The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study

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Augmentation of superoxide levels has been linked to impaired relaxation in hypertension, diabetes and hypercholesterolaemia. Purified endothelial nitric oxide synthase (eNOS) generates superoxide under limited availability of 5,6,7,8-tetrahydrobiopterin (BH_4). Thus alterations in endothelial BH_4 levels have been postulated to stimulate superoxide production from eNOS. This possibility was examined by determining the concentration-dependent effects of BH_4, and its analogues, on superoxide formation by eNOS. Superoxide was quantified by EPR spin trapping, which is the only available technique to quantify superoxide from eNOS. Using 5-ethoxycarbonyl-5-methyl-pyrroline *N*-oxide, we show that only fully reduced BH_4 diminished superoxide release from eNOS, with efficiency BH_4 > 6-methyl-BH_4 > 5-methyl-BH_4. In contrast, partially oxidized BH_4 analogues, 7,8-dihydrobiopterin (7,8-BH_2) and sepiapterin had no effect. Neither L-arginine nor N^G-nitro-L-arginine methyl ester (L-NAME) abolished superoxide formation. Together, BH_4 and L-arginine stimulated ·NO production at maximal rates of 148 nmol/min per mg of protein. These results indicate that BH_4 acts as a 'redox switch', decreasing superoxide release and enhancing ·NO formation. This role was verified by adding 7,8-BH_2 or sepiapterin to fully active eNOS. Both 7,8-BH_2 and sepiapterin enhanced superoxide release while inhibiting ·NO formation. Collectively, these results indicate that the ratio between oxidized and reduced BH_4 metabolites tightly regulates superoxide formation from eNOS. The pathological significance of this scenario is discussed.

Key words: L-arginine, 7,8-dihydrobiopterin, electron paramagnetic resonance.

INTRODUCTION

Vasorelaxation is elicited by ·NO [1,2] derived from endothelial cells in response to agonist-mediated activation of endothelial nitric oxide synthase (eNOS). This enzyme catalyses the oxidation of L-arginine to generate L-citrulline and ·NO upon activation with calcium/calmodulin. ·NO diffused from endothelial cells to vascular smooth muscle cells enhances cGMP levels and regulates intracellular calcium ion concentrations required for vasorelaxation. Thus there exist several checkpoints in the ·NO–cGMP pathway that can modulate ·NO-dependent vasorelaxation. Loss of endothelial-dependent vasorelaxation (endothelial dysfunction) occurs from decreased basal concentrations of ·NO [3,4]. The mechanisms modulating ·NO concentration in the vascular wall, however, are not fully understood. Regulation of the ·NO–cGMP pathway *in vivo* may be attributed to either impaired formation of ·NO and/or increased reaction with superoxide [5,6]. Increased formation of superoxide anion has been linked to the pathogenesis of chronic vascular diseases, such as atherosclerosis, diabetes and hypertension [7–9]. Endothelial dysfunction associated with these conditions has been attributed to the rapid reaction between superoxide and ·NO generating peroxynitrite, a potent oxidant [10] that can induce endothelial damage [11,12].

5,6,7,8-Tetrahydrobiopterin (BH_4), an essential cofactor in ·NO biosynthesis, has been shown to improve endothelium-dependent vasodilation in patients with coronary artery disease [13], type II diabetes mellitus [14], smokers [15,16], atherosclerosis [17] and several other disease models [18,19]. BH_4 deficiency reportedly impairs ·NO-dependent vasodilation. How intracellular BH_4 concentrations modulate the ·NO–cGMP pathway is an area of active investigation. Scavenging of superoxide by BH_4 was suggested to be a mechanism by which BH_4 enhances ·NO concentrations. We recently determined the rate constant for the reaction between BH_4 and superoxide to be approx. 10^5 $M^{-1} \cdot s^{-1}$. Our results indicate that BH_4 reacts with superoxide at rates very close to that of ascorbate [20]. However, BH_4 mediates vasorelaxation at much lower concentrations than ascorbate [21]. Thus it is likely that BH_4 acts through mechanisms other than superoxide scavenging.

Previously, we showed that BH_4 decreases the formation of superoxide from the oxygenase domain of NOS [22,23]. This suggested that BH_4, by controlling superoxide generation from eNOS, is important for modulating the ·NO–cGMP pathway. In the present study, we examined the mechanisms by which BH_4 and its analogues (Figure 1) inhibit superoxide release from BH_4-free eNOS. We demonstrated that BH_4, but not L-arginine or L-arginine analogues, such as N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA), completely abolishes superoxide release from eNOS. Unlike BH_4, the oxidized BH_4 analogues 7,8-dihydrobiopterin (7,8-BH_2) and sepiapterin augment superoxide release from eNOS even in the presence of L-arginine. Both 7,8-BH_2 and sepiapterin were shown to antagonize the effects of BH_4 and inhibit ·NO formation. Based on these results, we conclude that the ratio between reduced and oxidized BH_4 metabolites tightly controls superoxide

Abbreviations used: 7,8-BH_2, 7,8-dihydrobiopterin; BH_4, 5,6,7,8-tetrahydrobiopterin; 5Me-BH_4, 5-methyl-BH_4; 6Me-BH_4, 6-methyl-BH_4; DTPA, diethylenetriaminepenta-acetic acid; EMPO, 5-ethoxycarbonyl-5-methyl-pyrroline *N*-oxide; eNOS, endothelial nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine.

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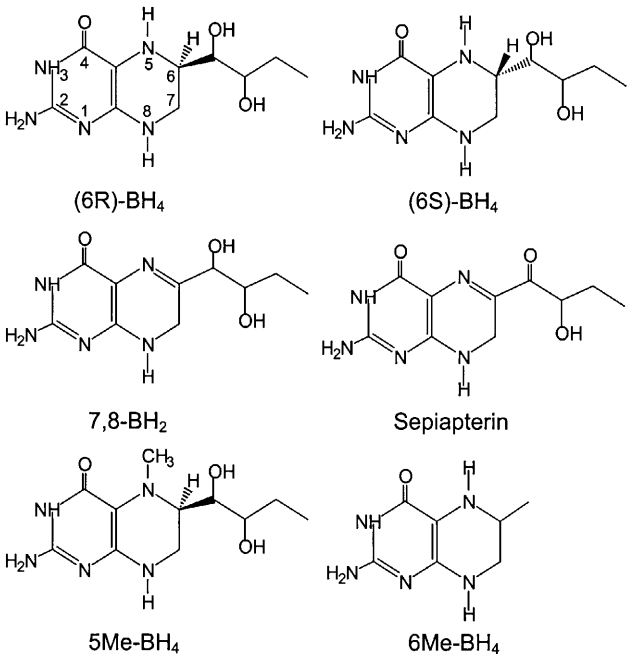


Figure 1 Chemical structures of natural and synthetic pteridines

formation from eNOS. This mechanism suggests that oxidant-induced alterations in intracellular BH_4 levels and/or decreased BH_4 formation play a key role in the aetiology of endothelial dysfunction associated with conditions such as hypertension, hypercholesterolaemia, diabetes and atherosclerosis [7–9].

EXPERIMENTAL

Materials

(6*R*)-BH_4, (6*S*)-BH_4 and 5-methyl-BH_4 (5Me-BH_4) were obtained from Schircks Laboratories (Jona, Switzerland), and 6-methyl-BH_4 (6Me-BH_4) and 7,8-BH_2 were obtained from Alexis Co. (San Diego, CA, U.S.A.). L-[^{14}C]Arginine was obtained from Dupont NEN, and L-arginine, NADPH, calcium chloride, GSH, imidazole and BSA were obtained from Sigma. Haemoglobin was purchased from Calbiochem, and diethylenetriaminepenta-acetic acid (DTPA) was obtained from Fluka Chemika-BioChemika. 5-Ethoxycarbonyl-5-methyl-pyrroline *N*-oxide (EMPO) was synthesized as previously described [24], and kept at -80 °C until used. Recombinant wild-type eNOS was purified in the absence of BH_4 as previously described [22], and its protein concentration was determined, based on haem content, by reduced carbon monoxide difference spectra using an absorption coefficient of 100 $mM^{-1} \cdot cm^{-1}$ for an absorbance difference between 444 and 475 nm. The average haem content of the protein was $>85\%$. Three different protein batches that had an enzyme activity ranging from 180 to 100 nmol of L-[^{14}C]citrulline/min per mg of protein were used in the present study.

EPR measurements

EPR measurements were recorded at approx. 25 °C on a Varian E-109 spectrometer operating at 9.01 GHz and 100 kHz field modulation equipped with a loop gap resonator [25]. This cavity enables the analysis of a sample volume of $<10\ \mu$l. Reactions (20 μl, final volume) were initiated by the addition of enzyme, BH_4-free eNOS or xanthine oxidase, to incubation mixtures containing 50 mM EMPO spin trap. EPR spectra were scanned for 100 G (*x*-axis) with a signal (*y*-axis) receiver gain of 5×10^4. The concentration of superoxide adduct was calculated by double integration of the simulated spectra, using EPR software by D. R. Duling [26]. 3-Carbamoyl-2,2,5,5,-tetrametlypyrrolidine *N*-oxyl was used as a standard.

Biochemical assays

eNOS activity

Enzyme activity was determined by either quantifying the conversion of L-[^{14}C]arginine into L-[^{14}C]citrulline as previously described [20], or by the haemoglobin assay. Briefly, eNOS was added to a reaction mixture containing NADPH (0.1 mM), L-arginine (40 μM), calcium (0.2 mM), calmodulin (20 μg/ml), GSH (0.1 mM), BSA (0.1 mg/ml), BH_4 (10 μM, unless otherwise specified), DTPA (0.1 mM), oxy-haemoglobin (approx. 8 μM) and Hepes (50 mM, pH 7.4). Oxidation of oxy-haemoglobin was followed at 401 nm for 1 min at approx. 25 °C and $^{\bullet}$NO formation was calculated using an absorption coefficient of 38 $mM^{-1} \cdot cm^{-1}$.

NADPH consumption

Initial rates of NADPH oxidation by eNOS were determined at 340 nm. NADPH concentrations were calculated using an absorption coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$. Reactions were initiated by adding NADPH (approx. 0.18 mM) to reaction mixtures (final volume, 0.25 ml) containing eNOS (approx. 2.5 μg), DTPA (0.1 mM), calcium (0.2 mM), calmodulin (20 μg/ml) and other additions in Hepes buffer (50 mM, pH 7.4)

RESULTS

Only fully reduced BH_4 inhibits superoxide release from eNOS

Figure 2(A) shows the EMPO-superoxide adduct (EMPO-OOH) detected upon activation of BH_4-free eNOS with calcium/calmodulin. This result is consistent with our previous data using the phosphorylated spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide ('DEPMPO') [21]. In the present study we used EMPO because it produces a more simple EPR spectrum, facilitating superoxide quantification [24,27]. Formation of EMPO-OOH from eNOS was inhibitable by 1 mM cyanide (results not shown). This further corroborates our previous findings, which indicate that superoxide is released from the haem group in the oxygenase domain of the enzyme [22].

An important role of BH_4 in the catalytic cycle of NOS is to control haem reactivity [28,29]. To examine how pteridines (Figure 1) affect the release of superoxide from the haem group in eNOS, BH_4-free enzyme was reconstituted with BH_4 and synthetic BH_4 analogues, such as 6Me-BH_4 and 5Me-BH_4. The activity of these reduced pteridines on superoxide release was compared with partially oxidized BH_4 analogues, such as 7,8-BH_2 and sepiapterin. As shown in Figure 2, 7,8-BH_2 (Figure 2B) and sepiapterin (results not shown) did not diminish superoxide from eNOS. By contrast, 5Me-BH_4 (Figure 2C), 6Me-BH_4 (Figure 2D) and BH_4 (Figure 2E) significantly diminished superoxide in a concentration-dependent fashion. At a concentration of 10 μM, 5Me-BH_4 inhibited superoxide by approx. 7% (results not shown), whereas inhibition by 6Me-BH_4 was approx. 80%, and BH_4 completely abolished superoxide formation. These results demonstrate that only fully reduced pteridines inhibit superoxide release from eNOS and suggest that the affinity of the pteridine for the eNOS binding

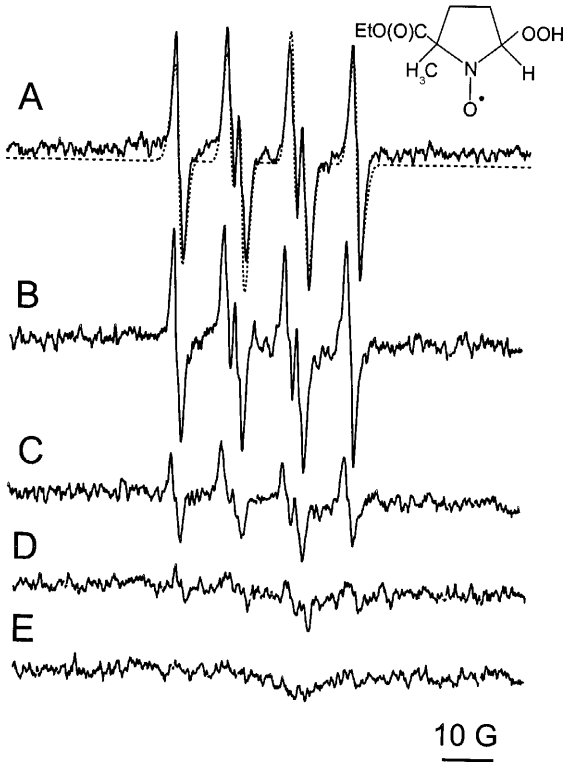


Figure 2 Inhibition of superoxide release from eNOS by BH_4 and its analogues

(**A**) Superoxide was detected with EMPO (50 mM) in a 20 μl incubation mixture of eNOS (1 μg), calcium (0.2 mM), calmodulin (20 μg/ml), NADPH (0.1 mM) in Hepes buffer (50 mM, pH 7.4) containing DTPA (0.1 mM) at approx. 25 °C. The broken line corresponds to the experimental spectrum which was computer-simulated considering two diastereomers with the following hyperfine coupling constants (in Gauss): isomer 1 (94%), $a^N = 12.8$ and $a^H_\beta = 9.83$; isomer 2 (6.1%), $a^N = 12.8$, $a^H_\beta = 12.1$ and $a^H_\gamma = 0.15$. (**B**) As in (**A**) with 0.1 mM 7,8-BH_2. (**C**) As in (**A**) with 50 μM 5Me-BH_4. (**D**) As in (**A**) with 10 μM 6Me-BH_4. (**E**) As in (**A**) with 10 μM BH_4. Instrumental conditions: microwave power, 2 mW; modulation amplitude, 1 G; time constant, 0.128 s; scan rate, 1.67 G/s; gain, 5×10^4; number of scans, 3.

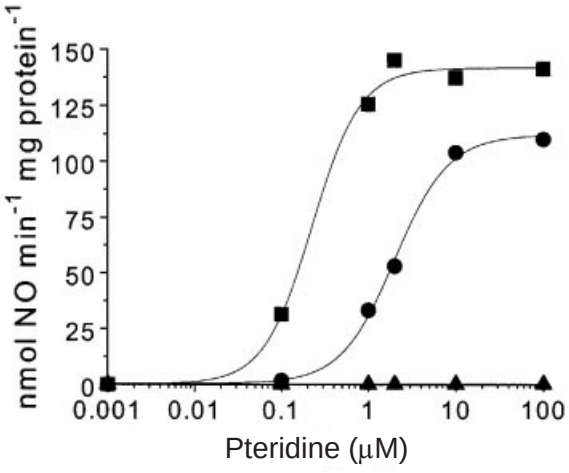


Figure 3 Effect of BH_4 diastereomers and 7,8-BH_2 on the formation of •NO by eNOS

Pterin-free eNOS (2.6 μg) was incubated in the presence of (■) (6*R*)-BH_4, (●) (6*S*)-BH_4 or (▲) 7,8-BH_2 at the indicated concentrations. Formation of •NO was measured by the haemoglobin assay as described in the Experimental section. Data represent the means of at least two independent experiments using different enzyme batches.

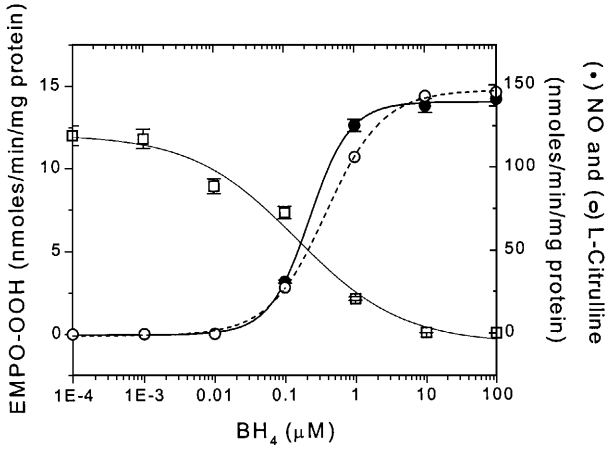


Figure 4 BH_4-dependent superoxide and •NO formation from eNOS

Superoxide formation (□; left-hand axis) was quantified with EMPO (50 mM) added to eNOS incubation mixtures containing calcium (0.2 mM), calmodulin (20 μg/ml), NADPH (0.1 mM), DTPA (0.1 mM) and (6*R*)-BH_4 at the indicated concentrations in Hepes buffer (50 mM, pH 7.4), in a final volume of 20 μl, at approx. 25 °C. Rates of EMPO-OOH formation were determined as described in the Experimental section. •NO (●; right-hand axis) and L-citrulline (○; right-hand axis) were measured upon addition of L-arginine (0.1 mM) to identical eNOS incubations used in the superoxide measurements as described in the Experimental section.

site determines the efficiency with which these analogues inhibit superoxide.

To characterize the role of pteridine binding in the regulation of both superoxide and •NO generation from eNOS, the effects of (6*R*)- and (6*S*)-BH_4 diastereomers were examined. As shown in Figure 3, (6*R*)-BH_4 maximally stimulated eNOS to generate 145.2 ± 4.1 nmol of •NO/min per mg of protein, whereas its diastereomer, (6*S*)-BH_4, supported only 75% of the maximal activity even when added at concentrations approx. 30-fold higher than eNOS. Parallel experiments demonstrated that a 2-fold higher concentration of (6*S*)-BH_4 was necessary to diminish superoxide at a similar level to (6*R*)-BH_4 (results not shown). These results demonstrate that (6*R*)-BH_4 specifically inhibits superoxide release from eNOS with an efficiency that correlates with its high affinity for the enzyme. In addition, these results suggest that there is a strong association between the potency of inhibition of superoxide and stimulation of •NO formation. This relationship was further confirmed with 5Me-BH_4, which did not efficiently inhibit superoxide release from eNOS. Accordingly, 5Me-BH_4 (10 μM) stimulated only 20% of the maximal rates of •NO formation obtained with BH_4 (10 μM). Higher 5Me-BH_4 concentrations (100 μM) were necessary to reach 80% of the maximal activity.

The quantitative relationship between superoxide, •NO formation and BH_4 was examined in parallel incubations. Rates of superoxide formation were quantified by measuring EMPO-OOH. Activation of L-arginine and BH_4-free eNOS with calcium/calmodulin in the presence of EMPO generated 11.8 nmol of EMPO-OOH/min per mg of protein. This was equivalent to the rate of EMPO-OOH formation by a xanthine/xanthine oxidase system producing 0.126 nmol of superoxide/min. BH_4-free eNOS was subsequently shown to generate 97.7 nmol of superoxide/min per mg of protein at approx. 25 °C. Reconstitution of eNOS with BH_4 diminished superoxide formation, with an IC_{50} of approx. 0.14 μM; at a concentration 10 times higher, BH_4 abolished superoxide formation (Figure 4). In the presence of L-arginine, BH_4 stimulated the formation of •NO with a half-

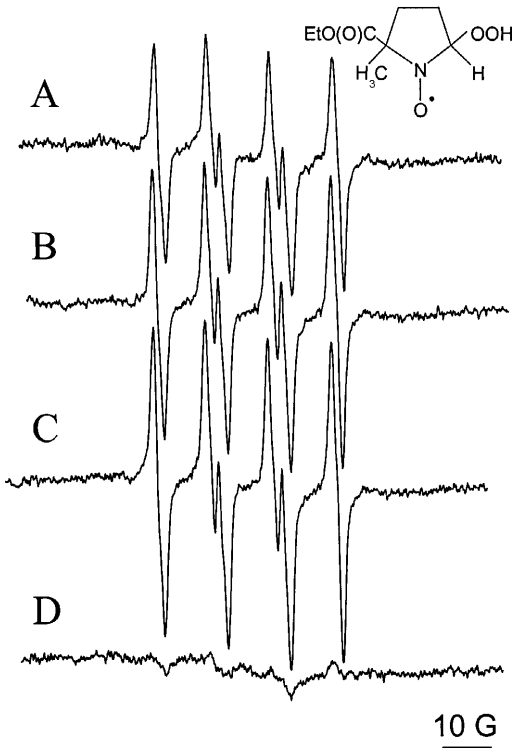


Figure 5 L-Arginine enhances superoxide generation from eNOS

(**A**) eNOS (1.3 μg) was incubated with calcium (0.2 mM), calmodulin (20 μg/ml), NADPH (0.1 mM), EMPO (50 mM) and DTPA (0.1 mM) in Hepes buffer (50 mM, pH 7.4). (**B**) As in (**A**), except eNOS (1.3 μg) was incubated with L-arginine (0.1 mM) for 10 min prior to activation. (**C**) As in (**B**) plus 7,8-BH_2 (10 μM). (**D**) As in (**B**) plus BH_4 (10 μM). Instrument conditions were identical with those described in the legend to Figure 2.

maximal effective dose of approx. 0.25 μM; maximal rates of $^{\bullet}$NO formation were attained at BH_4 concentrations of 10 μM. This result suggested that BH_4 inhibits superoxide formation from eNOS more efficiently than it stimulates $^{\bullet}$NO formation. Since detection of $^{\bullet}$NO by the haemoglobin assay can be underestimated under conditions where both $^{\bullet}$NO and superoxide are generated, we investigated the effect of BH_4 on eNOS activity by following L-[^{14}C]citrulline formation. Essentially the same pattern observed for $^{\bullet}$NO formation (Figure 4) was obtained when L-[^{14}C]citrulline was quantified (Figure 4), indicating that the concomitant formation of $^{\bullet}$NO and superoxide by eNOS is unlikely.

L-Arginine and its analogues do not prevent superoxide release from eNOS

Spin trapping experiments demonstrated that L-arginine did not inhibit superoxide release from BH_4-free eNOS (Figure 5A). By contrast, formation of superoxide was enhanced by approx. 60% when the enzyme was incubated with L-arginine prior to activation with calcium/calmodulin (Figure 5B). In combination with 7,8-BH_2 (Figure 5C) or sepiapterin (results not shown), L-arginine stimulated superoxide formation to the same extent as in the absence of the pteridines (Figure 5B). Previous results by others showed that L-arginine enhances uncoupled oxidation of NADPH [29]. Thus it is likely that L-arginine binding facilitates iron haem reduction that facilitates oxygen activation and superoxide formation. In the presence of BH_4, the process results in increased oxidation of L-arginine to $^{\bullet}$NO and L-citrulline, thereby preventing superoxide formation (Figure 5D).

To understand whether occupation of the L-arginine binding site controls the rate of electron transfer from the reductase domain to the haem group, we examined the effects of L-arginine analogues on the rate of NADPH consumption. L-NAME and L-NMMA are competitive eNOS inhibitors that have a high affinity for eNOS (IC_{50} = 0.5–0.7 μM). As shown in Table 1, L-NAME inhibited NADPH consumption in a concentration-dependent manner, reaching a maximum of approx. 32% inhibition at approx. 1 mM concentration. L-NMMA had a

Table 1 Effect of L-arginine and L-arginine analogues on NADPH oxidation by eNOS

eNOS (approx. 2.5 μg) was incubated with calcium (0.2 mM), calmodulin (20 μg/ml) and DTPA (0.1 mM) in Hepes buffer (50 mM, pH 7.4), in a final volume of 0.25 ml, at room temperature. Two different eNOS batches were used in these experiments, which had an activity of approx. 130 nmol of L-[^{14}C]citrulline/min per mg of protein. BH_4 (1 μM) was added immediately before activation of eNOS with calcium/calmodulin. n.d., not determined.

	NADPH consumption (nmol/min per mg of protein)	
Additions	$-BH_4$	$+BH_4$
None	16.0	16.0
eNOS	154	138
eNOS + L-NAME (0.1 mM)	114	n.d.
eNOS + L-NAME (1.0 mM)	106	96
eNOS + L-NMMA (0.1 mM)	160	125
eNOS + imidazole (0.1 mM)	68	78
eNOS + 7-nitroindazole (0.1 mM)	74	76
eNOS + L-arginine (0.1 mM)	215	205

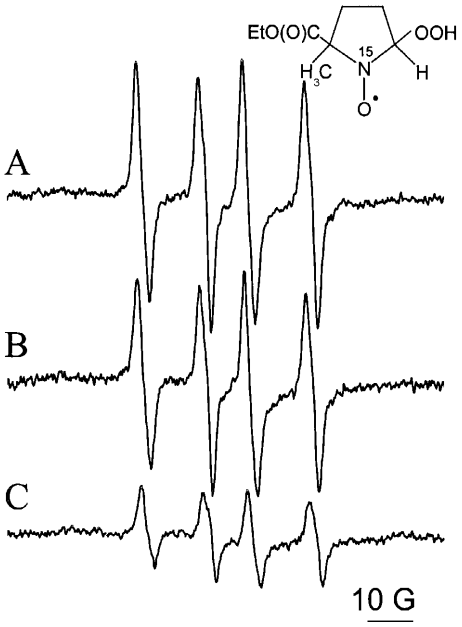


Figure 6 Effect of NOS inhibitors on superoxide release from eNOS

(**A**) eNOS (1.3 μg) was incubated with calcium (0.2 mM), calmodulin (20 μg/ml), NADPH (0.1 mM), DTPA (0.1 mM) and [^{15}N]EMPO (25 mM) in Hepes buffer (50 mM, pH 7.4). (**B**) As in (**A**) plus 1 mM L-NAME. (**C**) As in (**A**) plus 0.1 mM imidazole. Instrument conditions were identical with those described in the legend to Figure 2.

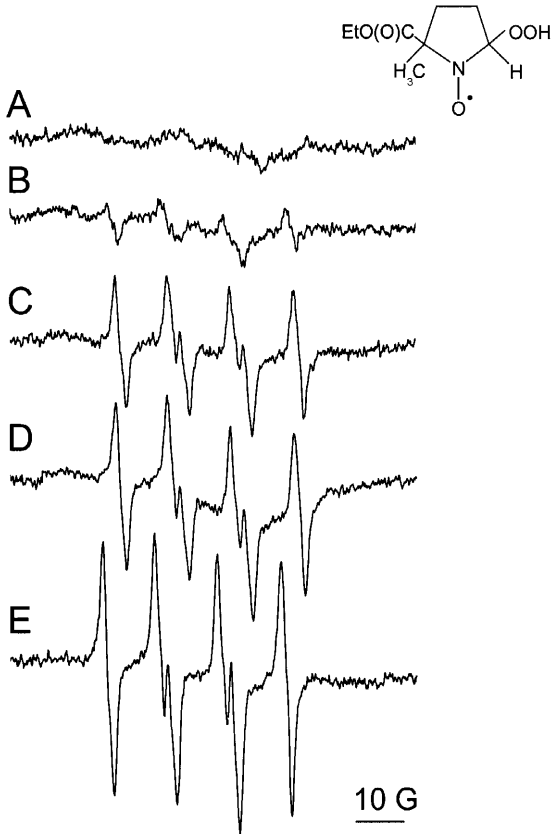


Figure 7 Augmentation of superoxide formation from eNOS by 7,8-BH_2 and sepiapterin

(**A**) eNOS (2.5 μg) was incubated with L-arginine (0.1 mM), BH_4 (2 μM), NADPH (0.1 mM), calmodulin (20 μg/ml), calcium (0.2 mM), DTPA (0.1 mM) and EMPO (50 mM) in Hepes buffer (50 mM, pH 7.4). (**B**) As in (**A**) plus 7,8-BH_2 (10 μM). (**C**) As in (**A**) plus 7,8-BH_2 (50 μM). (**D**) As in (**A**) plus sepiapterin (150 μM). (**E**) eNOS (1.5 μg) incubated with sepiapterin (100 μM), NADPH (0.1 mM), calcium (0.2 mM) and calmodulin (20 μg/ml) in Hepes buffer. Instrument conditions were identical with those described in the legend to Figure 2.

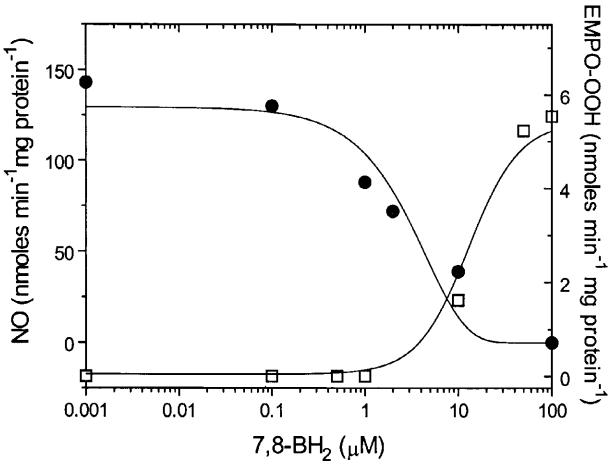


Figure 8 7,8-BH_2 enhances superoxide and inhibits $^{\bullet}$NO formation from eNOS

Superoxide (□, right-hand axis) was quantified with EMPO (50 mM) added to eNOS incubation mixtures containing BH_4 (2 μM), L-arginine (0.1 mM), NADPH (0.1 mM), calcium (0.2 mM), calmodulin (20 μg/ml), DTPA (0.1 mM), Hepes (50 mM, pH 7.4) and 7,8-BH_2 at the indicated concentrations. Rates of EMPO-OOH formation were calculated as described in the Experimental section. Rates of $^{\bullet}$NO formation (●, left-hand axis) were determined using the haemoglobin assay as described in the Experimental section.

marginal effect on the rates of NADPH consumption at all the concentrations tested (0.1–1 mM). These results suggest that occupation of the L-arginine binding site does not enhance NADPH oxidation and consequently superoxide formation from eNOS. Alternatively, this finding suggested that modifications in the iron spin state control the rates of haem reduction. This was verified with imidazole and 7-nitroindazole (IC_{50} values of approx. 0.8 μM), two NOS inhibitors that bind to the iron haem group blocking its reactivity [30]. Both inhibitors efficiently diminished NADPH oxidation (>50% at 0.1 mM; Table 1). These results demonstrate that L-arginine enhances NADPH oxidation by facilitating haem reduction and thereby oxygen binding. Identical experiments using BH_4-replenished enzyme were performed to examine how BH_4 modifies NADPH consumption in the presence of L-arginine analogues. As shown in Table 1, addition of BH_4 did not greatly affect NADPH oxidation under any of the tested conditions.

In contrast with the results described above, we have shown that L-arginine and its analogues inhibit superoxide release from the neuronal isoform of NOS [21]. To investigate whether the inhibitory effects of L-arginine analogues on superoxide release from eNOS were more discrete than with the neuronal isoform of NOS, superoxide measurements were performed using [^{15}N]-EMPO [27]. This labelled spin trap enhances the sensitivity of the assay by producing a four-line EPR spectrum of the [^{15}N]EMPO superoxide adduct ([^{15}N]EMPO-OOH), compared with the six lines of [^{14}N]EMPO-OOH. As shown in Figure 6, L-NAME (1 mM) inhibited [^{15}N]EMPO-OOH by only 20%, whereas imidazole (0.1 mM) inhibited [^{15}N]EMPO-OOH by approx. 65%. These results indicate that L-NAME is not an efficient inhibitor of haem iron reduction and superoxide release from eNOS. Also, these results demonstrate that the effects of L-arginine and L-arginine analogues on the control of superoxide release depend on the particular isoform of NOS.

Oxidized BH_4 analogues stimulate superoxide production from eNOS

Dihydrobiopterins, such as 7,8-BH_2 and sepiapterin, bind to eNOS [31], emulating several of the allosteric and electronic effects of BH_4, but they do not support $^{\bullet}$NO formation [32]. Thus we anticipated that displacement of BH_4 from eNOS by its analogues would increase superoxide formation. To examine this possibility, minimal amounts of BH_4 were used to ensure that free BH_4 concentrations were kept at a minimum while stimulating $^{\bullet}$NO formation at rates close to the V_{max}. From the experiments in Figure 4, it was determined that this condition was attained with 2 μM BH_4, which supported >97% $^{\bullet}$NO synthesis with no superoxide formation (Figure 7A). Supplementation with 7,8-BH_2 and sepiapterin, however, caused a robust increase in superoxide formation (Figures 7B–7D). This result indicates that 7,8-BH_2 and sepiapterin compete with BH_4 for the same binding site in eNOS that inhibits $^{\bullet}$NO and enhances superoxide formation. As shown in Figure 8, 7,8-BH_2 inhibited $^{\bullet}$NO formation from an eNOS–BH_4 (2 μM) system with an IC_{50} of 2 μM. This indicates that 7,8-BH_2 has an affinity for the pterin-binding site similar to BH_4, which makes it an efficient uncoupling agent for eNOS. Higher concentrations of 7,8-BH_2 were necessary to detect superoxide, reaching a half-maximum effective dose at

14 μM with maximum amounts of superoxide attained at a concentration of 100 μM 7,8-BH_2 (Figure 8). Superoxide formation from uncoupled eNOS was saturable, reaching 50 % of the maximum rate of superoxide formation corresponding to BH_4-free eNOS.

DISCUSSION

Augmented superoxide formation is becoming increasingly recognized as an aetiological agent of vascular dysfunction associated with chronic conditions such as hypertension, hypercholesterolaemia, diabetes and atherosclerosis [7–9]. Thus there is much interest in identifying the sources and factors that control superoxide formation in the vasculature. In the present study we examined how BH_4 and BH_4 analogues contribute to the regulation of superoxide formation from eNOS. Superoxide was measured by EPR spin trapping, which is the only suitable methodology available to quantify superoxide from eNOS. We used newly synthesized spin traps, EMPO and [^{15}N]EMPO, that produce more simple superoxide spectra and thereby enhance the sensitivity of superoxide measurement.

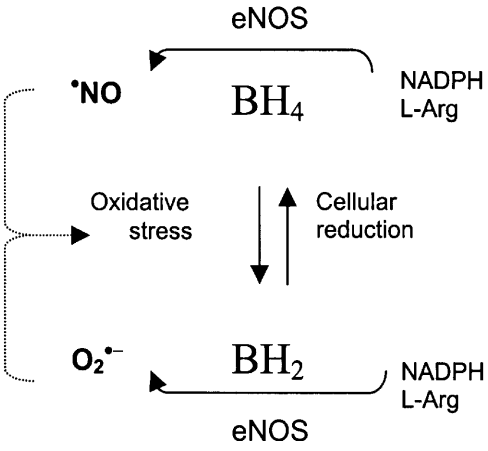
Inhibition of superoxide from eNOS by BH_4

Given that superoxide is the dissociation product of the ferrous-dioxygen species, it is possible to consider that by increasing the stability of the complex BH_4 inhibits superoxide release. This effect should be mirrored by diminished NADPH oxidation from eNOS due to the slower turnover of the haem iron group. However, BH_4 at concentrations approx. 7-fold higher than that necessary to inhibit superoxide release by 50 % (Figure 4) caused only a discrete diminution of the initial rates of NADPH oxidation. This indicates that neither reduction of the haem group nor dissociation of the ferrous-dioxygen complex greatly contributes to the mechanism by which BH_4 inhibits superoxide formation.

BH_4 may also accelerate the reduction of the ferrous-dioxygen species, generating the haem-peroxo complex that can decompose to generate hydrogen peroxide [22,33]. However, hydrogen peroxide formation was marginal at all of the BH_4 concentrations tested (0–100 μM; results not shown). In addition to increasing hydrogen peroxide formation, the reduction of the ferrous-dioxygen complex by BH_4 has been suggested to generate the trihydrobiopterin cation radical ($BH_4^{\bullet+}$). This radical has been detected from the oxygenase domain of eNOS with yields significantly lower in the absence of substrate [34]. Formation of $BH_4^{\bullet+}$ from the reaction between BH_4 and the ferrous-dioxygen complex is suggested by the inhibition of superoxide release from eNOS in the presence of BH_4 and fully reduced BH_4 analogues, such as 5Me-BH_4 and 6Me-BH_4. The parallel between inhibition of superoxide and formation of $BH_4^{\bullet+}$ suggests that BH_4 should increase formation of the haem-peroxo compound. However, the fate of the haem-peroxo species in the absence of substrate remains unclear. Together, our results suggest that BH_4 inhibits superoxide release from eNOS by two concerted mechanisms, stabilization and reduction of the haem-dioxygen complex.

Augmentation of superoxide release by oxidized BH_4 analogues

Superoxide formation from eNOS was stimulated by oxidized BH_4 analogues, even in the presence of BH_4. Competition experiments demonstrate that displacement of BH_4 from eNOS by 7,8-BH_2 and sepiapterin inhibits $^{\bullet}$NO formation while enhancing superoxide release. However, 7,8-BH_2-supplemented eNOS generated half of the superoxide yields that were detected from BH_4-free enzyme. It is possible that the competition between 7,8-BH_2 and BH_4 will favour both $^{\bullet}$NO and superoxide generation. As superoxide reacts with EMPO (approx. 10 $M^{-1} \cdot s^{-1}$) more slowly than with $^{\bullet}$NO (approx. 10^9 $M^{-1} \cdot s^{-1}$), it is likely that superoxide will be detected only when $^{\bullet}$NO formation has been significantly inhibited. This reaction could explain why 7,8-BH_2 appears to inhibit $^{\bullet}$NO formation more efficiently than it simulates superoxide formation.



Scheme 1 BH_4/BH_2 ratio controls $^{\bullet}$NO and superoxide generation from eNOS

A second explanation for the 50 % yield of superoxide at saturating 7,8-BH_2 concentrations could be that the two pterin-binding sites of the eNOS dimer present different affinities for pteridines. If one site presents a higher affinity for BH_4 than the second site, 7,8-BH_2 will displace only one of the cofactor molecules. This competition would lead one BH_4–eNOS monomer to generate $^{\bullet}$NO and citrulline while the other monomer containing 7,8-BH_2 generates superoxide. However, this possibility was ruled out because L-citrulline could not be detected in incubations with 7,8-BH_2 20 μM (results not shown). Altogether, our results demonstrate that full occupancy of BH_4–eNOS binding sites is critical for $^{\bullet}$NO formation from eNOS.

BH_4 and endothelial dysfunction

It has been shown that eNOS activity in endothelial cells can be enhanced by increasing intracellular BH_4 concentrations [35–37]. How increases in BH_4 stimulate eNOS activity is not known. We have shown that BH_4 critically controls superoxide release from eNOS regardless of L-arginine concentrations. Thus supplementation with BH_4 will prevent the formation of reactive oxygen species and optimize the formation of $^{\bullet}$NO. By contrast, accumulation of oxidized BH_4 analogues stimulates the formation of superoxide from eNOS. Thus elevation of BH_4 concentrations may be crucial either to saturate eNOS that is partially deficient in BH_4, or to compete with oxidized BH_4 metabolites for the eNOS binding site, thereby decreasing superoxide formation and enhancing $^{\bullet}$NO generation.

Sepiapterin and 7,8-BH_2 stimulate superoxide formation from eNOS while inhibiting $^{\bullet}$NO generation from an otherwise fully-coupled enzyme. This suggests that it is the balance between reduced and oxidized BH_4 that critically controls eNOS activity (Scheme 1), and indicates that BH_4 is a key redox switch controlling superoxide formation from eNOS. The importance of this mechanism to the pathogenesis of vascular diseases warrants investigation. It can be anticipated that in hypertension,

hypercholesterolaemia and diabetes, increased oxidation of BH_4 and/or impaired BH_4 formation will enhance superoxide formation by an eNOS-dependent mechanism.

Summary

Superoxide release from eNOS is critically controlled by the ratio between fully reduced and oxidized BH_4 analogues regardless of L-arginine concentrations. The role of the BH_4 pathway in the regulation of superoxide formation from eNOS and the pathogenesis of vascular diseases remains to be established.

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