FAST TRACK
Stimulation of Nitric Oxide Synthase in Cerebral Cortex Due to Elevated Partial Pressures of Oxygen: An Oxidative Stress Response

Stephen R. Thom,[1,2] Veena Bhopale,[2] Donald Fisher,[2] Yefim Manevich,[2] Paul L. Huang,[3] Donald G. Buerk[2,4]

[1] Department of Emergency Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

[2] Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

[3] Division of Cardiology, Massachusetts General Hospital, Boston, Massachusetts 02114

[4] Department of Physiology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

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ABSTRACT: **The purpose of this investigation was to determine the impact of elevated partial pressures of O_2 on the steady state concentration of nitric oxide ($^{\bullet}NO$) in the cerebral cortex. Rodents with implanted O_2- and $^{\bullet}NO$-specific microelectrodes were exposed to O_2 at partial pressures from 0.2 to 2.8 atmospheres absolute (ATA) for up to 45 min. Elevations in $^{\bullet}NO$ concentration occurred with all partial pressures above that of ambient air. In rats exposed to 2.8 ATA O_2 the increase was 692 ± 73 n*M* (S.E., $n = 5$) over control. Changes were not associated with alterations in concentrations of nitric oxide synthase (NOS) enzymes. Based on studies with knock-out mice lacking genes for neuronal NOS (nNOS) or endothelial NOS (eNOS), nNOS activity contributed over 90% to total $^{\bullet}NO$ elevation due to hyperoxia. Immunoprecipitation studies indicated that hyperoxia doubles the amount of nNOS associated with the molecular chaperone, heat shock protein 90 (Hsp90). Both $^{\bullet}NO$ elevations and the association between nNOS and Hsp90 were inhibited in rats infused with superoxide dismutase. Elevations of $^{\bullet}NO$ were also inhibited by treatment with the relatively specific nNOS inhibitor, 7 nitroindazole, by the ansamycin antibiotics herbimycin and geldanamycin, by the antioxidant *N*-acetylcysteine, by the calcium channel blocker nimodipine, and by the *N*-methyl-D-aspartate inhibitor, MK 801. Hyperoxia did not alter eNOS association with Hsp90, nor did it modify nNOS or eNOS associations with calmodulin, the magnitude of eNOS tyrosine phosphorylation, or nNOS phosphorylation via calmodulin kinase. Cerebral cortex blood flow, measured by laser Doppler flow probe, increased during hyperoxia and may be causally related to elevations of steady state $^{\bullet}NO$ concentration. We conclude that hyperoxia causes an increase in $^{\bullet}NO$ synthesis as part of a response to oxidative stress. Mechanisms for nNOS activation include augmentation in the association with Hsp90 and intracellular entry of calcium.** © 2002 Wiley Periodicals, Inc. J Neurobiol 51: 85–100, 2002;

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INTRODUCTION

The purpose of this investigation was to determine the impact of elevated partial pressures of O_2 on the steady state concentration of nitric oxide ($^{\bullet}NO$) in the cerebral cortex. Previous investigations have sug-

Correspondence to: S.R. Thom (sthom@mail.med.upenn.edu).
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gested that elevations in brain O_2 tension alter concentrations of •NO. However, conclusions were based on measurements of the products produced as a consequence of •NO production, rather than by direct measurements, and the results have been contradictory (Demchenko et al., 2000a, b; Elayan et al., 2000; Wang et al., 1998).

The quest to understand CNS O_2 toxicity has fueled interest in whether the •NO concentration may be altered by elevated partial pressures of O_2. Rats exposed to extreme pressures of O_2 [5 atmospheres absolute (ATA)] were found to have increased intrasynaptosomal free Ca^{++} concentrations, which secondarily increased NOS activity (Wang et al., 1998). NOS activity was assessed indirectly as cyclic GMP level. The occurrence of seizures, a manifestation of O_2 toxicity, was inhibited by a NOS inhibitor and also by a Ca^{++} channel blocker. More recently, Elayan et al. (2000) reported four- to sixfold elevations in nitrite and nitrate, decomposition products of •NO, in microdialysate perfused through the brains of rats exposed to 3 ATA O_2. They found the K_m for O_2 of nNOS to be 260 μM, and suggested that increased nNOS activity in brain may be a direct biochemical consequence of increased substrate. In contrast to these results, Demchenko et al. (a, b) reported a 31 to 36% decrease in nitrite and nitrate concentrations in brain microdialysate when rats were exposed to 5 ATA O_2. They proposed that •NO was depleted in brain due to reactions with superoxide radicals ($O_{2-}^{\bullet}$). Ito et al. (1996) speculated that hyperbaric O_2 may promote synthesis of •NO because of an apparent inhibition of arginase activity in brain, and exposures to 6 ATA O_2 have been found to cause elevations in brain arginine levels (Zhang et al., 1993).

Nitric oxide has been shown to have a stimulatory action on neurotransmitter release in the mammalian CNS (Ohkuma et al., 1998; Kuriyama and Ohkuma, 1995). Glia, neuronal and endothelial cells, and vascular nerves, are the major sites where •NO is synthesized, and neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) are the predominant isoforms that are involved (Brendt and Snyder, 1996). When agonists bind to membrane receptors, they elevate intracellular Ca^{++}, which causes calmodulin to bind and activate both nNOS and eNOS (Ignarro et al., 1987). Calcium influx through neuronal *N*-methyl-D-aspartate (NMDA) receptors, in particular, efficiently stimulates nNOS (Christopherson et al., 1999). Agonists also cause the molecular chaperone, heat shock protein 90 (Hsp90), to bind and activate eNOS and nNOS (Bender et al., 1999; Garcia-Cardena et al., 1998). Elevation of vascular shear stress will cause eNOS activation via Hsp90 binding and Akt protein kinase-dependent phosphorylation (Dimmeler et al., 1999; Garcia-Cardena et al., 1998). Phosphorylation of nNOS by calcium/calmodulin-dependent protein kinase IIα inhibits nNOS activity and may serve a feed-back regulatory function in neuronal cells (Komeima et al., 2000).

The aims of this study were to directly measure brain •NO and O_2 levels under hyperbaric conditions using selective microelectrodes and to identify the mechanism for alterations in •NO concentration. The majority of experiments were conducted in rats, with a complementary series carried out using "knock-out" mice lacking functional genes for eNOS or nNOS.

MATERIALS AND METHODS

Materials

Wistar male rats (Charles River Laboratories, MA) weighing 220–240 g were fed a standard diet and water *ad libitum*. Female house mice (*Mus musculus*) of the C57B6J strain were raised at the animal facilities of Massachusetts General Hospital. Mice used in the study were either wild type or lacked a functional nNOS gene (nNOS −/−) or a functional eNOS gene (eNOS −/−). Unless otherwise specified, reagents were purchased from Sigma Chemical Company (St. Louis, MO). Geldanamycin was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). MK 801 was purchased from RBI Research Biochemicals, Inc. (Natick, MA). Rabbit antibody to nNOS used for both immunoprecipitation and staining Western blots was purchased from Cayman Chemical Company (Ann Arbor, MI). Rabbit antibody to eNOS and against postsynaptic density protein 95 (PSD-95), used for immunoprecipitation, and herbimycin were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Anti-iNOS and anti-Hsp90 were purchased from StressGen, Inc. (Victoria, British Columbia). Mouse antibody against PSD-95 and phosphotyrosine used for staining Western blots were purchased from BD Transduction Laboratories (Lexington, KY). Mouse antibody against calmodulin was from Upstate Biotechnology (Lake Placid, NY). Rabbit antibody to Akt and phospho-Akt were purchased from Cell Signaling Technology (Beverly, MA). The following horseradish peroxidase-conjugated antibodies were purchased from Chemicon, Inc. (Temecula, CA): goat antimouse IgG, goat antirabbit IgG, and goat anti-guinea pig IgG. Rabbit antibody recognizing nNOS phosphorylated at serine 847 was generously provided by Dr. Yasuo Watanabe, Nagoya University School of Medicine, Nagoya, Japan. Guinea pig antibody against phosphofructokinase-M was provided by Dr. Robert G. Kemp, Finch University of Health Sciences, Chicago Medical School. ECL reagents were from Amersham Pharmacia Biotech. All aspects of this investigation were reviewed and approved by the Institutional Animal Care and Use Committee.

Microelectrode Fabrication

Microelectrodes selective for •NO were fabricated from either borosilicate or flint glass micropipettes, beveled at

approximately a 60° angle after pulling. Micropipettes were partially filled with heated Wood's metal alloy, leaving a ≈100 μm deep recess at the tip after cooling. A 20 to 40 μm thick layer of gold was electroplated into the recess over the metal alloy. After thorough cleaning, microsensors were tested for stability, and any electrodes with erratic polarographic currents were rejected. A thin layer of Nafion polymer was applied to each electrode by dip coating and allowed to dry. Two-point calibrations for each electrode were made at 37°C in physiological buffer equilibrated with either 100% N_2 or 1800 ppm NO (balance N_2). The electrodes were polarized at an oxidation potential of +850 mV relative to an Ag/AgCl reference electrode. Electrochemical oxidation currents were amplified with a sensitive electrometer (Keithley, model 610). The electrometer voltage output was low-pass filtered (analog circuit with 5 Hz cutoff) and digitized (1 sample/s) by computer. Current sensitivities ranged between 0.5 and 5 pA/μM. Sensitivities to ascorbate in the concentration range from 0.2 to 1 mM have been tested, and have been found to be 100 to 1000 times smaller than •NO sensitivities. L-tyrosine, another possible interfering chemical species in the brain, was also tested at concentrations up to 10 mM, and was found to have no effect on the current. Electrodes used in this study had tip diameters ranging between 5 and 15 μm, with recesses ≈50 μm deep (Buerk et al., 1996).

Electrode Studies

The standard procedure was to anesthetize both rats and mice with intraperitoneal ketamine (83 mg/kg) and xylazine (11 mg/kg). In a number of studies, to determine whether results may be affected by choice of anesthetic, rats were anesthetized with thiobutabarbital (Inactin, 80 mg/kg). After animals were anesthetized, the scalp was removed, a 4 mm hole drilled through the skull with a bone drill, and in an adjacent area the skull bone was thinned but not completely removed. If ketamine/xylazine was used for surgical anesthesia, a second dose of ketamine/xylazine amounting to 3/4 of the initial dose was given just prior to placing the animal in the hyperbaric chamber. Electrodes were placed on micromanipulator arms above the head and slowly advanced through the dura and into cerebral cortex. In the adjacent area where skull bone had been thinned, the laser Doppler flowmeter (a 3 mm probe attached to Model BLF 21 flowmeter; Transonic Systems, Ithaca, NY) was fixed using an additional manipulator arm. The hyperbaric chamber used in this study was rated for a maximum pressure of 3.0 ATA and has been described in a prior publication (Thom and Elbuken, 1991). In some studies arterial blood pressure was monitored by placing a saline-filled catheter into a femoral artery. Polyethylene tubing was passed through penetrations in the hyperbaric chamber wall and connected to a Gould Stratham physiological pressure transducer. Once in the closed chamber, animals were monitored for approximately 30 min until electrode and cerebral blood flow recordings became stable. During this time, pure, compressed air was flowed through the chamber to remove exhaled gases, but no additional pressure was applied. Where specified, rats were injected with 7-nitroindazole (12 mg/kg i.p.), *N*-acetylcysteine (NAC; 40 mg/kg i.p.), nimodipine (1 mg/kg i.p.), or geldanamycin (0.3 mg/kg i.p.) 30 min prior to pressurization in the hyperbaric chamber. Others received MK 801 (5 mg/kg) or bovine erythrocyte (copper-zinc) superoxide dismutase (SOD; 25,000 U/kg) intravenously immediately prior to pressurization through a catheter and intravenous line that passed through the hyperbaric chamber wall. Herbimycin was prepared as a 1 mM solution in DMSO, diluted to 5 μM in saline, and 100 μL was placed on the brain surface just before the chamber door was closed at the site where the •NO-specific electrode had been placed. Parallel control studies performed by placing just DMSO in saline on the brain demonstrated that the carrier solution had no effect on the •NO or blood flow responses.

Tissue Preparation for Immunochemical and Biochemical Assays

Rats were anesthetized with intraperitoneal ketamine (83 mg/kg) and xylazine (11 mg/kg), decapitated, and brains were removed. Brains were immediately homogenized in 25 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 100 μM DETPAC, 40 μM PMSF, 0.5 μg/mL leupeptin, and 0.01% BHT. For each 1 g of brain, 2 mL buffer was added, and tissue was homogenized with two 30 s pulses in a Polytron and then two strokes with a Teflon plunger. Homogenates were centrifuged (12,000 × g for 5 min) and supernatants used in subsequent assays.

Arginine Concentration

Measurements of arginine concentration were performed using supernatants from brain homogenates precipitated with 10% trichloroacetic acid and centrifuged at 3000 × g at 4°C for 15 min. The supernatant was dried and mixed with 20 μL of standard phenylisothiocyanate (PITC) derivatization mixture for 20 min at room temperature and then redried according to the standard PICO*TAG Amino Acid analysis procedure [PICO*TAG Amino Acid Analysis System (Operators Manual, revision 4, Waters, Milford, MA)]. The precipitate was resuspended in 100 μL standard diluent solution (Waters) and a 10 μL injection was analyzed using a 2690 Alliance separation unit (Waters) equipped with a NovaPack C_{18} (3.9 × 300 mm, particle size 4 μm, pore size 60Å) reversed phase high resolution column (Waters) at 46°C. The mobile phase was 60:40:5 = acetonitryl:water: 0.3 M sodium acetate, pH 6.0. Elution was performed in an isocratic regime at 1 mL/min and results were quantified by PDA 996 (Waters) at 254 nm using authentic l-arginine derivatized under identical conditions as an internal standard. All data were processed using Millenium32 software (Waters).

Immunoprecipitation

Brain supernatants containing 250 μg protein were incubated overnight at 4°C with antibodies (10 μg of anti-

HSP90, 10 μg of anti-n- or eNOS) in 500 μL precipitation buffer [20 m*M* MES ([4-morpholine] ethanesulfonic acid) pH 7.6 containing 100 m*M* NaCl, 0.1 m*M* dithiothreitol, 0.1 m*M* EDTA, 10% glycerol, 0.4% Triton X-100, 1 m*M* PMSF, 10 m*M* sodium fluoride, 10 μg/mL aprotinin, 1 m*M* sodium orthovanadate, 10 m*M* sodium fluoride, 10 μ*M* tetrahydrobiopterin, 1 m*M* arginine, and 20 m*M* sodium molybdate]. The next day, 30 μL 20% (w/v) protein G-sepharose was added to the suspensions, which were incubated 1.5 h at 4°C, and then centrifuged at 8,200 $\times$ *g* for 1 min. The immune pellets were washed twice with wash buffer (10 m*M* MES, pH 7.6 containing 50 m*M* NaCl, 20 m*M* sodium molybdate, 10% glycerol, and 0.4% Triton X-100), pellets were suspended with 40 μL sample buffer (100 m*M* sodium phosphate, pH 7.4 containing 2% SDS, 10% glycerol, 5% β mercaptoethanol, and 0.00125% bromophenol), and the suspension heated to 95°C for 10 min. After centrifugation at 8,200 $\times$ *g* for 1 min, aliquots (30 μL) of supernatant were electrophoresed using a 12% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes and probed with 1:1000 dilutions of antibody (anti-Hsp90, eNOS, nNOS, or PSD-95).

Data Analysis

Statistical significance was determined by ANOVA followed by Scheffe's test. The level of significance was taken as $p < .05$. Results are expressed as means $\pm$ S.E.

RESULTS

Elevation in Brain •NO and O_2 Concentration with Hyperbaric Oxygen

The dose-response relationship between O_2 partial pressure and brain •NO concentration is shown in a representative experiment (Fig. 1). Anesthetized rats with •NO- and O_2-selective microelectrodes in the cerebral cortex were monitored while breathing air and 100% O_2 at ambient pressure, followed by observations after pressurization with O_2 to 2 ATA and then 2.8 ATA. There were significant elevations in steady state •NO and O_2 concentrations in response to hyperoxia, even at ambient pressure. Mean changes in brain •NO concentration for rats exposed to 1.0, 2.0, and 2.8 ATA O_2 were, respectively, 292 $\pm$ 36 n*M* (S.E., $n = 5$), 641 $\pm$ 48 n*M* ($n = 5$), and 692 $\pm$ 73 n*M* ($n = 9$). If the gas flow at 2.8 ATA was changed from pure O_2 to a mixture of nitrogen and just 7.46% O_2 so that the O_2 partial pressure was the same as breathing air at ambient pressure (0.21 ATA), •NO concentration decreased (Fig. 1). This indicates that elevated •NO synthesis was not a consequence of increased pressure *per se*.

The relationship between the elevation in brain •NO concentration and local blood flow measured by

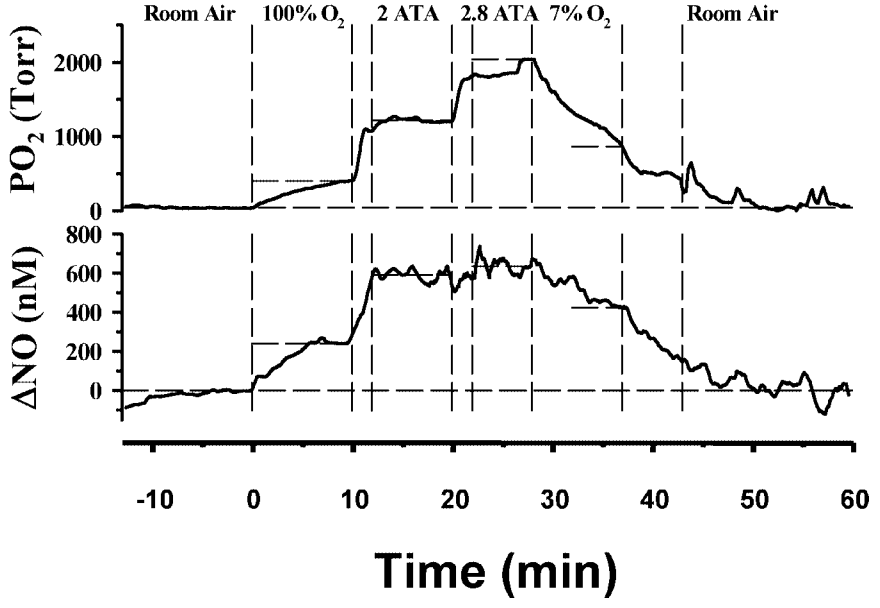


Figure 1 Representative experiment showing results of a rat with implanted O_2 and •NO-specific electrodes. Elevations in brain O_2 tension and •NO concentration are shown in response to changing the breathing gas from air (O_2 partial pressure of ≈0.21 ATA) to 100% O_2 at ambient pressure, then to 100% O_2 at 2.0 ATA, then to 100% O_2 at 2.8 ATA, then to 7.46% O_2 at 2.8 ATA (O_2 partial pressure 0.21 ATA), and then back to air at ambient pressure. Narrow intervals identified by vertical dashed lines were times when the O_2 partial pressure was being changed. Intervals where a particular O_2 partial pressure was breathed are identified on the figure.

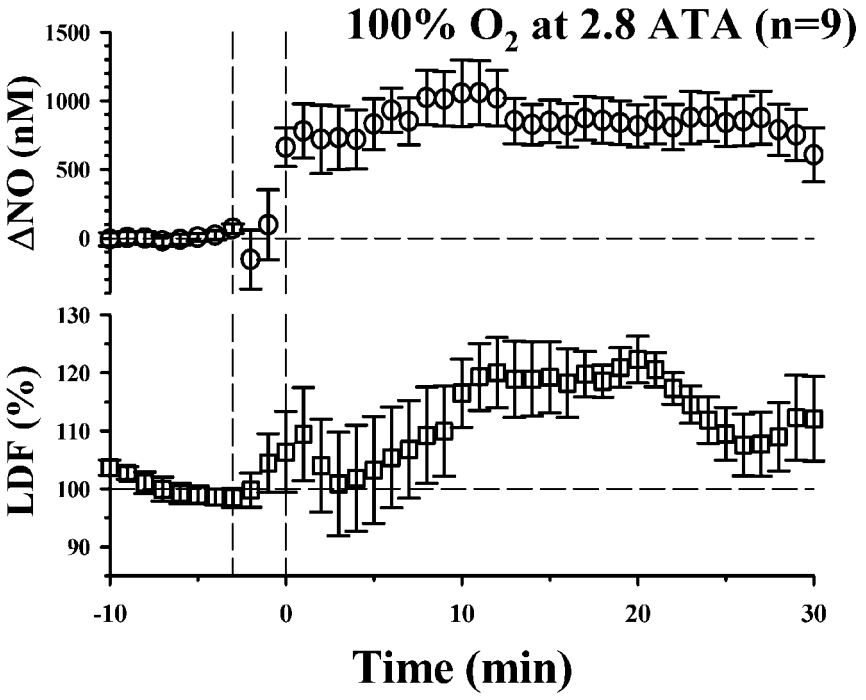


Figure 2 Mean values for changes in brain •NO concentration and laser Doppler blood flow among nine rats exposed to 2.8 ATA O_2. The vertical dashed lines illustrate the time required to increase chamber pressure using pure O_2.

laser Doppler flowmeter in rats exposed to 2.8 ATA O_2 is shown in Figure 2. The •NO concentration changed immediately upon elevation of ambient O_2 tension and stabilized shortly after reaching 2.8 ATA. We found that blood flow vacillated during exposure to hyperbaric O_2 but that after exposure to hyperoxia for 10 min all values were significantly elevated over control ($p < .05$, repeated measures ANOVA). Brain •NO level and cerebral blood flow decreased to the baseline level when rats were decompressed to ambient pressure and breathed air.

Our standard procedure was to anesthetize animals with ketamine/xylazine (see Materials and Methods). A second series of studies was conducted to evaluate whether responses may be influenced by a particular type of anesthesia. We found that rats anesthetized with Inactin (thiobutabarbital) exhibited virtually the same responses as those observed under the standard procedure (Fig. 3).

Hyperoxia did not change the concentrations of NOS isoforms in brains over the duration of these studies. Western blots performed on brain homogenates from control rats and rats killed immediately following exposure for 45 min to 2.8 ATA O_2 were probed for the three NOS isoforms. Relative band densities, normalized to the amount of glucose transporter protein 3 (GLUT 3) oxidase present on each blot, were not significantly different (Fig. 4).

Blood pressure was monitored during the hyperbaric O_2 exposure in some rats and was found to be unchanged. Arterial blood gas values could not be obtained during the O_2 exposures, but comparisons were made between samples obtained before and immediately after exposure to 2.8 ATA O_2 for 30 min. Arterial O_2 tension prior to pressurization was 94.6 $\pm$ 10.6 S.E., ($n = 8$) mmHg. Although it was expected that the value would drop rapidly following decompression, it was significantly greater than control, 164.9 $\pm$ 6.8 ($n = 8$, $p < .05$) mmHg, in arterial blood obtained within 5 min after the hyperbaric O_2 exposure. Blood pH and partial pressure of CO_2 were not significantly different.

Investigation of NOS Isoforms in Mice

The role of nNOS and/or eNOS in brain •NO elevations was examined in knock-out mice (Fig. 5). The average increase in brain •NO during the final 5 min of exposure to 2.8 ATA O_2 was 890 $\pm$ 108 n*M* (S.E., $n = 5$) in wild-type mice, 623 $\pm$ 99 n*M* ($n = 4$) in eNOS knock-out mice, and only 63 $\pm$ 32 n*M* ($n = 6$) in nNOS knock-out mice. These values were significantly different from each other (ANOVA). Treatment of wild-type mice with 7-nitroindazole (7-NI), a relatively specific nNOS inhibitor, gave results consistent with those of the nNOS knock-out mice (Fig.

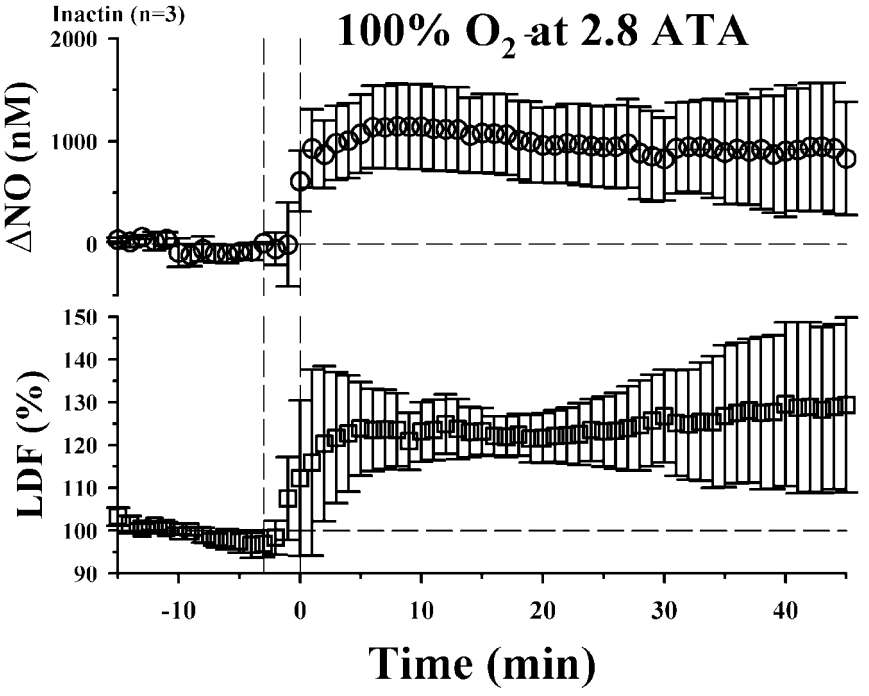
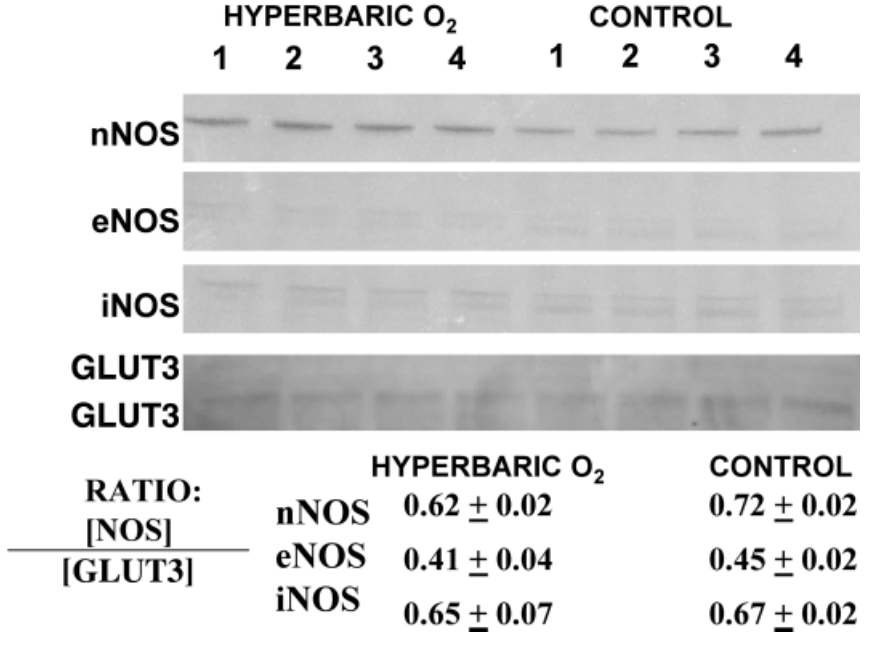


Figure 3 Mean values for changes in brain •NO concentration and laser Doppler blood flow among three rats exposed to 2.8 ATA O_2 after anesthesia with Inactin (thiobutabarbital) versus ketamine/xylazine, as was used in all other studies. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.



RATIO: [NOS]/[GLUT3]	HYPERBARIC O_2	CONTROL
nNOS	0.62 ± 0.02	0.72 ± 0.02
eNOS	0.41 ± 0.04	0.45 ± 0.02
iNOS	0.65 ± 0.07	0.67 ± 0.02

Figure 4 Representative Western blots of brain homogenates from four control rats and four rats killed immediately after exposure to 2.8 ATA O_2 for 30 min. The blot was probed for each NOS isoform and also glucose transporter protein 3 (GLUT3), as an internal control for protein loaded onto each column. Band densities were measured and the ratio of each NOS isoform versus GLUT3 was determined. No significant differences were found between control and hyperbaric O_2-exposed brain samples.

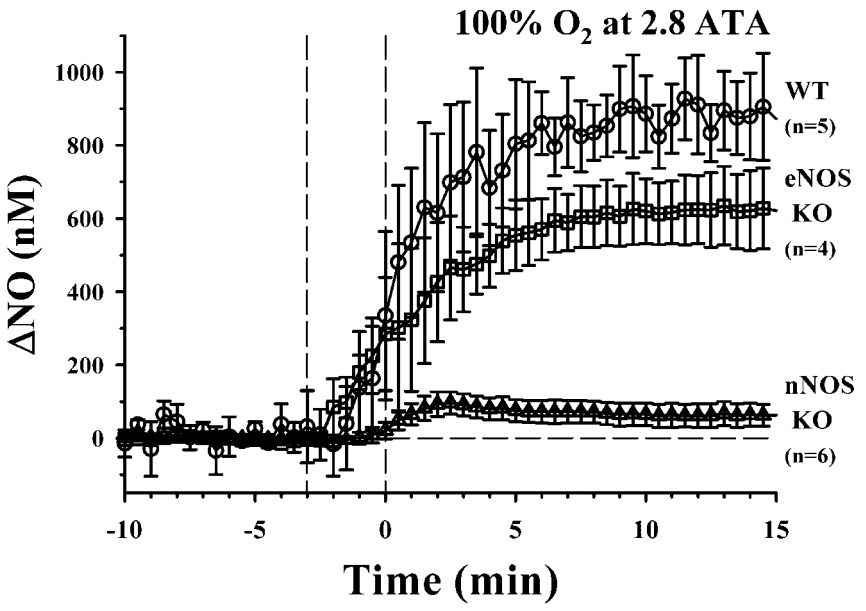


Figure 5 Mean values for changes in brain •NO concentration among mice exposed to 2.8 ATA O_2. Values shown are for changes in wild-type mice (WT), mice with no functional eNOS genes (eNOS), and those with no functional nNOS genes (nNOS). The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.

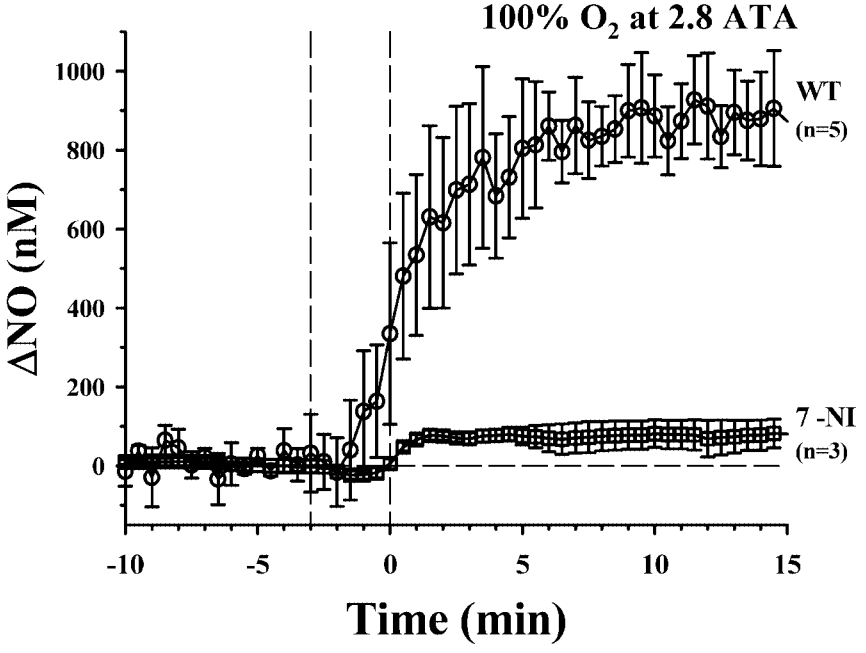


Figure 6 Mean values for changes in brain •NO concentration among wild-type mice. Where indicated, some mice were injected with 7-nitroindazole (12 mg/kg i.p.) before being exposed to 2.8 ATA O_2. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2. Data identified with a "WT" are the same as shown for wild-type mice in Figure 5, and were included to ease comparison.

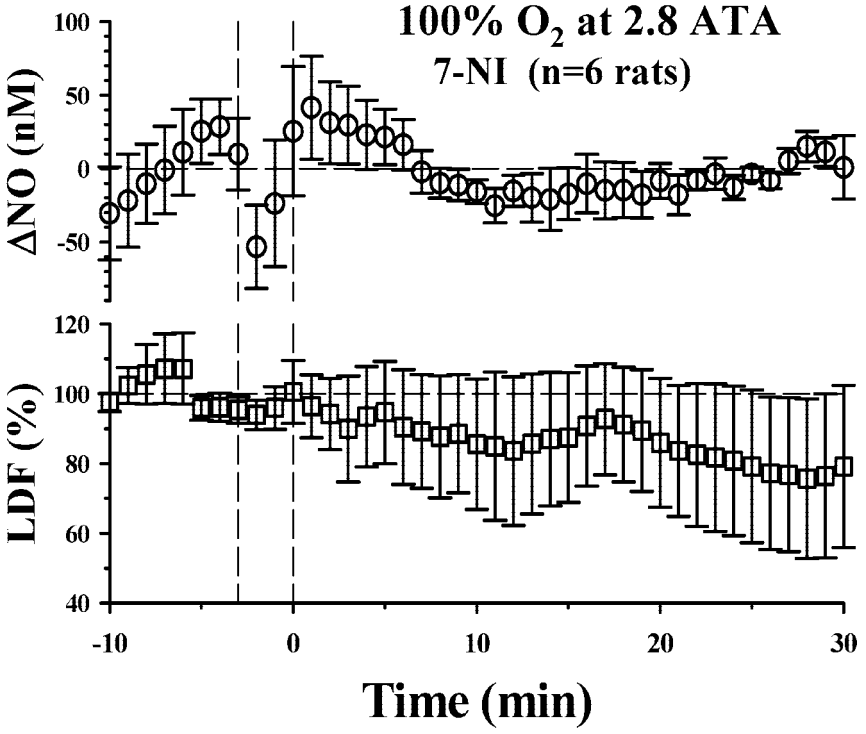


Figure 7 Mean values for changes in brain •NO concentration and laser Doppler blood flow among rats injected with 7-nitroindazole (12 mg/kg i.p.) before being exposed to 2.8 ATA O_2. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.

6). The increase in •NO concentration with exposure to 2.8 ATA O_2 was only 77 ± 39 n*M* ($n = 3$). From these studies we conclude that nNOS activity was responsible for approximately 90% of the •NO synthesis that occurred in response to hyperoxia.

Pharmacological Inhibitors of NOS Activation in Rats

Treatment with 7-NI inhibited increases in brain •NO concentration of rats exposed to 2.8 ATA O_2 and also blocked elevations in cerebral blood flow (Fig. 7). One mechanism by which constitutive NOS isozymes can be activated is by association with the molecular chaperone, Hsp90, and the effect can be inhibited by ansamycin antibiotics (Bender et al., 1999). Infusion of geldanamycin caused significant inhibition of hyperoxia-induced •NO elevation and also prevented the elevation in blood flow (Fig. 8). We found that administration of an alternative ansamycin antibiotic, herbimycin, onto the brain surface near the electrode also reduced the steady state concentration of •NO in response to 2.8 ATA O_2 (data not shown).

To assess the possible role of oxidative stress in NOS responses, rats were infused with either SOD or NAC. SOD was an effective inhibitor, blocking elevation in brain •NO concentration by over 80% (Fig. 9) for the first 20 min following infusion. Brain •NO concentration rose slightly during the last 10 min of the experiment, presumably due the short plasma half-life of SOD (Turrens et al., 1984). Laser Doppler blood flow also rose slightly during the final 10 min by 109 ± 11% (not significant). NAC reduced the initial elevation in brain •NO to approximately 500 n*M*, and the level dropped progressively over the remainder of the 30 min experiments (Fig. 10). Overall, NAC caused a modest, statistically significant (≈70%) inhibition in •NO elevation. Cerebral blood flow exhibited greater variability in the NAC studies than among untreated rats exposed to hyperbaric O_2. Changes in laser Doppler blood flow were not significantly different from those measured prior to exposure to 2.8 ATA O_2.

Figure 11 shows the effects of infusion of the calcium channel blocker, nimodipine. The magnitude of •NO elevation observed at the time of initial pressurization with O_2 to 2.8 ATA was reduced to approximately 450 n*M* and the •NO level dropped progressively for the duration of the experiment. It was just 160 ± 44 n*M* ($n = 4$) over baseline by the end of the exposure, significantly lower than in untreated rats (ANOVA). There were no significant changes in laser Doppler blood flow due to O_2 among nimodipine-

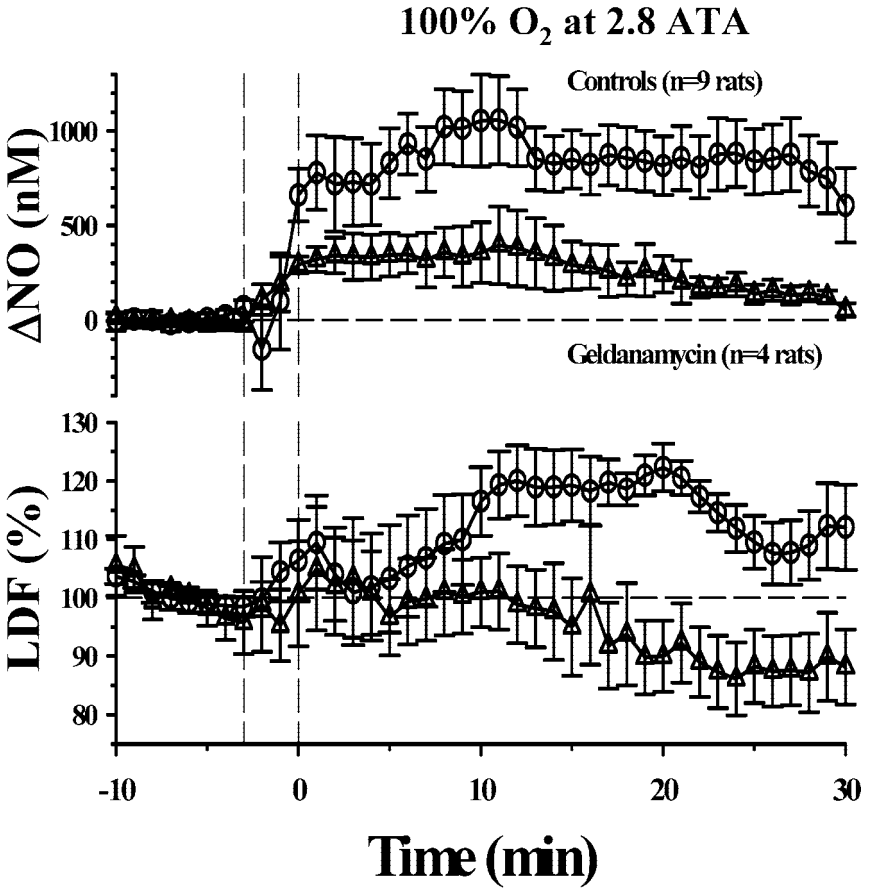


Figure 8 Mean changes in brain •NO concentration and laser Doppler blood flow for rats treated with geldanamycin (0.3 mg/kg i.p.) and exposed to 2.8 ATA O_2. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2. Data for untreated (control) rats, the same as shown in Figure 2, were included to ease comparison.

treated rats (Fig. 11). Infusion of MK 801, which binds to the calcium channel of the NMDA receptor, significantly reduced hyperoxia-induced •NO elevation and also the associated increase in laser Doppler blood flow (Fig. 12).

Immunoprecipitation Studies

Brain homogenates were subjected to immunoprecipitation to examine possible associations among proteins. Exposure to 2.8 ATA O_2 caused a twofold increase in association between nNOS and Hsp90 in Western blots generated following immunoprecipitations with antibody directed against either nNOS or Hsp90 (Fig. 13). Consistent with the inhibitory effect of SOD infusion on •NO synthesis shown in Figure 9, if rats were first infused with SOD, the association between nNOS and Hsp90 no longer occurred when rats were exposed to hyperbaric O_2 (Fig. 13).

There was no significant change in the association between eNOS and Hsp90 based on immunoprecipitation studies. When antibody against Hsp90 was used for immunoprecipitation, the band density ratio of eNOS to Hsp90 normalized to the density of the Hsp90 band was 0.33 ± 0.02 (n = 5) for control and 0.4 ± 0.02 (n = 5) for hyperbaric O_2-exposed samples. When anti-eNOS was used for immunoprecipitation, the Hsp90/eNOS band density ratio normalized to the density of eNOS on the blots was 0.68 ± 0.2 (S.E., n = 5) for control and 1.0 ± 0.2 (n = 5, not significant) for brains from rats exposed to 2.8 ATA O_2 for 45 min.

Immunoprecipitation studies were also done to assess associations between eNOS or nNOS with calmodulin. When using antibody directed against nNOS, the calmodulin/nNOS band density ratio normalized to the density of nNOS on the blots was 0.87 ± 0.03 (n = 5) for control samples and 0.86 ± 0.05 (S.E., not significant) for hyperbaric O_2 samples. In blots generated after immunoprecipitations were performed with anti-eNOS, the ratio of calmodulin to eNOS normalized to the eNOS band density on the

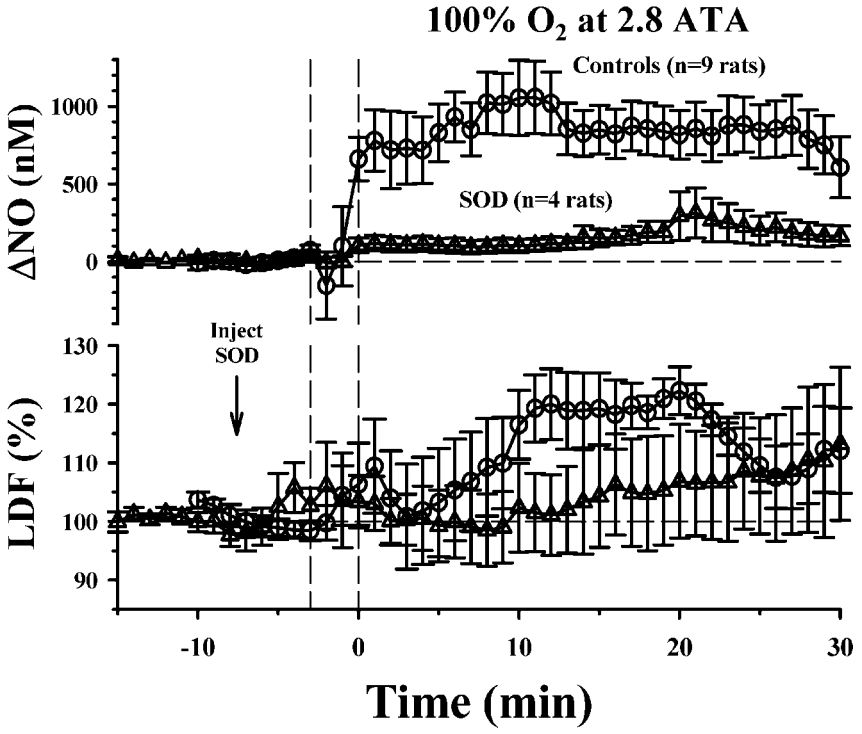


Figure 9 Mean changes in brain •NO concentration and laser Doppler blood flow for rats treated with superoxide dismutase (SOD; 25,000 U/kg i.v.) and exposed to 2.8 ATA O_2. Data from untreated (control) rats, the same as in Figure 2, were included to ease comparison. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.

blots was 1.4 ± 0.03 (S.E., $n = 5$) for control samples and 1.2 ± 0.05 ($n = 5$, not significant) for hyperbaric O_2-exposed samples.

Evidence was also sought for a number of alternative mechanisms by which NOS activity can be regulated. Blots prepared following immunoprecipitation of brain homogenates with anti-nNOS were probed with an antibody that recognizes nNOS phosphorylated at serine 437. Phosphorylation of serine 437 by calcium/calmodulin-dependent protein kinase IIα inhibits nNOS activity (Komeima et al., 2000). The band density ratio of phosphorylated nNOS to total nNOS of control brain homogenates was 0.39 ± 0.03 (S.E., $n = 4$), and in samples from rats first exposed to 2.8 ATA O_2 for 45 min the ratio was 0.40 ± 0.02 ($n = 4$, not significantly different). Phosphorylation of tyrosine residues in eNOS will reduce enzyme activity (Garcia-Cardena et al., 1996). Western blots generated from brain homogenates immunoprecipitated with anti-eNOS were probed with an antiphosphotyrosine antibody. The ratio of phosphorylated eNOS to total eNOS in control brains was 0.74 ± 0.07 (S.E., $n = 4$), and in brains from rats exposed to 2.8 ATA O_2 for 45 min the ratio was 0.84 ± 0.01 ($n = 4$, not significantly different).

Akt protein kinase-dependent phosphorylation will activate eNOS by phosphorylating serine 1177. In cell cultures, this process has been monitored by phosphoamino acid analysis and by identifying Akt activation (Dimmeler et al., 1999; Garcia-Cardena et al., 1998). We sought evidence for Akt activation by probing Western blots of rat brain homogenates with antibodies to both Akt and phosphorylated Akt. When expressed as a band density ratio, phospho-Akt/Akt, and normalized against the density of the Akt band, the value for control brains was 1.3 ± 0.08 (S.E., $n = 4$), and the value was 1.5 ± 0.21 ($n = 4$, not significantly different) in brain homogenates from rats exposed to 2.8 ATA O_2 for 45 min.

Due to the observation that MK 801 inhibited hyperoxia-induced •NO synthesis (Fig. 12), we examined the association between nNOS and the PSD-95 protein. PSD-95 is a scaffolding protein required for efficient coupling between NMDA receptors and nNOS (Sattler et al., 1999; Takagi et al., 2000). Immunoprecipitations were carried out with brain homogenates incubated with either anti-nNOS or anti-PSD-95. When using antibody against PSD-95 to probe Western blots of precipitated protein, the nNOS/PSD ratio normalized to the density of PSD

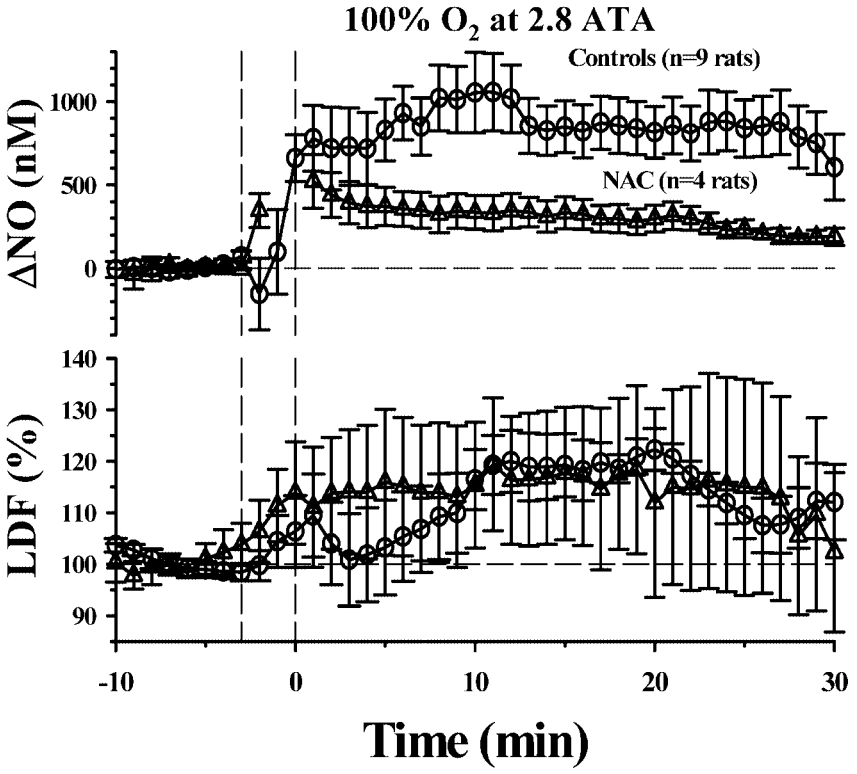


Figure 10 Mean changes in brain •NO concentration and laser Doppler blood flow for rats injected with *N*-acetylcysteine (NAC; 40 mg/kg i.p.) and exposed to 2.8 ATA O_2. Data from untreated (control) rats, the same as in Figure 2, were included to ease comparison. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.

was 0.42 ± 0.07 (S.E., $n = 5$) for control brains and 0.42 ± 0.08 ($n = 5$, not significantly different) for brains from rats exposed to 2.8 ATA O_2 for 45 min. If immunoprecipitations were performed with anti-nNOS, the ratio of PSD-95/nNOS was 0.59 ± 0.07 (S.E., $n = 5$) for control brains and 0.54 ± 0.06 ($n = 5$, no significant difference) for brains from rats exposed to 2.8 ATA O_2.

An association between nNOS and phosphofructokinase-M has been described which is thought to play a protective role from some forms of oxidative stress in NMDA-containing neurons (Firestein and Bredt, 1999). Western blots generated following immunoprecipitation of brain homogenates with anti-nNOS were probed with an antibody against phosphofructokinase-M. The densitometry ratio of PFK-M to nNOS, normalized to the band density of nNOS on these blots, was 1.3 ± 0.5 (S.E., $n = 4$) for control and 1.3 ± 0.4 ($n = 4$, no significant difference) for samples exposed to hyperbaric O_2.

Arginine Concentration

Arginine concentration was measured in brain homogenates from control rats and from rats killed immediately after exposure to 2.8 ATA O_2 for 45 min. We found no significant difference in concentration between the two groups. Arginine concentration in control rat brain was 211 ± 14 nmol (S.E., $n = 3$)/gram brain, and 165 ± 6 nmol (S.E., $n = 3$)/gram brain from rats exposed to hyperbaric O_2.

DISCUSSION

The findings outlined in this article are as follows:

1. Hyperbaric O_2 causes an elevation in steady state •NO concentration in cerebral cortex of both rats and mice.
2. No changes in concentration of NOS isoforms occur during a single 45 min exposure to hyperbaric O_2.
3. Results in mice show that nNOS activity contributes over 90% to total •NO elevation due to hyperoxia and the predominant importance of nNOS activity in rats was supported by the inhibitory effect of 7-NI.
4. •NO elevation due to hyperoxia can be inhibited by infusion of antioxidants such as SOD

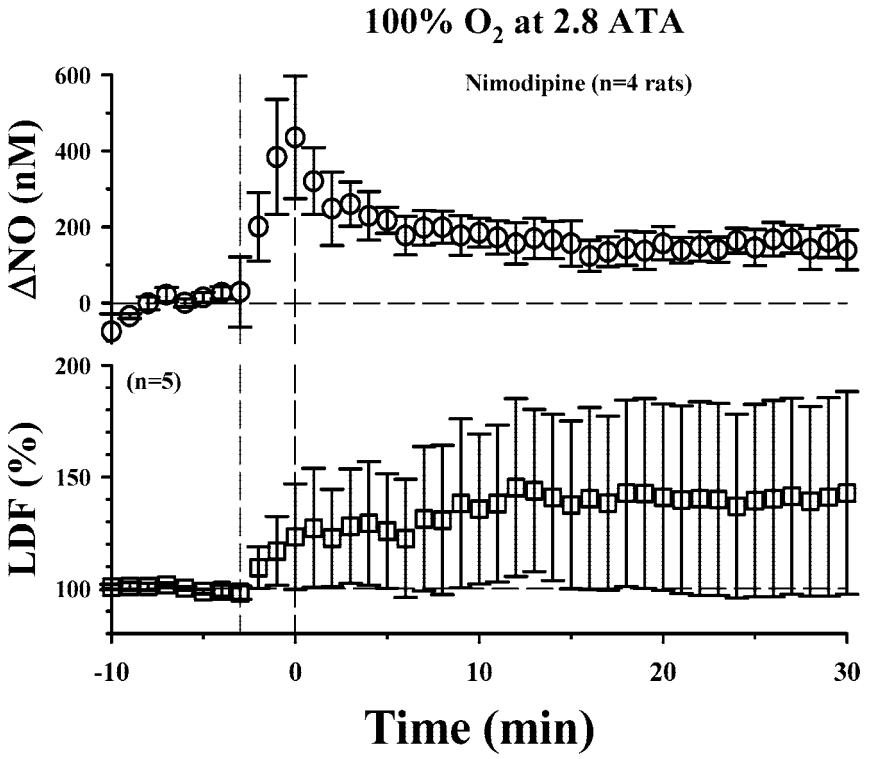


Figure 11 Mean changes in brain •NO concentration and laser Doppler blood flow for rats injected with nimodipine (1 mg/kg i.p.) and exposed to 2.8 ATA O_2. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.

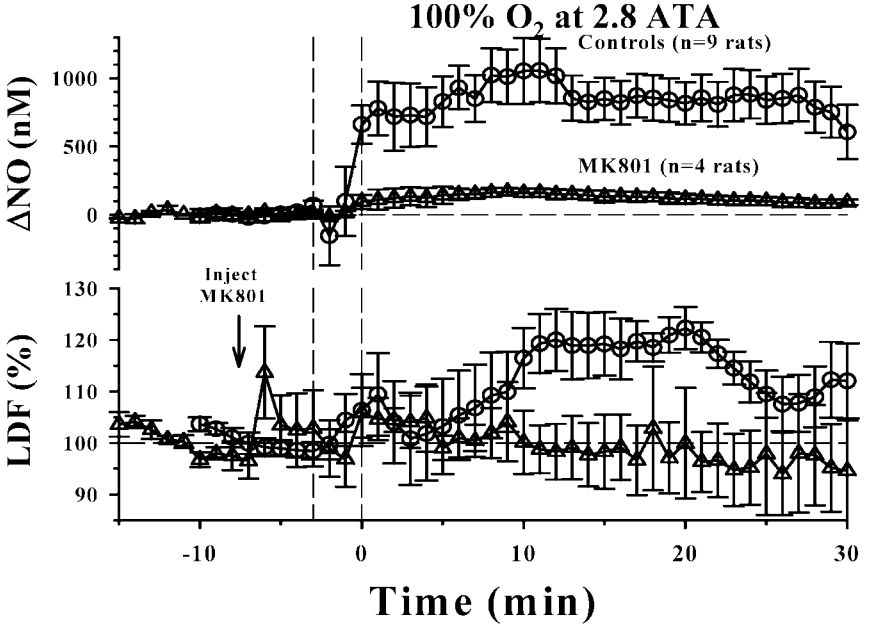


Figure 12 Mean changes in brain •NO concentration and laser Doppler blood flow for rats injected with MK 801 (5 mg/kg i.p.) and exposed to 2.8 ATA O_2. Data from untreated (control) rats, as shown in Figure 2, were included to ease comparison. The vertical dashed lines illustrate the time taken to increase chamber pressure using pure O_2.

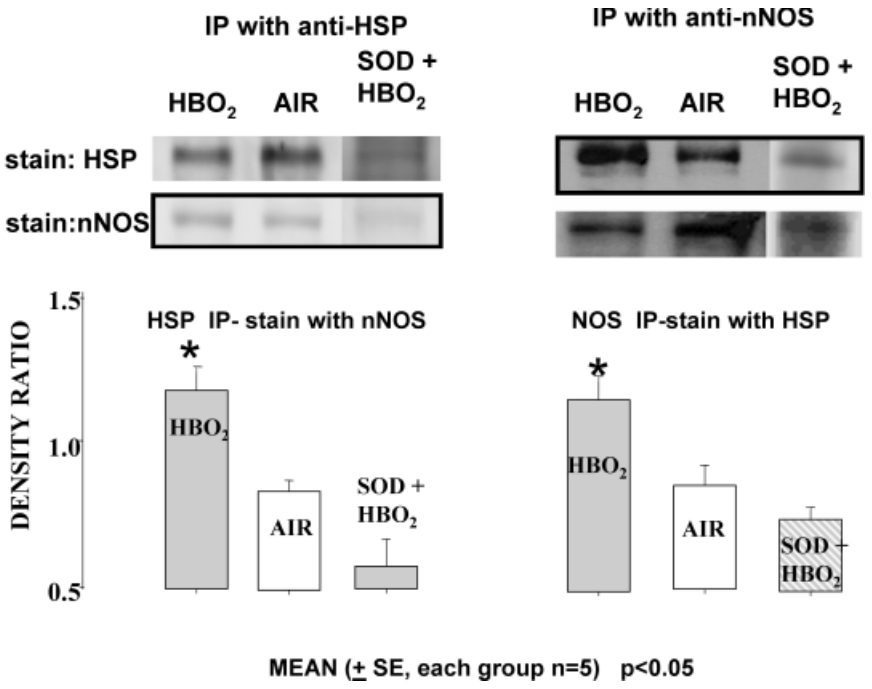


Figure 13 Results following immunoprecipitation (IP) of brain homogenates performed with antibody against heat shock protein 90 (HSP) or against nNOS. The top portion of the figure shows representative Western blots from samples obtained from a control rat (air) or rats killed immediately after 45 min exposure to 2.8 ATA O_2. Where indicated, one rat was injected with SOD (25,000 U/kg i.v.) immediately prior to the hyperbaric oxygen exposure. Band volumes were assessed on blots probed with anti-HSP and anti-nNOS and normalized to the band density obtained for the protein that was immunoprecipitated. The lower portion of the figure shows normalized mean band densities for blots stained with anti-nNOS after immunoprecipitations were done with anti-HSP (left panel of three bars) or mean band densities for blots stained with anti-HSP after immunoprecipitations were done with anti-nNOS (right panel of three bars). Three groups of rats were studied: control (air, $n = 5$), rats first exposed to 2.8 ATA O_2 ($n = 5$), and rats injected with 25,000 U SOD/kg and then exposed to 2.8 ATA O_2 ($n = 5$).

and NAC, by the NMDA receptor antagonist MK801, and by the calcium-channel blocker, nimodipine.

5. Immunoprecipitation studies indicate that hyperbaric O_2 triggers an increased association between nNOS and Hsp90 and this appears to be one mechanism for nNOS activation based on the inhibitory effect of SOD and ansamycin antibiotics in the electrode studies.
6. No alteration in eNOS association with Hsp90 occurs due to hyperoxia.
7. No modifications occur with nNOS or eNOS associations with calmodulin, in the magnitude of eNOS tyrosine phosphorylation, or nNOS phosphorylation via calmodulin kinase.
8. There is an elevation in laser Doppler measurements of blood flow in the cerebral cortex during hyperoxia and this may be causally related to elevations of steady state •NO concentration.

Seizures due to hyperbaric O_2 can be diminished by NOS inhibitors, but it is not clear whether this is due to a reduction of •NO concentration or simply due to lower O_2 delivery caused by the reduced cerebral blood flow from NOS inhibitors (Bitterman and Bitterman, 1998; Chavko et al., 1998; Oury et al., 1992; Zhang et al., 1995). Data in this report clearly demonstrate that elevated O_2 tensions will increase the production of •NO in brain. It is important to recognize that seizures as a manifestation of O_2 toxicity were not expected in our studies. Rats were exposed for only 30 to 45 min to O_2 partial pressure up to 2.8 ATA. Even at a pressure of 4 ATA O_2, the minimum duration of exposure to induce convulsions in rats has been reported to be nearly 2 h, and the mean time for onset of convulsions in rats exposed to 3.0 ATA O_2 is 7.3 h (Clark, 1984).

Hyperbaric O_2 was found to increase Hsp90 association with nNOS. This is the first report of a manipulation that changes this association *in vivo.* Sev-

eral agonists and also fluid shear stress have been shown to elevate Hsp90-NOS associations in cell cultures (Bender et al., 1999; Garcia-Cardena et al., 1998). In endothelial cells, Hsp90 appears to act as an allosteric modulator to increase eNOS activity (Garcia-Cardena et al., 1998). Augmentation of nNOS activity by Hsp90 association has been shown in transfected cell cultures (Bender et al., 1999). The mechanism by which Hsp90 activates nNOS appears more complex than that for eNOS, and may include enhanced opening of the heme binding cleft as well as slowing nNOS proteolysis (Bender et al., 1999). Geldanamycin binds to the nucleotide-binding site of Hsp90 and specifically blocks Hsp90 function (Whitesell et al., 1994; Grenert et al., 1997; Sullivan et al., 1997). The inhibitory effects of geldanamycin and herbimycin on O_2-induced elevations in $^{\bullet}NO$ concentration suggest that augmented nNOS activity occurs in response to increased association between nNOS and Hsp90.

The association of Hsp90 with nNOS does not abrogate nNOS activation related to increases in intracellular Ca^{++}, although ansamycin antibiotics will inhibit nNOS activity in the face of elevated intracellular Ca^{++} (Bender et al., 1999). Our findings suggest that a component to NOS activation by hyperbaric O_2 is related to elevations of intracellular Ca^{++}. Nimodipine, a calcium channel blocker, inhibits Ca^{++} entry via voltage-dependent channels and, to a lesser extent, via NMDA receptors (Sattler et al., 1998). Calcium influx through non-NMDA receptors does not efficiently activate nNOS, which may explain why nimodipine had only a relatively modest inhibitory effect in our study (Kiedrowski et al., 1992). Inhibition by MK 801 implicates a role for Ca^{++} entry via the NMDA channel in O_2-induced NOS activation.

Infusion of SOD, and to a lesser extent NAC, inhibited the elevation in $^{\bullet}NO$ during exposures to hyperbaric O_2. Because SOD cannot be expected to escape the circulation, these results suggest that nNOS is activated in response to perivascular changes mediated by one or more reactive species related to superoxide ($O_{2-}^{\bullet}$). Oxidative stress mediated by hydrogen peroxide was shown to stimulate NOS activity in cultured endothelial cells by increasing the intracellular Ca^{++} concentration (Shimizu et al., 1994). The increased Hsp90-nNOS association caused by hyperbaric O_2 was inhibited by SOD infusion. Hence, elevation of the Hsp90-nNOS association appears to be an oxidative stress response that contributes to nNOS activation.

Provision of higher concentrations of substrate has been suggested as the mechanism for elevated $^{\bullet}NO$ synthesis due to exposure to hyperbaric O_2 (Elayan et al., 2000). The Michelis constant (K_m) for O_2 of purified nNOS has been reported to be between 260 and 400 μM (Abu-Soud et al., 1996; Elayan et al., 2000). Oxygen tension is thought to influence NOS activity by mediating heme ferrous-to-ferric conversion at the active site (Abu-Soud et al,, 1996; Hurshman and Marletta, 1995). The propensity for oxidation in the intracellular milieu is likely to be limited, however, so the physiological relevance of this process is unclear. For example, iNOS has a K_m for O_2 of $\approx$190 μM (Dweik et al., 1998), and under reducing conditions it exhibits a negligible increase in activity with super-normal O_2 concentrations (Hurshman and Marletta, 1995). The inhibitory effects of ancamycin antibiotics, nimodipine, MK 801, and antioxidants argue against the importance of this mechanism in nNOS activation *in vivo* by hyperoxia.

Others have reported that elevated O_2 tensions will decrease the concentration of $^{\bullet}NO$ in brain, and clearly our findings contradict this conclusion (Demchenko et al., 2000a, b). A possible explanation for the contradiction is because the former conclusions were based on measurements of nitrite and nitrate, which would detect only a fraction of the total $^{\bullet}NO$ produced. Responses of transgenic mice overexpressing extracellular SOD have also been interpreted in a manner somewhat contrary to our conclusions. Whereas Oury et al. (1992) did report that NOS inhibition conferred resistance to O_2-induced seizures, they also found that the transgenic mice exhibited heightened sensitivity to O_2 toxicity versus wild-type mice. Although neither $^{\bullet}NO$ nor its products were measured, the authors suggested that $^{\bullet}NO$ synthesis was increased by hyperoxia. They speculated that $O_{2-}^{\bullet}$ protected against CNS O_2 toxicity by reacting with $^{\bullet}NO$ to reduce the steady state $^{\bullet}NO$ concentration. Therefore, SOD exacerbated O_2 toxicity by elevating the $^{\bullet}NO$ concentration in the face of O_2-induced $^{\bullet}NO$ synthesis. Based on direct measurements, we have now shown that SOD will decrease rather than increase the $^{\bullet}NO$ concentration associated with exposure to 2.8 ATA O_2. The seeming paradoxical effect of SOD in the study by Oury et al. (1992) may be related to the high pressure, 6 ATA O_2, used in this study. The large flux of $O_{2-}^{\bullet}$ that is likely to be produced at this pressure can be expected to yield a high concentration of hydrogen peroxide, due in part to the activity of SOD. If the accumulating reactive O_2 species activated guanylate cyclase independent of changes in $^{\bullet}NO$ concentration, a phenomenon reported by Mittal and Murad (1977), vasodilatation may increase O_2 delivery and hasten development of toxic responses. It is also possible that $^{\bullet}NO$ is only indirectly related to seizures or that there are uniden-

tified idiosyncratic processes present in the SOD-overexpressing transgenic mice.

Ito et al. (1996) speculated that exposure to 3.0 ATA O_2 for 2 h may promote nitric oxide synthase activity in rats by increasing the concentration of arginine. Zhang et al. (1993) also found approximately 25% elevation in brain arginine concentration when rats were exposed to 6 ATA O_2 for 20 min. The nitric oxide synthase K_m for arginine is about 2.9 μM and the normal intracellular arginine concentration is on the order of 800 μM (Forstermann et al., 1998). Therefore, it seems unlikely that an elevated arginine concentration would influence NOS kinetics. At least with regard to our findings, this does not have great bearing, as we found no significant effect of 2.8 ATA O_2 for 45 min on brain arginine level. The reason our findings differ from those of Ito et al. (1996) and Zhang et al. (1993) is likely to be because we used a lower pressure of O_2 for a shorter period of time.

Finally, comment is warranted on the increase in cerebral blood flow measured by laser Doppler when rats were exposed to hyperbaric O_2. Hyperoxia has been shown to reduce brain blood flow when measured by hydrogen clearance, [C^{14}] antipyrine, microspheres, and Doppler-detected flow through the carotid artery (Bean et al., 1971; Bergo and Tyssebotn, 1992; Demchenko et al., 2000a,b; Jacobson et al., 1963; Omae et al., 1998; Tindall et al., 1965; Torbati et al., 1979). The vascular response to hyperoxia is quite heterogeneous, however, and changes in flow to the cerebral cortex measured by microspheres are quite small (Bergo and Tyssebotn, 1992). The laser Doppler device measures relative flow, not total blood flow. As O_2-induced vasoconstriction is heterogeneous, regional perfusion pressure to the cortex may have been elevated by hyperbaric O_2. This would be detected as increased flow by laser Doppler although perfusion of the whole brain may actually have been diminished. Others have also observed a small increase in cerebral flow during hyperbaric O_2 exposures using laser Doppler (Zhang et al., 1995). Stimulation of •NO synthesis by hyperbaric O_2 appears to be the mechanism for elevations of laser Doppler blood flow. This is supported by the inhibitory effects of 7-NI, geldanamycin, SOD, NAC, nimodipine, and MK 801.

Our results clearly show that hyperoxia increases •NO production in cerebral cortex. We cannot predict whether similar changes occur in deeper brain structures. However, identification of discrete biochemical effects related to hyperoxia is expected to improve understanding of both the toxicity and therapeutic potential for hyperbaric O_2.

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