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Reactive Oxygen and Nitrogen Species Regulate Inducible Nitric Oxide Synthase Function Shifting the Balance of Nitric Oxide and Superoxide Production

Jian Sun, Lawrence J. Druhan, and Jay L. Zweier*

Davis Heart and Lung Research Institute, and the Division of Cardiovascular Medicine, Department of Internal Medicine, The Ohio State University, College of Medicine, Columbus, OH 43210, USA

Abstract

Inducible NOS (iNOS) is induced in diseases associated with inflammation and oxidative stress, and questions remain regarding its regulation. We demonstrate that reactive oxygen / nitrogen species (ROS/RNS) dose-dependently regulate iNOS function. Tetrahydrobiopterin (BH₄)-replete iNOS was exposed to increasing concentrations of ROS/RNS and activity was measured with and without subsequent BH₄ addition. Peroxynitrite (ONOO⁻) produced the greatest change in NO generation rate, ~95% decrease, and BH₄ only partially restored this loss of activity. Superoxide (O₂⁻) greatly decreased NO generation, however, BH₄ addition restored this activity. Hydroxyl radical (·OH) mildly decreases NO generation in a BH₄-dependent manner. iNOS was resistant to H₂O₂ with only slightly decreased NO generation with up to millimolar concentrations. In contrast to the inhibition of NO generation, ROS enhanced O₂⁻ production from iNOS, while ONOO⁻ had the opposite effect. Thus, ROS promote reversible iNOS uncoupling, while ONOO⁻ induces irreversible enzyme inactivation and decreases both NO and O₂⁻ production.

Keywords

inducible nitric oxide synthase; nitric oxide; superoxide; peroxynitrite; hydroxyl; hydrogen peroxide; dose-dependent; uncoupling; tetrahydrobiopterin; monomerization

Introduction

Nitric oxide (NO) is a critical signaling molecule involved in control of vasomotor tone, vascular homeostasis; neuronal and immunological function [1-4]. Endogenous NO is produced through the conversion of L-arginine to L-citrulline by NO synthase (NOS) [5-7]. There are three major isoforms of NOS. The neuronal (nNOS) and endothelial (eNOS)

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*Address for correspondence: Dr. Jay L. Zweier, 110G Davis Heart & Lung Research Institute, 473 West 12th Avenue, Columbus, OH 43210-1252. jay.zweier@osumc.edu.

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isoforms are constitutively expressed and require Ca^{2+} and calmodulin for activation, whereas the inducible isozyme (iNOS) is largely Ca^{2+} independent [5]. The expression of iNOS is induced in a wide variety of tissues in response to endotoxin, endogenous mediators of inflammation, and other stimuli such as hypoxia [8]. Relative to the constitutive isoforms, iNOS has ~5-fold higher NO production.

The active forms of all NOS isozymes are homodimeric. Each monomer of the homodimer is associated with calmodulin (CaM) and contains the bound cofactors BH_4 , FAD, FMN and iron protoporphyrin IX (heme) [9]. Each monomer consists of the heme-binding oxygenase domain that also contains the BH_4 and L-arginine binding sites, and the reductase domain that contains the NADPH-binding site, FAD and FMN. When CaM is bound to the dimeric NOS, electrons flow from the reductase of one monomer to the oxygenase domain of the other monomer, which produces an activated oxygen species at the heme, leading to substrate monooxygenation. The production of NO from L-arginine by NOS occurs via two sequential monooxygenation events, consuming 1.5 equivalents of NADPH for every NO produced [10].

All three NOS isoforms can generate $\text{O}_2^{\cdot-}$, depending on substrate and cofactor availability [11-15]. When NOS is not saturated with the cofactor BH_4 , each NOS isoform has been shown to catalyze the reduction of oxygen to $\text{O}_2^{\cdot-}$ [14-19]. In the postischemic heart, BH_4 depletion triggers endothelial dysfunction with loss of NO but gain of superoxide production [16]. In L-arginine-depleted macrophages, iNOS generates both $\text{O}_2^{\cdot-}$ and NO leading to peroxynitrite-mediated cell injury [13]. Moreover, iNOS has been implicated in many diseases associated with inflammation [20].

Oxidative stress occurs at low levels normally, but it is greatly enhanced in a variety of diseases associated with inflammation. Reactive oxygen species (ROS) that are commonly formed include $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$ and H_2O_2 . The reactive nitrogen species (RNS) ONOO^- is formed when $\text{O}_2^{\cdot-}$ combines with NO. Under normal physiological conditions these species are detoxified by several mechanisms, however, when the ROS and/or RNS are overproduced as occurs in many diseases involving chronic inflammation, these reactive species can cause oxidative damage to cellular proteins, membranes and DNA [21-24]. The induction of both iNOS and ROS during inflammation is well established [25,26], however little is known regarding how cellular oxidants affect iNOS function.

In order to characterize the effects of specific reactive oxygen or nitrogen species on iNOS function, the purified enzyme was pre-exposed to known amounts of $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$, H_2O_2 and ONOO^- , and the dose-dependent effects of each oxidant on NO and $\text{O}_2^{\cdot-}$ generation rates were quantified. We observe that $\text{O}_2^{\cdot-}$, $\cdot\text{OH}$, H_2O_2 and ONOO^- all trigger a dose-dependent decrease in NO production. In marked contrast, each of the three ROS species led to increased $\text{O}_2^{\cdot-}$ generation from iNOS, while ONOO^- had little effect. Overall, we observe that ROS and RNS regulate the function of iNOS shifting the balance of NO and $\text{O}_2^{\cdot-}$ production. The dose dependence and reversibility of this process are characterized.

Materials and methods

Materials

Rat iNOS was expressed in *E. coli* as described [27]. Peroxynitrite and degraded peroxynitrite were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Tetrahydro-L-biopterin (BH₄) was obtained from Cayman Chemical Company. HisTrap affinity column, HiTrap desalting column, Hiload 16/60 Superdex 200, Superdex 200 10/300 GL Tricorn™ high performance chromatography column, and gel filtration calibration kits were purchased from Amersham Pharmacia Biosciences (Pittsburgh, PA). 5-(Di-isopropoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DIPPMPO) was from Alexis Biochemicals, Inc. (San Diego, CA). N-methyl-D-glucamine dithiocarbamate (MGD) was synthesized in our laboratory. Xanthine oxidase (XO) and complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Sciences (Indianapolis, IN). All other chemicals were obtained from Sigma unless noted otherwise.

iNOS enzyme purification

Plasmids containing 65 iNOS (a gift from Dr. Dennis Steuhr, The Cleveland Clinic) and CaM/pACYC (a gift from Dr. Ortiz de Montellano, UCSF) were transformed into the protease-deficient *E. coli* strain BL21(DE3). 100 ml of an overnight bacterial culture grown from a single colony of the iNOS/CaM-transformed cells was inoculated to one liter terrific broth (GibcoBRL) containing 125 µg/ml carbenicillin, 35 µg/ml chloramphenicol, and 8 ml of glycerol. The cultures were grown with shaking at 200 rpm at 25 °C. Expression was induced when the optical density at 600 nm reached 0.8, by addition of isopropylthio-β-D-galactoside (IPTG) to the culture (1 mM final concentration); the heme precursor δ-aminolevulinic acid was added concurrently with the IPTG. Cells were harvested 20 h after induction by centrifugation. The cells from 4 L of culture were suspended in minimum volume of lysis buffer containing 40 mM HEPES, 150 mM NaCl, 20 mM imidazole, 10% glycerol and protease inhibitor cocktail tablets at pH 7.4 (Buffer A). The cells were lysed by two passes through an Emulsiflex C3 at 12-15 Kpsi, and the lysate was centrifuged at 48,000 g for 60 min to remove cell debris. The supernatant was loaded onto a 5 mL HisTrap column (GE Biosciences) equilibrated with buffer A. The column was extensively washed with buffer B (40 mM HEPES, 450 mM NaCl, 10 % glycerol, 40 mM imidazole, pH 7.4). Bound protein was eluted with buffer C containing 40 mM HEPES, 450 mM NaCl, 10 % glycerol, 250 mM imidazole, pH 7.4. Fractions containing iNOS were pooled and concentrated using an Amicon Ultra 100,000 MW cut off concentrator (Millipore). The concentrated proteins were applied to a Superdex 200 Hiload size exclusion column equilibrated in 40 mM HEPES, 150 mM NaCl, 10 % glycerol pH 7.4. The iNOS fractions were concentrated and stored in liquid nitrogen. The iNOS concentration was determined using the Bradford assay (Bio-Rad) with bovine serum albumin as the standard. The purity of iNOS was above 90% as determined by SDS-PAGE. The typical activity of iNOS was ~800 nmol mg⁻¹ min⁻¹.

Binding BH₄ to iNOS

As isolated, the iNOS is devoid of BH₄. Thus to prepare BH₄-replete iNOS, purified iNOS (25 µM) was incubated with 20-fold excess of BH₄ (500 µM) and DTT (3 mM) on ice for 4 hours. To remove the free BH₄, the incubated mixture was applied to a HiTrap desalting

column (Amersham) using an AKTA™ FPLC system (Amersham Pharmacia Biotech). BH₄-prebound iNOS was eluted in buffer containing 40 mM HEPES, 150 mM NaCl, and 10% glycerol, pH 7.4 at a flow rate of 0.5 ml/min. The fractions containing the BH₄-prebound iNOS were pooled, concentrated, and stored in liquid nitrogen.

Peroxynitrite treatment of iNOS

BH₄-prebound iNOS at 0.5 µg/µl (~1.0 µM monomer) was exposed to increasing fluxes of ONOO⁻ by infusing stock oxidant solutions with a 100 µl Hamilton syringe driven by Harvard PHD 2000 infusion pump (Harvard Apparatus) at a rate of 4 µl/min for 5 min in the cold box (at 4°C). ONOO⁻ concentrations were determined by absorbance at 302 nm ($\epsilon_{302} = 1.67 \text{ mM}^{-1}\text{cm}^{-1}$) as previously reported [28]. The ONOO⁻ stock, ~150 mM in 0.3 M NaOH, was diluted in 10 mM NaOH to increasing concentrations (0.1, 0.5, 1, 5, 10, 50, 100, 200, 500, 1000, 2000, 5000 µM) just before infusion to 180 µl of iNOS solution in 40 mM HEPES pH 7.4 with constant stirring. As such, the total concentration of ONOO⁻ infused over the 5 minute injection was from 0.01 µM to 500 µM, and there was no detectable pH change induced by the infusion. As a control, degraded ONOO⁻ (1000 µM and 5000 µM) was infused in an identical fashion. Since the half-life of peroxynitrite in neutral solution is less than 2 sec, there was no need for a quenching step for the termination of ONOO⁻ treatment.

ROS treatment of iNOS

BH₄-prebound iNOS at 0.5 µg/µl (~1.0 µM monomer) was exposed to increasing concentrations of each of the three reactive oxygen species: O₂⁻, ·OH and H₂O₂. For O₂⁻ exposure, the generating system consisted of 0.1 unit/ml XO, with xanthine in concentrations ranging from 0.01 µM to 1000 µM, along with 100 µM diethylenetriaminepentaacetic acid (DTPA) to chelate any adventitial iron and 20 unit/ml catalase to remove any H₂O₂ formed. The iNOS was incubated for 20 min at room temperature with this xanthine-XO system. Control experiments using the cytochrome c reduction assay [29] demonstrated that O₂⁻ production from XO at the xanthine concentrations used was complete at 20 min and that the total amount of O₂⁻ produced corresponded to ~50% of the xanthine concentration. Control experiments were also done, pre-exposing iNOS to XO only and uric acid only, with no significant change in NOS activity. For ·OH exposure, ·OH was generated from H₂O₂ via the iron mediated Fenton reaction as reported previously [30]. The ferric iron chelate Fe³⁺ - nitrilotriacetate (Fe-NTA) (1:2) was prepared as described previously [31]. The iNOS was incubated with 10 µM Fe-NTA and H₂O₂ (0.01 µM to 500 µM H₂O₂) on ice for 20 min. The reaction was terminated by the addition of catalase (20 unit/ml). Control experiments were done, exposing iNOS to the Fe-NTA only. For H₂O₂ exposure, the BH₄-prebound iNOS was incubated with given amounts of H₂O₂ in the presence of 100 µM DTPA. After 20 min incubation on ice, catalase (20 unit/ml) was added to terminate the exposure. For all exposures, 5 min was allowed between completion of exposure and measurement of iNOS activity.

EPR spin trapping of NO

Spin trapping measurements of NO release from iNOS were performed using a Bruker EMX EPR spectrometer with HS cavity. The assay mixture contained 40 µg/ml purified BH₄-

bound iNOS, 0.1 mM Fe-MGD (1:10) spin trap (ammonium iron (II) sulfate 0.1 mM, MGD 1 mM), 200 μ M CaCl_2 , 10 μ g/ml CaM, 1 mM NADPH, and 2 mM ^{15}N -L-arg in 40 mM HEPES, pH 7.4. For oxidant treatment, iNOS was treated as above to generate 50 μ M final concentrations except that ONOO^- was added as a bolus, and that 500 μ M oxypurinol was added 1 min before starting the spin trap measurements to ensure termination of the XO-mediated $\text{O}_2^{\cdot-}$ generation. All EPR spectra were obtained in a 50 μ l capillary tube at room temperature (23°C) using the following parameters: microwave power 20 mW, modulation frequency 100 kHz, microwave frequency 9.87 GHz, time constant of 163.84 mSec, and scan time 84 Sec. The modulation amplitude used for NO and $\text{O}_2^{\cdot-}$ detection was 4.0 G and 1.0 G, respectively. All EPR spectra shown are accumulations of 5 scans. Quantitation of NO trapping was determined from the intensity of NO adduct signal recorded after mixing of Fe-MGD with aqueous solutions equilibrated with NO gas of known concentration [32].

Measurement of NO generation rate

The initial rate of NO generation from iNOS was measured using NO-induced oxidation of oxyhemoglobin to methemoglobin. Using this assay, the amount of NO generated from the enzyme is calculated from the change in absorption at 401 nm minus 411 nm as a function of time ($\epsilon_{401-411} = 38 \text{ mM}^{-1} \text{ cm}^{-1}$) [33]. The assay mixture consisted of control or oxidant exposed iNOS (as described above) in 40 mM HEPES, pH 7.4, with 200 μ M CaCl_2 , 10 μ g/ml CaM, 150 μ M DTT, 200 μ M L-arginine and 50 μ M oxyhemoglobin. 200 μ M NADPH was added to start the reaction (Note that no excess BH_4 was added). To determine if BH_4 repletion could rescue the oxidant-induced decrease in NOS activity, matched experiments were performed with addition of 100 μ M BH_4 after the oxidant treatment. For all assays, oxidant treatments of the iNOS were taken to completion, or quenched, prior to addition of the treated enzyme to the kinetic assay mixture. These assays were carried out on a SPECTRA Max Plus 384 from Molecular Devices, using 96-well plates at 37°C. The NO generation rate was determined using the data collected in the linear phase within the first two minutes of the reaction.

Measurement of $\text{O}_2^{\cdot-}$ generation rate

Spin trapping measurements of $\text{O}_2^{\cdot-}$ were performed using a Bruker EMX EPR spectrometer. The assay mixture consisted of 40 μ g/ml purified BH_4 -prebound (control or oxidant treated) iNOS in 40 mM HEPES, pH 7.4, containing 20 unit/ml catalase, 200 μ M CaCl_2 , 10 μ g/ml CaM, 200 μ M NADPH and 10 mM DIPPMPO. All EPR spectra were obtained in a 50 μ l capillary tube at room temperature (23°C) using the following parameters: microwave power 20 mW, modulation amplitude 1.0 G, modulation frequency 100 kHz, microwave frequency 9.84 GHz. The rate of $\text{O}_2^{\cdot-}$ generation was recorded using the time scan function of the EMX software (Win-EPR Acquisition 3.03). The magnetic field was kept fixed at the 4th peak of the DIPPMPO-OOH spectrum and EPR intensity was monitored as a function of time. The parameters used for time scan were as above except time constant of 5242.88 mSec and conversion time of 2621.44 mSec. At the end of the time scan, a field sweep was run to insure that the static field of the time scan remained at the spectral peak ($\pm 0.05\text{G}$). The $\text{O}_2^{\cdot-}$ generation rate was calculated with data collected from the linear increase of EPR arbitrary intensity within the first 200 seconds of EPR acquisition. A constant 30 second delay prior to the initiation of data acquisition was included to allow

for differences in sample preparation time. For all assays, oxidant treatments of the iNOS were taken to completion, or quenched, prior to addition of the treated enzyme to the EPR assay mixture. Quantitations of $O_2^{\cdot-}$ generation with correction for trapping efficiency were performed as reported previously [34,35].

Detection of iNOS dimer and monomer

The iNOS samples were subjected to gel filtration chromatography on a Superdex 200 HR column using an AKTA™ design fast protein liquid chromatography (FPLC) system (GE Biosciences). The column was equilibrated with 50 mM Tris, 150 mM NaCl and 3 mM DTT, pH 7.4 at 4° C. Control and oxidant treated iNOS was injected manually and eluted at a flow rate of 0.3 ml/min and monitored by UV/Visible absorbance at 280 nm, 254 nm and 400 nm. The column was calibrated using protein standards of known molecular weight (thyroglobulin, ferritin, catalase, aldolase, albumin, and ovalbumin), and the void volume was determined with Dextran Blue 2000. The areas of the peaks of dimer and monomer were integrated using the chromatography software package, Unicorn 4.0 (GE Biosciences).

Statistical Analysis

Results were expressed as mean $\pm$ SE.

Results

Oxidant pre-exposure decreases NO production from BH₄-replete iNOS

Initial experiments were carried out to directly determine if exposure of BH₄-replete iNOS to the four biologically relevant oxidants ($ONOO^-$, $\cdot OH$, H_2O_2 or $O_2^{\cdot-}$) would alter NO production. For these experiments, we exposed iNOS to a 50 fold molar excess of each oxidant. After each oxidant exposure was allowed to run to completion, or quenched, we used the NO spin trap Fe-MGD and ^{15}N isotopically labeled L-arginine to measure NO derived from the guanidino group of L-arginine, ensuring that all measured NO production was NOS-specific. As expected, control iNOS generated a strong NO signal exhibiting the characteristic doublet spectrum of the ^{15}NO -Fe-MGD complex, with a 47 μM concentration observed (Figure 1). All four oxidants tested significantly decreased the measured NO production. $ONOO^-$ exhibited the greatest inhibition, decreasing the NO production by > 80% (Figure 1). $O_2^{\cdot-}$ and $\cdot OH$ also clearly inhibited NO formation to about half that of the control untreated enzyme. H_2O_2 exhibited much weaker inhibition of NO production with ~25% decrease observed. Thus, it is clear that exposure to this level of ROS or RNS, as can be formed in disease pathogenesis, decreases NO production from iNOS. As such, oxidant exposure in inflammatory diseases, could inhibit iNOS-mediated NO production.

In vivo, the basal physiological fluxes of each of the highly reactive oxidants we tested are in the submicromolar per second range, and the steady state concentrations in the picomolar range, and these fluxes and concentrations can be considerably higher under various physiologic and pathophysiologic conditions [36]. Therefore, further experiments were performed to measure and quantitate the dose-dependent effect of each oxidant on the NO synthase activity of iNOS over a large range of oxidant concentrations. For these

measurements we used the standard met-hemoglobin formation assay to determine the maximal velocity of the formation of NO from iNOS.

Peroxynitrite dose-dependently inhibits NO generation independent of BH₄ oxidation

In these experiments a flux of peroxynitrite was generated using an infusion pump over a time period of 5 minutes. In order to span the physiological range of ONOO⁻, we generated fluxes of from 1.67 nM/sec to 1.67 μM/sec. We generated these fluxes over a period of 5 minutes, as such they represent exposures of iNOS to a total concentration of 0.5 μM to 500 μM, integrated over the exposure time. For the following, we will refer to this total integrated oxidant concentration. After exposure to 0.5 μM ONOO⁻ the NO generation rate decreased to 82% of basal levels, with 5 μM to 48%, with 50 μM it decreased to 14% of basal iNOS, and with 500 μM total ONOO⁻ (equivalent to a flux of 1.67 μM/sec) it decreased to less than 5% of basal iNOS activity (Figure 2 A). As a control experiment we subjected iNOS to a 1.67 μM/sec flux of degraded ONOO⁻, and no decrease in NO production was observed. Subsequent addition of BH₄ was not effective in restoration of ONOO⁻-induced enzyme dysfunction, only increasing the EC₅₀ from 4.6 μM to 8.4 μM (total integrated ONOO⁻ exposure). With a maximum BH₄-dependent restoration of +17.5 % of basal NO generation seen following 5 μM total ONOO⁻ exposure, and at the highest total oxidant concentration studied, 500 μM ONOO⁻, addition of BH₄ was completely ineffective. This indicates that BH₄-depletion only accounts for a small part of the loss of NO synthase activity and as such ONOO⁻ - induced loss of activity can not be effectively reversed by BH₄ supplementation.

Reactive oxygen species dose-dependently inhibit NO generation mainly via BH₄ oxidation

O₂⁻ also dose-dependently decreased the NO generation rate (Figure 2 B). In order to generate O₂⁻, we kept the XO concentration constant, generating a maximal flux of O₂⁻ equivalent to ~0.4 μM/sec, and increased the amount of total oxidant exposure by increasing the xanthine available for conversion to O₂⁻. In the following we refer to the total O₂⁻ to which the iNOS was exposed during the course of the oxidant flux. Exposure to 20 μM xanthine, which is converted by XO to ~10 μM total measured O₂⁻, produced a statistically significant decrease in NO production, decreasing NOS activity to 78% of baseline. The observed O₂⁻-induced inhibition in NO generation rate increased to a maximum of 86% inhibition at 500 μM total O₂⁻. In stark contrast to the ONOO⁻-induced decrease in iNOS activity, subsequent addition of BH₄ almost completely reversed the O₂⁻-induced loss of iNOS activity, increasing the EC₅₀ from 27.7 μM to 278.6 μM. Even with 250 μM O₂⁻, subsequent addition of BH₄ restored the activity from 16% to 88% of basal levels. Thus, BH₄ was highly effective in reversing the O₂⁻-induced loss of iNOS activity indicating that the majority of the loss of activity was due to O₂⁻-induced BH₄ depletion.

Exposure to ·OH from the H₂O₂/Fe-NTA generating system, producing the same total integrated oxidant concentrations as above, resulted in loss of NO production which was more mild compared to that induced by O₂⁻ treatment, with 72% of basal activity remaining at 50 μM total ·OH (Figure 2 C), and more than 20% of the baseline activity remaining after exposure to a total of 500 μM ·OH. Prominent BH₄-induced rescue of enzyme activity was

observed, but was incomplete suggesting that processes other than BH₄ release/oxidation contribute to the ·OH-induced loss of activity.

Of all the oxidants tested, H₂O₂ had the smallest effect on iNOS activity, 80% of baseline activity was still present even after exposure to 200 μM H₂O₂ (Figure 2 D). H₂O₂ treatment had no significant effect on the NO generation rate at concentrations below 200 μM (Figure 2 D). Even with 500 μM H₂O₂, NO generation only decreased to 44% of the control activity, and subsequent addition of BH₄ almost completely restored this loss of activity.

Effects of oxidative stress on iNOS O₂^{·-} generation

In addition to NO, all NOS isoforms can produce O₂^{·-}, and this ROS generation is increased when BH₄ is depleted [15-19]. BH₄ is susceptible to oxidation and our results above indicate that ROS (and perhaps ONOO⁻) deplete BH₄, limiting the NO synthase function of iNOS. Therefore, we sought to determine the dose-dependent effects of each oxidant on the rate of O₂^{·-} generation from iNOS. Exposure of iNOS to ONOO⁻ had practically no effect on the rate of O₂^{·-} generation at low levels and significant decreases were seen following exposure to total ONOO⁻ concentrations 20 M (Figure 3 A). Following exposure to the highest total ONOO⁻, the rate of O₂^{·-} production concentration studied was decreased by 26%, in stark contrast to the NO generation which was almost totally abolished with a >95% decrease. Exposure to degraded ONOO⁻ did not affect the iNOS O₂^{·-} generation rate.

Conversely, all three ROS treatments dose-dependently increased the O₂^{·-} generation rate of iNOS (Figure 3 B-D), with O₂^{·-} and ·OH producing a biphasic response. With exposure to 10 μM total O₂^{·-} over the time course (20 μM xanthine), the iNOS O₂^{·-} generation rate increased by 33% above baseline and with 50 μM O₂^{·-} by 55% (Figure 3 B). However, exposures to higher O₂^{·-} concentrations, lead to a decrease from this maximal value, returning the iNOS O₂^{·-} generation back to the rate of control enzyme when treated with 500 μM xanthine.

Exposure to ·OH generated from the H₂O₂/Fe-NTA generating system resulted in an overall increase in iNOS-derived O₂^{·-}, with a slight biphasic response. The observed ·OH-dependent increase in iNOS O₂^{·-} generation reached a maximum of 47% of basal activity at 20 μM ·OH, and decreased by 26% of control activity with 500 μM ·OH (Figure 3 C). Of the four oxidants tested, H₂O₂ had the smallest effect on O₂^{·-} generation rate, and like the other ROS species H₂O₂ treatment increased iNOS O₂^{·-} output, with a gradual increase by 24% of basal activity at 20 μM H₂O₂ (Figure 3 D).

Effects of Oxidants on the iNOS monomer/dimer equilibrium

It has been established that monomerization of the NOS enzymes leads to inactivation [37,38]. To determine if the observed oxidant-induced decreases in iNOS NO synthesis activity was due too to changes the iNOS monomer/dimer equilibrium, we used FPLC to quantify the amount of monomer and dimer in control and oxidant treated iNOS. Purified iNOS (25 μg) in 100 μl volume was injected to a Superdex 200 10/300 mm size exclusion column. As seen in figure 4, the untreated iNOS is almost totally dimer, with a retention volume of 11.4 ml. To obtain iNOS monomer, iNOS was incubated with 3 M urea on ice for 2 hours [39]. This urea treated iNOS gave 62% monomer and 38% dimer according to the

peak integration of the absorbance at 280 nm. The retention volume for the monomer was 12.7 ml (Figure 4 A). For these experiments we chose to use oxidant concentrations that demonstrated the most significant changes in iNOS activity, and as such potentially induce the most significant change in the monomer/dimer ratio. A clear increase in monomer occurred with $O_2^{\cdot-}$ -treated (1000 μ M xanthine/0.1 XO unit/ml, 500 μ M total $O_2^{\cdot-}$) and ONOO⁻ (1000 M)-treated iNOS, with levels of 68% and 43% respectively (Figure 4 B). $\cdot$ OH and H_2O_2 treatment (1000 μ M H_2O_2) induced less but notable monomer formation of 27% and 25%, respectively.

Discussion

In the absence of L-arginine or with BH_4 depletion, production of NO from iNOS becomes uncoupled from the oxidation of NADPH, resulting in prominent $O_2^{\cdot-}$ generation. This uncoupling of the enzyme with $O_2^{\cdot-}$ formation would also further shift the balance of NO and $O_2^{\cdot-}$ present and likely induce cellular injury. However, the precise sensitivity of the coupling of iNOS to specific oxidant stress had not been determined. This study demonstrates that in the absence of additional BH_4 both RNS and ROS markedly reduce iNOS NO production. ROS treatment led to an increase in the $O_2^{\cdot-}$ generation capacity of the enzyme, and while the RNS ONOO⁻ tended to decreased the superoxide output of the enzyme, it did not completely inhibit. Thus, oxidative stress leads to inhibition of NO synthase function as well as the uncoupling of iNOS, and this shifts the balance of its product formation from NO synthesis to $O_2^{\cdot-}$ generation (Table 1).

Under various pathophysiological conditions involving oxidative stress, it can be inferred that the total integrated fluxes of ROS formation of $O_2^{\cdot-}$, $\cdot$ OH, or H_2O_2 result in total oxidant concentrations from several μ M to 100 μ M [40-42]. Similar values would also be expected for ONOO⁻ [43]. In this study, we observed that both ONOO⁻ and $O_2^{\cdot-}$ significantly alter iNOS function at levels above 0.5 – 10 μ M, while levels $\geq$ 100 μ M induce 70% reduction of NO synthase activity. In contrast to this, for H_2O_2 -derived $\cdot$ OH, relatively high ($\geq$ 50 μ M) exposures were required to induce significant alterations in iNOS activity. Moreover, treatment with H_2O_2 had very little effect even at very high concentrations, and $\geq$ 200 μ M H_2O_2 was required to produce any significant inhibition. This resistance to oxidation by H_2O_2 is consistent with previous data demonstrating that iNOS can use H_2O_2 as a substrate, with formation of the reactive heme-oxy species, followed by reaction with N^{ω} -hydroxyl-L-arginine or L-arginine [44,45].

The cofactor BH_4 is required for normal NO synthase function but it is highly sensitive to oxidant stress. When BH_4 is depleted iNOS uncoupling occurs, and BH_4 repletion has the potential to restore NO synthase function [17]. With iNOS that was pre-exposed to ONOO⁻, subsequent addition of BH_4 produced only a small partial restoration of NO generation and this BH_4 -induced restoration of function decreased at high levels of exposure. Thus, our data demonstrate that oxidation of BH_4 is not the mechanism by which ONOO⁻ induces iNOS inhibition, and previous work has indicated that oxidation of the iNOS bound calmodulin is also not responsible [46]. Recently it has been shown that iNOS activates ONOO⁻ decomposition, leading to an increase in one-electron oxidation power and protein nitration [47]. This activation of ONOO⁻ was shown to be dependent on the iNOS heme, and the

subsequent iNOS inactivation was hypothesized to correlate with the oxidation of amino acids in the heme binding site, perhaps via nitration of specific tyrosine residues. Our data agree with this hypothesis, moreover, our data imply that $\cdot\text{OH}$ -treated iNOS is at least partially inactivated by similar mechanisms.

Thus, the loss of NO generation by iNOS induced by either ONOO^- or $\cdot\text{OH}$ is via mechanisms involving more than BH_4 oxidation. In contrast, we find that the decrease in NO synthase activity induced by $\text{O}_2^{\cdot-}$ or H_2O_2 is almost completely due to BH_4 oxidation until very high concentrations are reached. We have previously reported the dose-dependent oxidant sensitivity for nNOS, but BH_4 was much less effective in restoration of the oxidant-induced loss of nNOS NO synthase function [17]. Thus nNOS is more prone to BH_4 -independent loss of function than iNOS, indicating that residues critical to NOS activity are more readily oxidized in nNOS than in iNOS.

It is well known that NOS enzymes can produce $\text{O}_2^{\cdot-}$, either in lieu of or in addition to NO, and that the balance of these two processes is related to the levels and oxidation state of the bipterin cofactor. In the absence of BH_4 , the activated oxygen species generated at the heme iron of the NOS oxygenase domain decays with a one electron reduction of molecular oxygen, producing $\text{O}_2^{\cdot-}$ rather than leading to substrate monooxygenation. BH_4 not only increases NO generation by iNOS, but also decreases $\text{O}_2^{\cdot-}$ and subsequent H_2O_2 formation. When BH_4 is added to a viable “uncoupled” NOS, the oxidation of NADPH is again coupled to the production of NO. As such, any oxidant-induced decrease in NO synthase activity that can be rescued by the addition of BH_4 is an indication of the presence of uncoupled NOS. Indeed, we observed that ROS exposure induced this BH_4 -dependent uncoupling, with observed prominent increase in $\text{O}_2^{\cdot-}$ production (coinciding with a decrease in NO synthase activity). Interestingly, we observed that the ROS-induced alterations in iNOS dependent $\text{O}_2^{\cdot-}$ generation is biphasic, and we conclude that the higher levels of ROS exposure lead to oxidation of both BH_4 and other targets in the protein.

In contrast to ROS treatment, the drastic decrease in NO synthase elicited by ONOO^- treatment was practically not reversible with subsequent BH_4 addition, and was accompanied by a decrease in iNOS derived $\text{O}_2^{\cdot-}$ with a modest inhibition seen at the highest levels tested. While treatment with 500 μM ONOO^- , which left the enzyme largely incapable of generating NO even when supplemented with additional BH_4 , $\text{O}_2^{\cdot-}$ generation persisted at ~74% of basal levels (Table I). These data, together with the demonstrated inability of BH_4 to rescue ONOO^- induced enzyme dysfunction, supports our conclusion that ONOO^- -dependent uncoupling (and other oxidants at high levels of exposure) occurs by mechanisms independent of BH_4 oxidation.

Electron transfer from the reductase domain to the oxygenase domain is a requisite inter-subunit transfer [48]. As such, NO synthase activity of NOS requires that the enzyme be in the dimeric form. We found that oxidant exposure led to the degradation of the iNOS dimer. Our FPLC results showed that a large amount of iNOS monomer was generated by treatment with $\text{O}_2^{\cdot-}$, while $\cdot\text{OH}$, H_2O_2 or ONOO^- treatment produced a lesser amount of monomer. Our data indicate that the decrease in iNOS activity produced by $\text{O}_2^{\cdot-}$ is due almost exclusively to the oxidation of BH_4 . It is well known that BH_4 stabilizes the NOS

dimer [49-52], and it is clear that NOS monomerization is in general reversible [51]. Thus, our data indicate that the inactivation of iNOS produced by $O_2^{\cdot-}$ is via the oxidation of BH_4 accompanied by the reversible dissociation of the iNOS dimer. $ONOO^-$ also significantly decreases iNOS NO synthase activity, but our FPLC data clearly demonstrate that this decrease in activity is not solely due to the monomerization of the enzyme, and like the biochemical data this data implies that the oxidation of BH_4 is not the mechanism by which $ONOO^-$ induces iNOS dysfunction. Not unexpectedly, the two ROS species that have the least effect on iNOS activity, $\cdot OH$ and H_2O_2 , also produced the least dissociation of the iNOS dimer.

iNOS is of critical importance in immune function and is induced in a variety of diseases with inflammation and oxidative stress [8,53,54]. In contrast to the other NOS isoforms iNOS is not regulated by Ca^{2+} /Calmodulin [55]. Once synthesized, in the presence of L-arginine and NADPH, iNOS will generate NO at a high rate. In view of this, questions have remained as to how iNOS is regulated. We observe that oxidant stress serves as a brake on NO generation inducing a prominent dose-dependent decrease in the rate of NO formation. With $O_2^{\cdot-}$ and other ROS, this is largely mediated through BH_4 depletion and can be reversed with BH_4 addition. This inhibition was shown to be associated with shift of the enzyme from the active dimer to the inactive monomer. However, with the RNS species $ONOO^-$, reversibility is lost. Thus, ROS and RNS can exert both reversible and irreversible inhibition of iNOS function. This oxidant mediated alteration in iNOS function provides a mechanism for the regulation of iNOS enabling modulation of the amount of NO synthesis and the balance of NO and $O_2^{\cdot-}$ generation from the enzyme.

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The abbreviations used are

NO	nitric oxide
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
eNOS	endothelial nitric oxide synthase
ROS	reactive oxygen species
RNS	reactive nitrogen species
$ONOO^-$	peroxynitrite
$O_2^{\cdot-}$	superoxide
$\cdot OH$	hydroxyl radical
H_2O_2	hydrogen peroxide
BH_4	tetrahydrobiopterin
L-arg	L-arginine

CaM	calmodulin
DIPPMPO	5-(Di-isopropoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide
MGD	N-methyl-D-glucamine dithiocarbamate
XO	xanthine oxidase
Fe-NTA	Fe ³⁺ - nitrilotriacetate
DTPA	diethylenetriaminepentaacetic acid
DTT	dithiothreitol
EPR	electron paramagnetic resonance

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Figure 1. H_2O_2 , $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$ and ONOO^- decrease iNOS NO generation

iNOS was pre-exposed to 50 μM total H_2O_2 , $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$ (100 μM xanthine–0.1 unit/ml XO) or ONOO^- at room temperature for 10 min. EPR spin trapping measurements of NO production from iNOS were performed with 0.2 mM Fe–MGD in the presence of 2 mM ^{15}N -L-arginine with 200 μM CaCl_2 , 10 $\mu\text{g/ml}$ CaM, 1 mM NADPH. EPR spectra were acquired over with parameters as described in methods. Each oxidant treatment was performed in triplicate. The left panel shows representative spectra, exhibiting the characteristic doublet signal of the $^{15}\text{NO-Fe-MGD}$ adduct. The right panel shows a graph of the mean $\pm$ SE value, $n=3$, of the measured NO adduct concentrations from each of the oxidant-treated iNOS samples.

Figure 2. Dose-dependent effect of ONOO^- and ROS on NO generation rate from iNOS
 NO generation rate was measured using the oxyhemoglobin assay in 40 mM HEPES, pH 7.4, with 200 μM CaCl_2 , 10 $\mu\text{g/ml}$ CaM, 150 μM DTT, 200 μM L-arginine and 50 μM oxyhemoglobin, with or without subsequent addition of 100 μM BH_4 . 200 μM NADPH was added to start the reaction. **A**, BH_4 saturated iNOS (1 μM) was incubated with ONOO^- (0-500 μM) through infusion, 4 l/min for 5 min at 4°C. **B**, BH_4 saturated iNOS (1 μM) was incubated with the $\text{O}_2^{\cdot-}$ generation system, xanthine (0-1000 μM) – XO (0.1 unit/ml) – catalase (20 unit/ml), for 20 min at room temperature. $\text{O}_2^{\cdot-}$ produced from xanthine-XO was quantitated using the cytochrome c reduction assay and the concentration of $\text{O}_2^{\cdot-}$ generated corresponded to ~50% of the xanthine concentration. Control experiments were performed to assure that the $\text{O}_2^{\cdot-}$ production from xanthine-XO was completed within 20 min at room temperature. **C**, BH_4 saturated iNOS (1 μM) was incubated with 10 μM Fe-NTA and increasing concentrations of H_2O_2 (0-500 μM) for 20 min on ice and 20 unit/ml catalase was added to remove the residual H_2O_2 before the assay. **D**, BH_4 saturated iNOS (1 μM) was incubated with 100 μM DTPA and increasing concentrations of H_2O_2 (0-500 μM) for 20 min on ice and 20 unit/ml catalase was added to remove the residual H_2O_2 before the assay. The NO generation rates were calculated from the initial rates and are given as percentage of

the activity of the un-treated iNOS. Data are presented as mean $\pm$ S.E. of triplicate experiments. X-axis is the oxidant concentration (μ M) plotted in the log scale.

Figure 3. Effect of biological oxidants on O₂⁻ generation rate from iNOS

BH₄ saturated iNOS was incubated with each of the three ROS tested (0 - 500 μM) for 20 min. ONOO⁻ was infused into iNOS solution at 4 μl/min for 5 min at 4°C. O₂⁻ generation rate was then measured by EPR spin trapping. Each sample contained 40 μg/ml purified BH₄-replete iNOS in 40 mM HEPES, pH 7.4, containing 20 unit/ml catalase, 200 μM DTPA, 200 μM CaCl₂, 10 μg/ml CaM, 200 μM NADPH and 10 mM DIPPMPO. Results for treatment with ONOO⁻ (panel A), O₂⁻ (panel B), ·OH (panel C), and H₂O₂ (panel D) are given at each concentration as the mean ± S.E. of three independent measurements. X-axis is the oxidant concentration (μM) plotted in the log scale.

Figure 4. FPLC measurement of the oxidant-induced monomerization of iNOS

iNOS 25 μ g was applied to each analysis. Elution buffer consisted of 50 mM Tris, 150 mM NaCl and 3 mM DTT, pH 7.4 at 6°C. Supedex 200 column and elution buffer were well equilibrated at ~6 °C. **A**, shows the chromatograms for untreated iNOS, 3 M urea-treated iNOS (3 M urea; 2 hours on ice), O_2^- -treated iNOS (1000 μ M xanthine; 0.1 unit/ml XO; 20 min at RT), $ONOO^-$ -treated iNOS (1000 μ M $ONOO^-$; 10 min on ice), $\cdot OH$ -treated iNOS (1000 μ M H_2O_2 ; 10 μ M Fe-NTA; 20 min on ice), and H_2O_2 -treated iNOS (1000 μ M H_2O_2 ; 200 μ M DTPA; 20 min on ice). Solid lines show the absorbance at 280 nm wavelength and

the dashed lines the absorbance at 400 nm wavelength. **B**, shows a graph summarizing the results from a series of triplicate measurements. Each bar shows the percentage monomer for each condition.

Table 1

Oxidants shift the balance of NO and O₂^{•-} generation

Rate (nmol/mg/min)	Control + BH ₄	Control	ONOO ⁻ (μM)			O ₂ ^{•-} (μM)			·OH (μM)			H ₂ O ₂ (μM)		
			5	50	500	5	50	500	5	50	500	5	50	500
NO ^a	828	431	207	59	20	376	192	59	449	312	95	457	408	189
O ₂ ^b	N.D.	28	30	25	20	34	43	26	38	40	35	33	33	33
NO / O ₂ ^{•-}	-----	15.4	6.9	2.4	1.0	11	4.5	2.3	11.8	7.8	2.7	13.8	12.4	5.7

BH₄ replete iNOS (μM) was exposed to given peroxynitrite and ROS levels. NO and O₂^{•-} generation rates and their ratio are shown. The first column shows values in the presence of 100 μM BH₄ (excess BH₄).

N.D. – not detected

^a Measured by the oxyhemoglobin conversion to methemoglobin

^b Measured by EPR spin trapping using DIPPMPO with quantitation and correction for trapping efficiency performed as previously reported [35].