Stimulation of perivascular nitric oxide synthesis by oxygen

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The purpose of this investigation was to determine the effect of hyperoxia in vivo on perivascular nitric oxide (NO) synthesis by using NO-specific microelectrodes implanted adjacent to the abdominal aorta were exposed to O2 at partial pressures from 0.2 to 2.8 atmospheres absolute (ATA). Exposures to 2.0 and 2.8 ATA O2 stimulated neuronal (type I) NO synthase (nNOS) and significantly increased steady-state NO concentration, but the mechanism for enzyme activation differed at each partial pressure. At both pressures, elevations in NO concentration were inhibited by the nNOS inhibitor 7-nitroindazole and the calcium channel blocker nifedipine. Enzyme activation at 2.0 ATA O2 appeared to be due to an altered cellular redox state. Exposure to 2.8 ATA O2, but not 2.0 ATA O2, increased nNOS activity by enhancing nNOS association with calmodulin, and an inhibitory effect of gelynaminic acid indicated that the association was facilitated by heat shock protein 90. Infusion of superoxide dismutase inhibited NO elevation at 2.8 but not 2.0 ATA O2. Hyperoxia increased the concentration of NO associated with hemoglobin. These findings highlight the complexity of oxidative stress responses and may help explain some of the dose responses associated with therapeutic applications of hyperbaric oxygen.

neuronal nitric oxide synthase; heat shock protein 90; calmodulin; hyperbaric oxygen

THE PURPOSE of this investigation was to determine the effect of hyperoxia in vivo on perivascular nitric oxide (NO) synthesis by using NO-specific microelectrodes. The impact of elevated O2 tension on NO synthesis has not been clearly established by studies with cell cultures and isolated enzymes. We considered that inconsistencies in the literature may be due to reliance on indirect measurements of NO production, such as nitrite and nitrate, and because the incubation conditions used in model systems may not accurately reflect those that exist in vivo.

Among the three NO synthase (NOS) enzymes, the activity of inducible (type II) NOS (iNOS) is controlled at the level of gene transcription, whereas the activities of neuronal (type I) NOS (nNOS) and endothelial (type III) NOS (eNOS) are controlled by intracellular calcium/calmodulin, several different phosphorylation mechanisms, and by binding of the molecular chaperone heat shock protein 90 (HSP90) (5, 12, 13, 16, 36). Studies with all three NOS enzymes in vitro have shown that enzyme activity is influenced by the redox state and specifically by O2 tension. During NOS-mediated arginine catalysis, some of the self-generated NO reduces ferric heme in the active site to the ferrous form. Ferrous-state NOS has ~10% activity compared with ferric NOS (2, 3, 20). Elevated O2 tension influences NOS activity by hastening conversion of ferrous heme back to the native ferric conformation. Conversion of ferric heme to the ferrous state is influenced by the rate of NO synthesis. Therefore, this process appears less important with eNOS, which exhibits catalytic activity between four and eight times lower than the other two NOS isoforms (1). The effect of O2 on catalytic activity is reflected by values for the apparent Michaelis-Menten constant (Km) for O2 of each enzyme. Purified nNOS exhibits an apparent Km of ~400 μM and saturation at 800 μM (2), values far greater than the Km for binding O2. This contrasts sharply with the value for eNOS, 4 μM (1). iNOS has catalytic activity similar to that of nNOS, and its apparent Km for O2 is ~190 μM (11). Incubation conditions can alter these findings; however, as Hurshman and Marletta (20) reported that under reducing conditions, there was little enhancement of iNOS activity by super-normal O2 concentrations.

Hypoxic conditions diminish synthesis of NO in cells from both pulmonary and systemic circulations, likely because of the enzyme O2 requirement (8, 11, 30, 31, 39, 46, 48). Elevated O2 tensions above ambient will increase NO production by pulmonary endothelial
cells and intact lungs (8, 11, 30–32, 38). In contrast, O₂ tensions above ~55 mmHg were reported to have little effect on ÀNO production by cells obtained from the systemic circulatory system (31, 48). In the central nervous system, elevated partial pressures of O₂ increase the steady-state concentration of ÀNO by stimulating nNOS activity (42). This action is mediated by binding HSP90 and is an oxidative stress response elicitable by geldanamycin and infusion of superoxide dismutase (SOD) (42).

This study examined ÀNO production in the vicinity of the abdominal great vessels. We avoided studies of the microvasculature because ÀNO synthesis in this region is influenced by variations in blood flow controlled by proximal resistance vessels and by diffusion of an array of chemical mediators (6, 7, 19, 30, 38). We examined the dose response between elevated partial pressures of O₂ and the steady-state concentrations of ÀNO, the mechanism for NOS activation, and changes in concentration of ÀNO-carrying substances in the blood. The majority of experiments were conducted in rats, with a complementary series carried out using “knockout” mice lacking functional genes for eNOS or nNOS.

This study also examined whether mechanisms for perturbing NOS activity differed with O₂ partial pressure. There is an increasing interest in the use of hyperbaric O₂ therapy for a variety of disorders such as refractory wounds, radiation injury, and decompression sickness (18). Dosing protocols have been based on anecdotal experience because underlying mechanisms for benefit have not been elucidated. Hyperbaric O₂ has been shown to cause angiogenesis and to inhibit neutrophil b₂-integrin adhesion, two potentially beneficial actions that may be influenced by changes in steady-state ÀNO concentration (4, 18, 26, 34, 40, 41).

METHODS AND MATERIALS

Materials. Wistar male rats (Charles River Laboratories) weighing 220–240 g were fed a standard diet and water ad libitum. House mice (Mus musculus) of the C57B6J strain were raised at the animal facilities of the University of Pennsylvania. Mice used in the study were either wild type or lacking a functional eNOS gene (eNOS1/1). Mice lacking an additional pressure was applied. Where specified, rats were injected with 7-nitroindazole (12 mg/kg ip), N(G)-nitro-arginine methyl ester (L-NAME, 40 mg/kg ip), nimodipine (1 mg/kg ip), aminoxyguanidine (100 mg/kg ip), or geldanamycin (0.3 mg/kg ip) 30 min before pressurization in the hyperbaric chamber. The hyperbaric chamber used in this study was rated for a maximum pressure of 3.0 atmospheres absolute (ATA) and has been described in a prior publication (43). Once in the closed chamber, animals were monitored for ~30 min until electrode recordings became stable. During this time, 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 ip), N(G)-nitro-arginine methyl ester (L-NAME, 40 mg/kg ip), nimodipine (1 mg/kg ip), aminoxyguanidine (100 mg/kg ip), or geldanamycin (0.3 mg/kg ip) 30 min before pressurization in the hyperbaric chamber. Tissue preparation for immunochemical and biochemical assays. Rats were anesthetized with intraperitoneal ketamine (83 mg/kg) and xylazine (11 mg/kg). After the animals were anesthetized, the abdomen was opened and the peritoneum was reflected to allow placement of the electrode between aorta and vena cava. Abdominal contents were then replaced, and a sterile saline-soaked piece of gauze was placed over the abdominal incision. A second dose of ketamine-xylazine amounting to three-fourths of the initial dose was given just before the animal was placed in the hyperbaric chamber. The hyperbaric chamber used in this study was rated for a maximum pressure of 3.0 atmospheres absolute (ATA) and has been described in a prior publication (43). Once in the closed chamber, animals were monitored for ~30 min until electrode recordings became stable. During this time, 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 ip), N(G)-nitro-arginine methyl ester (L-NAME, 40 mg/kg ip), nimodipine (1 mg/kg ip), aminoxyguanidine (100 mg/kg ip), or geldanamycin (0.3 mg/kg ip) 30 min before pressurization in the hyperbaric chamber or with N-3(aminomethyl)benzyl acetamide (1400-W, 1 mg/kg ip) 2 h before pressurization. Others received bovine erythrocyte (copper-zinc) SOD concentration (25,000 U/kg) intravenously immediately before the pressurization. Toft from the Mayo Graduate School (Rochester, MN). Rabbit antibody to Akt, phospho-Akt, eNOS phosphorylated at serine-1177, and calmodulin were purchased from Cell Signaling Technology (Beverly, MA). Anti-iNOS and anti-nNOS were purchased from Cayman Chemical (Ann Arbor, MI). Rabbit antibody to Akt, phospho-Akt, eNOS phosphorylated as serine-1177, and calmodulin were purchased from Cell Signaling Technology (Beverly, MA). Anti-iNOS and anti-HSP90 were purchased from StressGen (Victoria, British Columbia, Canada). Mouse antibody against the 10-kDa protein inhibitor of nNOS (PIN) was purchased from BD Transduction. Horseradish peroxidase-conjugated antibodies (goat anti-mouse IgG and goat anti-rabbit IgG) were purchased from Chemicon (Temecula, CA). Mouse monoclonal antibody J33 recognizing p23 was generously provided by Dr. David Toft from the Mayo Graduate School (Rochester, MN). Rabbit antibody recognizing phosphorylated nNOS (serine-847) was a gift from Dr. Yasuo Watanabe, Nagoya University School of Medicine (Nagoya, Japan). ECL reagents were from Amerham Pharmacia Biotech. Sodium salt of N-methyl-n-glucamine dithiocarbamate (MGD) was synthesized at the Oklahoma Medical Research Foundation from N-methyl-n-glucamine and carbon disulfide by using the procedure of Shinobu et al. (34). It was crystallized twice from ethanol and stored in small aliquots (0.5–1 g) under argon and at ~8°C.

Microelectrode fabrication. Studies performed with dual electrodes selective for ÀNO and for O₂ were fabricated by Dr. Buerk’s laboratory according to published methods (42). The majority of studies were performed with ÀNO-selective microelectrodes obtained commercially from World Precision Instruments (Sarasota, FL). Studies in rats were done with the ISO-NOP200 electrode (sensor surface area 200 µm x 20,000 µm in length), and studies in mice were done with the ISO-NOP3020 (sensor surface area 30 x 2,000 µm).

Electrical studies. The conditions present during pressurization to anesthetize both rats and mice with intraperitoneal ketamine (83 mg/kg) and xylazine (11 mg/kg). After the animals were anesthetized, the abdomen was opened and the peritoneum was reflected to allow placement of the electrode between aorta and vena cava. Abdominal contents were then replaced, and a sterile saline-soaked piece of gauze was placed over the abdominal incision. A second dose of ketamine-xylazine amounting to three-fourths of the initial dose was given just before the animal was placed in the hyperbaric chamber. The hyperbaric chamber used in this study was rated for a maximum pressure of 3.0 atmospheres absolute (ATA) and has been described in a prior publication (43). Once in the closed chamber, animals were monitored for ~30 min until electrode recordings became stable. During this time, 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 ip), N(G)-nitro-arginine methyl ester (L-NAME, 40 mg/kg ip), nimodipine (1 mg/kg ip), aminoxyguanidine (100 mg/kg ip), or geldanamycin (0.3 mg/kg ip) 30 min before pressurization in the hyperbaric chamber or with N-3(aminomethyl)benzyl acetamide (1400-W, 1 mg/kg ip) 2 h before pressurization. Others received bovine erythrocyte (copper-zinc) SOD concentration (25,000 U/kg) intravenously immediately before the pressurization.

Tissue preparation for immunochemical and biochemical assays. Rats were anesthetized with intraperitoneal ketamine (83 mg/kg) and xylazine (11 mg/kg), and abdominal aortas were removed. Tissue was immediately homogenized in 25 mM Tris·HCl, pH 7.5, containing 100 mM NaCl, 100 µM diethylene triminepentaacetic acid, 40 µM PMSF, 0.5 µg/ml leupeptin, and 0.01% butylated hydroxytoluene. For each 1 g of aorta, 2 ml buffer were 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 were frozen at ~8°C until used in subsequent assays.

Immunoprecipitation. Supernatants containing 250 µg protein were suspended in 500 µl of precipitation buffer [20 mM MES, pH 7.6 containing 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.4% Triton X-100, 1 mM PMSF, 10 mM sodium fluoride, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 µM tetrahydrobiopterin, 1 mM arginine, and 20 mM sodium molybdate]. Suspensions were all preclarified by incubation
for 2 h at 4°C with 30 μl 20% (wt/vol) protein G-Sepharose and then centrifuged at 8,200 g for 1 min. The supernatant was saved, antibodies (10 μg of anti-HSP90, anti-nNOS, or anti-eNOS) were added, and the suspension was incubated overnight at 4°C. The next day, 30 μl of 20% (wt/vol) protein G-Sepharose were added to the suspensions, incubated 1.5 h at 4°C, and then centrifuged at 8,200 g for 1 min. The immune pellets were washed twice with wash buffer (10 mM MES, pH 7.6 containing 50 mM NaCl, 20 mM sodium molybdate, 10% glycerol, and 0.4% Triton X-100), pellets were suspended with 40 μl of sample buffer (100 mM sodium phosphate, pH 7.4, containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.00125% bromophenol), and the suspension was heated to 95°C for 10 min. After centrifugation at 8,200 g for 1 min, aliquots (30 μl) of the supernatant were electrophoresed by using a 12% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes and probed with 1:1,000 dilutions of antibody (anti-HSP90, eNOS, nNOS, or calmodulin). Where indicated, aortic homogenate preparations containing 250 μg protein were incubated for 30 min at 30°C with 50 μl of phenyl-Sepharose in 10 mM Tris-HCl pH 7.5, KCl, 5 mM MgCl₂, and 1 mM dithiothreitol. The phenyl-Sepharose was washed three times with Tris-HCl, pH 7.5, plus 1 mM EDTA, suspended with 40 μl sample buffer, and subjected to electrophoresis as described above.

Measurement of NO-containing substances in blood. Heparinated blood was obtained by puncturing the abdominal aorta of anesthetized rats. Where indicated, rats were exposed to 2.8 ATA O₂ for 45 min immediately before anesthesia. Assays were performed exactly as described by Sonoda et al. (35).

Electron paramagnetic resonance measurements. Deionized water used for stock solutions was bubbled with nitrogen to remove dissolved O₂, and stock solutions of 1 M cysteine-HCl and 0.45 M FeSO₄·7H₂O were bubbled a second time before storage at −80°C. A solution of MGD-Fe-cysteine was prepared by dissolving 3.4 g MGD in 3.4 ml H₂O to which 0.13 ml FeSO₄ stock solution was added. After several minutes to allow equilibration, 0.6 ml cysteine stock solution was added, and the solution was kept under an argon flow until aliquots of 0.12 ml were distributed to Eppendorf tubes and stored at −80°C.

For electron paramagnetic resonance (EPR) measurements, rats had catheters placed in a femoral veins and were maintained under ketamine-xylazine anesthesia. Initial measurements, obtained while rats breathed air, were performed by collecting 0.25 ml blood into an Eppendorf tube, to which was added 1 ml 0.15 M citrate buffer (pH 4.5). The tube was centrifuged at 4,000 g for 2 min, the serum was removed, and 0.1 ml MGD-Fe-cysteine solution was added to the erythrocyte pellet. After the mixing was completed, the solution was transferred to small glass tubes with an internal diameter of 1.13 mm (50 μl Wiretrol pipette, Thomas Scientific; Swedeboro, NJ) to a length of ~40 mm. The bottom of the capillary tube was sealed by hematocrit sealant and placed inside a quartz tube for EPR measurements. These steps were also performed after rats had been exposed to 2.8 ATA O₂ for 45 min or after exposure to 2.8 ATA by using 7.6% O₂ as a normoxic control.

EPR spectra were recorded by using a Varian E-109 spectrometer at the Johnson Research Foundation of the University of Pennsylvania. The EPR settings were 9.15 GHz microwave frequency, 10 mW microwave power, 100 kHz modulation frequency, 0.32 mT modulation, and 10⁵ gain at room temperature. The signal increased over 15 min and remained stable for 10 min, after which it slowly decayed. During the 10-min stabilization period, three scans were performed and signals were averaged. The spin concentration was obtained by double integration using commercial software.

A standard curve was generated by adding a known amount 1.91 mM NO (generated by bubbling deionized water with pure NO gas) into 5 ml of deaerated 150 mM sodium citrate buffer (pH 4.5) and then by placing 30 μl of this mixture in 100 μl of MGD-Fe-cysteine solution.

Data analysis. Statistical significance was determined by ANOVA followed by Scheffe’s test. The level of significance was taken as P < 0.05. Results are expressed as means ± SE.

RESULTS

Temporal changes in tissue O₂ and ·NO elevations. A dual-barrel electrode placed adjacent to the wall of the abdominal aorta was used to examine the relationship between elevation of tissue O₂ tension and steady-state ·NO concentration. As shown in a representative experiment (Fig. 1), tissue O₂ tension rose rapidly from a value between 10 and 40 Torr while air was breathed to a mean value of 1,900 Torr ± 100 (n = 4) when rats were pressurized to 2.8 ATA while breathing pure O₂. The steady-state ·NO concentration also increased rapidly, and the mean concentration while O₂ was breathed at 1, 1.5, 2.0, and 2.8 ATA is shown in Fig. 1. The concentration at 2.0 and 2.8 ATA O₂ were both greater than control, and 2.8 ATA O₂ had a significantly greater effect than 2.0 ATA O₂ (P < 0.05, ANOVA). The markedly higher number of samples for air and 2.8 ATA O₂ studies occurred because the results in Fig. 2 included studies in which only the effects of O₂ were examined and studies related to pharmacological inhibitors (see Pharmacological inhibitors of NOS activation in rats).

Changes in ·NO concentration lagged behind the rise in tissue O₂ tension during pressurization to 2.8 ATA. The average time to achieve half-maximal O₂ concentration in four experiments was 1.7 min, whereas for ·NO, it took 3.5 min to achieve half-maximal concentration (Fig. 3A). On decompression to ambient pressure while air was breathed, the O₂ tension dropped by...
one-half in 4.6 min, and the \( \cdot \)NO concentration decreased to one-half in 10 min (Fig. 3B).

To assess the effect of pressure per se versus elevated \( \text{O}_2 \) partial pressure, a series of studies was performed with rats exposed to 2.8 ATA by using 7.46% \( \text{O}_2 \) versus pure \( \text{O}_2 \). Breathing hypoxic gas at this pressure achieves the same partial pressure of \( \text{O}_2 \) as with breathing air at ambient pressure (0.21 ATA). A representative experiment, shown in Fig. 4, demonstrates that this exposure had a negligible effect on measured \( \cdot \)NO, but the level rose significantly when ventilation gas was changed from 7.46% to 100% \( \text{O}_2 \). In studies performed with seven rats exposed to 7.46% \( \text{O}_2 \) at 2.8 ATA pressure, \( \cdot \)NO concentration was 81 ± 13 nM over the baseline (no significant difference versus control). We conclude that increases in \( \text{O}_2 \) partial pressure caused the elevations in \( \cdot \)NO concentration rather than an increase in pressure per se.

To assess whether the increases in \( \cdot \)NO differed among alternative locations around the great vessels, we carried out a series of studies with the electrode placed along the lateral abluminal surface of the vena cava or aorta. The elevation in \( \cdot \)NO concentration was insignificantly different from that observed with the electrode in its standard placement along the wall of the aorta in a pocket created between the aorta and vena cava. When adjacent to the vena cava, the elevation in steady-state \( \cdot \)NO concentration due to exposure to 2.8 ATA \( \text{O}_2 \) was 315 ± 41 nM (means ± SE, \( n = 3 \)). When the electrode was placed adjacent to the aorta, the elevation was 326 ± 41 nM (\( n = 3 \)).

**NOS enzymes and regulatory proteins present in aorta.** Protein amounts, normalized to actin present on Western blots, were measured for the three NOS isoforms and for calmodulin and HSP90. Homogenates of the abdominal aorta were made from control animals, and rats were killed immediately after a 45-min exposure to 2.8 ATA \( \text{O}_2 \). A representative group of images from Western blots is shown in Fig. 5. The concentrations of proteins were not significantly different between control and 2.8 ATA \( \text{O}_2 \) groups (Table 1). No iNOS was detected on the blots.

**Pharmacological inhibitors of NOS activation in rats.** Pharmacological manipulations were performed in rats to gain insight into the mechanism by which \( \text{O}_2 \) elevated the perivascular steady-state \( \cdot \)NO concentration. Injection with the nonspecific NOS inhibitor \( \text{l-NAME} \) or with the 7-nitroindazole, a relatively specific inhibitor of nNOS, significantly reduced \( \cdot \)NO production due to exposure to 2.8 ATA \( \text{O}_2 \) (Fig. 6). In contrast, there was no significant inhibition when rats were pressurized to 2.8 ATA \( \text{O}_2 \) (values are means ± SE of 4 trials).
were treated with aminoguanidine or 1400-W, two inhibitors of iNOS. Significant inhibition was observed with infusions of geldanamycin, an inhibitor of HSP90, with nimodipine, a calcium channel blocker, and with SOD. Inhibition due to SOD was reversible. In three trials, the experiment was repeated 1 h after SOD infusion, and the response to hyperoxia returned to 102.3 ± 9% of the response observed before SOD infusion. SOD did not change the temporal pattern of elevations in O2 tension and steady-state NO concentration (as shown in Fig. 3, A and B). Times to achieve half-maximal O2 tension and NO concentration were within 3 ± 2% (n = 4) of those observed with exposure to 2.8 ATA O2 on trials conducted before SOD infusion.

Experiments with inhibitors were also performed at 2.0 ATA O2 because there was a significant difference in NO concentration between 2.0 and 2.8 ATA O2 exposures. In studies conducted at 2.0 ATA O2, infusion with nimodipine inhibited NO production by 68 ± 17% (n = 3, P < 0.05, ANOVA), and 7-NI inhibited the production by 88 ± 14% (n = 3, P < 0.05, ANOVA), results similar to those with 2.8 ATA O2 (Fig. 6). In contrast, geldanamycin and SOD did not significantly inhibit NO production in studies at 2.0 ATA O2. Geldanamycin inhibited NO elevation by 0.1 ± 0.1%

Table 1. Protein concentration in aortic homogenates

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<tr>
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<th>Control</th>
<th>2.8 ATA O2</th>
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<tr>
<td>eNOS</td>
<td>0.83 ± 0.31</td>
<td>0.46 ± 0.03</td>
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<tr>
<td>nNOS</td>
<td>0.79 ± 0.18</td>
<td>0.79 ± 0.10</td>
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<tr>
<td>HSP90</td>
<td>0.63 ± 0.21</td>
<td>0.46 ± 0.03</td>
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<tr>
<td>CaM</td>
<td>2.6 ± 0.66</td>
<td>2.0 ± 0.23</td>
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</table>

Values are expressed as means ± SE of band density ratios versus the density of the actin band present on each blot; n = 4 for all samples. eNOS, endothelial nitric oxide (NO) synthase (NOS); nNOS, neuronal NOS; HSP90, heat shock protein 90; CaM, calmodulin; ATA, atmospheres absolute.

Fig. 6. Changes in NO elevation due to pharmacological agents. Elevation in perivascular NO concentration due to hyperbaric O2 in these studies was 384 ± 39 nM. The 27 "HBO2-only" values were a subset of the 2.8 ATA O2 values reported in Fig. 2. After elevation in NO concentration due to 2.8 ATA O2 was measured, drug was infused, and the study was then repeated. Effect of drug administration was expressed as the percent rise in NO when drug was present compared with the concentration measured before drug administration. 1-NAME, Nω-nitro-l-arginine methyl ester; 7-NI, 7-nitroindazole. Numbers in parentheses are n values. *Significantly less than the value for 2.8 ATA O2 (P < 0.05, ANOVA).

Investigation with knockout mice. Production of NO in mice in response to elevations in O2 partial pressure is shown in Fig. 7. No significant difference was observed in the periaortic NO concentration between wild-type and eNOS knockout mice exposed to 2.8 ATA O2 (n = 3), and SOD inhibited NO elevation by 3.2 ± 3.1% (n = 3).

Fig. 7. Elevations in perivascular NO in mice. Studies were performed with wild-type mice or mice lacking functional genes for eNOS or nNOS mice (knockout [KO] mice). Numbers in parentheses are n values. *Significantly less than wild-type mice exposed to 2.8 ATA O2.
There were significantly lower steady-state NO concentrations in wild-type mice exposed to 1.5 and 2.0 ATA O2 and in nNOS knockout mice exposed to 1.5, 2.0, and 2.8 ATA O2. We conclude that nNOS was the predominant isoform activated in response to hyperoxia.

Protein associations and NOS phosphorylation. Aortic tissue homogenates were subjected to immunoprecipitation to examine the associations among proteins. Samples from rats exposed to 2.8 ATA O2, but not 2.0 ATA O2, had twice as much calmodulin associated with immunoprecipitated nNOS compared with control, air-breathing rats (Fig. 8). A representative group of images from Western blots is shown in Fig. 9. The elevation of calmodulin-nNOS association was inhibited by treatment with geldanamycin but not by SOD. There was no significant increase in the association between HSP90 and nNOS among samples from rats exposed to 2.0 or 2.8 ATA O2 (Fig. 10).

We examined whether nNOS activity may be altered via serine-437 phosphorylation or by binding to the 10-kDa protein PIN. Aortic homogenates from 2.8 ATA O2-exposed rats were immunoprecipitated with anti-nNOS and probed with an antibody that recognizes nNOS phosphorylated at serine-437 or an antibody against PIN. No bands were detected with either antibody. Therefore, we conclude that neither phosphorylation of serine-437 by calcium/calmodulin-dependent protein kinase II, nor association with PIN, played a role in aortic nNOS inactivation.

Alterations were not found in the associations between eNOS and HSP90 or eNOS and calmodulin after hyperbaric oxygen exposure.

Table 2. Protein concentrations relative to that of eNOS

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<th>Band Densities Relative to eNOS</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>HSP90</td>
<td>0.21 ± 0.07(7)</td>
</tr>
<tr>
<td>CaM</td>
<td>0.24 ± 0.03(7)</td>
</tr>
<tr>
<td>Phospho-eNOS</td>
<td>0.40 ± 0.02(3)</td>
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Values are means ± SE; sample numbers in parentheses. Immunoprecipitation with anti-eNOS antibody was performed, blots were probed with antibody against HSP90 and CaM, and the band densities were normalized to that of eNOS to assess relative amount of proteins. In other studies, aortic homogenates were subjected to SDS-PAGE and Western blots were probed for eNOS and phosphorylated eNOS. The band density of phosphorylated eNOS was normalized to that of eNOS to assess the relative amount of phosphorylated protein. There are no significant differences.
exposures to 2.8 ATA O₂ in aortic homogenates immunoprecipitated with anti-eNOS (Table 2). A representative group of images from Western blots is shown in Fig. 11. Akt protein kinase-dependent phosphorylation will activate eNOS by phosphorylating serine-1177 (10, 13). There was no difference in the ratio of phospho-eNOS to total eNOS on Western blots of aortic homogenates probed with an antibody that recognizes serine-1177-phosphorylated eNOS (Table 2 and Fig. 11). Blots were also probed for Akt and its activated form phospho-Akt. The ratio of phosphorylated Akt to total Akt was 0.5 ± 0.2 (n = 4) for aortic homogenates from control rats and 0.48 ± 0.2 (n = 4) for samples from rats exposed to 2.8 ATA O₂ (no significant difference).

**Immunoprecipitates**

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<tr>
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<th>CONTROL</th>
<th>2.8 ATA O₂</th>
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<tr>
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<td>Phospho eNOS</td>
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<tr>
<td>eNOS</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
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Fig. 11. Representative group of images from Western blots probed for eNOS and phosphorylated eNOS (bottom) or blots probed for HSP90 and calmodulin after immunoprecipitation of aortic homogenates with antibody against eNOS (top). Top portion shows calmodulin and HSP90 coprecipitated with nNOS.

Elevations of NO-carrying substances in blood. The concentration of NO-carrying substances in the blood by a fluorometric method was 4.7 ± 0.7 μM (n = 6) for control rats and 4.4 ± 0.4 (n = 6, no significant difference versus control) after 2.8 ATA O₂. The limit of detection for this assay was ~0.5 μM, so a more sensitive technique was sought. We developed an EPR method by using erythrocyte lysates from which NO was extracted using the spin trap MGD (see MATERIALS AND METHODS). Examples of NO-MGD EPR spectra ob-

HSP90 binding characteristics. Because of the inhibitory effects of geldanamycin on O₂-mediated NO production, we examined whether some binding functions attributed to HSP90 may be altered by exposure to 2.8 ATA O₂. Geldanamycin binds at the amino-terminal portion of HSP90 where ATP binding occurs (17). ATP binding regulates the HSP90 conformational change required for binding p23, a cochaperone that confers stability to protein heterocomplexes (22, 28). Aortic homogenates were subjected to immunoprecipitation using anti-HSP90 and Western blots probed for p23 that coprecipitated with HSP90. The band density ratios of p23 to HSP90 on the blots were 0.91 ± 0.23 (n = 4) for control samples and 0.99 ± 0.41 (n = 4, no significant difference) for samples obtained from rats exposed to 2.8 ATA O₂ for 45 min.

The ADP-bound form of HSP90 exhibits an elevated affinity for binding to phenyl-Sepharose (17, 40). The mean band density of HSP90 was measured on Western blots prepared from aortic homogenate samples eluted from phenyl-Sepharose, and this was compared with the total amount of HSP90 in the homogenate expressed as the band density from lanes loaded with samples that had not been eluted from Sepharose. The band density ratio of control samples was 0.18 ± 0.02 (n = 4), and for samples obtained from rats exposed to 2.8 ATA O₂ for 45 min it was 0.19 ± 0.03 (n = 4, no significant difference).

**Fig. 12.** Examples of electron paramagnetic resonance spectra of methyl-d-glucamine dithiocarbamate (MGD)-NO spin adducts for different experiments. A: control, air-breathing rat; B: after exposure to 2.8 ATA O₂ for 45 min; C: after exposure to 2.8 ATA while breathing 7.46% O₂ (normoxic control); D: rat injected with l-NAME and then exposed to 2.8 ATA O₂ for 45 min.
tained from control rats and rats exposed to 2.8 ATA using either pure O2 or hypoxic gas (7.46% O2) are shown in Fig. 12. Where indicated, rats were pre-treated with L-NAME. Approximately a 50% increase in spin concentration occurred with exposure to 2.8 ATA O2 but not by exposure to normoxic gas at 2.8 ATA or in rats infused with L-NAME before exposure to 2.8 ATA O2 (Table 3). The NO concentration in erythrocyte lysates was estimated by determining the amount of NO required to cause a ~50% increase in spin concentration in standard curves. The concentration following exposure to 2.8 ATA O2 was estimated to be 910 ± 105 nM (means ± SE, n = 4), whereas the concentration in control, air-breathing rats was 590 ± 66 nM (means ± SE, n = 4).

DISCUSSION

This investigation was focused on extending the understanding of how NO synthesis in vivo is altered by elevated partial pressures of O2. The array of inhibitor studies in rats (Fig. 6) and results in knockout mice (Fig. 7) indicate that nNOS is the isoform responsible for elevations of steady-state NO concentration due to increases in tissue oxygenation. We exclude iNOS activity based on a lack of effect of specific iNOS inhibitors. The inhibitory effect of nimodipine on O2-mediated stimulation of NO synthesis is consistent with the role of calcium/calmodulin in nNOS activation (5, 39).

Geldanamycin prevented the 2.8 ATA O2-mediated elevation in steady-state NO concentration (Fig. 6) and inhibited the O2-mediated increase in association between nNOS and calmodulin (Fig. 8). This indicates that HSP90 plays a role in nNOS activation. HSP90 is a molecular chaperone that interacts with its substrate proteins by forming transient multiprotein complexes. Two mechanisms have been identified for how HSP90 can activate nNOS. HSP90 can lower the dissociation constant (Kd) of calmodulin binding and sustain the opening of the heme-binding cleft to allow substrate interaction by reacting with NO in the periaortic microenvironment. The circulatory half-life of SOD is only ~6 min, and it cannot escape the circulation (47). This is consistent with the reversible nature of the SOD effect in our study. Our data do not refute the possibility that some NO could be removed from the perivascular zone due to reactions with O2. The results suggest that at 2.8 ATA O2, either O2 or reactive species produced by O2 initiate changes leading to nNOS stimulation. This mechanism does not occur when the O2 partial pressures was only 2.0 ATA (Fig. 8), which may explain why elevated NO concentrations were not observed in prior studies with aortic rings or endothelial cells exposed to elevated partial pressures of O2 up to 1.0 ATA (27, 40). SOD infusion did not change the temporal pattern for elevation of tissue O2 tension (Fig. 3) as might be considered if local blood flow was modified (9). Moreover, SOD infusions did not alter the elevated nNOS-calmodulin association triggered by hyperoxia (Fig. 8). This indicates that the elevation in the calmodulin-nNOS association was not sufficient by itself to increase perivascular steady-state NO concentration and that additional processes linked to O2 were required.

The steady-state concentration of NO due to exposure to 2.0 ATA O2 was significantly greater than the control, but also significantly less than that caused by 2.8 ATA O2 (Fig. 2). At 2.0 ATA O2, there was no elevation in the nNOS-calmodulin association (Fig. 8), and neither geldanamycin nor SOD prevented the elevation in NO concentration. These data suggest that the mechanism for nNOS activation was not as complex as with 2.8 ATA O2 exposure. Better oxygenation, and thus maintaining a higher percentage of enzyme in the more active ferric conformation, may be the predominant mechanism at 2.0 ATA O2 (2, 3, 20).

Whereas the inhibitory effect of geldanamycin indicates that HSP90 binding to nNOS is important for responses to 2.8 ATA O2, there were no apparent alterations in HSP90-binding characteristics. Hyperbaric O2 did not increase HSP90 binding to eNOS or nNOS (Figs. 10 and 11, and Table 2). In addition, interactions between HSP90 and p23 and HSP90 and phenyl-Sepharose were not altered by hyperoxia. The small protein p23 confers stability to many HSP90 heterocomplexes, and p23 is thought to bind specifi-
concentration by standard curve has several assumptions. Globin-trapped Hb was devised a method by which a small amount of hemoglobin can be injected without causing side effects, and we found this method was not adequately sensitive to detect a small change of blood NO level during hyperbaric O₂. We also tried spin trapping NO by using blood hemoglobin but found no difference with hyperoxia, perhaps because the amount of NO was too small compared with the heme concentration in the red blood cells (20 mM as heme concentration). Therefore, we compared with the heme concentration in the red blood cells is also assumed to be the same as that in the steady-state concentration of total NO-containing substances using a fluorescence technique, but an elevation in hemoglobin-associated NO by hyperoxia was identified with a sensitive EPR technique.

It is well established that low-molecular-weight thiols, albumin and hemoglobin, can carry NO in the blood stream (14, 15, 22). We did not find an elevation in the steady-state concentration of total NO-containing substances using a fluorescence technique, but an elevation in hemoglobin-associated NO by hyperoxia was identified with a sensitive EPR technique. We have previously used diethylthiocarbamate (DTC) for NO trapping in animal tissues (28, 45). However, DTC is water insoluble and not suitable for blood experiments. Kotake (25) used water-soluble MGD for detecting the serum concentration of NO. This method is limited because of the concentration of MGD that can be injected without causing side effects, and we found this method was not adequately sensitive to detect a small change of blood NO level during hyperbaric O₂. We also tried spin trapping NO by using blood hemoglobin but found no difference with hyperoxia, perhaps because the amount of NO was too small compared with the heme concentration in the red blood cell (20 mM as heme concentration). Therefore, we devised a method by which a small amount of hemoglobin-trapped NO was extracted ex vivo by MGD at a high concentration (~0.5 M, which cannot be reached in vivo). This method provided us with sufficient sensitivity to detect differences caused by hyperbaric O₂. It must be recognized that estimating the actual NO concentration by standard curve has several assumptions. It was assumed that NO was efficiently trapped with hemoglobin and extracted completely by MGD. The efficiency of adduct formation in the presence of red blood cells is also assumed to be the same as that in the pure buffer solution used for calibration. Therefore, the results are best viewed as an approximation. The NO concentration measured with the EPR technique was approximately twice the concentration measured by microelectrode, which may also have underestimated the concentration achieved by hyperoxia because the electrode measured values from outside of the blood vessel.

The studies with inhibitors and knock-out mice offer a consistent impression of the relative importance of nNOS. However, NO from eNOS associated with vascular endothelium may not have been detected as readily, given that most of the NO would diffuse to the vascular lumen away from the electrodes on the abluminal surface of blood vessels. Therefore, our methodology could have underestimated the apparent contribution of eNOS to NO synthesis in response to hyperoxia.

The physiological relevance of elevations in NO concentration due to hyperoxia require added investigation. These changes may contribute to augmentation of angiogenesis and inhibition of neutrophil β₂-integrin function that have been reported with hyperbaric O₂ (33, 41, 44). Differences in the mechanism for NO production at 2.0 versus 2.8 ATA O₂, as well as differences in magnitude of NO synthesis, may offer insight into dose-response phenomena. Our findings also indicate the complex influence that O₂ has on steady-state NO concentration.

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REFERENCES


