Tetrahydrobiopterin-dependent Inhibition of Superoxide Generation from Neuronal Nitric Oxide Synthase*

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Jeanette Vásquez-Vivar‡§, Neil Hogg‡, Pavel Martásek‡, Hakim Karouï, Kirk A. Pritchard Jr. §, and Balarama Kalyanaraman**

From the ‡Department of Pathology, Cardiovascular Research Center and §Biophysics Research Institute, Medical College of Wisconsin, Milwaukwe, Wisconsin 53226, ‡Laboratoire Structure et Réactivité des Éspèces Paramagnétiques, CNRS URA 1412, Université de Provence, 13397, Marseilles Cedex 20, France, and the **Biochemistry Department, University of Texas Health Science Center, San Antonio, Texas 78284-7760

The binding of calcium/calmodulin stimulates electron transfer between the reductase and oxygenase domains of neuronal nitric oxide synthase (nNOS). Here, we demonstrate using electron spin resonance spin trapping with 5-dietioxyphosphoryl-5-methyl-1-pyrroline N-oxide that pterin-free nNOS generates superoxide from the reductase and the oxygenase domain by a calcium/calmodulin-dependent mechanism. Tetrahydrobiopterin (BH₄) diminishes the formation of superoxide by a mechanism that does not cause inhibition of NADPH consumption. In contrast, BH₄ analogs 7,8-dihydrobiopterin and sepiapterin do not affect superoxide yields. L-Arginine alone inhibits the generation of superoxide by nNOS but not by C331A-nNOS mutant that has a low affinity for L-arginine. A greater decrease in superoxide yields is observed when nNOS is preincubated with L-arginine. This effect is in accordance with the slow binding rates of L-arginine to NOS in the absence of DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DTPA, diethylenetriaminepentaacetic acid; eNOS, endothelial nitric oxide synthase; NOS, neuronal nitric oxide synthase; DMPO, 5,5′-dimethyl-1-pyrroline N-oxide.

Tetrahydrobiopterin (BH₄) plays important roles in several biological processes such as metabolism (1, 2), brain function (3), immune response (4, 5), cell proliferation (6), and vascular homeostasis (7–10). These roles have been linked to the ability of BH₄ to regulate the production of active metabolites by several enzymes. Tetrahydrobiopterin and pterin analogs including 6-methyltetrahydrobiopterin (Scheme I) serve as reductase-active cofactors of hydroxylases such as phenylalanine, tyrosine, and tryptophan hydroxylases (11, 12). Tetrahydrobiopterin is also an important cofactor of nitric oxide synthase (NOS). However, the role of this cofactor in the control of NOS activity is still unclear (13–15).

The catalytic domain of nitric oxide synthase (NOS) comprises an NADPH-binding reductase, a heme-binding oxygenase domain, and a calmodulin binding sequence. Upon binding of the calcium/calmodulin complex, electrons flow from the reductase domain to the oxygenase domain resulting in the activation of the enzyme. We recently have demonstrated that activation of the endothelial isoform of NOS (eNOS) under limited availability of BH₄ leads to the generation of superoxide from the oxygenase domain by a calcium/calmodulin-dependent mechanism (16). Previous studies with the neuronal isoform (nNOS) showed that activation of the enzyme in the absence of L-arginine generates superoxide (17–19). Because the formation of superoxide was calcium/calmodulin-dependent, it was suggested that superoxide was formed at the oxygenase domain of nNOS (17–19). However, by using genetically engineered nNOS constructs it has been shown that the reductase domain of nNOS generates superoxide, by a calcium/calmodulin-dependent mechanism (20). Recently, it has been demonstrated that the P450 reductase-like activity of the nNOS reductase domain is comparable to iNOS (21). This raises the question of what regulates superoxide generation from the reductase domain of nNOS.

The ability of NOS to reduce and/or release superoxide in one-electron reduction reactions with substrates such as cytochrome c, lucigenin, and nitro blue tetrazolium has precluded the use of superoxide assays that involve these compounds. Although superoxide dismutase-inhibitable inactivation of acinetase enzyme can be used to quantify superoxide, recent data indicate that peroxynitrite also can inhibit acinetase activity. Thus, it appears that electron spin resonance (ESR) technique remains as the only viable alternative to detect and to quantify superoxide generated from NOS.

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**Scheme 1.** Chemical structure of tetrahydropterin and analogs.

commonly used trap. In addition, unlike the superoxide adduct of DMPO, DEPMPO-OOH does not spontaneously decay to the corresponding DEPMPO-hydroxyl radical adduct (DEPMPO-OH). As a result, the steady state concentration of DEPMPO-OOH is higher than DMPO-OOH adduct at the same rate of superoxide generation. In order to conserve the spin trap and NOS enzyme, which are both expensive and scarce, we have utilized the loop gap resonator (23). The loop gap resonator is a device that allows ESR measurements of exceedingly small reaction volumes ($\approx 10\mu l$) which brings the sensitivity limit down from 250 to 2–5 pmol. This allows many more experiments to be performed with small amount of enzyme preparation.

Evidence indicates that BH$_4$ plays a critical role not only in increasing the rate of nitric oxide (NO) (16, 24, 25) generation by NOS but also in controlling the formation of superoxide (16). Tetrahydrobiopterin and 7,8-dihydroxybiopterin and N$_2$H$_4$-hydroxy-L-arginine were added to nNOS incubation mixtures (24, 25). Low hydrogen peroxide levels were detected in incubations of nNOS supplemented with non-saturating concentrations of BH$_4$ (24, 25). In this work, we investigated the effects of BH$_4$ and BH$_3$ analogs (Scheme I) on the generation of superoxide by nNOS using recombinant wild type BH$_4$-free enzyme. Our results indicate that in the absence of L-arginine, BH$_4$ inhibits the generation of superoxide without inhibiting NADPH consumption. In the presence of L-arginine, BH$_3$ enhances L-arginine binding and couples NADPH to L-arginine oxidation, which enhances NO generation. The effects of BH$_4$ on superoxide generation by nNOS indicate that this cofactor may be essential to drive the synthesis of L-citrulline by preventing the dissociation of the heme-ferrous dioxygen complex and by promoting the generation of the heme-peroxo species.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bovine Cu,Zn-superoxide dismutase (0.1 unit/mg) was obtained from Roche Molecular Biochemicals, and diethylenetriaminepentacetic acid (DTPA) was obtained from Fluka Chemika-BioChemika. (6R)-Tetrahydrobiopterin and 7,8-dihydroxybiopterin and N$_2$H$_4$-hydroxy-L-arginine were obtained from Alexis Co. (San Diego, CA). Sepiapterin was obtained from Cayman (Ann Arbor, MI). L-[1$^{13}$C]Arginine was obtained from NEN Life Science Products. NADPH, L-arginine, calcium chloride, EGTA, FMN, FAD, GSH, imidazole, and bovine serum albumin were obtained from Sigma. DEPMPO was synthesized as described (22). Recombinant wild type neuronal nitric oxide synthase (nNOS) was purified in the absence of BH$_4$ as described previously (26, 27). Plasmid construction, protein expression, and purification of the nNOS-C331A mutant were performed as described (26). The purified protein isolated was BH$_4$-free due to the absence of GTP hydroxylase in *Escherichia coli*. Protein concentrations of nNOS and C331A-nNOS were determined, based on heme content, by reduced carbon monoxide difference spectra using an extinction coefficient of 100 m$^{-1}$ cm$^{-1}$ for an absorbance difference between 444 and 475 nm. The average heme content of the protein was 80%.

**Biochemical Assays**

**nNOS Activity**—nNOS activity was determined by quantifying the conversion of L-[1$^{13}$C]arginine to L-[1$^{13}$C]citrulline as described previously (16). Briefly, nNOS (66.7 nM) was added to reaction mixtures (final volume, 0.20 ml) containing Hapes (50 mM, pH 7.4), DTPA (0.1 mM), L-[1$^{13}$C]arginine (0.1 mM, 0.625 nCi), NADPH (0.3–0.5 mM), calcium chloride (0.2 mM), calmodulin (20 $\mu$g/ml), BH$_4$ (10 $\mu$M), GSH (100 $\mu$M), and bovine serum albumin (200 $\mu$g/ml). To stop the reaction, an aliquot of the reaction mixture (50 $\mu$l) was added to aliquots (75 $\mu$l) of nNOS incubations mixed with DTPA (1 mM) and chilled on ice. L-[1$^{13}$C]Citrulline was isolated from the excess of L-[1$^{13}$C]arginine using a Dowex 50W-cation exchange column, and its concentration was determined by liquid scintillation counting.

**Hydrogen Peroxide Generation**—Generation of hydrogen peroxide by nNOS was quantified as described previously (24, 25). Briefly, 100 $\mu$l of H$_2$SO$_4$ (0.3 N) was added to aliquots (75 $\mu$l) of nNOS incubations mixed with DTPA (1 mM) and sodium thiocyanate (NaSCN, 6 nM). Absorbances were read at 480 nm, and the concentration of hydrogen peroxide was calculated from calibration curves.

**NADPH Consumption**—Initial rates of NADPH oxidation were determined spectrophotometrically at 340 nm. NADPH concentration was calculated using a molar extinction coefficient of 6.22 m$^{-1}$ cm$^{-1}$. Reactions were initiated by adding NADPH (~ 18 mmoles) to reaction mixtures (final volume, 0.25 ml) containing nNOS (16.7 nM), DTPA (0.1 mM), L-arginine, BH$_4$, and BH$_3$ analogs in Hapes buffer (50 mM, pH 7.4).

**Electron Spin Resonance Measurements**

Electron spin resonance (ESR) spectra were recorded at room temperature on a Varian E-109 spectrometer operating at 9.03-GHz and 100-kHz field modulation equipped with a loop-gap resonator (22). Typically samples were analyzed using a microwave power, 2 milliwatts; modulation amplitude, 1 G; time constant, 0.064 s; scan rate, 1.67 G/s; number of scans, 5 unless otherwise specified. nNOS was added to incubation mixtures (20 $\mu$l, final volume) containing DEPMPO, and the sample was examined after incubation for 1 min. Quantification of ESR data has an accuracy of 2.5%.

**RESULTS**

**Generation of Superoxide from BH$_4$-Free nNOS**—Activation of BH$_4$-free nNOS with calcium/calmodulin in the presence of the spin-trap DEPMPO led to detection of the DEPMPO-OOH (Fig. 1, trace A), whereas no superoxide adduct was detected in incubation mixtures of resting enzyme (Fig. 1, trace D). Superoxide dismutase (10 $\mu$g/ml) abolished the ESR signal, catalase (10 $\mu$g/ml) had no effect on the signal intensity (data not shown), and simulation of experimental data agreed well with theoretical parameters for DEPMPO-OOH (Fig. 1, trace A) (22). This indicates that the trapped species is superoxide.

To investigate the contribution of the oxygenase domain of nNOS to the total amount of superoxide generated by the enzyme, saturating concentrations of iron ligands such as cyanide and imidazole were added to nNOS incubation mixtures. As shown in Fig. 1 (trace B and trace C) each compound decreased the generation of superoxide by approximately 50%.

This result suggested that nNOS generates superoxide, from the reductase and oxygenase domains, by a calcium/calmodulin-dependent mechanism. To exclude the possibility that cyanide inhibits electron transfer reactions at the reductase domain of nNOS, we compared the levels of superoxide stimulated by FMN acceptor, FMN, in the presence and absence of cyanide. Addition of FMN, but not FAD, to nNOS stimulated the generation of superoxide from the reductase domain of nNOS by a calcium/calmodulin-independent mechanism (Fig. 1, trace E). In the presence of cyanide FMN-stimulated superoxide generation by nNOS was not significantly inhibited (Fig. 1, trace F). This indicates that cyanide at a final concentration of 1 mM did not
NADPH (0.1 mM), DEPMPO (50 mM) in Hepes buffer (50 mM, pH 7.4) containing DTPA (0.1 mM) and calcium/calmodulin. Previous studies have demonstrated that BH₄ decreases the contributions of both reductase and oxygenase domain activities to superoxide formation. Two possible mechanisms for explaining this observation are as follows. (i) Nitric oxide generated from enzyme turnover effectively competes with DEPMPO for superoxide and therefore prevents detection. As shown in Fig. 2 (trace E), superoxide was barely detected in incubations containing 0.1 µM BH₄, a condition that stimulates the generation of NO at a rate of 117.3 nmol min⁻¹ mg protein⁻¹. (ii) The combination of BH₄ and L-arginine may more tightly couple electron transfer between the reductase and oxygenase domains, preventing leakage of electrons to oxygen from the reductase domain.

Previous studies have demonstrated that BH₄ decreases the generation of hydrogen peroxide by nNOS while increasing L-citrulline generation. As shown in Fig. 3, BH₄ dramatically decreased superoxide generation by nNOS (Fig. 3, open squares). In the absence of L-arginine, however, BH₄ did not interfere with electron transfer within the reductase domain. Therefore, inhibition of superoxide from nNOS (i.e., trace B in Fig. 1), in the presence of cyanide, is due to blockage of superoxide generation at the oxygenase domain.

Effect of BH₄ and L-arginine on Superoxide Generation—To investigate the effect of BH₄ on superoxide generation by nNOS, BH₄ was added to the pterin-free enzyme. BH₄ (100 nM) decreased nNOS-dependent DEPMPO-OOH yields by approximately 59% (Fig. 2, trace B; as A in the presence of BH₄, C, as A in the presence of L-arginine (1 mM), D, as A in the presence of BH₄ and L-arginine, E, as A in the presence of BH₄ (100 nM)). As shown in Fig. 2 (trace B), the combination of BH₄ and L-arginine decreased the contributions of both reductase and oxygenase domain activities to superoxide formation. Two possible mechanisms for explaining this observation are as follows. (i) Nitric oxide generated from enzyme turnover effectively competes with DEPMPO for superoxide and therefore prevents detection. As shown in Fig. 2 (trace B), superoxide was barely detected in incubations containing 0.1 µM BH₄, a condition that stimulates the generation of NO at a rate of 117.3 nmol min⁻¹ mg protein⁻¹. (ii) The combination of BH₄ and L-arginine may more tightly couple electron transfer between the reductase and oxygenase domains, preventing leakage of electrons to oxygen from the reductase domain.

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diminish the generation of hydrogen peroxide. nNOS generated 2.0 and 2.3 μmol of H₂O₂ min⁻¹ mg protein⁻¹ in the absence and presence of 1 μM BH₄, respectively. Similar results were obtained in the presence of higher amounts of BH₄ (10 μM) indicating that under our experimental conditions, i.e. in the presence of the iron chelator DTPA, hydrogen peroxide formation was not enhanced due to BH₄ autoxidation. In the presence of L-arginine (0.1 mM), BH₄ further decreased superoxide generation (Fig. 3, closed circles) and increased the specific activity of nNOS from 68.4 to 267.2 nmol of L-citrulline min⁻¹ mg protein⁻¹ at 0.01 and 10 μM BH₄, respectively. These results demonstrate that generation of nitric oxide diminishes superoxide levels from nNOS via a minor pathway and that the major effect is due to binding of BH₄. It is likely that BH₄ controls superoxide generation by nNOS as a result of the stabilization of the heme-ferrous dioxygen complex and/or by the coupling of electron transfer between the reductase and oxygenase domain, thereby preventing reduction of oxygen at the reductase domain of the enzyme. Thus, it would be expected that both mechanisms lead to a decreased NADPH consumption by the enzyme.

The effect of L-arginine and BH₄ alone on superoxide levels indicates that binding of these compounds to the oxygenase domain of the enzyme controls oxygen reduction at the heme. To assess this possibility further, we examined the generation of superoxide by C331A-nNOS, which possesses a low affinity for L-arginine and BH₄ (27). As shown in Fig. 4, trace A, BH₄-free C331A-nNOS generated lower levels of superoxide than wild type nNOS (approximately 31%, cf. Fig. 2, trace A). Preincubation of the enzyme with L-arginine (1 mM) for 10 min decreased superoxide by 28% (Fig. 4, trace B) rather than 67% with wild type enzyme. In addition, BH₄ (100 nM) decreased superoxide yields by 44% (Fig. 4, trace C) rather than 59% with wild type enzyme. This result demonstrated that occupation of both BH₄- and L-arginine-binding sites at the oxygenase domain of nNOS is a key step to inhibit the generation of superoxide. This suggests that L-arginine modulates the heme reduction and therefore the electron transfer rate between the reductase and oxygenase domains.

**Effect of BH₄ Analogs on Superoxide Yields—**To demonstrate further that occupation of the BH₄-binding site is important to control formation and stability of the heme-ferrous dioxygen complex in nNOS, we compared the effect of BH₄ analogs such as sepiapterin and 7,8-dihydrobiopterin (7,8-BH₂) on superoxide formation. Addition of 10 μM 7,8-BH₂ (Fig. 5, trace B) caused a 30% decrease of superoxide yields, compared with a 59% decrease caused by 0.1 μM BH₄ (Fig. 2, trace B). In the presence of L-arginine, 7,8-BH₂ decreased the generation of DEPMPO-OOH by approximately 57% (Fig. 5, trace C), compared with the control (Fig. 5, trace A). Under these conditions, the citrulline forming activity of nNOS was 14.8 nmol min⁻¹ mg protein⁻¹, a value 18 times lower than that measured with BH₄. This suggests that minimal amounts of nitric oxide are generated in the presence of 7,8-BH₂. Parallel control experiments demonstrated that 7,8-BH₂ alone or in combination with L-arginine did not decrease superoxide levels generated by the xanthine/xanthine oxidase system (not shown). Taken together, these results suggest that 7,8-BH₂, in the presence of L-arginine, does not inhibit superoxide detection by either direct scavenging of superoxide or by stimulating the generation of NO. It is likely, therefore, that inhibition occurs by increasing the binding of L-arginine to nNOS. Similar results were obtained for incubations of nNOS with sepiapterin. As shown in Fig. 5 (trace D) sepiapterin did not decrease superoxide yields from nNOS. In combination with L-arginine (0.1 mM), however, sepiapterin decreased superoxide yields by approximately 30% (Fig. 5, trace E), and this inhibition was comparable to that caused by L-arginine alone. Studies on the effect of BH₄ on L-arginine binding to nNOS have demonstrated that BH₄ acts as an allosteric effector of nNOS increasing the rate of binding and decreasing the rate of dissociation of L-arginine from the oxygenase domain of nNOS (25, 28). Our results demonstrate that BH₄ analogs such as 7,8-BH₂ and sepiapterin also facilitate the binding of L-arginine and that binding of L-arginine inhibits superoxide generation by nNOS.

**Effect of BH₄ and Analogs on NADPH Consumption—**We
have demonstrated that BH4 and L-arginine inhibit superoxide production from nNOS, although at vastly different concentrations. In addition, these compounds in conjunction have a greater effect than each compound alone. To investigate whether BH4 and L-arginine inhibit total electron flow through the enzyme, the effects of these compounds on NADPH consumption were examined. As shown in Fig. 6 (trace ●), NADPH consumption by BH4-free nNOS was enhanced by calcium/calmodulin. Under these conditions, the rate of NADPH consumption was nonlinear, indicating apparent inactivation of nNOS over 20 min. Addition of BH4 did not affect initial rates of NADPH consumption but slowed the rate of inactivation. Consequently, the total amount of NADPH consumed over 20 min was increased by 24% with 0.1 mM BH4 (Fig. 6, □) and 44% with 10 μM BH4 where the rate of NADPH consumption was linear (not shown). To investigate whether this effect was due only to occupation of the bipterin-binding site, the effect of the BH4 analog 7,8-BH2 was examined as a control. As shown in Fig. 6 (trace □), 7,8-BH2 at concentrations 100-fold higher than BH4 slightly decreased the initial rates of NADPH consumption. However, as observed above for BH4, 7,8-BH2 increased the total amount of NADPH consumed by nNOS (Fig. 6, trace □). After incubating for 20 min, 7,8-BH2 (10 μM) increased the total amount of NADPH consumed approximately 26% compared with calcium/calmodulin. In contrast, activated nNOS incubated with L-arginine (0.1 mM) alone (Fig. 6, trace ●) or in the presence of BH4 (Fig. 6, trace □) decreased the rate of NADPH consumption by approximately 3 times (Fig. 6; trace □ compared with trace ●).

In conclusion, these results indicate that BH4 inhibits superoxide generation by a mechanism that does not involve decreased NADPH oxidation. L-Arginine, however, inhibits the generation of superoxide by reducing the NADPH consumption by the enzyme. In combination, L-arginine and BH4 inhibit superoxide generation by decreasing the rate of NADPH consumption and by generating NO.

**DISCUSSION**

Activation of nNOS with calcium/calmodulin increases the rate of electron transfer between flavin cofactors of the reductase domain and also enhances the rate of reduction of the heme-ion group at the oxygenase domain (29, 30). Evidence indicates that nNOS generates superoxide from the reductase domain of the enzyme (20). Here we report, using a wild type BH4-free enzyme, that nNOS generates superoxide from both the reductase and oxygenase domains by a calcium/calmodulin-dependent mechanism.

**Mechanism of NOS Catalysis—**As shown in Scheme II activation of molecular oxygen is one of the first steps in the catalytic cycle of NOS (31, 32) which is similar in many aspects to the mechanism of catalysis of P450 cytochromes (33). Purified nNOS contains a mixed population of high and low spin states of heme-iron [Fe^{III}\(\nu\)] (Scheme II). L-Arginine and/or BH4 binding to pterin-free nNOS promotes the transition from low to high spin (Scheme II, step I) that facilitates the reduction of heme-ion from ferric [Fe^{III}]1 to the ferrous form [Fe^{II}]2 (Scheme II, step II) (23, 34–36). Oxygen binds to the ferrous-heme group, forming the ferrous-heme-dioxygen complex, [FeO2]32+ , the most stable intermediate of the reaction cycle (Scheme II, step III). This complex is isoelectronic with the ferric superoxide complex [Fe^{III}\(O_2\)]2+. The Effect of BH4—Our results indicate that in the absence of L-arginine and BH4, the [Fe^{III}\(O_2\)]2+ species (Scheme II, step IV) readily dissociate to generate superoxide, regenerating the [Fe^{II}\(H\)]3+ form of the enzyme (Scheme II, step V). Reduction of the heme-ion at the expense of NADPH establishes a cycle that results in the generation of superoxide (Fig. 1 and Fig. 6).

Pterin-free enzyme efficiently binds BH4 to generate a form that exhibits spectral characteristics similar to those observed for the enzyme isolated from eukaryotic cells that contain tightly bound BH4 (25). Recently, Pou et al. (37) reported that BH4 only decreases superoxide generation, using NOS from purified mammalian cell nNOS, at a concentration 1000-fold higher than that used in this study. The addition of 0.01–10 mM

**FIG. 6.** Effect of tetrahydrobiopterin, 7,8-dihydrobiopterin, and L-arginine on NADPH consumption by nNOS. NADPH was added to pterin-free nNOS incubations mixtures in the absence (●) and in the presence (●) of calcium (0.2 mM) and calmodulin (20 μg/ml), DTPA (1 mM) in Hepes buffer (50 mM, pH 7.4) as shown in Fig. 6 (trace ●), in the presence of 7,8-BH2 (10 μM) (●), as in the presence of L-arginine (0.1 mM) (trace ●). As shown in Fig. 6 (trace ●), in the presence of L-arginine (0.1 mM) and BH4 (100 mM).
BH$_4$, in the absence of a metal ion chelator, is likely to lead to artifactual spin-adduct formation (37). Furthermore, the DMPO-OOH radical adduct is unstable making the quantification of superoxide from nNOS more difficult (22, 38). The exact role of BH$_4$ in NOS catalysis is a matter of intense debate in the literature. It has been established that BH$_4$ promotes the transition of the heme group from low to high spin, stabilizes the conformation of the homodimer, and acts as an allosteric effector of NOS enhancing l-arginine binding (25, 39, 40). A combination of these effects may explain why BH$_4$ prevented the inactivation of the enzyme during NADPH consumption in the absence of substrate (Fig. 6). It may be expected that BH$_4$, by preventing the inactivation of the enzyme, should increase the generation of superoxide. However, superoxide formation was dramatically inhibited by very low amounts of BH$_4$ (Figs. 2 and 3). This decrease in superoxide formation cannot be attributed to a decrease in NADPH-dependent oxygen reduction by nNOS as the rate of NADPH oxidation and the formation of hydrogen peroxide were almost unaffected by the addition of BH$_4$. The most plausible mechanism to explain the effect of BH$_4$ on superoxide generation is that BH$_4$ enhances the rate of reduction of the [Fe$^{III}$-O$_2$]$^{2+}$ complex to generate the peroxyl iron complex [Fe$^{III}$-OOH]$^{2+}$ (Scheme II, step VI). By this mechanism, the steady state concentration of the [Fe$^{III}$-O$_2$]$^{2+}$ is decreased, and superoxide generation is reduced (Fig. 3).

In support of this mechanism, it has been recently proposed that BH$_4$ participates in oxygen activation during the catalytic cycle of NOS mediating the one-electron reduction of the [Fe$^{III}$-O$_2$]$^{2+}$ to generate [Fe$^{III}$-OOH]$^{2+}$ (Scheme II, step VI) (35). This mechanism indicates that increasing concentrations of BH$_4$ would decrease the generation of superoxide in favor of the generation of the peroxyl iron intermediate [Fe$^{III}$-OOH]$^{2+}$, (Scheme II step VI). Dissociation of the [Fe$^{III}$-OOH]$^{2+}$ species will result in the generation of hydrogen peroxide, which has been detected as the product of uncoupled oxygen reduction under limited BH$_4$ and/or l-arginine concentrations (24, 25). This implies that hydrogen peroxide may be formed by nNOS by two different mechanisms depending on the availability of BH$_4$. At low BH$_4$ concentrations, nNOS will generate only superoxide that, by dismutation, will produce hydrogen peroxide and oxygen. In the presence of BH$_4$, however, no superoxide will be formed, and the enzyme will generate hydrogen peroxide by a mechanism involving a two-electron reduction of oxygen.

BH$_4$ may participate in oxygen activation by two mechanisms. The first is that BH$_4$ directly reduces the [Fe$^{III}$-O$_2$]$^{2+}$, by hydrogen atom donation or by electron transfer, to generate [Fe$^{III}$-OOH]$^{2+}$ and the highly reactive trihydrobiopterin radical (‘BH$_3$’), which must be quickly reduced by reductase domain flavins (35). Raman et al. (41) have suggested that for eNOS to utilize a radical intermediate, both BH$_4$ and ‘BH$_3$’ need to be protonated. Although the exact location of BH$_4$ at the oxygenase domain of nNOS is unknown, by analogy to iNOS and eNOS, it is unlikely that BH$_4$ can interact directly with the heme iron, favoring electron transfer rather than hydrogen abstraction (41–43). An alternative mechanism to explain the effects of BH$_4$ on superoxide generation is that occupation of the BH$_4$-binding site accelerates the flavin-dependent reduction of the [Fe$^{III}$-O$_2$]$^{2+}$ species to generate [Fe$^{III}$-OOH]$^{2+}$. To discriminate between these two possibilities, it would be necessary to detect the formation of the trihydrobiopterin radical under single turnover conditions.

BH$_4$ is essential to prime the enzyme for catalysis, and it is plausible to propose that it promotes the formation of a catalytically competent oxidation state of heme iron. Characterization of the product of two-electron oxidation of other heme proteins indicates the presence of a tetravalent heme-iron with a full electron octect oxygen [Fe$^{IV}$-O]$^{2+}$ (33). For some peroxidases, the extra electron necessary to render such configuration is donated by the porphyrin (P) or by adjacent amino acid residue (33). It is possible to speculate that the extra electron necessary to form the oxoferryl species $\cdot X[Fe^{IV}=O]$ is drawn from BH$_4$, which is coordinated to the heme propionate groups (41–43) as shown in Equation 1.

\[
\begin{align*}
\text{BH}_4 + \text{H}_2\text{O}_2 &\rightarrow \text{[Fe}^{III}\text{OH}]^2+ + \text{BH}_3 + \text{H}_2\text{O} \quad \text{(Eq. 1)}
\end{align*}
\]

By this mechanism BH$_4$ would stabilize the oxoferryl state and ensure, by rechlorinating an electron, that the heme iron returns to the ferric form after a catalytic cycle. In addition the formation of BH$_3$ may be intrinsically more stable than the formation of a porphyrin cation radical and consequently may prevent enzyme inactivation. One advantage of this hypothesis is that
The Effect of l-Arginine—The inhibition of superoxide elicited by l-arginine alone in nNOS (Fig. 2) but not in C331A-nNOS (Fig. 4) is mirrored by a decrease in NADPH oxidation.2 This suggests that l-arginine decreases NADPH-dependent oxygen reduction by nNOS. One possible mechanism for this observation is that occupation of the l-arginine-binding site stabilizes the [FeHL2O2]+ intermediate (Scheme II, step V) (44). It has been demonstrated that l-arginine binding to NOS induces a transition from low to high spin ferric iron, which favors the reduction of heme-iron by reduced flavins upon binding of calcium/calcmodulin. This effect of l-arginine will decrease the leakage of superoxide from the reductase domain of nNOS. It is unlikely that l-arginine favors the formation of hydrogen peroxide by reducing the [FeHL2O2]+ intermediate, since l-arginine binding caused a significant decrease of the rates of NADPH consumption by nNOS (Fig. 6). This is in agreement with previous studies demonstrating that l-arginine stabilizes the ferrous-dioxygen complex (45). This result indicates that l-arginine by preventing the dissociation of the [FeHL2O2]+ intermediate decreases the generation of superoxide both from the oxygenase and reductase domains of nNOS (Fig. 1).

Concluding Remarks—The present data indicate that l-arginine controls the generation of superoxide by decreasing the rate of NADPH consumption, whereas BH4 controls superoxide formation by promoting the formation of heme-peroxo species. In the presence of both BH4 and l-arginine, the formation of the oxoferryl will occur (Scheme II, step VII) thereby facilitating the oxidation of l-arginine to generate N5-hydroxy-l-arginine (46) (Scheme II, step IX).

Our results also reveal that generation of superoxide is tightly controlled by BH4. This effect is achieved at nanomolar concentrations of BH4 (Fig. 3). The kinetic constant of the BH4 binding to nNOS is $K_d = 0.25 \mu M$ indicating that the enzyme has a high affinity for BH4 (39). Considering that this affinity is about 2–3 orders lower than that reported for aromatic biopterin, and only a single route of electron transfer from the flavin domain to the heme is required. As l-arginine can bind to nNOS (Fig. 4) is mirrored by a decrease in NADPH oxidation.2 This suggests that l-arginine decreases NADPH-dependent oxygen reduction by nNOS. It is unlikely that l-arginine favors the formation of hydrogen peroxide by reducing the [FeHL2O2]+ intermediate, since l-arginine binding caused a significant decrease of the rates of NADPH consumption by nNOS (Fig. 6). This is in agreement with previous studies demonstrating that l-arginine stabilizes the ferrous-dioxygen complex (45). This result indicates that l-arginine by preventing the dissociation of the [FeHL2O2]+ intermediate decreases the generation of superoxide both from the oxygenase and reductase domains of nNOS (Fig. 1).

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C331A-nNOS consumed 63.7 and 60.7 nmol of NADPH min $^{-1}$ mg protein $^{-1}$ in the absence and presence of l-arginine, respectively.

2 C331A-nNOS consumed 63.7 and 60.7 nmol of NADPH min $^{-1}$ mg protein $^{-1}$ in the absence and presence of l-arginine, respectively.