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Reactive oxygen and nitrogen species regulate inducible nitric oxide synthase function shifting the balance of nitric oxide and superoxide production

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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 (BH_4)-replete iNOS was exposed to increasing concentrations of ROS/RNS and activity was measured with and without subsequent BH_4 addition. Peroxynitrite ($ONOO^-$) produced the greatest change in NO generation rate, ~95% decrease, and BH_4 only partially restored this loss of activity. Superoxide ($O_2^{\cdot-}$) greatly decreased NO generation, however, BH_4 addition restored this activity. Hydroxyl radical ($^{\cdot}OH$) mildly decreases NO generation in a BH_4-dependent manner. iNOS was resistant to H_2O_2 with only slightly decreased NO generation with up to millimolar concentrations. In contrast to the inhibition of NO generation, ROS enhanced $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ production.

Introduction

Nitric oxide (NO)[1] 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) 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 equiv. of NADPH for every NO produced [10].

All three NOS isoforms can generate $O_2^{\cdot-}$, 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 $O_2^{\cdot-}$ [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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$, $^{\cdot}OH$, and H_2O_2. The reactive nitrogen species (RNS) $ONOO^-$ is

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[1] *Abbreviations used:* 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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formed when $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$, $^{\cdot}OH$, H_2O_2, and $ONOO^-$, and the dose-dependent effects of each oxidant on NO and $O_2^{\cdot-}$ generation rates were quantified. We observe that $O_2^{\cdot-}$, $^{\cdot}OH$, 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ production. The dose dependence and reversibility of this process are characterized.

Materials and methods

Materials

Rat iNOS was expressed in *Escherichia coli* as described [27]. Peroxynitrite and degraded peroxynitrite were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Tetrahydro-L-biopterin (BH_4) 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 Δ65 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). One hundred milliliters 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 BH_4 to iNOS

As isolated, the iNOS is devoid of BH_4. Thus to prepare BH_4-replete iNOS, purified iNOS (25 μM) was incubated with 20-fold excess of BH_4 (500 μM) and DTT (3 mM) on ice for 4 h. To remove the free BH_4, the incubated mixture was applied to a HiTrap desalting column (Amersham) using an AKTA™ FPLC system (Amersham Pharmacia Biotech). BH_4-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 BH_4-prebound iNOS were pooled, concentrated, and stored in liquid nitrogen.

Peroxynitrite treatment of iNOS

BH_4-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 a Harvard PHD 2000 infusion pump (Harvard Apparatus) at a rate of 4 μl/min for 5 min in cold box (at 4 °C). $ONOO^-$ concentrations were determined by absorbance at 302 nm ($\varepsilon_{302} = 1.67\ mM^{-1}cm^{-1}$) 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, and 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 min injection was from 0.01 to 500 μM, and there was no detectable pH change induced by the infusion. As a control, degraded $ONOO^-$ (1000 and 5000 μM) was infused in an identical fashion. Since the half-life of peroxynitrite in neutral solution is less than 2 s, there was no need for a quenching step for the termination of $ONOO^-$ treatment.

ROS treatment of iNOS

BH_4-prebound iNOS at 0.5 μg/μl (~1.0 μM monomer) was exposed to increasing concentrations of each of the three reactive oxygen species: $O_2^{\cdot-}$, $^{\cdot}OH$, and H_2O_2. For $O_2^{\cdot-}$ exposure, the generating system consisted of 0.1 U/ml XO, with xanthine in concentrations ranging from 0.01 to 1000 μM, along with 100 μM diethylenetriaminepentaacetic acid (DTPA) to chelate any adventitial iron and 20 U/ml catalase to remove any H_2O_2 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 $O_2^{\cdot-}$ production from XO at the xanthine concentrations used was complete at 20 min and that the total amount of $O_2^{\cdot-}$ 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 $^{\cdot}OH$ exposure, $^{\cdot}OH$ was generated from H_2O_2 via the iron mediated Fenton reaction as reported previously [30]. The ferric iron chelate Fe^{3+}–nitrilotriacetate (Fe–NTA) (1:2) was prepared as described previously [31]. The iNOS was incubated with 10 μM Fe–NTA and H_2O_2 (0.01–500 μM H_2O_2) on ice for 20 min. The reaction was terminated by the addition of catalase (20 U/ml). Control experiments were done,