

Effect of Hyperbaric Oxygen Treatment on Nitric Oxide and Oxygen Free Radicals in Rat Brain

IKRAM M. ELAYAN, MILTON J. AXLEY, PARUCHURI V. PRASAD, STEPHEN T. AHLERS, AND CHARLES R. AUKER

Naval Medical Research Center, National Naval Medical Center, Bethesda, Maryland 20889-5607

Elayan, Ikram M., Milton J. Axley, Paruchuri V. Prasad, Stephen T. Ahlers, and Charles R. Auker. Effect of hyperbaric oxygen treatment on nitric oxide and oxygen free radicals in rat brain. *J. Neurophysiol.* 83: 2022–2029, 2000. Oxygen (O_2) at high pressures acts as a neurotoxic agent leading to convulsions. The mechanism of this neurotoxicity is not known; however, oxygen free radicals and nitric oxide (NO) have been suggested as contributors. This study was designed to follow the formation of oxygen free radicals and NO in the rat brain under hyperbaric oxygen (HBO) conditions using *in vivo* microdialysis. Male Sprague-Dawley rats were exposed to 100% O_2 at a pressure of 3 atm absolute for 2 h. The formation of 2,3-dihydroxybenzoic acid (2,3-DHBA) as a result of perfusing sodium salicylate was followed as an indicator for the formation of hydroxyl radicals. 2,3-DHBA levels in hippocampal and striatal dialysates of animals exposed to HBO conditions were not significantly different from controls. However, rats treated under the same conditions showed a six- and fourfold increase in nitrite/nitrate, break down products of NO decomposition, in hippocampal and striatal dialysates, respectively. This increase was completely blocked by the nitric oxide synthase (NOS) inhibitor L-nitroarginine methyl ester (L-NAME). Using neuronal NOS, we determined the NOS $O_2 K_m$ to be 158 ± 28 (SD) mmHg, a value which suggests that production of NO by NOS would increase approximately four- to fivefold under hyperbaric O_2 conditions, closely matching the measured increase *in vivo*. The increase in NO levels may be partially responsible for some of the detrimental effects of HBO conditions.

INTRODUCTION

Animals exposed to pressures of oxygen (O_2) in excess of two atmospheres absolute (ATA) experience abnormal motor activity such as shivering, jerks, and “wet-dog” shakes. These manifestations can progress to tonic-clonic seizures and death if animals continue to be exposed to these hyperbaric oxygen (HBO) conditions for a prolonged period (Balentine 1973). Humans who are exposed to HBO conditions can experience visual changes, nausea, muscle twitching, irritability, dizziness, and/or convulsions. Because of these toxic symptoms, human exposure to HBO conditions, such as in diving or treatment of decompression sickness and several other medical conditions (Gabb and Robin 1987), are purposely limited in pressure and duration.

The toxic effects of HBO were first described by Bert (1878); however, the mechanisms responsible for the toxicity are still not fully understood. Hyperbaric oxygen conditions produce a variety of biochemical changes in the brain, such as

inactivation of intracellular enzymes, formation of lipid hydroperoxides, depletion of GABA, and a decrease in mitochondrial respiratory rate (Clark 1982; Mayevsky 1984). One or more of these changes might be responsible for HBO-induced toxicity. Formation of oxygen free radicals, as measured by H_2O_2 production, has also been proposed as one mechanism by which HBO produces its toxicity (Jerrett et al. 1973; Piantadosi and Tatro 1990). The gaseous compound nitric oxide (NO) has also been implicated in HBO-induced toxicity (Zhang et al. 1993). Nitric oxide plays important roles in diverse physiological processes, such as neurotransmission, vasodilatation, and inhibition of platelet aggregation (Bredt and Snyder 1992; Garthwaite 1991; Knowles and Moncada 1992). In addition, NO increases cytosolic concentration of free calcium ion (Richter et al. 1994) and affects functions of various enzymes (Gopalakrishna et al. 1993; Michetti et al. 1995). It is possible that an increase in NO levels as a result of HBO treatment might mediate HBO toxicity.

In vivo microdialysis affords the advantage of detecting ongoing changes in response to a certain treatment in comparison to a single timepoint from a postmortem tissue sample. Previous studies that have investigated the role of oxygen free radicals and NO under HBO conditions were unable to utilize *in vivo* microdialysis to follow the formation of these radicals because of technical difficulties imposed by hyperbaric conditions. We used a specially designed hyperbaric chamber system adapted for microdialysis experiments to detect changes in hydroxyl radical ($\cdot OH$) and NO levels that occur during HBO exposure before the onset of seizures. To follow the formation of $\cdot OH$, we perfused sodium salicylate through the dialysis probe and measured levels of 2,3-dihydroxybenzoic acid (2,3-DHBA), the product of salicylate trapping of $\cdot OH$. We also measured the levels of nitrite and nitrate, breakdown products of NO, in the dialysates as an indicator of NO formation. In a companion set of experiments, nitric oxide synthase (NOS) activity was determined *in vitro* at various O_2 partial pressures (pO_2) to test whether the level of O_2 affects NOS activity in a way consistent with the changes in NO formation observed *in vivo*.

METHODS

Apparatus used to perform microdialysis under HBO conditions

To avoid any combustion hazard while performing microdialysis under HBO conditions, the electrical equipment, principally the microinfusion pump and fraction collector, was isolated from HBO. To

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

accomplish this we modified a standard Bethlehem hyperbaric chamber (Bethlehem Foundry NB973, Bethlehem, PA) with through-hull fittings designed to accommodate two separate hyperbaric gas delivery systems: one for O₂, the other for nitrogen (N₂). In this system, O₂ is piped into a movable internal Plexiglas box (27.2 × 25.4 × 27.2 cm) in which the rat is tethered and N₂ is piped into the Bethlehem chamber, which houses the Plexiglas box and the dialysis equipment. A 20-cm diam cylindrical compartment extending above the square portion of the box housed the swivel mechanisms through which the perfusate and dialysate flowed to and from the rat. A two-channel swivel was affixed along a Teflon rod extending across the diameter of the cylindrical compartment to allow the rat freedom of movement in all directions. The O₂ box was securely closed during compression by a round door that was fastened with screws. This allowed for a complete separation between the O₂ environment inside the box and the equipment housed in the Bethlehem chamber. The O₂ box and the microdialysis equipment were mounted on a sliding tray for easy insertion and removal from the chamber.

ELECTRICAL MODIFICATIONS. The microinfusion pump (CMA/100), fraction collector (CMA/170), and remote infusion switch (CMA/111) were modified to utilize DC current. We also used the Bioanalytical System (BAS) small Worker Bee fraction collector when immediate refrigeration of samples was not absolutely required. Cooling for the CMA/170 was provided by a water-circulating bath located outside the chamber, but piped through the chamber to the fraction collector. Pressure switches on some of the equipment were modified to negate their sensitivity to the pressure produced by the hyperbaric chamber.

Animal preparation for microdialysis

Male Sprague-Dawley rats (weighing 300–350 g), obtained from Charles River Laboratory (Wilmington, MA), were used in these studies. The animals were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle. Three days before the experiment, animals were anesthetized with xylazine (10 mg/kg) and ketamine (50 mg/kg) and guide cannulas (CMA 12) were implanted above either the striatum (coordinates, A: 0.7, L: 2.7, and V: –3.2) or the hippocampus (coordinates, A: –5.3, L: 4.8, V: –3.0) using Kopf stereotaxic apparatus. The guide cannula was secured to the skull using screws and dental cement. The animals were allowed to recover from surgery for two days. The night before the experiment, a dialysis probe (CMA 12, 4-mm tip, 0.5-mm OD) was inserted through the cannula and implanted into the brain structure. The animals were then connected to the dialysis apparatus. They were attached with a neck collar to a rotating swivel to accommodate for their free movement and to decrease the tension on tubes connected to the dialysis probe. To prevent pulling of the probe during animal movement, the probe was taped to the guide cannula using a strong adhesive tape. Ringer's buffer containing (in mM) 147 NaCl, 3 KCl, 1.2 CaCl₂, and 1.0 MgCl₂ was perfused through the dialysis probe during the night at a rate of 0.5 μl/min using a CMA/100 pump.

Compression profile

When animals were ready for compression, the door of the box was tightly closed and a flow of 100% O₂ or a gas mixture of 20% O₂ and 80% N₂ was flushed into the box. The box and the other microdialysis equipment were introduced into the Bethlehem hyperbaric chamber. The chamber was flushed with N₂ to lower O₂ levels in the chamber to <5% and then compressed to 3 ATA (304 kPa) by delivering N₂ at a rate of 60 feet of seawater (fsw)/min (184 kPa/min, 1.8 atm/min). Balancing the pressure between the O₂ box and the N₂ chamber was accomplished via a valve that dumps the excess O₂ from the box by way of a through-hull into the room. The animals were kept under these conditions for 2 h. This compression profile (3 ATA for 2 h) is not expected to result in motor seizure activities. During the compression

time the animals were monitored by cameras located on the observation windows of the hyperbaric chamber. Oxygen levels in the box and the chamber were monitored continuously with a Servomex O₂ analyzer (model 540A; Syborn/Taylor, Norwood, MA) using a manually controlled switch that allowed alternating sampling from either the chamber or the box. At the end of the 2 h compression the chamber was decompressed at a rate of 60 fsw/min (184 kPa/min, 1.8 atm/min) and the chamber and the box were opened.

Experimental designs

OXYGEN FREE RADICALS. On the day of the experiment, the flow rate of the perfusing Ringer's buffer was increased to 1 μl/min and collections were made for 2 h before the compression. At the end of the 2 h baseline collections, salicylate (5 mM) dissolved in Ringer solution was perfused through the dialysis probe. At this time, the treatment group was compressed under 100% O₂ at 3 ATA (pO₂ = 3.0 ATA) for 2 h according to the compression profile described earlier. The control group was kept in the box with the door open breathing room air at 1 ATA (pO₂ = 0.21 ATA).

In the control group, salicylate was perfused through the dialysis probe at the end of the 2 h baseline collections to coincide with the time of O₂ exposure in the treatment group. Preliminary studies indicated no difference in 2,3-DHBA levels between control animals inserted into the chamber with air flushing through the box to simulate the noise effect and control animals housed in the box outside the chamber. Therefore control animals in the current study were left outside the chamber in the box with the door closed with a porous screen. Dialysates were collected in tubes containing 2 μl of 0.1 M EDTA for an additional hour after the compression and subsequently analyzed for 2,3-DHBA, as will be described later.

NO FORMATION. On the day of the experiment, the dialysis probe was perfused with Ringer's buffer at the rate of 2 μl/min for 2 h before the compression to establish baseline levels of nitrite/nitrate. The following four groups of rats were used: 1) one group was compressed under 100% O₂ at 3 ATA (pO₂ = 3 ATA), 2) a control group breathing room air at 1 ATA (pO₂ = 0.21 ATA), 3) a group compressed under a mixture of 20% O₂ and 80% N₂ (representing air) at 3 ATA (pO₂ = 0.63 ATA), and 4) a group compressed under 100% O₂ at 3 ATA (pO₂ = 3 ATA) after the NOS inhibitor L-nitroarginine methyl ester (L-NAME, 1 mM) was perfused through the dialysis probe for 1 h before the compression. Collections were continued after the compression for an additional 2 h. Samples were stored at –80°C until assayed for nitrite/nitrate levels, as will be described later.

At the end of the experiments, animals were decapitated and the position of the microdialysis probe was confirmed.

Analytic methods

ASSAY FOR 2,3-DHBA. Samples were immediately assayed after their collection for 2,3-DHBA levels according to the method of Globus et al. (1995). Dialysate (5 μl) was injected onto a C18 5-μm (particle size) microbore column (150 × 1 mm, BAS Sepstick) using a refrigerated microsampler (CMA 200). A mobile phase containing 0.027 mM EDTA, 14.7 mM NaH₂PO₄, 30 mM sodium citrate, 10 mM diethylamine-HCl, 10 mM NaCl, 476 mg of octane sulfonic acid, and 30 ml of acetonitrile in a total volume of 1 l (pH 3.35) was delivered by a Hewlett Packard 1050 pump. 2,3-DHBA was detected using a UniJet Amperometric detector (BAS) attached to a LC-4C amperometric controller (BAS). The UniJet detector utilizes an Ag/AgCl reference electrode and a glassy carbon-working electrode (MF-1003). The working electrode was set at 650 mV against the reference electrode. Standards of different concentrations of 2,3-DHBA were run daily with experimental samples.

ASSAY FOR NO. Levels of NO in the dialysates were determined indirectly by measuring levels of nitrite and nitrate according to the method of Misko et al. (1993). Dialysate (100 μl) was transferred to

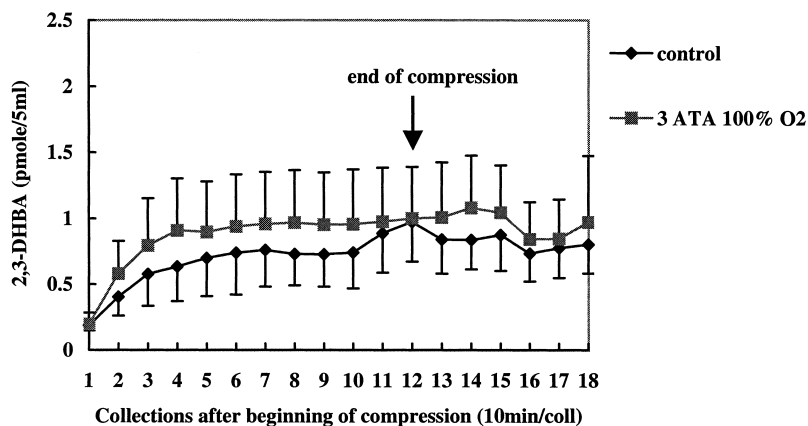


FIG. 1. 2,3-dihydroxybenzoic acid (2,3-DHBA) levels in dialysates collected from striatum of rats compressed under 100% O₂ at 3 atm absolute (ATA) for 2 h or control animals breathing room air at 1 ATA. Compression started with collection 1 and ended with collection 12 (total 2 h, 10 min/collection). Salicylate (5 mM) was perfused at the beginning of the compression for the treated group and at the same time in the control group. Results are expressed as pmol of 2,3-DHBA/5 μ l of dialysate. Values are mean \pm SE for $n = 6$ for the control group and 9 for the treatment group.

a nitrate reductor (World Precision Instrument) containing 5 μ l of 0.5 M ammonium hydroxide (pH 9). The mixture was then shaken for 5 min to allow the conversion of nitrate to nitrite. The mixture was then centrifuged for 5 min and used for the determination of nitrite levels using 2,3-diaminonaphthalene (DAN). DAN reacts with nitrite under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent product. DAN does not react with nitrate, therefore the conversion of nitrate to nitrite was necessary. Twenty μ l of DAN, 0.05 mg/ml in 0.62 M HCl, was added to 100 μ l of sample or standard and incubated for 10 min at room temperature. At the end of the incubation time, 10 μ l of 2.8 N NaOH was added to the mixture and fluorescence was read using a fluorometric plate reader (Dyntech, Chantilly, VA) with excitation at 356 nm and emission at 410 nm. Nitrite standards were used to obtain a standard curve for the measurement of nitrite levels in samples. To eliminate the possibility that our fluorescent assay for nitrite could be confounded by the presence of endogenous fluorescent compounds, we measured the fluorescence of samples obtained both before and after the compression, untreated with DAN, and found no fluorescence produced by these samples.

ENZYME. Purified rat neuronal NOS (nNOS) (specific activity >500 nmol/min/mg) was purchased from BioMol Laboratories (Plymouth Meeting, PA). Where noted, partially purified nNOS was also prepared from rat cerebella with DEAE and 2',5'-ADP-agarose chromatography (Bredt and Snyder 1990).

RADIOISOTOPE METHOD OF NOS ACTIVITY DETERMINATION. L-[2,3-³H] arginine (36.8 Ci/mmol) was purified on a Dowex -50-[Na⁺] column (Komori et al. 1993) and diluted with unlabeled arginine before use. The reaction mixture (1.2 ml) consisted of 50 mM HEPES (pH 7.0), 0.02 mM L-arginine (45 mCi/mmol), 3.5 mg of purified recombinant rat brain NOS (Biomol, Plymouth Meeting, PA), 0.1 mM NADPH, 1 mM CaCl₂, 10 mg/ml calmodulin, 1 mM FMN,

10 mM FAD, and 10 mM tetrahydrobiopterin (H₄B). The reaction mixture without enzyme was preincubated at 37°C in a disposable glass tube closed with a tight rubber stopper and equilibrated with a calibrated mixture of N₂ and O₂ gases to obtain the desired O₂ concentration. The pO₂ of the gas mixture was measured using a Clark-type electrode and an O₂ monitor (Model 5300 Biological Oxygen Monitor, Yellow Springs Instruments, Yellow Springs, OH). After equilibrating the incubation mixture for 6 min, the reaction was initiated by the addition of enzyme with a syringe (Hamilton, Reno, NV). At 1-min intervals, 0.1 ml of the reaction mixture was withdrawn with a syringe and diluted into 0.9 ml of stop buffer (50 mM sodium acetate, 2 mM EDTA, and 0.5 mM EGTA; pH 5.5). The levels of ³H-citrulline present in the stopped reaction samples were determined as described (Bredt and Snyder 1990) by separation of labeled arginine and citrulline on 1-ml columns of Dowex AG-50W-X8 resin (Bio Rad, Richmond, CA). Ready Gel scintillation fluid (10 ml; Beckman, Fullerton, CA) was added to the eluate and radioactivity was determined by counting for 1 min in a liquid scintillation counter (Model 1410, Wallac, Gaithersburg, MD). The radioactivity at different timepoints was plotted against time and initial velocities were calculated by fitting the data to a straight line.

OXYHEMOGLOBIN ASSAY OF NOS ACTIVITY. Nitric oxide synthase activity was determined by measuring the change in absorbance at 401 nm resulting from NO binding to oxyhemoglobin at 22°C as described by Hevel and Marletta (1994). The typical reaction mixture (1 ml) consisted of 50 mM HEPES (pH 7.0), 1 mM CaCl₂, 1 mM FMN, 10 mM FAD, 5 mM H₄B, 50 mM L-arginine, 10 mg calmodulin, 10 mM oxyhemoglobin, and 0.1 mM NADPH. Reactions were initiated by the addition of NADPH and absorbance change was recorded for 5–10 min.

HYPERBARIC CUVETTE. A spectrophotometer cuvette was constructed that was capable of being pressurized with gas during absor-

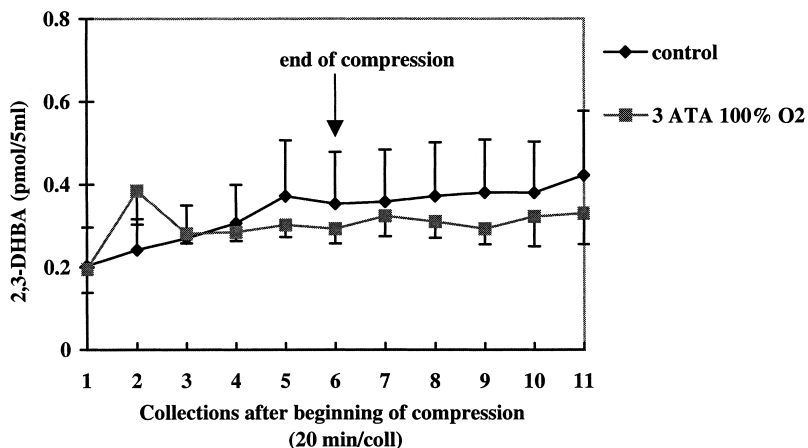


FIG. 2. Levels of 2,3-DHBA in dialysates collected from hippocampus of rats compressed under 100% O₂ at 3 ATA for 2 h or control animals breathing room air at 1 ATA. Compression started with collection 1 and ended with collection 6 (total 2 h, 20 min/collection). Salicylate (5 mM) was perfused at the beginning of the compression for the treated group and at the same time in the control group. Results are expressed as pmol of 2,3-DHBA/5 μ l of dialysate and they represent the mean \pm SE of $n = 4$ for the O₂ treated group and 3 for the control.

bance measurements. The cuvette starting material was a 7.3-cm cylinder of clear acrylic (2.2 cm diam). The lower 4.8 cm of the acrylic was shaped into a 1.25-cm square to fit a standard cuvette holder. A 1/8-inch NPT-threaded hole was drilled into the acrylic from the top to within 0.5 cm of the bottom. A stainless steel T-fitting with two 1/8-inch Swagelok connectors was screwed onto the top of the acrylic sealed with Teflon tape. One 1/8-inch Swagelok connector was used for connection to high-pressure gas lines. A Swagelok cap with an open center was attached to the other connector and a 2-mm thick silicone septum was cut to fit snugly inside the cap between the cap and the connector. The cuvette was pressure-tested to a working pressure of 150 pounds per square inch gauge (11.2 ATA). Nitric oxide synthase activity measurements with this cuvette were performed as with regular cuvettes, except that the reaction mixture without enzyme was equilibrated with gas provided by the high pressure connection before reaction, and the reaction was initiated by addition of enzyme with a syringe through the silicone septum. Concentrations of dissolved oxygen in the reaction mixture under hyperbaric conditions were not directly measured, rather they were calculated using known solubility of oxygen at 1 atm (Weathersby and Homer 1980) and Henry's Law, which states that the concentration of gas dissolved in a solution is proportional to the partial pressure of the gas over the solution.

DATA ANALYSIS FOR THE ENZYME ACTIVITY. One NOS activity unit is defined as the production of 1 nmol of NO per minute. The reaction velocity at different O₂ concentrations was determined by time course measurements as described above. The data points were fit to the Michaelis-Menten equation using the Enzfitter (Biosoft, Cambridge, UK) computer program to obtain the kinetic parameters. Only data points <760 mmHg O₂ were used for fitting to the Michaelis-Menten equation and for the determination of the kinetic parameters.

UNITS. Oxygen levels are reported as pO₂ in mmHg. Conversion of pO₂ to concentration units was based on the solubility of O₂ in water at 37°C (Weathersby and Homer 1980) and therefore 760 mmHg = 1.25 mM.

STATISTICAL ANALYSIS FOR ANIMAL GROUPS. The data from each group was expressed as mean ± SE. Statistical comparisons were made relative to the appropriate control group by two-way analysis of variance (ANOVA) and post hoc Tukey's test using commercially available software (Statistica, StatSoft, Tulsa, OK). A value of *P* < 0.05 was accepted as significant.

RESULTS

Hydroxyl radical levels as a result of exposure to 100% O₂ at 3 ATA

Hydroxyl radical levels in the brains of control and HBO-treated rats were determined by measuring 2,3-DHBA in the

dialysates (see METHODS). The HBO-treated rats were exposed to 100% O₂ at 3 ATA, whereas control animals breathed room air at 1 ATA. Perfusion of salicylate through the microdialysis probe began simultaneously with exposure to 100% O₂ and continued until the end of the experiment. Results are shown in Figs. 1 and 2 for the striatum and hippocampus, respectively. In both cases, 2,3-DHBA levels in dialysates from the treatment group were not significantly different from those of control animals at any timepoint. Although there appeared to be a trend of a slightly higher levels of 2,3-DHBA in the striatum of the treated group, this difference was not statistically significant.

NO levels in response to exposure to 100% O₂ at 3 ATA

The effect of HBO treatment on NO levels in rat brains was determined by measuring nitrite and nitrate in the dialysates (see METHODS). The HBO-treated and control animals were exposed as described in the previous paragraph. Nitrite/nitrate levels were also measured in two additional treatment groups: rats treated with a gas mixture of 20% O₂ and 80% N₂ at 3 ATA and rats pretreated with the NOS inhibitor L-NAME perfused through the dialysis probe for 1 h before exposure to 100% O₂ at 3 ATA. The effects of these treatments on nitrite/nitrate levels are shown in Figs. 3 and 4 for the striatum and hippocampus, respectively. Nitrite/nitrate levels increased in HBO-treated animals by four- and sixfold in the striatum and hippocampus, respectively. These increases returned to baseline levels following decompression. Increases in nitrite/nitrate levels were not observed in animals treated with 20% O₂ and 80% N₂ at 3 ATA, indicating that this increase was caused by HBO and not pressure per se. Perfusion of L-NAME completely blocked the HBO-induced increase in nitrite/nitrate levels.

Determination of *K_m* for O₂

Neuronal NOS activity was determined using two different assays: measuring the rate of conversion of ³H-labeled arginine to citrulline (Bredt and Snyder 1990) and measuring the increase in absorbance of oxyhemoglobin at 401 nm (Hevel and Marletta 1994). Using our hyperbaric cuvette setup and the oxyhemoglobin assay, it was possible to determine the activity of partially purified NOS from rat cerebella with O₂ levels varied into the hyperbaric range. To obtain initial velocities at varying O₂ concentrations, time course measurements were

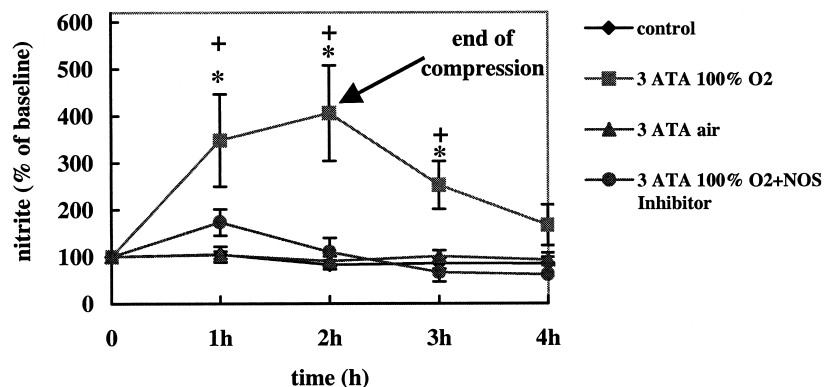


FIG. 3. Nitrite levels in dialysates collected from the striatum of control rats breathing room air at 1 ATA, rats compressed under either 100% O₂ at 3 ATA or a gas mixture of 20% O₂ and 80% N₂ (representing air) at 3 ATA and a group compressed under 100% O₂ at 3 ATA after pretreatment with L-nitroarginine methyl ester (L-NAME). L-NAME (1 mM) was perfused through the dialysis probe for 1 h before the compression at a rate of 2 μ l/min. Results are expressed as percent of baseline and they represent the mean ± SE of *n* = 4 for the control, 5 for the 100% O₂ group, 4 for the air group, and 3 for the 100% O₂ pretreated with L-NAME. Baseline levels of nitrite/nitrate in striatal dialysates were 7.3 ± 0.7 nM. *, statistical significance from the control value at that time point (*P* < 0.05). +, significantly different from the air compression at that time point (*P* < 0.05).

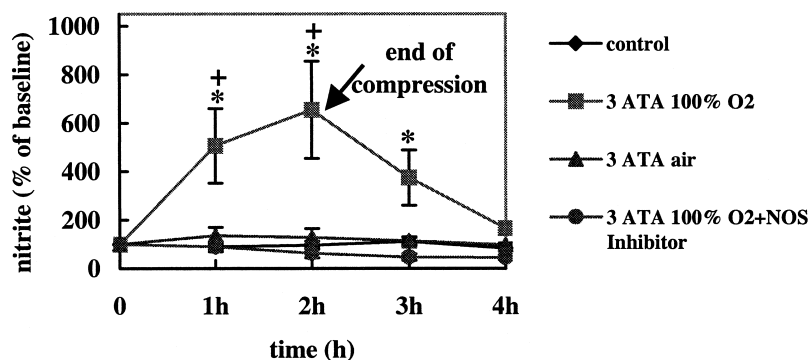


FIG. 4. Nitrite levels in dialysates collected from the hippocampus of control rats breathing room air at 1 ATA, rats compressed under either 100% O₂ at 3 ATA or a gas mixture of 20% O₂ and 80% N₂ (representing air) at 3 ATA, and a group compressed under 100% O₂ at 3 ATA after pretreatment with L-NAME. L-NAME (1 mM) was perfused through the dialysis probe for 1 h before the compression at a rate of 2 μ l/min. Results are expressed as percent of baseline and they represent the mean \pm SE of $n = 4$ for the control, 5 for the 100% O₂ group, 4 for the air group, and 3 for the 100% O₂ pretreated with L-NAME. Baseline levels of nitrite/nitrate in hippocampal dialysates were 5.4 ± 0.6 nM. *, statistical significance from the control value at that time point ($P < 0.05$). +, significantly different from the air compression at that time point ($P < 0.05$).

done at different O₂ levels and reaction velocities were calculated from linear portions of time course measurements. Results of NOS activity measurements for O₂ substrate levels from 15 mmHg (0.025 mM) to 5,900 mmHg (8.33 mM) are shown in Fig. 5. The data points at hyperbaric oxygen levels were not used for fitting to the Michaelis-Menten equation and determination of the kinetic parameters because the oxygen levels could not be directly measured. However, these hyperbaric data points are shown in Fig. 5 (\blacktriangle) for illustrative purposes. The data obtained using the two different assays were combined into a single figure. Because two different enzyme preparations whose specific activities were not equal were used for the two assays, enzyme activity at different O₂ levels was expressed as a percentage of V_{\max} for that enzyme preparation. The V_{\max} for each enzyme preparation was separately calculated by fitting the O₂ saturation data to the Michaelis-Menten equation for a single substrate to these combined data gave an O₂ K_m of 158 ± 28 mmHg (260 ± 35 μ M). The solid line in Fig. 5 represents a plot of the Michaelis-Menten equation using these parameters and illustrates the fit of the data. The NOS enzyme was near saturation at 1 ATA O₂ and no substrate inhibition was observed at higher pO₂.

DISCUSSION

Using *in vivo* microdialysis, we report here a six- and fourfold increase in NO levels in the hippocampus and the striatum, respectively, of rats treated with 100% O₂ at 3 ATA for 2 h. In contrast, we did not observe an increase in hydroxyl radical levels in the striatum or the hippocampus of rats treated

similarly with HBO. We also report the O₂ K_m value of nNOS to be 158 ± 28 mmHg (260 ± 35 μ M).

Nitric oxide has been proposed to mediate HBO effects on the nervous system. Zhang et al. (1995) reported that the NOS inhibitor, L ^{ω} -nitro-L-arginine, protected against HBO-induced seizures. Also, Ito et al. (1996) observed an increase in arginine levels and a decrease in arginase activity under HBO conditions, implicating an increase in NO. Nitric oxide could have a detrimental effect under HBO conditions by increasing blood flow to the area and thus increasing O₂ delivery. Initially, cerebral blood flow (CBF) decreases in response to HBO exposure (Bergo and Tyssebotn 1992; Torbati et al. 1978), but recently Chavko et al. (1998) have shown in awake animals that as HBO exposure continues there is a subsequent large increase in CBF that was attenuated by NOS inhibitor, N-nitro-L-arginine. However, in anesthetized animals, Zhang et al. (1995) demonstrated that the protective effect of NOS inhibitor under HBO conditions was not because of a decrease in CBF or O₂ tension, at least not in the cerebral cortex. It is possible that the increase in NO can produce toxic effects by other means such as mediating excitotoxicity of glutamate and aspartate (Dawson et al. 1991). Nitric oxide also can produce toxicity by inhibiting aconitase and complex I and II in the electron transport chain (Stadler et al. 1991). This can inhibit mitochondrial respiration leading to ATP depletion and eventually disruption of the active transport systems of the cell. These changes might then lead to membrane potential disturbances and depolarization, which could be responsible for the observed seizures. Another mechanism by which NO might cause toxicity is by increasing intracellular free calcium by activating the ryanodine receptor (Clementi et al. 1996) and by

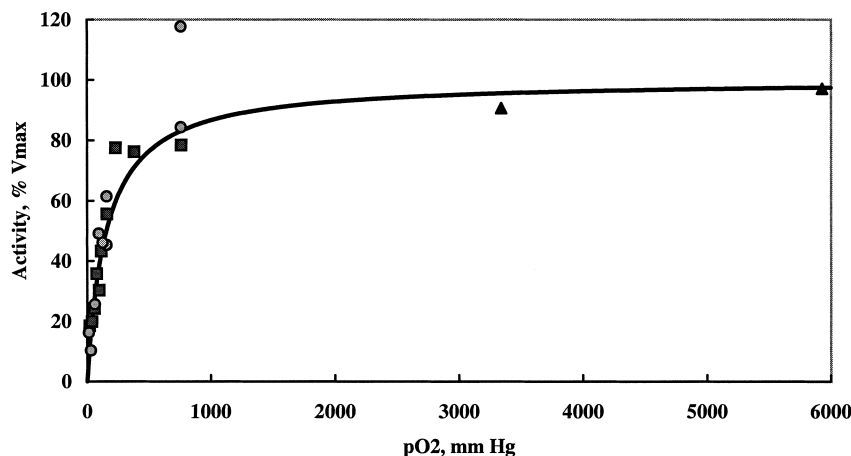


FIG. 5. Neuronal NOS (nNOS) activity as a function of pO₂ determined with citrulline and oxyhemoglobin assays. nNOS activity was determined by measuring the conversion of ³H-arginine to ³H-citrulline (\blacksquare) or by oxyhemoglobin assay (\bullet and \blacktriangle) at various O₂ partial pressures as described in METHODS. The solid line represents the best fit of the Michaelis-Menten equation to the nonhyperbaric data. Data at hyperbaric conditions (\blacktriangle) were not used for fitting to the equation (see RESULTS for more details). Activity at each O₂ concentration is represented as percent V_{\max} for the enzyme preparation used in a given assay. The V_{\max} for the enzyme used in radioactive assay was 198.1 nmol NO/min/mg protein and the V_{\max} for the enzyme used in oxyhemoglobin assay was 68.8 nmol NO/min/mg protein.

the activation of the L and P Ca^{2+} channels (Ohkuma et al. 1998). An increase in calcium levels in the cell can result in the activation of a variety of enzymes and the disruption of membrane potential, which might lead to cellular dysfunction and seizures.

Using rat nNOS, we determined the nNOS O_2 K_m value to be 158 ± 28 mmHg (260 ± 35 μM), and there was no evidence of substrate inhibition at O_2 levels up to 5,930 mmHg (8.33 mM, indirectly measured). This nNOS O_2 K_m value is similar to an estimate previously reported by Abu-Soud et al. (1996). An apparent K_m for O_2 for bovine brain NOS has been reported as 23 μM (Rengasamy and Johns 1996). We have shown here that the K_m for rat nNOS is 11 times that value at saturating levels of arginine (20 μM) and NADPH (100 μM). The other study (Rengasamy and Johns 1996) does not state the concentration of arginine used in NOS reactions, and the use of subsaturating levels of arginine in the reactions might result in apparent K_m values for O_2 lower than the true values. More importantly, it appears that nNOS activities were not measured with O_2 levels sufficient to saturate the enzyme. The highest level of O_2 substrate at which they report nNOS activity was ~ 125 μM (equivalent to a solution saturated with 12% O_2 at 1 atm) and replotting their data as a direct rather than reciprocal plot suggests that saturation of the enzyme with O_2 was not achieved or approached at this $p\text{O}_2$. Furthermore, bovine brain NOS activity was determined in the presence of higher O_2 levels in a previous publication by the same authors (Rengasamy and Johns 1991). They found that nNOS displayed 42% higher activity under "hyperoxic" $p\text{O}_2$ (460 mmHg or 645 μM) as compared with "normoxic" $p\text{O}_2$ (130 mmHg or 182 μM). The results of this earlier report are inconsistent with a K_m of 23 μM published in their subsequent report, if it is a true K_m , as the enzyme should have been near saturation under normoxic conditions and the activity under hyperoxic conditions should have been no more than 8.8% higher. Our K_m determination of 260 ± 35 μM predicts activity under their hyperoxic conditions to be 50% higher than normoxic, similar to the results reported in their previous publication (Rengasamy and Johns 1991). The difference in reported K_m values does not alter the authors' conclusion that lowering tissue $p\text{O}_2$ below normoxic levels could decrease NOS activity and production of NO.

The O_2 K_m determined here suggests that nNOS activity could increase significantly under HBO conditions. The O_2 K_m value we determined can be used to estimate the effect of increasing brain O_2 levels on production of NO by nNOS. The $p\text{O}_2$ of brain tissue under normoxic conditions (breathing air at 1 atm) is ~ 30 mmHg, whereas the $p\text{O}_2$ of brain tissue for an animal breathing pure O_2 at 3 atm pressure is >450 mmHg (Jamieson and Brenk 1963). Using the Michaelis-Menten equation and assuming the availability of O_2 substrate limits enzymatic activity, breathing HBO would cause NO production to increase ~ 4.6 -fold, which is very close to the increase in NO levels we observed with in vivo microdialysis.

Oxygen free radical formation has been proposed as a mechanism by which HBO produces its effect on the nervous system. To the best of our knowledge, this is the first study to investigate the formation of oxygen free radicals under HBO conditions using in vivo microdialysis. Several studies have investigated the role of oxygen free radicals under HBO con-

ditions using indirect methods, such as lipid peroxidation (Chavko and Harabin 1996; Ito et al. 1996; Noda et al. 1983), protein oxidation (Chavko and Harabin 1996), or catalase activity in the presence of aminotriazole (Piantadosi and Tatro 1990; Yusa et al. 1987). Others have looked at the formation of oxygen free radicals by measuring spin trapping adducts in whole brain tissues subsequent to HBO exposure (Torbati et al. 1992). Our results indicate that oxygen free radicals in the striatum and the hippocampus, as reflected by the levels of 2,3-DHBA levels measured during HBO exposures, were not significantly different from those formed in control animals not exposed to HBO conditions. These structures are believed to be involved in HBO-induced toxicity. In the previously mentioned reports, investigators performed their experiments either in the whole brain (Torbati et al. 1992; Yusa et al. 1987) or in pooled brain parts (Piantadosi and Tatro 1990), which might reflect an increase in brain structures other than the hippocampus and the striatum.

Chavko and Harabin (1996) reported an increase in lipid peroxidation in the hippocampus and the frontal cortex, and protein oxidation in all the brain structures studied including the hippocampus and the basal ganglia. These results are not in agreement with our findings that 2,3-DHBA levels, formed as a result of HBO treatment, were not different from controls. This cannot be explained by the fact that we used an O_2 pressure of 3 ATA and they used 6 ATA, because we also measured 2,3-DHBA levels under 6 ATA and failed to observe any significant difference between treated and control animals (data not shown). However, our experimental conditions were different from those in the previously mentioned reports, because we used in vivo microdialysis and they used lipid peroxidation, protein oxidation, or spin trapping as an indicator of reactive free radical formation. Microdialysis affords the advantage of detecting ongoing changes during exposure. One possible explanation for the differences in results might be that the data presented here did not involve posttreatment effects or decapitation. Another possibility is that the presence of the dialysis probe in our study interfered with the formation of free radicals; however, when we perfused hydrogen peroxide into the striatum we observed a significant increase in 2,3-DHBA levels in comparison to control animals (data not shown). One potential disadvantage of the salicylate trapping method is the low efficiency by which salicylate traps hydroxyl radicals. Floyd et al. (1984) observed that 1 mM of salicylate is able to trap only 10% of the formed hydroxyl radicals. It is possible that we were unable to detect an increase in hydroxyl radical levels due to this disadvantage. Nevertheless, this method has been extensively used by others (Althaus et al. 1993; Chiueh et al. 1992; Piantadosi et al. 1997), where relative changes in free radical formation were detected despite low absolute recovery.

Our results showed an increase in NO levels in response to HBO treatment. One possible mechanism by which HBO results in this increase is by increasing O_2 availability, thus driving the reaction forward. This hypothesis assumes that NOS activity is affected by O_2 levels and that O_2 delivery is increased under HBO conditions. Previous studies have reported modulation of NOS activity by O_2 tension both in vitro (Abu-Soud et al. 1996; Kwon et al. 1990; Rengasamy and Johns 1991) and in vivo (Dweik et al. 1998). As for the

increase in O₂ levels in brain tissue, Jamieson and Brenk (1963) and Zhang et al. (1995) reported an increase in brain tissue pO₂ levels in response to HBO treatment. Therefore an increase in O₂ levels under HBO conditions might be a factor in increasing NO production under these conditions. This increase in NO levels under HBO conditions might be responsible for some of the abnormal observations, such as seizures, by any of the previously mentioned mechanisms.

We thank G. Goehring and W. Long for producing the hyperbaric cuvette and the hyperbaric chamber for microdialysis experiments. We also thank W. Koller for technical assistance.

This research was supported by Naval Medical Research and Developmental Command Work Units 62233N MM33P30.004-1519 and 61153N MR04101.001-1508 and Uniform Services University of the Health Sciences Grant G1927501. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, National Academy Press 1996. The opinions expressed in this paper are those of the authors and do not reflect the official policy of the Department of Navy, Department of Defense, or the U. S. Government.

Present addresses: M. J. Axley, MedImmune Inc., 35 W. Watkins Mill Rd., Gaithersburg, MD 20878; P. V. Prasad, Data Sciences Inc., 2150 Industrial Parkway, Silver Spring, MD 20904; S. T. Ahlers, CCCADP, Medical Science and Technology Division (code 341), Office of Naval Research, 800 N. Quincy, Arlington, VA 22217.

Address for reprint requests: I. M. Elayan, Naval Medical Research Center, 8901 Wisconsin Ave., Bethesda, MD 20889-5607.

Received 9 November 1998; accepted in final form 30 September 1999.

REFERENCES

- ABU-SOUD, H. M., ROUSSEAU, D. L., AND STUEHR, D. J. Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. *J. Biol. Chem.* 271: 32515–32518, 1996.
- ALTHAUS, J. S., ANDRUS, P. K., WILLIAMS, C. M., VONVOIGTLANDER, P. F., CAZERS, A. R., AND HALL, E. D. The use of salicylate hydroxylation to detect hydroxyl radical generation in ischemic and traumatic brain injury. *Mol. Chem. Neuropathol.* 20: 147–162, 1993.
- BALENTINE, J. D. Selective vulnerability of the central nervous system to hyperbaric oxygen. *Adv. Exp. Med. Biol.* 37A: 293–298, 1973.
- BERGO, G. W. AND TYSSEBOTN, I. Cerebral blood flow distribution during exposure to 5 bar oxygen in awake rats. *Undersea Biomed. Res.* 19: 339–354, 1992.
- BERT, P. Action of compressed air on animals. In: *Barometric Pressure*, edited by M. A. Hitchcock and F. A. Hitchcock. Columbus, OH: College Book, 1878, [English trans. by M. A. and F. A. Hitchcock.] Chapter 4, 1943: 709–779.
- BRETT, D. S. AND SNYDER, S. H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* 87: 682–685, 1990.
- BRETT, D. S. AND SNYDER, S. H. Nitric oxide, a novel neuronal messenger. *Neuron* 8: 3–11, 1992.
- CHAVKO, M., BRAISTED, J. C., OUTSA, N. J., AND HARABIN, A. L. Role of cerebral blood flow in seizures from hyperbaric oxygen exposure. *Brain Res.* 791: 75–82, 1998.
- CHAVKO, M. AND HARABIN, A. L. Regional lipid peroxidation and protein oxidation in rat brain after hyperbaric oxygen exposure. *Free Rad. Biol. Med.* 20: 973–978, 1996.
- CHUEH, C. C., KRISHNA, G., TULSI, P., OBATA, T., LANG, K., HUANG, S. J., AND MURPHY, D. L. Intracranial microdialysis of salicylic acid to detect hydroxyl radical generation through dopamine autooxidation in the caudate nucleus: effects of MPP⁺. *Free Rad. Biol. Med.* 13: 581–583, 1992.
- CLARK, J. M. Oxygen toxicity. In: *Physiology and Medicine of Diving* (3rd ed.), edited by P. B. Bennett and D. H. Elliot. London: Bailliere, Tindal & Cox, 1982, p. 200–238.
- CLEMENTI, E., RICCIO, M., SCIORATI, C., NISTICO, G., AND MELDOLESI, J. The type 2 ryanodine receptor of neurosecretory PC12 cells is activated by cyclic ADP-ribose. Role of the nitric oxide/cGMP pathway. *J. Biol. Chem.* 271: 17739–17745, 1996.
- DAWSON, V. L., DAWSON, T. M., LONDON, E. D., BRETT, D. S., AND SNYDER, S. H. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. USA* 88: 6368–6371, 1991.
- DWEIK, R. A., LASKOWSKI, D., ABU-SOUD, H. M., KANEKO, F., HUTTE, R., STUEHR, D. J., AND ERZURUM, S. C. Nitric oxide synthesis in the lung. Regulation by oxygen through a kinetic mechanism. *J. Clin. Invest.* 101: 660–666, 1998.
- FLOYD, R. A., WATSON, J. J., AND WONG, P. K. Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and salicylate hydroxylation products. *J. Biochem. Biophys. Methods* 10: 221–235, 1984.
- GABB, G. AND ROBIN, E. D. Hyperbaric oxygen. A therapy in search of disease. *Chest* 92: 1074–1082, 1987.
- GARTHWAITE, J. Glutamate, nitric oxide and cell-cell signaling in the nervous system. *Trends Neurosci.* 14: 60–67, 1991.
- GLOBUS, M. Y., BUSTO, R., LIN, B., SCHNIPPERING, H., AND GINSBERG, M. D. Detection of free radical activity during transient global ischemia and recirculation: effects of intracerebral brain temperature modulation. *J. Neurochem.* 65: 1250–1256, 1995.
- GOPALAKRISHNA, R., CHEN, Z. H., AND GUNDIMEDA, U. Nitric oxide and nitric oxide generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *J. Biol. Chem.* 268: 27180–27185, 1993.
- HEVEL, J. M. AND MARLETTA, M. A. Nitric-oxide synthase assays. *Meth. Enzymol.* 233: 250–258, 1994.
- ITO, T., YUFU, K., MORI, A., AND PACKER, L. Oxidative stress alters arginine metabolism in rat brain: effects of sub-convulsive hyperbaric oxygen exposure. *Neurochem. Int.* 29: 187–195, 1996.
- JAMIESON, D. AND VAN DEN BREK, H.A.S. Measurement of oxygen tensions in cerebral tissues of rats exposed to high pressures of oxygen. *J. Appl. Physiol.* 18: 869–876, 1963.
- JERRETT, S. A., JEFFERSON, D., AND MENGEL, C. E. Seizures, H₂O₂ formation and lipid peroxides in brain during exposure to oxygen under high pressure. *Aerospace Med.* 44: 40–44, 1973.
- KNOWLES, R. G. AND MONCADA, S. Nitric oxide as a signal in blood vessels. *Trends Biochem. Sci.* 17: 399–402, 1992.
- KOMORI, Y., CHIANG, K. T., AND FUKUTO, J. M. The effect of nonionic detergents on the activity and/or stability of rat brain nitric oxide synthase. *Arch. Biochem. Biophys.* 307: 311–315, 1993.
- KWON, N. S., NATHAN, C. F., GILKER, C., GRIFFITH, O. W., MATTHEWS, D. E., AND STUEHR, D. J. L-citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen. *J. Biol. Chem.* 265: 13442–13445, 1990.
- MAYEVSKY, A. Invited review: brain oxygen toxicity. In: *Undersea Physiology VIII*, edited by A. J. Bachrach and M. M. Matzen. Bethesda, MD: Undersea Medical Society, 1984, p. 69–89.
- MICETTI, M., SALAMINO, F., MELLONI, E., AND PONTREMOLI, S. Reversible inactivation of calpain isoforms by nitric oxide. *Biochem. Biophys. Res. Commun.* 207: 1009–1014, 1995.
- MISKO, T. P., SCHILLING, R. J., SALVEMINI, D., MOORE, W. M., AND CURRIE, M. G. A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* 214: 11–16, 1993.
- NODA, Y., MCGEER, P. L., AND MCGEER, E. G. Lipid peroxide distribution in brain and the effect of hyperbaric oxygen. *J. Neurochem.* 40: 1329–1332, 1983.
- OHKUMA, S., KATSURA, M., HIBINO, Y., XU, J., SHIROTANI, K., AND KURIYAMA, K. Multiple actions of nitric oxide on voltage-dependent Ca²⁺ channels in mouse cerebral cortical neurons. *Mol. Brain Res.* 54: 133–140, 1998.
- PIANTADOSI, C. A. AND TATRO, L. G. Regional H₂O₂ concentration in rat brain after hyperoxic convulsions. *J. Appl. Physiol.* 69: 1761–1766, 1990.
- PIANTADOSI, C. A., ZHANG, J., AND DEMCHENKO, I. T. Production of hydroxyl radical in the hippocampus after CO hypoxia or hypoxic hypoxia in the rat. *Free Rad. Biol. Med.* 22: 725–732, 1997.
- RENGASAMY, A. AND JOHNS, R. A. Characterization of endothelium-derived relaxing factor/nitric oxide synthase from bovine cerebellum and mechanism of modulation by high and low oxygen tensions. *J. Pharmacol. Exp. Ther.* 259: 310–316, 1991.
- RENGASAMY, A. AND JOHNS, R. A. Determination of K_m for oxygen of nitric oxide synthase isoforms. *J. Pharmacol. Exper. Ther.* 276: 30–33, 1996.
- RICHTER, C., GOGVADZE, V., SCHLAPBACH, R., SCHWEIZER, M., AND SCHLEGEL, J. Nitric oxide kills hepatocytes by mobilizing mitochondrial calcium. *Biochem. Biophys. Res. Commun.* 205: 1143–1150, 1994.

- STADLER, J., BILLIAR, T. R., CURRAN, R. D., STUEHR, D. J., OCHOA, J. B., AND SIMMONS, R. L. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. *Am. J. Physiol.* 260: C910–C916, 1991.
- TORBATI, D., CHURCH, D. F., KELLER, J. M., AND PRYOR, W. A. Free radical generation in the brain precedes hyperbaric oxygen-induced convulsions. *Free Rad. Biol. Med.* 13: 101–106, 1992.
- TORBATI, D., PAROLLA, D., AND LAVY, S. Blood flow in rat brain during exposure to high oxygen pressure. *Aviat. Space Environ. Med.* 49: 963–967, 1978.
- WEATHERSBY, P. K. AND HOMER, L. D. Solubility of inert gases in biological fluids and tissues: a review. *Undersea Biomed. Res.* 7(4): 277–296, 1980.
- YUSA, T., BECKMAN, J. S., CRAPO, J. D., AND FREEMAN, B. A. Hyperoxia increases H₂O₂ production by brain in vivo. *J. Appl. Physiol.* 63: 353–358, 1987.
- ZHANG, J., SAM, A. D., KLITZMAN, B., AND PIANTADOSI, C. A. Inhibition of nitric oxide synthase on brain oxygenation in anesthetized rats exposed to hyperbaric oxygen. *Undersea Hyperbaric Med.* 22: 377–382, 1995.
- ZHANG, J., SU, Y., OURY, T. D., AND PIANTADOSI, C. A. Cerebral amino acid, norepinephrine and nitric oxide metabolism in CNS oxygen toxicity. *Brain Res.* 606: 56–62, 1993.