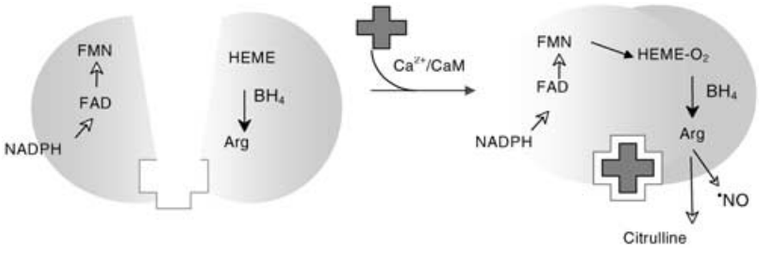
Scheme 1 shows a representation of the catalytic domains of eNOS. The active enzyme is a homodimer composed of subunits each containing a reductase domain and an oxygenase domain. These two domains are linked by a sequence that has a high affinity for calmodulin. The C-terminal reductase domain possesses binding sequences for NADPH, flavin-adenine dinucleotide (FAD) and flavin-adenine mononucleotide (FMN). At the sequence level, this domain has a close homology to the NADPH-cytochrome P450 reductase (CPR). However, a distinctive characteristic between eNOS and CPR is the presence of an additional sequence of about 40 residues neighboring the eNOS-FMN module. Deletion of this sequence greatly diminishes calcium-dependence to enhance the maximal activity of the enzyme.[15] Therefore, this sequence is thought to be an auto-inhibitory element in eNOS. The N-terminal oxygenase domain has binding sites for the heme-Fe group that is coordinated to a cysteine thiolate group in the protein. Coordinated to heme propionate is BH_4 cofactor that interacts with several other residues close to the heme pocket through H-bonding and van der Waals contacts; altogether these interactions appear to account for the high cofactor affinity. L-Arginine binding site is in close proximity to the heme group. The guanidino nitrogen that participates in catalysis is 4.0 Å from the heme group and is H-bonded to Glu363 and to the heme propionate. These interactions appear to be critical for the control of catalytic activity. Upon binding of calcium-activated calmodulin, electron transfer reaction from NADPH-derived electrons at the reductase domain to the heme group in the oxygenase domain is activated. This enables the heme group to catalyze the two-step conversion of L-arginine to L-citrulline and $^{\bullet}NO$ free radical.

OXYGEN ACTIVATION IN THE CATALYTIC MECHANISM OF eNOS

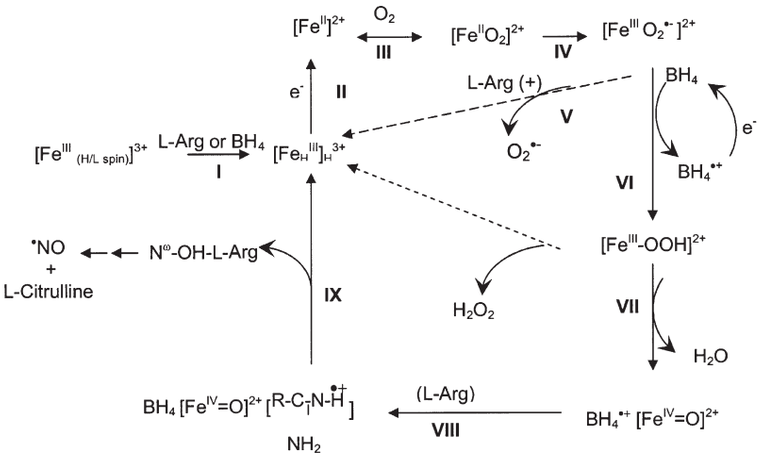
Reduction of the heme-Fe^{III} group to heme-Fe^{II} that has a high affinity for oxygen is the first step in the catalytic mechanism of NOS (Scheme 2).[16,17] This step appears to dictate the maximal activity of the different isoforms. Stop-flow studies showed that the rate of heme-Fe^{III} reduction follows the order iNOS > nNOS > eNOS[18,19] indicating that heme-Fe^{III} reduction directly correlates with turnover number. Domain swapping studies demonstrated that the rate limiting-step in the reduction of heme-Fe^{III} is controlled by electron transfer reaction from reduced FMN to the heme group.[20,21] Examination of NOS mutants indicates that this reaction is controlled by structural features at the C-terminus (tail) and at the N-terminus of the reductase domain (auto-inhibitory element).

The heme-Fe^{III} in the purified enzyme is found as a mixture of 5-coordinated/low spin heme and 6-coordinated/high spin. The binding of L-arginine and/or BH_4 promotes the conversion of all heme to 6-coordinated high spin heme (Scheme 2, step I). This transition is predicted to reduce heme redox potential facilitating its reduction to heme-Fe^{II}[22–24] oxygen binding to the heme-Fe^{II} generates the ferrous-dioxygen complex that re-arranges to the isoelectronic species ferric-superoxide complex (Scheme 2, step III and IV). Formation of this species has been documented by spectrophotometric studies showing transitions in the UV-region.[25] The reduction of the ferric-superoxide complex will generate the heme-peroxo complex that is further oxidized to the heme-Fe^{IV}= O species. The latter is the active species that oxidizes L-arginine (Scheme 2, step VI) to generate *N*-hydroxy-L-arginine intermediate.

The second step in the oxidation of L-arginine proceeds through a mechanism different from the above. It has been modeled after the mechanism of heme-peroxidases. That is, in the second step, hydrogen peroxide can provide the electrons



SCHEME 1 Subunit composition of eNOS. Left panel shows the enzyme in the resting state. Right panel shows the enzyme in an active form where electrons flow from reductase to oxygenase domain enabling the conversion of L-arginine to $^{\bullet}NO$ and L-citrulline. Adapted from Ref. [1].



SCHEME 2 Formation of activated heme species in the catalytic mechanism of eNOS. Representation of the reactions involved in the first step of oxidation of L-arginine to *N*-hydroxy-L-arginine.

necessary for *N*-hydroxy-L-arginine oxidation to generate •NO and L-citrulline. This reaction has been demonstrated in purified systems. However, there are conflicting ideas in regard to whether BH_4 is involved in this step of the catalytic cycle. Although of major importance, this discussion is not included in this work.

ROLE OF BH_4 IN SUPEROXIDE GENERATION FROM eNOS

The BH_4 cofactor directly activates oxygen promoting hydroxylation of aromatic amino acids by non-heme Fe-containing hydroxylases.[26] Direct oxygen activation by BH_4 in NOS catalysis, however, was shown to be very unlikely. Since direct activation of oxygen implicates two-electron oxidation of BH_4 to generate 5,6-quinonoid or 7,8-dihydrobiopterin, a coupled enzymatic reduction of these oxidized forms would be necessary to sustain enzyme activity. However, NOS lacks dihydrobiopterin reductase activity.[27] Thus, it was anticipated that the role of BH_4 in NOS catalysis is at the structural and/or electronic level. In fact, BH_4 was shown to be important in the stabilization of the dimeric structure of NOS although is not necessary for dimerization itself[28] and that BH_4 inhibits monomerization of the enzyme during catalysis. Other recognized functions include facilitating the transition of the heme group from 5-coordinated low spin to 6-coordinated high spin, to augment L-arginine binding and decrease its dissociation from the active site of the enzyme.[29] Because of the latter activity, BH_4 is considered a positive allosteric cofactor of NOS.

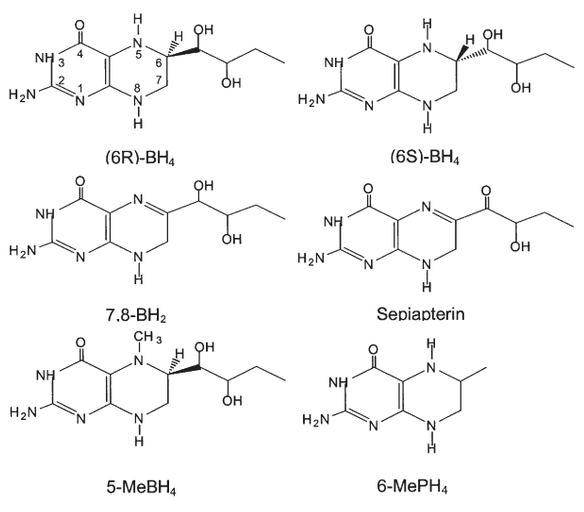
Although BH_4 does not directly activate oxygen, it was shown to play a key role in controlling superoxide formation from eNOS. Activation of BH_4-free eNOS with calcium/calmodulin stimulates superoxide generation that is inhibited by addition of BH_4.[14,30] In the presence of iron ligands that alter heme reactivity such as cyanide and imidazole, a remarkable diminution of superoxide from BH_4-free eNOS was observed. Superoxide formation by resting enzyme was barely detectable.[14] Altogether these data provided the first direct evidence that BH_4 participates in the steps involving oxygen activation by the heme group at the oxygenase domain of eNOS. Quantitative electron paramagnetic resonance spin trapping experiments indicated that evolution of superoxide from activated BH_4-free eNOS is lower than for BH_4-free nNOS. The reason for this difference likely reflects the slower rate of heme-Fe^{III} reduction and therefore oxygen activation, since superoxide yields correlate well with rates of NADPH oxidation by these two isoforms.

Low temperature spectroscopic studies showed that BH_4 promoted the formation of blue-shifted heme species that were attributed to formation of heme-peroxo species (Scheme 2, step VI).[31] It was concluded that BH_4 reduces the heme-dioxygen species and thus that the cofactor plays a redox role in the catalytic cycle of the enzyme.[31] This model anticipated that in the absence of BH_4 the enzyme will catalyze the oxidation of NADPH to generate superoxide which is fully supported by the spin trapping studies.[14,30] Another implication of this model is that in the presence of BH_4 and in the absence of L-arginine, hydrogen peroxide, but not superoxide, will be generated (Scheme 2, step VI). In other words, in the presence of BH_4, NOS catalyzes the two-electron reduction of oxygen. However, experimental data have shown that in the presence of BH_4 little or no hydrogen peroxide is generated by eNOS, whereas significant amounts are detected from nNOS in the same conditions.[32]

These results suggest that BH_4-mediated inhibition of superoxide release from eNOS involves more than one mechanism. In support of this idea, we and others have demonstrated that BH_4 decreases NADPH oxidation from eNOS but not from nNOS. This result suggests that another mechanism by which BH_4 inhibits superoxide is by stabilizing the ferrous-dioxygen complex.

Studies using reduced and oxidized BH_4 indicated that only BH_4 and fully reduced BH_4-analogs such as 6-methyltetrahydropterin (6-MePH_4) and 5-methyltetrahydrobiopterin (5-MeBH_4) (Scheme 3) were able to inhibit superoxide from eNOS. In contrast, oxidized BH_4 analogs 7,8-dihydrobiopterin (7,8-BH_2) and sepiapterin, (Scheme 3) were ineffective in diminishing superoxide release from BH_4-free eNOS even at millimolar concentrations. Both 7,8-BH_2 and sepiapterin do not support enzyme activity. The lack of $^{\bullet}$NO-cofactor activity may be explained by their inability to promote formation of high oxidation states of heme necessary for catalysis. Interestingly, BH_4 is by far the most potent superoxide inhibitor. Thus, the rule appears to be the more efficient the cofactor is at inhibiting superoxide, the better it is at stimulating $^{\bullet}$NO formation.

To further demonstrate that the lack of superoxide inhibiting activity of 7,8-BH_2 was specific, 7,8-BH_2 was added to fully coupled eNOS with BH_4 and L-arginine. Under these conditions, 7,8-BH_2 caused a dose-dependent increase of superoxide generation from eNOS.[30] Concomitant to increasing superoxide, 7,8-BH_2 inhibited $^{\bullet}$NO formation from eNOS.[30] Taken together these results indicate that (1) only reduced BH_4 inhibits superoxide release from eNOS; (2) oxidized BH_4 analogs are efficient eNOS-uncoupling agents enhancing superoxide while inhibiting $^{\bullet}$NO formation; (3) BH_4-binding site is relatively accessible to BH_4 analogs.



SCHEME 3 Chemical structures of natural and synthetic pteridines.

Unlike BH_4, L-arginine at saturating concentrations increased superoxide production from BH_4-free eNOS.[14,30] This effect is likely due to the facilitated reduction of the heme-Fe^{III} and hence with oxygen binding to eNOS.[22,23] Another possibility is that L-arginine destabilizes the ferrous-dioxygen species assisting the dissociation of superoxide from the heme group (Scheme 2, step V). Conversely, L-arginine decreases superoxide generation from nNOS. This difference suggests that the geometry of interaction between eNOS heme-dioxygen complex and guanidino group of L-arginine is not as strong as for nNOS. This is also indicated by spin trapping data showing that L-arginine has little or no effect on the superoxide release from nNOS mutant C331A that has a much lower affinity for L-arginine than wild type enzyme.[32] This data demonstrates that eNOS coupling is only controlled by BH_4 and not by L-arginine as previously suggested.[33,34] The physiological implications of these observations are that depletion of L-arginine is not a mechanism to explain eNOS uncoupling. Also, eNOS is the most likely isoform responsible for superoxide generation under conditions of low BH_4 levels.

The importance of the interaction of substrate with heme group in the control of superoxide formation is also illustrated by the effect of L-arginine analogs on superoxide formation from eNOS. L-NAME had a marginal effect on superoxide formation from BH_4-free eNOS[30] whereas L-thiocitrulline significantly inhibits superoxide (unpublished results).

TETRAHYDROBIOPTERIN AND ENDOTHELIAL DYSFUNCTION

Low BH_4 levels have been associated with the onset of endothelial dysfunction. Evidence indicates that augmentation of BH_4 concentrations with BH_4 analogs (sepiapterin or 6-MePH_4, Scheme 3) ameliorates vasoconstriction in genetic hypertension,[35] hypercholesterolemic patients,[36] experimental diabetes and reperfusion injury.[37] The mechanism by which BH_4 controls vascular reactivity in these disease states, however, is largely unknown. The initial assumption was that BH_4 may increase $^{\bullet}$NO production by the endothelium since inhibition of the *de novo* BH_4 synthesis reduces the formation of $^{\bullet}$NO and cGMP levels. Alternatively, it was proposed that BH_4 supplementation may decrease superoxide production. Given that superoxide rapidly reacts with $^{\bullet}$NO,[7] reduction of superoxide levels will increase $^{\bullet}$NO levels. Data in the literature is confusing in regard to which of these mechanisms explain BH_4 effects in the vasculature. It was reported that BH_4 treatment of spontaneous hypertensive rats (SHR) decreases superoxide formation in aortic segments but did not increase L-citrulline,[35]

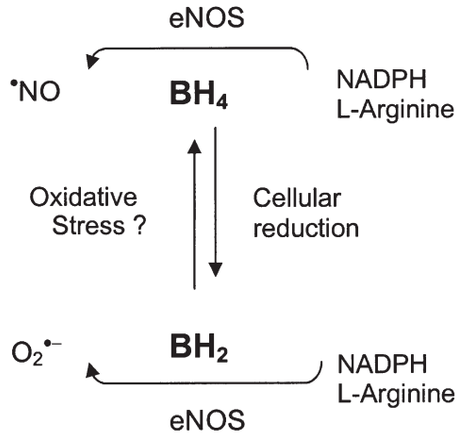
while normotensive control rats (WKY) increases L-citrulline formation. The conclusion is that a "dysfunctional eNOS" (eNOS lacking BH_4) enhances superoxide formation in SHR and that BH_4 inhibits its formation.[35] In contrast, it was shown that both endothelial-dependent relaxation and cGMP levels were reduced in BH_4 supplemented canine cerebral arteries.[38] Only in the presence of superoxide dismutase, were both endothelial-dependent vasorelaxation and cGMP levels significantly increased compared to untreated arteries. Unlike previous reports, this study indicated that BH_4 does not decrease superoxide formation in blood vessels, but supported the idea that BH_4 enhances eNOS-dependent increase of cGMP levels.

The demonstration that BH_4 enhances eNOS activity in blood vessels indicates that the enzyme may exist in a partially BH_4-free form. Although this is a reasonable conclusion, the exact mechanism by which increases in intracellular BH_4 levels influence eNOS activity is not understood. Depletion of BH_4 levels are easily reachable upon pharmacological inhibition of GTP-cyclohydrolase I, the rate limiting enzyme in de novo BH_4 synthetic pathway.[25] This indicates that BH_4 turnover in the endothelium is fairly active. The reactions consuming BH_4 and/or the signals controlling its synthesis/cycling, however, remain obscure.

Modulation of BH_4 levels by mechanisms other than supplementation suggests that oxidative stress may play a role in BH_4 homeostasis. This is indicated by augmentation of $^{\bullet}NO$ formation from endothelial cells upon supplementation with L-ascorbic, an activity that was attributed to stabilization of intracellular BH_4 levels.[39] Folate treatment is considered to improve vascular function by mimicking BH_4 activity,[40,41] although it is unclear which one. The most important lesson of these data is that local and transient modulation of the redox state of the endothelium is important for balancing BH_4 levels and thereby eNOS activity.

Whether BH_4 effects are related to superoxide scavenging was recently investigated by kinetic spin trapping.[42] It was determined that the second order reaction constant for the reaction between BH_4 and superoxide is $3.9 \pm 0.2 \times 10^5\,M^{-1}s^{-1}$ at pH 7.4 and 25°C. This constant is very similar to that of L-ascorbate with superoxide. However, BH_4 levels in the endothelium are much lower than ascorbate even in BH_4 supplemented cells. This indicates that the effects of BH_4 are unlikely to be related to its antioxidant potential but they are due to the specific inhibition of superoxide formation from eNOS.

In combination with L-arginine, BH_4 enhances the formation of $^{\bullet}NO$ and L-citrulline.[9,14] This control is disrupted by oxidized BH_4 analogs such as 7,8-BH_2 and sepiapterin to enhance superoxide formation from the enzyme.[14] This suggested that



SCHEME 4 BH_4/BH_2 ratio controls $^{\bullet}NO$ and $O_2^{\bullet-}$ generation from eNOS in the vasculature. Augmentation of BH_4 in the vasculature by supplementation and/or synthesis/recycling will enhance $^{\bullet}NO$ formation from eNOS. Low BH_4 levels or augmentation of oxidized BH_4 (BH_2) is predicted to enhance superoxide formation from eNOS. Adapted from Ref. [14].

the intracellular ratio of reduced and oxidized BH_4 metabolites is a key mechanism controlling superoxide and $^{\bullet}NO$ formation in the endothelium (Scheme 4).

The mechanism proposed in Scheme 4 indicates that under conditions of high BH_4 turnover, the ratio between BH_4 and oxidized BH_4 (BH_2) may tip in favor of increased formation of eNOS-dependent superoxide formation despite L-arginine concentrations. It is likely that BH_4 supplementation shifts the balance towards $^{\bullet}NO$ formation concomitantly to inhibit superoxide formation from eNOS. This mechanism may explain the apparent superoxide scavenging activity of BH_4. Thus, the balance between BH_4/BH_2 in the endothelium offers a biochemically and physiologically relevant pathway by which it would be possible to appraise the role of BH_4/eNOS in endothelial dysfunction associated with several cardiovascular diseases.

SUMMARY

Increased formation of superoxide in the vasculature impairs vasorelaxation. Clinical studies have suggested that superoxide levels are increased upon endothelial depletion of BH_4. We have shown that eNOS generates superoxide under limited availability of BH_4. In addition, we showed that augmentation of oxidized BH_4 concentrations (BH_2, Scheme 4) also increases superoxide formation from eNOS. This evidence strongly suggests that mechanisms depleting BH_4 or augmenting BH_4 oxidized metabolites in the vasculature will enhance superoxide formation from eNOS. How BH_4 levels are regulated in the endothelium is not known.

Understanding BH_4 turnover in vasculature appears of major importance considering the important regulatory effect on eNOS biochemistry. An interesting observation is that *in vitro* BH_4 display low reactivity with oxidants suggesting that *in vivo* mechanism regulating BH_4 concentrations may involve regulation of GTP cyclohydrolase I, the first enzyme in its biosynthetic pathway. All these possibilities may have enormous impact in endothelial function and they certainly warrant further investigation.

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