Mechanism of Superoxide Generation by Neuronal Nitric-oxide Synthase*

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Sovij Pou, Lori Keaton, Wanida Surichamorn, and Gerald M. Rosen‡

From the Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland 21201

Neuronal nitric-oxide synthase (NOS I) in the absence of L-arginine has previously been shown to generate superoxide (O2•−) (Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H., and Rosen, G. M. (1992) J. Biol. Chem. 267, 24173–24176). In the presence of L-arginine, NOS I produces nitric oxide (NO•). Yet the competition between O2•− and L-arginine for electrons, and by implication formation of O2•− has until recently remained undefined. Herein, we investigated this relationship, observing O2•− generation even at saturating levels of L-arginine. Of interest was the finding that the frequently used NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NMMA), monomethyl L-arginine enhanced O2•− production in the presence of L-arginine because this antagonist attenuated NO• formation. Whereas diphenylidionium chloride inhibited O2•− formation in the presence of L-arginine, this antagonist prevented the formation of superoxide (O2•−) even at concentrations that inhibited NO• formation from L-arginine. Taken together these data demonstrate that NOS I generates O2•− and the formation of this free radical occurs at the heme domain.

At a time before the physiologic properties of endothelium-derived relaxation factor were associated with nitric oxide (NO•)1 (1–3), it was found that this free radical activated soluble guanylate cyclase from crude homogenates of brain tissue (4). The significance of this observation would, surprisingly, remain dormant for nearly a decade, even though it was known that L-arginine was the endogenous activator of this enzyme (5). With the purification and characterization of a unique monooxygenase, NOS I, capable of oxidizing L-arginine to L-citrulline, and NO• (6, 7), a new class of small molecules (8) acting as transient second messengers in the brain was discovered (9). The versatility of this free radical in controlling a myriad of brain functions will undoubtedly result in new and provocative findings. Of special interest will be research that can distinguish NO• from NOS I-secreting neurons versus NO• from NOS III-containing endothelial cells and NO• from NOS II-stimulated microglial cells. Until the recent development of specific antagonists for each of the NOS isozymes (10–12), it has been difficult to address this question, which is of particular significance when one considers, as described in this article, that NOS I, NOS II, and NOS III may produce NO• and O2•− under differing cell conditions.

Nitric-oxide synthase is known to catalyze the production of NO• from L-arginine (13, 14). In the absence of substrate, we have previously demonstrated that purified NOS I can use O2•− as the terminal electron acceptor, generating O2•− (15). These findings have been confirmed because O2•− has been spin-trapped in L-arginine-depleted NOS I-transfected human kidney cells (16). During the course of our earlier studies (15), we noted to our surprise that purified NOS I appeared to produce O2•− even in the presence of L-arginine. The current study, therefore, explores this phenomenon in depth. Herein, we demonstrate that NOS I, like NOS II (17) and NOS III (18–20), can generate O2•− and NO• despite saturating levels of substrate. However, unlike NOS II, the heme of NOS I is the locus for the production of both free radicals. Finally, we discuss the implications of our findings; particularly relevant is the ability of L-NMMA to enhance NOS I-derived O2•− even in the presence of saturating concentrations of L-arginine.

EXPERIMENTAL PROCEDURES

Materials—NADPH, calmodulin, L-arginine, phenethylisulfonyl fluoride, dihydroxybenzosineptacetic acid, ferrocyanochrome c, xanthine, Nω-nitro-L-arginine methyl ester (L-NNAME), Nω-monomethyl L-arginine (L-NMMA), EGTA, HEPES, and penicillin G-streptomycin solution were purchased from Sigma. Imidazole, 1-phenylhydrazine, sodium cyanide, and diphenylidionium chloride (DPI) were obtained from Aldrich. Tetrahydrobiopterin was purchased from Alexis Biochemicals (San Diego, CA). Cation exchange resin Dowex 50W-X8 hydrogen form resin was obtained from Bio-Rad. 2′,5′-ADP-Sepharose was obtained from Pharmacia (Upsala, Sweden). L-[14C]Arginine was purchased from ICN Radiochemicals (Costa Mesa, CA). Dulbecco’s modified Eagle’s medium:nutrient mixture F-12 (1:1) and phosphate-buffered saline were obtained from Life Technologies, Inc. Bovine calf serum was purchased from Hyclone (Logan, UT). Superoxide dismutase and xanthine oxidase were obtained from Boehringer Mannheim. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was synthesized according to the procedure of Bonnett et al. (21).

NOS I Purification—NOS I-transfected kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium:nutrient mixture F-12 containing 10% fetal calf serum, penicillin G (100 units/ml), and streptomycin (100 µg/ml). NOS I was purified from these cells by the method of Bredt and Snyder (6). Briefly, cells were removed from the culture flasks and washed three times with phosphate-buffered saline via centrifugation. The pellet was resuspended in buffer containing phenethylisulfonyl fluoride (4 mg/ml) and homogenized with a Poltron (Brinkmann Instruments, model PCU-2 at setting 2 for 10 s). The remaining mixture was centrifuged at 15,000 rpm for 20 min to separate unbroken cells, and the supernatant was applied to a 2′,5′-ADP-Sepharose affinity column. After washing the column three times with 0.45 M NaCl and standard buffer, NOS was eluted with standard buffer containing 10 mM NADPH. Excess NADPH was removed by washing.

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‡ To whom correspondence should be addressed: Dept. of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 725 West Lombard St., Baltimore, MD 21201. Tel.: 410-706-0514; Fax: 410-706-8184; E-mail: grosen@umaryland.edu.

1 The abbreviations used are: NO•, nitric oxide; O2•−, superoxide; H2O2, hydrogen peroxide; NOS I, neuronal nitric-oxide synthase; NOS II, inducible nitric-oxide synthase; NOS III, endothelial nitric-oxide synthase; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EPR, electron paramagnetic resonance; L-NNAME, Nω-nitro-L-arginine methyl ester; L-NMMA, Nω-monomethyl L-arginine; DMPO-OH, 2′,5′-dimethyl-5-hydroperoxy-1-pyrroline-1-oxyl; DPI, diphenylidionium chloride; ONOO•−, peroxynitrite; SOD, superoxide dismutase; BSA, tetrahydrobiopterin.
and concentrating the eluate with CentriCell-30 (Polysciences, War-nington, PA) until the concentration of NADPH was approximately 1–1.5 mM as assessed spectrophotometrically at 340 nm (ε = 6.2 x 10^3 M^-1 cm^-1). Protein concentration was determined by the Bradford method (22) using bovine serum albumin as a standard. 

**Estimation of NOS I Activity—**NOS I activity was assayed by measuring the formation of L-[14C]citrulline from L-[14C]arginine as described previously (23). Briefly, purified NOS I was added into a reaction mixture containing L-[14C]arginine (0.6 μCi/mL), L-arginine (1 mM), NADPH (1 mM), calmodulin (100 units/ml; 23.6 μg/ml), free calcium ions (CaCl₂, 1 μM; calculated as described in Ref. 24), and standard phosphate buffer at pH 7.4 (final volume = 0.15 ml). After incubating at room temperature for exactly 10 min, the reaction was terminated by adding HEPES (20 mM, 2 ml) containing EDTA (2 mM, pH 5.5). L-[14C]Citrulline was separated by passage through an 0.5-ml column of Dowex-50-X8 cation exchange, and radioactivity was counted using a Beckman β counter.

**Spin Trapping and EPR Spectroscopy—**Spin trapping experiments with purified NOS I were conducted by mixing all the components described in the figure legends to a final volume of 0.25 ml. The experiment was initiated by adding freshly purified NOS I. Reaction mixtures were then transferred to a flat quartz cell and fitted into the cavity of the EPR spectrometer (Varian Associates, model E-109), and spectra were recorded at room temperature 1 min after addition of the enzyme. Instrumentation settings are presented in the figure legends.

**Superoxide Detection—**The assessment of the effect of l-NAME on the ability of xanthine/xanthine oxidase to produce O₂ was evaluated as described previously (25). Briefly, xanthine oxidase was added to a solution containing xanthine (400 μM) and ferricytochrome c (80 μM) such that the rate of O₂ formation, measured as the SOD-inhibitable reduction of ferricytochrome c at 550 nm (26) was 1 μM/min. The effect of various concentrations of l-NAME on the rate of O₂ generation was estimated.

**NADPH Oxidation—**The rate of NADPH oxidation by NOS I was determined spectrophotometrically at 340 nm (ε = 6.2 x 10^3 M^-1 cm^-1).

## RESULTS AND DISCUSSION

Before determining the ability of NOS I to generate O₂, our initial series of studies were devoted to determining the Kₘ and the Vₘₐₓ for NOS I, because these constants will help dictate future experimental designs. Based on these studies, the Kₘ and the Vₘₐₓ were found to be 3.47 μM and 216 nmol min⁻¹ mg⁻¹, respectively, well within the ranges (Kₘ of 2–4.3 μM and Vₘₐₓ of 74–3400 nmol min⁻¹ mg⁻¹) reported by others (27).

In the absence of L-arginine, NOS I has previously been shown to generate O₂ (15, 16), whereas at high concentrations of L-arginine (1 mM), complete inhibition of O₂ production had been noted (15). We therefore examined the effect that intermediate concentrations of L-arginine would have on the ability of NOS I to generate O₂. As shown in Fig. 1, l-arginine, in a dose-dependent manner, blocked O₂ secretion from NOS I, exhibiting an EC₅₀ = 5 μM. Inhibition of NOS I-generated O₂ at 100 μM l-arginine was nearly complete. Under these conditions, the rate of NADPH oxidation decreased as l-arginine concentration increased from 1.96 μmol/min/mg protein in the absence of substrate to 1.53 μmol/min/mg protein at 100 μM l-arginine. Thereafter, the rate of NADPH consumption remained constant. This result suggests that for NOS I the transfer of electrons from NADPH to O₂ in the absence of substrate is faster than the transfer of electrons to catalyze the formation of NO⁻ and L-citrulline from L-arginine. In contrast to NOS I, O₂ generation by purified NOS II was unaffected by 100 μM L-arginine. In fact, it was still possible to detect this free radical even in the presence of 1 mM L-arginine (17). Similar findings have been reported for NOS III (18, 19). These are surprising observations, considering the fact that the Kₘ for NOS II varies between 2.8 to 32 μM, whereas for NOS III, the Kₘ has been reported to be 2.9 μM (27). These data nevertheless point to a substantial difference between the three isozymes of NOS with respect to O₂ secretion. For NOS II, it was found that O₂ was generated by electron leakage from the flavin domain (17), based on the finding that 100 μM NaCN did not significantly inhibit O₂ generation as estimated by spin trapping experiments (17). In contrast, either 100 μM NaCN (19) or 1 mM NaCN (18) blocked O₂ secretion from NOS III. These data suggest that the heme domain of NOS III is the site of O₂ production (18, 19).

The initial step in the generation of NO⁻ by NOS is the transport of electrons from NADPH to the oxidized flavin, FAD, resulting in FADH₂, after abstraction of a proton from the surrounding milieu. Disproportionation between the flavins leads to FADH₂/FMNH. The electron donation from FMNH to Fe²⁺ gives the reduced heme, Fe⁺ and FADH/FMNH ↔ FAD/FMNH (28). Binding of O₂ in the sixth ligand position would give the hypothetical intermediate [Fe²⁺–O₂] (29). In the absence of l-arginine, O₂ accepts an electron from NOS, generating O₂⁻ (15, 17–19). When l-arginine is present, however, there is a binding of the guanidino nitrogen in an ordered position near the heme (30), which allows the oxidation of l-arginine to proceed. Based on a Kₘ of 3.47 μM for the NOS I oxidation of l-arginine and on data presented in Fig. 1, NO⁻ and O₂ are both generated. The rate of each free radical produced, however, cannot accurately be estimated by spin trapping. Fig. 1 indicates that NOS I at l-arginine concentration around the Kₘ is capable of producing O₂ at around 60% of the rate of that generated in the absence of l-arginine. For NOS II, electron transport from FMNH⁻ to Fe³⁺ appears not to be so tightly coupled, because some leakage from the flavin domain results in the formation of O₂⁻ even in the presence of 1 mM l-arginine (17). For NOS I, the site of O₂⁻ generation has not yet been defined. We suggest, however, that during the oxidation of l-arginine to l-citrulline and NO⁻, direct competition with O₂ results in O₂⁻ formation. At a fixed concentration of O₂⁻, the ratio of NO⁻ and O₂⁻ is dependent, therefore, on the concentration of l-arginine.

To explore the mechanism of O₂⁻ generation by NOS I, it is important to determine how electrons are transferred through the enzyme. Thus, we undertook a series of inhibition experiments exploring the effects of two well known inhibitors of NOS, l-NAME and l-NMMA, on O₂⁻ production. Before conducting these experiments, we estimated the capacity of these NOS inhibitors to block l-citrulline formation and, by implication,
the generation of NO. The dose-response curves for L-[14C]citrulline formation from L-[14C]arginine by NOS I in the presence of L-NAME and L-NMMA are shown in Fig. 2. L-NMMA was found to be slightly more potent (EC50 = 5 μM) than L-NAME (EC50 = 10 μM). For NOS III, L-NAME has been reported to be a more effective inhibitor of this isozyme than L-NMMA (31). With these data in mind, we then defined the ability of L-NAME and L-NMMA to prevent O2 generation by NOS I. Fig. 3 shows the effect of increased concentrations of L-NAME on the ability of NOS I to generate O2. Similar to L-arginine, L-NAME antagonized the spin trapping of this free radical by DMPO in a dose-dependent manner, with an EC50 = 40 μM. In contrast, L-NMMA did not appreciably depress the formation of O2 by NOS I even at concentrations as high as 10 mM (Fig. 3). Consistent with these findings, the rate of NADPH oxidation by NOS I was inhibited by >70% as the concentration of L-NAME reached 100 μM, whereas the rate of NADPH oxidation remained constant at about 50% of control with increasing concentration of L-NMMA up to 10 mM (Fig. 4). These results suggested that L-NAME, but not L-NMMA, inhibited the formation of O2 by impeding the electron transport to O2.

To confirm this hypothesis, the effect of L-NAME (1 mM) on the xanthine/xanthine oxidase production of O2 as measured by the SOD-inhibitable reduction of cytochrome c, was assessed. Within experimental error, this rate was unchanged. These data indicate that L-NAME did not scavenge O2 but rather that L-NAME acted specifically on NOS I, inhibiting generation of this free radical. Because L-NMMA is a potent antagonist of NOS-generated NO, we explored whether L-NMMA could block the ability of L-arginine to inhibit NOS I production of O2. These findings are presented in Fig. 5. As expected, L-arginine (100 μM) almost completely inhibited the NOS I formation of O2 (Fig. 5B), which confirmed earlier studies (15, 32). Surprisingly, L-NMMA, in a dose-dependent manner, reversed the inhibitory properties of L-arginine (Fig. 5, C–E), almost reaching control values, in the absence of L-arginine, at 1 mM (Fig. 5, E and F). Our findings support the theory that L-NAME antagonizes the transfer of electrons to either L-arginine or O2, whereas L-NMMA prevents the oxidation of L-arginine by competing for the same binding site on the enzyme (33). Although NO’ production is inhibited by the presence of L-NMMA, NOS I still has the capacity to transfer electrons from NADPH to O2 (Fig. 4 and 5).

Next, experiments were designed to further determine the site on NOS I where O2 production takes place. Because cytochrome P-450 and NOS I are members of the same superfamily of enzymes (34), we looked to cytochrome P-450 to gain insight as to potential loci for NOS I-derived O2. There are two sites on
cytochrome P-450 and NOS I where \( \text{O}_2^\bullet \) may be generated: the flavins of the reductase and the iron of the heme. For cytochrome P-450, the heme domain is the origin of \( \text{O}_2^\bullet \) formation (35). Would NOS I behave in a similar manner? First, we needed to demonstrate that our NOS I preparation was capable of transferring electrons from the flavin site to the heme domain, these experiments cannot establish whether \( \text{O}_2^\bullet \) is produced solely at this site or at the heme domain. To further address this query, we investigated the effect of NaCN and two imidazoles (known to inhibit cytochrome P-450 and NOS (37, 38) by blocking the heme site) on the generation of this free radical. First, however, we had to demonstrate that NaCN, imidazole, and 1-phenylimidazole inhibited the metabolism of L-arginine to \( \text{O}_2 \) by NOS I. As shown in Fig. 6, the findings for cytochrome P-450 (35) (Fig. 9) and similar to the findings for cytochrome P-450 (35) (Fig. 9). For NOS II, it appears that the reductase domain is not as tightly coupled to the heme domain as seen in NOS I and cytochrome P-450. This weak coupling allows some electron leakage to \( \text{O}_2 \), generating \( \text{O}_2^\bullet \), even though sufficient electron flow to the heme permits a.

![Figure 5](image-url)  
**Fig. 5.** Effect of L-NMMA on \( \text{O}_2^\bullet \) generation by purified NOS I in the presence of L-arginine as assessed by spin trapping/EPR spectroscopy. Typical EPR spectra corresponding to DMPO-OOH under a variety of experimental conditions are shown. The mixture in scan A consists of DMPO (100 mM), free calcium ion (1 mM), calmodulin (100 units/ml), NADPH (124 \( \mu \)M), and NOS (14.4 \( \mu \)g/ml). Scans B–E were recorded under identical conditions to scan A except for the addition of L-arginine (100 \( \mu \)M), L-arginine (100 \( \mu \)M) + L-NMMA (10 \( \mu \)M), L-arginine (100 \( \mu \)M) + L-NMMA (10 \( \mu \)M) + calmodulin (100 \( \mu \)M), and L-arginine (100 \( \mu \)M) + L-NMMA (1 mM), respectively. Scan F was recorded under identical conditions to scan A except for the addition of L-NMMA (1 mM). Microwave power was 20 megawatts, modulation frequency was 100 kHz with an amplitude of 1 G, sweep time was 12.5 G/min, response time was 1 s, and receiver gain was \( 10 \times 10^4 \).
high flux of NO· from the oxidation of L-arginine.

Finally, we investigated the role that BH₄ might play in regulating production of O₂⁻ by NOS I in the absence of L-arginine. Tetrahydrobiopterin, at 10 and 100 μM, inhibited O₂⁻ secretion by NOS I by >90%. As shown in Fig. 10, B and C, the EPR spectra are characteristic of DMPO-OH and to a lesser extent of DMPO-OOH. The source of DMPO-OH appears to be BH₄. At 1 mM BH₄ (Fig. 10D), we were still able to observe some DMPO-OOH, even though DMPO-OH dominated the EPR scan. At 10 mM BH₄, DMPO-OH (Fig. 10F) was the only spin-
trapped adduct recorded. These data suggest that at low concentrations (1–100 μM), the primary effect of BH₄ is to inhibit NOS I production of O₂⁻. However, at higher concentrations, in addition to the inhibition of NOS I-secreted O₂⁻, BH₄ generates O₂⁻ through autoxidation. The resultant H₂O₂, either through metal ion catalysis or direct oxidation, produced DMPO-OH (39, 40).

Several important observations are readily apparent from our studies. First, we were able to spin trap O₂⁻ even in the presence of saturating levels of L-arginine. This is remarkable, considering that O₂⁻ and NO combine at near diffusion controlled rates, producing ONOO⁻ (41, 42), whereas O₂⁻ reacts with DMPO at only 12 m⁻¹ sec⁻¹ (43). The ability to spin trap O₂⁻ under these experimental conditions may result from the fact that O₂⁻ and NO are generated sequentially at the heme iron site. After O₂⁻ is produced, NOS I must cycle twice before NO⁻ is secreted. This is a sufficiently long time to allow O₂⁻ to diffuse from the enzyme to the surrounding milieu where this free radical can react with the high concentration of DMPO included in the reaction mixture to give the observed spin-trapped adduct, DMPO-OOH. Under physiological conditions at which NOS I generates both O₂⁻ and NO⁻, SOD regulates cellular flux of O₂⁻ thereby preventing, or at least drastically limiting, ONOO⁻ generation (44).

Second, despite the above findings, there are pathologic states, such as ischemia/reperfusion injury, that might promote the formation of ONOO⁻ (45). Therefore, it is surprising that the fate of this peroxide still remains in doubt (44, 46). At the low L-arginine concentrations at which the steady-state flux of O₂⁻ would exceed NO⁻, in addition to ONOO⁻, H₂O₂ would arise from the dismutation of O₂⁻. Subsequent formation of HO²⁻, either through the metal ion catalyzed Haber-Weiss reaction (47) or from decomposition of ONOO⁻ (48–52), at sensitive cellular sites may result in cytotoxicity. Thus, under different experimental conditions, a variety of oxidants can be produced with an impact on cell function that is significant or in some cases remains to be defined.

Third, it should be noted that the reductase domain of NOS is, like cytochrome P-450 reductase, susceptible to uncoupling, shunting electrons away from the heme toward a xenobiotic. One recent example illustrates this point (53). It was discovered that o-quinones can promote O₂⁻ production by uncoupling NOS I at its reductase domain, yielding a semiquinone free radical. In agreement with the result obtained with NOS III (18–20), BH₄, in a dose-dependent manner, was found to inhibit the generation of O₂⁻ by NOS I. Interestingly, unlike NOS III, L-arginine, independent of added BH₄, inhibited O₂⁻ production by NOS I, which was reversed by L-NMMA (Fig. 5). This suggests that in the case of NOS I, BH₄ is not the sole pathway for controlling O₂⁻ formation but that the competition between L-arginine and L-NMMA is an important factor in determining the generation of O₂⁻.

How does BH₄ regulate the ratio of NO⁻ and O₂⁻? The answer, of course, is not simple and depends, to a large extent, on the isozyme of NOS. For instance, one would predict, based on the findings in Ref. 18, that for an activation of a NOS III-containing cell in which L-arginine and BH₄ are at normal levels, high fluxes of O₂⁻ and NO⁻ would result. The secretion of NO⁻ to the surrounding milieu would be tied to the ability of SOD to

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**Fig. 8.** Effect of NOS inhibitors on O₂⁻ generation by FMN (0.1 mM)/NADPH (1 mM). Scan A was obtained in the absence of inhibitors. Scans B–E were obtained in the presence of imidazole (10 mM), 1-phenylimidazole (10 mM), DPI (10 μM), and NaCN (10 mM), respectively. Microwave power was 20 megawatts, modulation frequency was 100 kHz with an amplitude of 1 G, sweep time was 12.5 G/min, response time was 1 s, and receiver gain was 5 × 10⁴.
scavenge $O_2^\cdot$. In contrast, activation of a NOS I-containing cell, under the conditions described above, would result primarily in the formation of NO'. Thus, under cellular conditions that would impact the availability of either l-arginine or BH$_4$, variable amounts of NO$^\cdot$ and $O_2^\cdot$ would be generated. Evidence in support of this thesis comes from studies with NOS I-containing transfected kidney 293 cells (16). Here, when these cells were placed in a complete medium, NO$^\cdot$ but not $O_2^\cdot$ was spin trapped (16). However, when the cells were cultured in l-arginine-depleted medium for 24 h, it was possible to spin trap $O_2^\cdot$ at the expense of NO$^\cdot$ (16).

Although the implications of our findings have yet to be fully realized, two recent publications may shed some light on the importance of our observations. First, during anoxia/reoxygenation of cardiomyocytes, generation of $O_2^\cdot$ was found to be markedly enhanced when l-NMMA was included (54), which supports the data presented in Fig. 5. Second, elevated levels of MnSOD were an essential element of viable NOS I-containing neurons exposed to N-methyl-d-aspartate (NMDA) (55). Even though the source of $O_2^\cdot$ in these studies (54, 55) was not identified, NOS must certainly be considered a possible contributor to the origin of this free radical.

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