Inducible Nitric-oxide Synthase Generates Superoxide from the Reductase Domain*

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Yong Xia,† Linda J. Roman,‡ Bettie Sue S. Masters,§ and Jay L. Zweier∥

From the †Molecular and Cellular Biophysics Laboratories, Department of Medicine, Division of Cardiology and the Electron Paramagnetic Resonance Center, The Johns Hopkins University School of Medicine, Johns Hopkins Bayview Medical Center, Baltimore, Maryland 21224 and the ‡Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284

In the absence of L-arginine, the heme center of the oxygenase domain of neuronal nitric-oxide synthase reduces molecular oxygen to superoxide (O$_2^-$). Our recent work has provided evidence that inducible NOS (iNOS) may also catalyze O$_2^-$ formation in macrophages. However, there has been a lack of direct evidence of superoxide generation from the purified iNOS, and it was previously hypothesized that significant O$_2^-$ production does not occur. Moreover, the mechanism and enzyme site responsible for O$_2^-$ generation is unknown. To determine whether iNOS produces O$_2^-$ and to identify the mechanism of this process, we performed electron paramagnetic resonance measurements on purified iNOS using the spin trap 5,5-dimethyl-1-pyrroline N-oxide. In the presence of NADPH, prominent O$_2^-$ adduct signals were detected from iNOS. These signals were totally abolished by superoxide dismutase but not affected by catalase. High concentrations of L-arginine decreased this O$_2^-$ formation, whereas its enantiomer d-arginine did not. Pre-incubation of iNOS with the flavoprotein inhibitor diphénylenedioïdium totally blocked these O$_2^-$ signals. Conversely, pretreatment of the enzyme with the heme blocker cyanide had no effect on O$_2^-$ generation. Furthermore, strong O$_2^-$ generation was directly detected from the isolated iNOS reductase domain. Together, these data demonstrate that iNOS does generate O$_2^-$ and this mainly occurs at the flavin-binding sites of the reductase domain.

Nitric oxide (NO),1 a gaseous free radical, has been identified as a ubiquitous signaling molecule in biological systems (1). In cells or tissues, NO is produced by a family of NO synthases (NOSs), which utilize L-arginine, oxygen, and NADPH as substrates to synthesize NO as well as the coproduct L-citrulline (2, 3). Three distinct isoforms of NOS have been cloned: neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (4). nNOS and eNOS are also referred to as constitutive NOS, while the expression of iNOS requires induction by microbial endotoxins or cytokines. With tightly bound calmodulin, iNOS is fully active at basal Ca$^{2+}$ levels, whereas constitutive nNOS and eNOS activity depend on the elevation of intracellular Ca$^{2+}$. In addition to calmodulin-binding sites, all three NOS isoforms also contain FAD, FMN, and tetrahydrobioperin (BH$_4$) binding sites and require these cofactors for their enzymatic function (5).

Besides synthesizing NO, purified nNOS can catalyze superoxide (O$_2^-$) formation when L-arginine is absent (6, 7). This is also a Ca$^{2+}$/calmodulin-dependent process. In L-arginine-depleted cells, nNOS was found to generate both O$_2^-$ and NO leading to peroxynitrite (ONOO$^-$)-mediated cell injury (8). Whereas the O$_2^-$ generation from nNOS was well documented both in vitro and in intact cells, controversy remains regarding whether iNOS is also capable of producing O$_2^-$. Considering the similarity in amino acid sequence and enzymatic function between these two isoforms, it would be expected that iNOS will also generate O$_2^-$. However, iNOS was reported to be much less prone to oxidize NADPH than nNOS under conditions of L-arginine depletion, and it was presumed that iNOS does not produce significant amounts of O$_2^-$. This highly limited O$_2^-$-generating capacity from iNOS has been proposed to be critical for its biological functions since O$_2^-$ could in turn react with and scavenge NO, and it was hypothesized that this would perturb iNOS-mediated immune defense actions (9, 10). Recently, our experiments on macrophages have provided evidence that iNOS may produce significant amounts of O$_2^-$ in cells (11). Under L-arginine depletion, iNOS was found to generate O$_2^-$ as well as ONOO$^-$, and these oxidants may contribute to the antibacterial activity of macrophages. However, these observations on macrophages cannot conclusively prove if iNOS syntheses O$_2^-$. So far, definitive oxygen radical measurements on purified iNOS have not been performed. Moreover, even if iNOS is indeed capable of synthesizing O$_2^-$, questions remain regarding whether the mechanism of O$_2^-$ generation from iNOS is similar to or different from that of nNOS.

To address these issues, we have applied electron paramagnetic resonance (EPR) spin trapping techniques to directly measure and characterize the process of O$_2^-$ generation from purified iNOS. We also explored the role of the reductase and oxygenase domains in this iNOS-catalyzed O$_2^-$ generation.

**EXPERIMENTAL PROCEDURES**

Materials—The NADPH, L- or D-arginine, BH$_4$, calmodulin, L-NAME, dithiothreitol, superoxide dismutase (SOD), catalase, and other reagents were purchased from Sigma unless otherwise noted. Diphenyleneiodionum (DPI) and sodium cyanide (NaCN) were from...
The total volume of 300 dtopyranoside. The heme and flavin precursors, troporation. Under selective pressure (50 L-[14C]arginine, 0.5 mM NADPH, 0.5 mM Ca\textsuperscript{2+}, 1 mM chloramphenicol), bacteria were grown to containing 100 mM imidazole.

The iNOS-containing fractions were pooled, concentrated, and stored at 0.1 mM dithiothreitol, and 10% glycerol. The column was washed with about 10 volumes of this buffer containing 500 mM NaCl and 5 mM 2-AMP, and is approximately 50% pure after this step. The iNOS-containing fractions were concentrated using a Centriprep 30 (Amicon). After reconstitution with BH\textsubscript{4} (250 \mu M), this fraction was applied to a S-200 gel filtration column. The iNOS-containing fractions were pooled, concentrated, and stored at −80 °C. iNOS concentration was determined by CO-difference spectrum, assuming an extinction coefficient of 75 mM\textsuperscript{-1} cm\textsuperscript{-1}. The purity of iNOS was determined by SDS-polyacrylamide gel electrophoresis and visualized with Coomassie Blue staining. iNOS activity was up to 1300 nmol/min/mg at 37 °C assayed by monitoring the conversion of L-[14C]arginine to L-[14C]citrulline, as described below.

The iNOS reductase domain construct is residues 499–1144 of the holoenzyme, encompassing the calmodulin binding site. An alanine and six histidine residues were added directly following the initiation methionine (MAH-MAH-MAH-MAH-MAH-MAHIII). The construct was made by inserting the amine chain reaction-amplified DNA, incorporating the above changes, from the iNOS-pCW plasmid into NcoI/HindIII re-stricted pCw vector. The resultant iNOS reductase domain plasmid was coexpressed with the calmodulin plasmid (pACMIP), as in the holoenzyme. The growth was the same as the above except that delta-ALA was not added at induction. The cells were harvested, and the supernatant of cell lysate was loaded on 2'5'-ADP-Sepharose 4B column. The iNOS-containing domain was pulled down and further purified by double DEPMPO was prepared as reported (12).

**RESULTS**

Recombinant mouse iNOS was isolated from an E. coli expression system. This system has been proven to be a powerful and efficient way to prepare large quantities of NOS proteins (13, 18). Recombinant eNOS or nNOS from this system has exhibited enzymatic properties indistinguishable from native enzymes isolated from mammalian cells (13, 19). iNOS was isolated by NADPH affinity chromatography, and the purity of the preparations was further improved by size exclusion chromatography. As shown in Fig. 1, top, purified protein preparations exhibited a prominent major band (>90% pure) on SDS-polyacrylamide gel electrophoresis with a molecular mass of 130 kDa, which is in agreement with the molecular mass for native iNOS as previously reported (4, 5). This recombinant protein possessed strong NOS activity as measured from conversion of L-[14C]arginine to L-[14C]citrulline (Fig. 1, bottom). The catalytic activity was independent of additional Ca\textsuperscript{2+} and could be blocked by the NOS inhibitor, l-NNAME (1 mM), confirming that it was derived from iNOS.

To determine whether iNOS generates O\textsubscript{2}·, EPR spin-trapping measurements were performed on iNOS using the well characterized spin trap DMPO. In the absence of the enzyme, no signals were observed from solutions containing DMPO and NADPH (Fig. 2, top, trace A). However, after adding purified iNOS (7.3 \mu M/ml), strong EPR signals were seen (Fig. 2, bottom, trace B). The purity of this fraction was determined by SDS-polyacrylamide gel electrophoresis and visualized with Coomassie Blue staining. iNOS activity was up to 1300 nmol/min/mg at 37 °C assayed by monitoring the conversion of L-[14C]arginine to L-[14C]citrulline (control values were up to 1300 nmol/min/mg at 37 °C). The preparations showed typical NOS characteristics with l-NNAME-inhibitory and Ca\textsuperscript{2+}-independent activity (**, p < 0.01, compared with control, n = 4).

**Fig. 1. Profile of recombinant iNOS isolated from E. coli expression system.** Top, SDS-polyacrylamide gel electrophoresis analysis of isolated iNOS preparations. Lanes: molecular mass markers (A); 0.5 \mu M purified iNOS (B); 1 \mu M purified iNOS (C). Proteins were separated on 7.5% polyacrylamide gels and visualized by Coomassie Blue staining. Bottom, enzymatic activity of purified iNOS preparations. NOS activity was assayed by monitoring the conversion of L-[14C]arginine to L-[14C]citrulline (control values were up to 1300 nmol/min/mg at 37 °C). The preparations showed typical NOS characteristics with l-NNAME-inhibitory and Ca\textsuperscript{2+}-independent activity (**, p < 0.01, compared with control, n = 4).
Superoxide Generation from iNOS

The presentation of oxygen free radicals generated from iNOS. The reaction system consists of 0.5 mM NADPH, 0.5 mM Ca^{2+}, 10 μg/ml calmodulin, and 50 mM DMPO in 50 mM Tris-HCl buffer, pH 7.4. Whereas no signal was observed in the reaction system without enzyme (trace A), a prominent spectrum of the DMPO-OOH adduct was seen after adding 7.3 μg/ml iNOS (trace B). These signals were totally abolished by SOD (200 units/ml, trace C) but not affected by catalase (300 units/ml, trace D). Spectra were recorded at room temperature with a microwave frequency of 9.785 GHz, 20 milliwatts of microwave power, and 0.5 G modulation amplitude. Each spectrum is the sum of five 1-min acquisitions. Bottom, time course of $O_2^−$ generation from iNOS in the absence (filled circles) and presence (unfilled circles) of SOD (200 units/ml). Spectra were continuously recorded at 1 min acquisition from the beginning of the reaction until 15 min. Results are the average of three experiments.

Trace B. These prominent signals exhibited the characteristic DMPO-OOH spectrum ($\alpha_N = 14.2$ G, $\alpha_H = 11.3$ G, $\alpha_H^x = 1.3$ G), indicative of trapped $O_2^−$. A small DMPO-OOH signal ($\alpha_H = \alpha_N = 14.9$ G), which can be derived from the breakdown of DMPO-OOH, was also observed. These signals were totally abolished by SOD (200 units/ml) but not affected by catalase (300 units/ml) (Fig. 2, top, traces C and D), demonstrating that $O_2^−$ was the primary oxygen radical generated by iNOS and that the small DMPO-OOH signals were derived from the decomposition of DMPO-OOH (20). EPR signals from iNOS persisted for at least 60 min, indicating that sustained $O_2^−$ generation occurred. The time course of $O_2^−$ generation from iNOS is shown in Fig. 2, bottom. As shown, the signals were detected immediately after the start of the reaction and reached peak levels after 4−5 min. In the presence of SOD, the process of $O_2^−$ generation was totally quenched.

To reconfirm and quantify the production of $O_2^−$ from iNOS, the recently developed spin trap DEPMPO was also used. The $O_2^−$ adduct, DEPMPO-OOH (half-life ~15 min), is much more stable than that of DMPO, which allows more accurate quantitative measurement of $O_2^−$ production (21). As shown in the Fig. 3 A, no EPR signals were observed in the reaction system with 25 mM DEPMPO in the absence of iNOS. After adding iNOS, prominent DEPMPO-OOH signals were seen (Fig. 3B). These signals were totally quenched by SOD (200 units/ml, Fig. 3C), reconfirming the process of $O_2^−$ generation from iNOS. By comparing the double integral of observed signals with that of known concentrations of the standard TEMPO measured under the same conditions, the initial rate of DEPMPO-OOH production was determined to be 0.4 μM/min. Using the previously reported value for the efficiency of $O_2^−$ trapping by DEPMPO of approximately 60%, we estimate the initial rate of $O_2^−$ generation to be 0.67 μM/min which for the 7.5 μM of iNOS protein used corresponds to 89 nmol/min/mg. Under similar room temperature conditions, the NOS activity measured from L[^14C]arginine to L[^14C]citrulline conversion was 290 ± 6 nmol/min/mg.

Because nNOS generates $O_2^−$ only in the absence of L-arginine, we studied the effect of L-arginine on the $O_2^−$ formation from iNOS. Interestingly, iNOS-catalyzed $O_2^−$ formation was not affected by low levels of L-arginine. With 100 μM L-arginine present, $O_2^−$ signals were essentially unchanged (Fig. 4, A, not shown). However, L-arginine at high concentrations (1-5 mM) markedly decreased the $O_2^−$ signals (Fig. 4, B and C). This inhibition was specifically elicited by L-arginine because its enantiomer, d-arginine, at the same concentration (5 mM) had no effects (Fig. 4D). The fact that iNOS-catalyzed $O_2^−$ is much less sensitive to L-arginine raised the question of whether $O_2^−$ is synthesized at the heme center of the oxygenase domain or the flavins bound at the reductase domain. To explore whether $O_2^−$ was generated from the oxygenase or the reductase domain of iNOS, the flavoprotein inhibitor DPI and heme blocker NaCN were used. $O_2^−$ generation from iNOS was completely blocked by DPI (20 μM, Fig. 4F) but not affected by NaCN (100 μM, Fig. 4E). These effects of DPI and NaCN strongly suggest that $O_2^−$ generation primarily occurs at the flavin-binding sites of the reductase domain, not the heme center of the oxygenase domain.

To directly prove that the iNOS reductase domain is responsible for this $O_2^−$ formation, measurements were also carried out using isolated iNOS reductase domain (residues 499–1144). In
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These O$_2^-$ signals were totally abolished by SOD (200 units/ml) (C); with DPI (20 $\mu$m) (D). EPR spectra were recorded under the same condition as illustrated in the legend to Fig. 2.

In the presence of L-arginine, simultaneous O$_2^-$ and NO generation may occur at the reductase and oxygenase domains, respectively.

Different from the process of O$_2^-$ generation from nNOS which is thought to occur primarily at the oxygenase domain (5–8). This may have important implications in the enzymatic and biological function of iNOS.

Although derived from separate genes and chromosomes, the three NOS isoforms share 50–60% identity in their amino acid sequence (4, 5). They are all bi-domain enzymes consisting of a C-terminal reductase and N-terminal oxygenase. The reductase domain contains NADPH, FAD, and FMN binding sites and exhibits 58% homology to NADPH-cytochrome P-450 reductase (22). Binding sites for heme, BH$_4$, and L-arginine are located at the oxygenase domain. A unified model has been proposed to explain the enzymatic mechanism of NOSs (5).

Their catalytic mechanisms involve flavin-mediated electron transport from C-terminal-bound NADPH and flavins to an N-terminal heme center, where oxygen is reduced and incorporated into the guanidino group of L-arginine giving rise to NO and L-citrulline. Calmodulin binds to a consensus sequence in the NOS enzymes and serves to position the two domains allowing the electron transfer from FMN to heme (5, 23). Although iNOS and nNOS may share similar NO-synthesizing mechanisms, our study reveals a dramatic difference in their mechanisms of O$_2^-$ generation. nNOS produces O$_2^-$ when the heme center is not occupied by L-arginine (5–8, 24). This process requires Ca$^{2+}$/calmodulin to facilitate the delivery of electrons from the reductase to oxygenase domains. In contrast, O$_2^-$ generation from iNOS was much less sensitive to L-arginine and cannot be blocked by cyanide, indicating that it does not occur primarily at the heme. On the other hand, the flavoprotein inhibitor DPI can totally prevent iNOS-catalyzed O$_2^-$ formation. Finally, the experiments performed on isolated iNOS reductase domain definitively demonstrate that O$_2^-$ synthesis occurs at the flavin-binding sites of the reductase domain (Fig. 6).

iNOS and nNOS exhibit very different L-arginine-dependent inhibition of O$_2^-$ generation. Inhibition of O$_2^-$ generation from iNOS was seen only at high L-arginine concentrations. Whereas 100 $\mu$m L-arginine can totally block O$_2^-$ generation.
from nNOS (7), iNOS-mediated O$_2^-$ formation was essentially unaltered by 100 μM L-arginine. Even in the presence of high levels of L-arginine (1 mM), iNOS-catalyzed O$_2^-$ was only partially blocked. In fact, the mechanisms underlying L-arginine-induced inhibition may also vary with these two isoforms. L-arginine blocks the O$_2^-$ generation from nNOS by occupying the heme center leading to NO and L-citrulline formation. From our experiments, it is not clear why large amounts of L-arginine are required to quench the O$_2^-$ generated from the reductase domain, especially in light of the low $K_m$ values for L-arginine with iNOS (1–10 μM). The fact that the signal was not affected by 100 μM L-arginine indicates that DMPO in the 50 mM concentrations used effectively outcompeted NO for reaction with O$_2^-$. The loss of signal at high L-arginine concentrations is most likely due to substrate inhibition. A nonspecific effect of the amino acid is less likely because identical concentrations of d-arginine had no effect. Similarly a reaction between arginine and DMPO-OOH is unlikely because D-arginine had no effect. We hypothesize that higher concentrations of L-arginine suppress the O$_2^-$ generation from iNOS either by altering the conformation of the protein and accessibility of the flavin or by rendering the flavin in a more oxidized state due to more rapid electron transfer to the heme.

Our data did show that substantial O$_2^-$ generation occurs even in the presence of 1 mM L-arginine, strongly suggesting that O$_2^-$ and NO synthesis can occur simultaneously within iNOS. It has been well established that O$_2^-$ reacts with NO at diffusion-limited rates to form another potent oxidant ONOO$^-$ (25). Therefore, O$_2^-$ and NO generated from iNOS may combine to form ONOO$^-$. Indeed, iNOS has been found to generate ONOO$^-$ in L-arginine-depleted macrophages (11).

Considering the structural similarity between the NO reductase domain and cytochrome P-450 reductase, and because cytochrome P-450 reductase is known to be capable of reducing oxygen to O$_2^-$ (26–28), it is not surprising that the iNOS reductase domain can generate O$_2^-$. A recent study showed that the reductase domain of eNOS can also utilize adriamycin as an electron acceptor to generate O$_2^-$ (29). However, nNOS-derived O$_2^-$ is highly sensitive to L-arginine and is thought to mainly arise from the oxygenase domain (5–8). This different O$_2^-$ generation mechanism between nNOS and iNOS appears to precisely match the requirements of their biological function. nNOS mainly generates NO that serves as a neurotransmitter. Because of the relative high L-arginine levels in cytosol (200–800 μM), under physiological conditions, O$_2^-$ generation would rarely occur. This will ensure that nNOS only produces NO and operates properly in signal transduction in neurons. On the other hand, host defense is the main role of iNOS. Simultaneous NO and O$_2^-$ generation may be more beneficial than NO formation alone, because these two free radicals will interact to form the more potent toxic oxidant, ONOO$^-$. Moreover, the combination of O$_2^-$ and NO will prevent the feedback inhibition on iNOS caused by NO or O$_2^-$ promoting sustained NO and O$_2^-$ generation which eventually enhances the killing activity of iNOS.

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