Vascular reactivity and endothelial NOS activity in rat thoracic aorta during and after hyperbaric oxygen exposure

Jonas Hink,1,2,6 Stephen R. Thom,4 Ulf Simonsen,5 Inger Rubin,3 and Erik Jansen6

1Royal Danish Naval Diving School, The Danish Armed Forces, Departments of 2Medical Physiology and 3Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, 4Hyperbaric Oxygen Treatment Unit, Department of Anaesthesia, The Centre of Head and Orthopaedics, Copenhagen University Hospital Righospitalitaet, Copenhagen, 5Department of Pharmacology, University of Aarhus, Aarhus, Denmark; and 6Department of Emergency Medicine, Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania

Submitted 9 February 2006; accepted in final form 31 March 2006

Hink, Jonas, Stephen R. Thom, Ulf Simonsen, Inger Rubin, and Erik Jansen. Vascular reactivity and endothelial NOS activity in rat thoracic aorta during and after hyperbaric oxygen exposure. Am J Physiol Heart Circ Physiol 291: H1988–H1998, 2006. First published April 28, 2006; doi:10.1152/ajpheart.00145.2006.—Accumulating evidence suggests that hyperbaric oxygen (HBO) stimulates neuronal nitric oxide (NO) synthase (NOS) activity, but the influence on endothelial NOS (eNOS) activity and vascular NO bioavailability remains unclear. We used a bioassay employing rat aortic rings to evaluate vascular NO bioavailability. HBO exposure to 2.8 atm absolute (ATA) in vitro decreased ACh relaxation. This effect remained unchanged, despite treatment with SOD-polyethylene glycol and catalase-polyethylene glycol, suggesting that HBO inhibited endothelium-derived NO bioavailability independent of superoxide production. In vitro HBO induced contraction of resting aortic rings with and without endothelium, and these contractions were reduced by the NOS inhibitor N^ω-nitro-L-arginine. In addition, in vitro HBO attenuated the vascular contraction produced by norepinephrine, and this effect was reversed by N^ω-nitro-L-arginine, but not by endothelial denudation. These findings indicate stimulation of extracellular NO production during HBO exposure. A radiochemical assay was used to assess NOS activity in rat aortic endothelial cells. Catalytic activity of eNOS in cell homogenates was not decreased by HBO, and in vivo HBO exposure to 2.8 ATA was without effect on eNOS activity and/or vascular NO bioavailability. We conclude that HBO reduces endothelium-derived NO bioavailability independent of superoxide production, and this effect seems to be unrelated to a decrease in eNOS catalytic activity. In addition, HBO increases the resting tone of rat aortic rings and attenuates the contractile response to norepinephrine by endothelium-independent mechanisms that involve extracellular NO production.

nitric oxide synthase; acetylcholine relaxation; autooxidation

HYPERBARIC OXYGEN (HBO) is used in clinical medicine in the management of different diseases and conditions, because it mediates some useful actions, such as stimulation of angiogenesis and inhibition of leukocyte adhesion. However, the underlying biochemical mechanisms have not been fully elucidated.

Nitric oxide (NO) bioavailability in rat and mouse cerebral cortex, measured by NO-specific microelectrodes, is increased early during HBO exposure and paralleled by an increase in regional blood flow (40). Thom et al. (40) reported that cerebral NO production was increased much more in knockout mice lacking genes for endothelial NO synthase (eNOS) than in those lacking genes for neuronal NO synthase (nNOS), suggesting that changes in eNOS activity during HBO exposure are minimal compared with changes in nNOS activity. However, because of the predominance of nNOS in the central nervous system (20), this might not be the case in the systemic circulation. Increased nNOS- but not eNOS-derived NO was also observed at the abluminal side of the rat abdominal aorta during HBO exposure, but an increase in endothelium-derived NO may not have been detected because of the relatively large diffusion distance and the scavenging effect of the flowing blood (41). So, although the mechanism whereby nNOS activity increases during HBO exposure has been well described, the degree to which eNOS activity is influenced remains to be clarified.

In the case of simultaneous increase in superoxide and NO, vascular tone is influenced by the balance between these two species, and the net result may be vasoconstriction or vasodilation. HBO exposure has been shown to induce an early cerebral vasoconstriction in mice due to inactivation of eNOS-derived NO by superoxide followed by late vasodilatation (2, 9). The late-HBO-induced vasodilation was dependent on eNOS and nNOS, and it has been speculated that HBO stimulates NOS activity by increasing cerebral PO2 (11). The Km and EC50 values of eNOS for oxygen measured in purified enzyme and intact endothelial cells have been reported to be 7.7–38 μM (31, 46), corresponding to 4.7–23.1 mmHg PO2 in aqueous solutions (45). These findings are inconsistent with an increase in eNOS activity and endothelium-derived NO bioavailability during HBO exposure compared with normobaric hyperoxia. However, none of the studies on Km values of eNOS for oxygen included oxygen levels in the hyperbaric range. Also, hydrogen peroxide, which is believed to increase during HBO (21), has been found to activate eNOS and enhance endothelium-dependent relaxation (36, 47).

Rat aortic ring endothelium-dependent relaxation has been found to be enhanced after 1 h of in vivo normobaric hyperoxia (38) but abolished after 6 h of in vivo normobaric (28) or hyperbaric (33) oxygen exposure. In vitro studies using cultured bovine endothelial cells, ovine fetal endothelial cells, and human umbilical vein endothelial cells have shown that norm-
mobaric (5, 25) or hyperbaric (7) hyperoxia increases the expression of eNOS after 8–24 h. However, neither eNOS activity nor endothelial NO release has been determined during or after HBO exposure.

In this study, a variety of different model systems were used to assess how HBO may alter eNOS activity and its physiological effects. Thus contractile and vasodilatory responses in rat aortic rings were used as an indirect measure of vascular NO production/release and bioavailability. We tested endothelium-dependent and -independent contractile and vasodilatory responses in the presence and absence of SOD-polyethylene glycol (PEG) and catalase-PEG during HBO exposure compared with responses during exposure to an oxygen level reflecting normobaric hyperoxia. Furthermore, to study the kinetic properties of eNOS, we measured NOS functional activity in rat aortic endothelial cell homogenate at different oxygen substrate levels during normo- and hyperbaric exposure.

Finally, we investigated rat aortic vasomotor responses and endothelial cell NOS activity at different times after in vivo HBO exposure.

MATERIALS AND METHODS

Animals and Reagents

All procedures for animal manipulation were reviewed and approved by the Danish Animal Experiments Inspectorate. A total of 119 male Wistar rats (Charles River Laboratories, Sulzfeld, Germany; 250–300 g body wt) were fed a standard diet and allowed water ad libitum. Unless otherwise indicated, reagents were purchased from Sigma-Aldrich Denmark.

Hyperbaric Exposures

For investigation of vascular reactivity and eNOS activity during in vitro HBO exposure, the experimental equipment (and the operator) were brought into a multiphase hyperbaric chamber that was pressurized to 2.8 atmospheres absolute (ATA).

For the in vivo experiments, awake and freely moving rats were exposed to pure oxygen at 2.8 ATA for 45 min in an animal hyperbaric chamber and killed immediately thereafter (to investigate acute changes) or 17 h after decompression. Untreated control rats were exposed to room air only.

Euthanasia and Anesthetic Regimen

Unanesthetized animals were decapitated in a guillotine. For investigation of eNOS at different oxygen levels, endothelial cell homogenate was harvested from separate unexposed rats anesthetized with one part fentanyl-fluanisone (Hypnorm: 0.2 mg fentanyl + 10 mg fluanisone per milliliter) + one part water and one part midazolam 5 mg/ml (Dormicum) + one part water (0.2 ml/100 g sc).

Preparation of Aortic Rings

For harvesting of endothelial cells, the entire aorta was opened longitudinally and carefully rinsed free of blood with cold saline. Endothelial cells were selectively isolated by gentle scraping of the inner surface with the tip of a scalpel in a single movement. For the in vivo experiments, awake and freely moving rats were exposed to pure oxygen at 2.8 ATA for 45 min in an animal hyperbaric chamber and killed immediately thereafter (to investigate acute changes) or 17 h after decompression. Untreated control rats were exposed to room air only.

Preparation of Endothelial Samples

For harvesting of endothelial cells, the entire aorta was opened longitudinally and carefully rinsed free of blood with cold saline. Endothelial cells were selectively isolated by gentle scraping of the inner surface with the tip of a scalpel in a single movement. For the in vivo experiments, awake and freely moving rats were exposed to pure oxygen at 2.8 ATA for 45 min in an animal hyperbaric chamber and killed immediately thereafter (to investigate acute changes) or 17 h after decompression. Untreated control rats were exposed to room air only.

Endothelial cells were selectively isolated by gentle scraping of the inner surface with the tip of a scalpel in a single movement. For the in vivo experiments, awake and freely moving rats were exposed to pure oxygen at 2.8 ATA for 45 min in an animal hyperbaric chamber and killed immediately thereafter (to investigate acute changes) or 17 h after decompression. Untreated control rats were exposed to room air only.

Preparation of Aortic Rings

Aortic rings from separate rats were prepared in a petri dish with cold oxygenated physiological salt solution containing (in mM) 119.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.17 MgSO4, 25.0 NaHCO3, 1.18 KH2PO4, 0.027 EDTA, and 5.5 glucose. The thoracic aorta was carefully cleaned of blood, fat, and connective tissue and cut into 2-mm-long ring segments.

Measurement of Isometric Contractile and Vasodilatory Responses: General Procedures

The aortic ring segments were mounted in a dual-wire myograph system (Danish Myo Technology) with 100-μm wire, kept at 37°C in organ baths containing physiological salt solution, and continuously bubbled with a gas mixture of 95% O2-5% CO2. Each ring was stretched to a passive force of 20 mN. In some experiments, endothelium was removed by gentle rubbing of the luminal side with a 40-μm stainless steel wire. After 60 min of equilibration, the contractile function of the vessels was tested twice with 5 μM norepinephrine (15 min) interspersed and followed by washout and rest periods of ~45 min in total. Only preparations showing a maximal response of ≥14 mN were included in the studies. All experiments were carried out in the presence of 3 μM indomethacin and 1 μM propranolol to minimize the contribution of cyclooxygenase products to vasodilatation and to eliminate a possible effect of norepinephrine on β-adrenoceptors. In the concentration-response studies, cumulative drug doses were added every 2 min (during in vitro exposure) or 3 min (after in vivo exposure). In the studies where the ACh concentration-response relation was not determined, a quick check of endothelial function was performed after precontraction with 0.1–0.4 μM norepinephrine. The endothelium was considered intact if stimulation with 10 μM ACh produced ≥60% relaxation. The endothelium was considered denuded if stimulation with 10 μM ACh produced <10% relaxation.

Contractile and Vasodilatory Responses During In Vitro Hyperbaric Exposures

Pressurization procedures.

The aortic rings were unstratched immediately before pressurization, and P02 in the organ bath was continuously measured using a Clark-type oxygen sensor (model OX-100, Unisense). Bubbling of the organ bath was stopped during the 2-min pressurization period. Once at pressure, the aortic rings were carefully restretched to a passive force of 20 mN, and bubbling of the organ bath was started with a gas mixture of 32.6% O2-1.8% CO2-65.6% N2 (corresponding to 95% O2-5% CO2 at atmospheric pressure in regard to PO2 and P02). Temperature was maintained at 37°C, and the rings were allowed to rest before one of several different experiments was carried out. The rings were exposed to hyperbaric conditions for ≤80 min. In previous calibration studies, we found that the myograph system was not affected by pressure. PO2 in the organ bath was increased at pressure by a change in the bubbling gas mixture to 98.2% O2-1.8% CO2. In control experiments, such a shift in the bubbling gas mixture did not change pH in the organ bath. Equilibration at pressure (2.8 ATA) with 32.6% O2-1.8% CO2-65.6% N2 (hyperbaric control) produced ~680 mmHg PO2, whereas equilibration at pressure with 98.2% O2-1.8% CO2 (HBO) produced ~2,040 mmHg PO2 in the organ bath.

Effect of increasing oxygen on resting aortic rings. To investigate the effect of increasing oxygen on baseline resting force, the rings were monitored for ≥7 min after a change in gas mixture at pressure to HBO (n = 9) and after the hyperbaric control gas mixture (n = 9) was turned off and on (sham change). Similar experiments were performed with resting endothelium-denuded rings (n = 5) and resting rings incubated for 30 min with 100 U/ml PEG-SOD + 3,000 U/ml PEG-catalase (n = 9) or with 0.3 mM Nω-nitro-l-arginine (l-NNA, n = 4), an NOS inhibitor.
ACh stimulation studies. The ACh concentration-response relation was determined and used as an indirect measure of endothelial NO production/release. The gas mixture was changed before or after precontraction of the rings and after ACh stimulation.

For change in the gas mixture before precontraction of the rings, the bubbling gas mixture in the organ bath was changed to HBO (n = 6) or to hyperbaric control (“sham,” n = 6), and aortic rings were stimulated with the concentration of norepinephrine that produced a precontraction level of ~60–80% of the maximal norepinephrine (5 μM) contraction obtained in the beginning of each experiment. ACh stimulation (10^{-8}–10^{-5} M) was started when the precontraction had stabilized. A similar experiment (10^{-8}–10^{-5} M ACh) was carried out after 30 min of incubation with 100 U/ml PEG-SOD and 3,000 U/ml PEG-catalase (n = 6 in each group).

For change in gas mixture after precontraction of the rings, the aortic rings were submaximally precontracted with 0.1 μM norepinephrine for 15 min, and the bubbling gas mixture in the organ bath was changed to HBO (n = 8) or to hyperbaric control (sham, n = 8). ACh stimulation (10^{-8}–10^{-5} M) was started after the gas mixture was changed. A similar experiment was carried out after 30 min of incubation with 100 U/ml PEG-SOD (n = 6 in each group).

For change in gas mixture after ACh stimulation, the time course and reversibility of the effect of high oxygen level on ACh-mediated relaxation were investigated at pressure after 30 min of incubation with 100 U/ml PEG-SOD and 3,000 U/ml PEG-catalase (n = 4). At stable precontraction with 0.25–0.4 μM norepinephrine, relaxation was induced by 10 μM ACh. After 5 min, the gas mixture was changed to HBO. When oxygen concentration in the organ bath had equilibrated, the gas mixture was restored to hyperbaric control (32.6% O2-1.8% CO2-65.6% N2).

Sodium nitroprusside concentration-response studies. The potency of sodium nitroprusside (SNP) at pressure was investigated to test the sensitivity of aortic smooth muscle cells to NO. The gas mixture in the organ bath was changed to HBO (n = 6) or to hyperbaric control (sham, n = 6), and the rings were stimulated with the concentration of norepinephrine that produced a precontraction level of ~60–80% of maximal norepinephrine (5 μM) contraction. Stimulation with 3 × 10^{-10}–10^{-8} M SNP was started when precontraction had stabilized.

Norepinephrine concentration-response studies. Reactivity to a vasoconstrictor was tested in aortic rings incubated for 30 min with 100 U/ml PEG-SOD and 3,000 U/ml PEG-catalase. Control experiments were carried out at atmospheric pressure. In the group at 2.8 ATA (n = 6), the bubbling gas mixture in the organ bath was changed to HBO, and stimulation with 10^{-10}–10^{-8} M norepinephrine was started 20 min later. In the normobaric control group (n = 6), the bubbling gas mixture in the organ bath was changed from/to 95% O2-5% CO2 (sham), and norepinephrine stimulation was started 20 min later. Similar experiments were carried out after 30 min of incubation with 0.3 mM L-NNa or with endothelium-denuded aortic rings.

Contractile and Vasodilatory Responses After In Vivo Hyperbaric Exposures

From each animal, two rings were tested, one of which was denuded of endothelium after initial testing of contractile function. Concentration-response relations produced by 10^{-8}–10^{-5} M ACh, 10^{-10}–10^{-8} M norepinephrine, and 10^{-8}–10^{-5} M SNP, in this order were determined in rings from rats killed 0 h (n = 6) or 17 h (n = 6) h after HBO exposure and from untreated control rats (n = 6). Each concentration-response experiment was followed by a washout and a ≥30-min rest period before the next stimulation. ACh and SNP concentration-response relations were determined in rings precontracted to a stable value with 0.1 μM norepinephrine.

Validation experiments. To assess whether ACh-induced relaxations are mediated by NO, endothelium-intact aortic rings from five untreated rats were precontracted with 0.03–0.1 μM norepinephrine with or without prior incubation for 30 min with 0.3 mM L-NNa and then stimulated with 10 μM ACh.

Effect of In Vivo HBO on eNOS Activity

NOS enzymatic activity was measured in samples of endothelial cells from rats killed 0 h (n = 6) and 17 h (n = 6) after HBO exposure and from untreated control rats (n = 6). NOS activity was determined by a modification of a method previously described (14). Conversion of L-[3H]arginine to L-[3H]citrulline was determined in triplicate for each sample. Ten microliters of the cell homogenate were incubated in a total volume of 150 μl of 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, 1.25 mM CaCl2, 1 μM calmodulin, 1 μM FAD, 1 μM FMN, 1 mM NADPH, 15 μM 6R-tetrahydrobiopterin, and 30 μM (~10 μg/ml) L-[3H]arginine (Amersham Pharmacia Biotech, Buckinghamshire, UK). The reaction was started by addition of NADPH, continued for 60 min at 37°C, and terminated by addition of 1 ml of ice-cold stop buffer: 100 mM HEPES buffer (pH 5.5) containing 10 mM EGTA. For separation of L-[3H]citrulline, the samples were applied to the columns with 0.5 ml of preequilibrated Dowex 50WX8 (sodium form). L-[3H]citrulline content was quantified by counting in a liquid scintillation counter (model LS 1801, Beckman). Protein concentration in 20 μl of cell homogenate was determined by duplicate spectrophotometry (Lambda Bio, Perkin-Elmer) at 224 and 236.5 nm, with serum albumin as a standard. NOS enzymatic activity was then calculated and expressed in picomoles per minute per milligram of protein.

Determination of eNOS Activity at Different Oxygen Levels

NOS enzymatic activity was measured in a pool of endothelial samples from 28 rats. The assay was carried out in a custom-built Plexiglas box at different PO2, including a hyperbaric level. Through-hull fittings allowed for the oxygen sensor (model OX-100, Unisense) in the box, which was ventilated with gases of different oxygen (in nitrogen) fractions or with pure oxygen for 45 min before initiation of an assay. A constant oxygen fraction in the box was confirmed throughout the experiments by measurement of oxygen in the exhaust gas with an oxygen monitor (model OM 871, Dameco). PO2 in the incubation medium was measured in a control sample before and during each run. The NOS assay was performed four times at each oxygen level. Average values of NOS activity and oxygen were used for determination of the Km for oxygen. Conversion of L-[3H]arginine to L-[3H]citrulline was determined in triplicate for each assay of 30 μl of the cell homogenate for 20 min. During two of the assays with HBO, SOD (13.3 U/ml) was added to the incubation medium in additional samples to test for a possible effect of superoxide on NOS activity. These measurements were not included in the Km calculations.

Immunocytochemistry

Aortic intimal scrapings from each of four rats were spun onto four glass slides, incubated with rabbit anti-human von Willebrand factor (catalog no. A0082, Dako), and visualized and photographed with a Nikon Eclipse E 600 microscope at a magnification of ×100. Four negative control preparations were incubated without the primary antibody.

Western Blotting

For determination of the NOS isoforms in the endothelial samples, four mixed pools were tested: a sample used for study of the kinetic properties of eNOS, samples from rats killed 0 h and 17 h after HBO exposure, and a pooled sample from untreated control rats. Briefly, equal amounts of diluted and boiled endothelial cell homogenate were electrophoresed onto polyvinylidene difluoride membranes (Bio-Rad). The blots were incubated with anti-eNOS antibody (1:2,500 dilution; catalog no. N30020, Transduction Laboratories), anti-iNOS antibody
(1:10,000 dilution; catalog no. N39120, Transduction Laboratories), and anti-nNOS antibody (1:2,500 dilution; catalog no. N41520, Transduction Laboratories), visualized via chemiluminescence, and radioassayed at various exposure times. Human endothelial lysate (eNOS), rat pituitary lysate (nNOS), and mouse macrophage lysate (iNOS; all from Transduction Laboratories) were used as positive controls.

Data Analysis

The contractile responses are expressed as percent contraction relative to the maximal norepinephrine (5 μM) contraction obtained in the beginning of each experiment. However, in the series testing responses after in vivo hyperbaric exposures, the contractile responses to norepinephrine are expressed as increases in force from basal levels. Dilator responses are expressed as percent relaxation relative to the preconstriction level. However, responses from the experiment testing the onset time and reversibility of the effect of HBO on ACh relaxation are expressed as changes in force.

The concentration-response curves were fitted to the Hill equation (variable slope) using GraphPad Prism (GraphPad Software, San Diego, CA), and the pD2 = −log (EC50) values were calculated for every ring individually, where EC50 is the concentration (M) of drug required to give half-maximal contraction or relaxation and pD2 is logarithm of the half-maximal effective dose. The NOS activity measurements were fitted to the Michaelis-Menten equation using GraphPad Prism.

Values are means ± SE, and the differences between groups were analyzed by unpaired and, when appropriate, paired Student’s t-test. In the case of unequal variance, the difference between groups was analyzed by the Mann-Whitney test. The level of significance was taken as P < 0.05.

RESULTS

Contractile and Vasodilatory Responses During Hyperbaric Exposures

Effect of increasing oxygen on resting aortic rings. PO2 in the organ bath was in equilibrium within 7 min after a change in the bubbling gas mixture at pressure to HBO, and the rise in PO2 was paralleled by a small but highly significant contractile response in the endothelium-intact resting aortic rings (Figs. 1 and 2). A similar contractile response was observed in endothelium-denuded rings but absent in endothelium-intact rings that had been incubated with PEG-SOD and PEG-catalase (Fig. 2), indicating that the contractile effect was endothelium independent and mediated by superoxide. Incubation with l-NNA significantly decreased the contractile response to HBO, indicating that the response was NO dependent (Fig. 2).

ACh stimulation studies. In all concentration-response experiments, there was no significant difference in level of preconstriction between HBO and control groups (data not shown).

When the oxygen level was changed before the rings were precontracted, the potency of ACh was significantly reduced during HBO compared with hyperbaric control exposure, but there was no difference in the maximal response between the groups (Table 1). In rings incubated with PEG-SOD and PEG-catalase, the potency was unaltered, but the maximal ACh relaxation was significantly lower during HBO than hyperbaric control exposure (Table 1). Average precontraction level was 70% (without PEG-SOD and PEG-catalase) and 60% (with PEG-SOD and PEG-catalase) of the maximal norepinephrine (5 μM) contraction. The first ACh dose was added 25–32 min after the gas mixture was changed to HBO/control.

When the oxygen level was changed after the rings were precontracted, the potency of ACh could not be established in the aortic rings without addition of PEG-SOD, because the last accumulated concentration of ACh was too low to ensure that the maximal relaxation had occurred (Fig. 3). However, the maximal response was significantly lower during HBO than hyperbaric control exposure (Table 1). In rings incubated with PEG-SOD, the potency of ACh was significantly reduced during HBO compared with hyperbaric control exposure, but there was no difference in maximal relaxation between the groups (Table 1). The effect of ACh was potentiated when PEG-SOD was added (Fig. 3) during HBO and hyperbaric control exposure. The average preconstriction level was 93% (without PEG-SOD) and 86% (with PEG-SOD) of the maximal norepinephrine (5 μM) contraction.

In the studies testing the time course and reversibility of the effect of HBO on ACh-mediated relaxation, precontracted rings incubated with PEG-SOD and PEG-catalase were stimulated with ACh and the bubbling gas mixture was changed to HBO. The resulting increase in PO2 was paralleled by an increase in isometric force (Fig. 4). Restoration of the gas mixture to hyperbaric control caused a decrease in isometric
tension, indicating that the HBO-induced hyporeactivity to ACh is immediate and reversible.

**SNP concentration-response studies.** The first SNP dose was added 25–32 min after the gas mixture was changed to HBO/ control. The responses were concentration dependent (Fig. 5). The potency and the maximal response (Table 1) of the HBO group were not significantly different from those of the hyperbaric control group. Therefore, there was no indication that HBO exposure influenced the sensitivity to NO in aortic smooth muscle cells.

**Table 1. Potency and maximal response (E) to ACh, norepinephrine, and SNP in aortic rings exposed to HBO and ~680 mmHg PO**

<table>
<thead>
<tr>
<th></th>
<th>pD2</th>
<th>Emax, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBO</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>HBO</td>
<td>Control</td>
</tr>
<tr>
<td><strong>ACh</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>7.38 ± 0.08*</td>
<td>7.66 ± 0.08</td>
</tr>
<tr>
<td>BP, SOD, CAT</td>
<td>7.60 ± 0.09</td>
<td>7.92 ± 0.14</td>
</tr>
<tr>
<td>AP</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>AP, SOD</td>
<td>6.74 ± 0.17*</td>
<td>7.01 ± 0.13</td>
</tr>
<tr>
<td><strong>SNP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>8.78 ± 0.15</td>
<td>8.78 ± 0.15</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD, CAT</td>
<td>7.73 ± 0.10</td>
<td>7.73 ± 0.06</td>
</tr>
<tr>
<td>SOD, CAT, l-NNA</td>
<td>8.41 ± 0.14</td>
<td>8.30 ± 0.18</td>
</tr>
<tr>
<td>SOD, CAT, E</td>
<td>8.47 ± 0.11</td>
<td>8.42 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6–8). BP, shift to HBO/sham before precontraction; AP, shift to HBO/sham after precontraction; SOD, incubation with 100 U/ml polyethylene glycol (PEG)-SOD; CAT, incubation with 3,000 U/ml PEG-catalase; l-NNA, incubation with 0.3 mM N-nitro-l-arginine (l-NNA); E - without endothelium; pD2, potency (logarithm of half-maximal dose); Emax, maximal response; N/A, not applicable. *P < 0.05; †P < 0.005 vs. control.

**Norepinephrine concentration-response studies.** Responses to norepinephrine obtained with HBO and normobaric control in the presence of PEG-SOD and PEG-catalase were concentration dependent (Fig. 6). The potency (Table 1) in endothelium-intact rings in the HBO group was not significantly different from that in the normobaric control group. Also, incubation for 30 min with 0.3 mM l-NNA or use of endothelium-denuded aortic rings did not result in differences in potency between exposure groups. However, the maximal response (Table 1) was significantly reduced in the HBO group in endothelium-intact and endothelium-denuded rings but not in endothelium-intact rings incubated for 30 min with 0.3 mM norepinephrine.
The finding that the hyporeactivity to high concentrations of norepinephrine was abolished by an NOS inhibitor, but not by endothelium denudation, suggests that the effect was due, at least in part, to nonendothelial NO production.

**Determination of eNOS Activity at Different Oxygen Levels**

NOS activity was measured at oxygen substrate levels of 13–2,130 mmHg (corresponding to oxygen concentrations of 21.4 –3,503.3 /H9262 M; Fig. 7). Changing oxygen from 746 to 2,130 mmHg did not significantly increase NOS activity, and there was no evidence of substrate inhibition. Addition of SOD (13.3 U/ml) to the incubation medium at 2,130 mmHg oxygen did not change NOS activity (data not shown). Fitting the results to the Michaelis-Menten equation by nonlinear regression gave a $K_m$ value for oxygen of 31.8 /H11006 6.5 mmHg (corresponding to 52.3 /H11006 10.6 M).

**Contractile and Vasodilatory Responses After In Vivo Hyperbaric Exposures**

Potency and maximal responses to ACh, norepinephrine, and SNP are given in Table 2. Responses to ACh were not significantly different between rings from rats killed 0 and 17 h after HBO exposure and those from untreated control rats ($P$ values are given in Table 2).

The potency of SNP was significantly increased in the endothelium-denuded rings from rats killed 17 h after HBO exposure compared with rings from untreated control rats ($P = 0.08$ and 0.06, respectively).

**Validation experiments.** Maximal relaxation to ACh without addition of L-NNA was 91 ± 2%, whereas it was 4 ± 2% after HBO exposure compared with rings from untreated control rats ($P = 0.08$ and 0.06, respectively).

Fig. 5. Concentration-response curves to sodium nitroprusside (SNP) of aortic rings at 2,040 mmHg (HBO) and 680 mmHg oxygen (control) at 2.8 ATA. Bubbling gas mixture in organ bath was changed from 32.6% O$_2$-1.8% CO$_2$-65.6% N$_2$ to 98.2% O$_2$-1.8% CO$_2$ or sham changed to 32.6% O$_2$-1.8% CO$_2$-65.6% N$_2$, and rings were stimulated with epinephrine concentration that produces a precontraction level of 60–80% of maximal norepinephrine (5 μM) contraction obtained at beginning of each experiment. SNP stimulation was started when precontraction had stabilized. There was no significant difference in level of precontraction between groups (data not shown). Dilator responses are expressed as percent relaxation relative to preconstriction level. Values are means ± SE ($n = 6$).

L-NNA. The finding that the hyporeactivity to high concentrations of norepinephrine was abolished by an NOS inhibitor, but not by endothelium denudation, suggests that the effect was due, at least in part, to nonendothelial NO production.

Fig. 6. A: norepinephrine concentration-response curves of aortic rings incubated with 100 U/ml PEG-SOD and 3,000 U/ml PEG-catalase at 2,040 mmHg (HBO) and 680 mmHg oxygen (control). In HBO group, bubbling gas mixture in organ bath was changed from 32.6% O$_2$-1.8% CO$_2$-65.6% N$_2$ to 98.2% O$_2$-1.8% CO$_2$, and rings were stimulated with norepinephrine 20 min later. In normobaric control group, bubbling gas mixture in organ bath was sham changed from/to 95% O$_2$-5% CO$_2$, and rings were stimulated with norepinephrine 20 min later. Contractile responses are expressed as described in Fig. 1 legend. Experiment was also performed with aortic rings incubated with 0.3 mM L-NNA (B) and with endothelium-denuded aortic rings (C). Values are means ± SE ($n = 6$).
Potency and maximal response to ACh, norepinephrine, and SNP in aortic rings from rats killed 0 and 17 h after in vivo HBO exposure and from untreated control rats

Table 2. Potency and maximal response to ACh, norepinephrine, and SNP in aortic rings from rats killed 0 and 17 h after in vivo HBO exposure and from untreated control rats

<table>
<thead>
<tr>
<th></th>
<th>pD2</th>
<th>Emax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated 0 h 17 h</td>
<td>Untreated 0 h 17 h</td>
</tr>
<tr>
<td>ACh E⁺</td>
<td>6.92±0.07</td>
<td>6.70±0.09</td>
</tr>
<tr>
<td></td>
<td>(P=0.39)</td>
<td>(P=0.08)</td>
</tr>
<tr>
<td>E⁻</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Norepinephrine E⁺</td>
<td>8.23±0.13</td>
<td>8.20±0.07</td>
</tr>
<tr>
<td></td>
<td>(P=0.94)</td>
<td>(P=0.82)</td>
</tr>
<tr>
<td>E⁻</td>
<td>8.43±0.08</td>
<td>8.39±0.07</td>
</tr>
<tr>
<td></td>
<td>(P=0.55)</td>
<td>(P=0.81)</td>
</tr>
<tr>
<td>SNP E⁺</td>
<td>7.80±0.08</td>
<td>8.04±0.08</td>
</tr>
<tr>
<td></td>
<td>(P=0.47)</td>
<td>(P=0.06)</td>
</tr>
<tr>
<td>E⁻</td>
<td>8.11±0.05</td>
<td>8.36±0.05*</td>
</tr>
<tr>
<td></td>
<td>(P=0.23)</td>
<td>(P=0.04)</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6). E⁺, with endothelium; E⁻, without endothelium; SNP, sodium nitroprusside. P values are for comparison with untreated.

30 min of incubation with 0.3 mM l-NAME (P < 0.0001), indicating that ACh-induced relaxations were mediated almost entirely by NO.

Effect of In Vivo HBO on eNOS Activity

NOS activity in endothelial samples from rats killed at 0 and 17 h (112 ± 11 and 103 ± 14 pmol·min⁻¹·mg protein⁻¹, respectively) after HBO exposure was not significantly different from that in endothelial samples from untreated control rats (110 ± 12 pmol·min⁻¹·mg protein⁻¹, P = 0.93 for 0 h compared with untreated and P = 0.71 for 17 h compared with untreated).

Immunocytochemistry

Identification of endothelial cells in aortic intimal scapings was based on immunocytochemical characterization using an antibody against von Willebrand factor, which is a carrier protein for coagulation factor VIII. Exact counting of cells in the cytospin preparations was not possible because of insufficient cell separation. However, randomly selected areas showed that virtually all the cells were positively stained, indicating that they originated in the endothelium (not shown). In control preparations where the primary antibody was omitted, no positive reaction product was observed. The results clearly indicate that the endothelial cell-harvesting method (aortic intimal scraping) is selective for endothelial cells.

Western Blotting

Western analysis of pooled aortic endothelial cell homogenates from rats killed at 0 and 17 h after in vivo HBO exposure and from untreated control rats. A single band of endothelial NOS (eNOS) protein (140 kDa) was detectable in all pools; no bands for inducible NOS (iNOS) and neuronal NOS (nNOS) could be detected in any of the three pools. Lysates of human endothelial cells, mouse macrophages, and rat pituitary tumor were used as positive (Pos) controls for eNOS, iNOS, and nNOS, respectively.

Fig. 7. Effect of PO₂ on nitric oxide synthase (NOS) activity in a pooled sample of aortic endothelial cell homogenate. PO₂ in incubation medium was measured in a control sample during each run. NOS assay was performed 4 times at each oxygen level. Average values of NOS activity and oxygen are plotted. Solid line, best fit of Michaelis-Menten equation.

Fig. 8. Western analysis of pooled aortic endothelial cell homogenates from rats killed at 0 and 17 h after in vivo HBO exposure and from untreated control rats.

AJP-Heart Circ Physiol • VOL 291 • OCTOBER 2006 • www.ajpheart.org
properties of eNOS also revealed a single band of eNOS protein (not shown). No band was detectable for iNOS. These findings indicate that eNOS is the isoform responsible for the measured NOS activity.

**DISCUSSION**

The main findings of the present study are as follows: 1) HBO induces contraction in resting aortic rings with and without endothelium. These contractions are abolished by the combination of PEG-SOD and PEG-catalase or reduced by l-NNA, an NOS inhibitor. 2) HBO decreases ACh relaxation, and the effect is not abolished by PEG-SOD or PEG-catalase. Addition of PEG-SOD increases ACh relaxation in control and HBO-exposed tissue, suggesting that the HBO-induced inhibition of ACh relaxation cannot be ascribed to increased superoxide formation. 3) HBO decreases maximal norepinephrine contraction, an effect that is reversed by l-NNA, but not by endothelial cell removal.

**HBO-Induced Contraction**

In our experiment, HBO caused an immediate but small contractile response in resting aortic rings, apparently due to superoxide-induced inactivation of nonendothelial NO, because PEG-SOD + PEG-catalase, as well as l-NNA, inhibited the contractions. However, we did not actually show scavenging of superoxide and hydrogen peroxide by PEG-SOD and PEG-catalase; therefore, we cannot rule out that other PEG-SOD/PEG-catalase-mediated effects may be involved.

Atochin et al. (2) and Demchenko and co-workers (9) found that in vivo HBO exposure to 5.0 ATA induces an early cerebral vasoconstriction due to superoxide-mediated inactivation of eNOS-derived NO. Their findings are, at first glance, different from our results. We found an HBO-induced contraction in endothelium-denuded aortic rings, whereas they found no significant cerebral vasoconstriction in eNOS-knockout mice during HBO. They did, however, report a reduced sensitivity to NO in the eNOS-knockout mice. Thus it is possible that a similar reduction in sensitivity to changes in NO could explain why these animals did not show significant cerebral vasoconstriction during HBO exposure. Another explanation could be that the response is not present in mice cerebral vessels. One of their main findings was a significant cerebral vasoconstriction in nNOS-knockout mice during HBO exposure. In our study, the degree of contraction in response to HBO was the same in rings with and without endothelium, suggesting that inactivation of endothelial NO did not play a role. This is, however, not surprising. Basal endothelial NO production in passively stretched resting aortic rings is believed to be negligible (15). Therefore, an HBO-induced contractile response as a result of scavenging of endothelial NO by superoxide is not to be expected in our conditions with resting aortic rings. Our results, on the other hand, suggest a nonendothelial vascular NO production during HBO exposure (see below).

The HBO-induced endothelium-independent contractile response was small, and the significance of the finding for the regulation of blood flow during in vivo HBO exposure depends on whether the same effect is also present in smaller arteries and arterioles.

**ACh Relaxation and eNOS Activity During HBO Exposure**

We have demonstrated that ACh-induced relaxation in rat aortic rings decreases during HBO exposure, even in the presence of PEG-SOD and PEG-SOD + PEG-catalase. This could be due to the inability of SOD to enter subendothelial layers of the aortic rings, but PEG-conjugated enzymes have been shown to be more likely than unconjugated enzymes to enter the vascular wall (3). Therefore, the data suggest that endothelium-derived NO bioavailability decreases during HBO exposure because of a mechanism independent of inactivation or reduced production/release by superoxide and hydrogen peroxide. We also showed that the effect is immediate and reversible.

In aqueous solution, NO reacts with oxygen, resulting in the formation of nitrite (16). The rate law for NO autooxidation in aqueous solution is second order with respect to NO and first order with respect to molecular oxygen, with a rate constant of \(-8 \times 10^9 \text{ M}^{-2}\text{s}^{-1}\) (16). It is therefore possible that an increase in P02 from 680 to 2,040 mmHg might shorten the lifetime and, hence, decrease the bioavailability of NO. The finding in our study that the effect of HBO exposure on ACh-induced relaxation is immediate and reversible is consistent with inactivation of NO by molecular oxygen. A low oxygen concentration has previously been found to enhance NO-dependent relaxation in endothelium-denuded aorta of the rat, suggesting a role of NO autooxidation (39), although a possible effect of superoxide was not tested. In vivo exposure to HBO at 5.0 ATA has been shown to reduce cerebral blood flow in rats, and the effect was completely abolished by prior administration of intravascular SOD (25 U/g iv) (10). In that study, the reduction in cerebral blood flow was paralleled by a reduction in brain interstitial nitrite/nitrate concentration. It has also indirectly been found that peroxynitrite increases in mouse brain during in vivo HBO exposure to 5.0 ATA and that the response is inhibited by pretreatment with an NOS inhibitor (11). These findings indicate inactivation of NO by superoxide but are inconsistent with autooxidation of NO as a mechanism behind cerebral vasoconstriction during HBO. However, several studies have demonstrated an increase in rat brain nitrite/nitrate concentration during HBO exposure to 5.0 ATA (18, 35) or 3.0 ATA (13). In addition, it has been proposed that autooxidation is of physiological importance when the concentration of oxygen greatly exceeds (by >6 orders of magnitude) that of superoxide. Taken together, it is likely that inactivation of NO by molecular oxygen and superoxide takes place during HBO exposure and that the relative contribution of each mechanism depends on oxygen concentration, superoxide generation, endogenous SOD activity, and NO production, factors that may differ according to species, tissue/organ, and type of vessel. Hence, inactivation of NO by molecular oxygen may play a role in the regulation of blood flow during in vivo HBO exposure. Studies have shown that exposure to HBO causes a general vasoconstriction and blood flow reduction in various organs in rats and humans (1, 4, 19, 27, 30, 44), and it is tempting to speculate that autooxidation of NO may be involved.

Another, but less likely, explanation for the hyporeactivity to ACh in the present study may be that the activity of eNOS decreases in an immediate and reversible manner. The activity of eNOS is controlled by a complex combination of factors,
such as calmodulin binding, phosphorylation/dephosphorylation, and protein interactions (32). When we determined eNOS activity in endothelial cell homogenate at different oxygen levels, we did not find a negative change in activity during HBO exposure compared with normobaric hyperoxia, suggesting no alterations in the function of enzyme or cofactors. However, it cannot be excluded that negative modulation of eNOS activity by phosphorylation/dephosphorylation, calcium-calmodulin-mediated processes, or protein interaction plays a role in the intact endothelial cell during HBO exposure.

Previous in vivo studies have shown that HBO exposure induces cerebral vasodilation and stimulates NO synthesis through increased nNOS activity (40, 41). It has also been found, however, that late-HBO-induced vasodilation depends on eNOS- and nNOS-derived NO (2, 9). The reason for the discrepancy between our results and the in vivo studies suggesting a role of eNOS in the late cerebral vasodilation during HBO exposure at 5.0 ATA could be the different HBO profile (i.e., pressure and exposure time) or the different vessels and species used in our study.

The $K_m$ value determined in the present study indicates that oxygen regulation of eNOS activity in the normal systemic arterial circulation is not an important determinant of NO production within the range of arterial $P_O_2$ during normobaric hyperoxia and HBO exposure. However, in vascular beds with $<50$ mmHg $P_O_2$, such as the pulmonary arterial circulation, or during arterial ischemia, HBO exposure may increase $P_O_2$ to a level at which eNOS activity and NO production are significantly increased. The same could be the case in the venous circulation, although this seems less likely, because NOS in venous endothelium is quiescent under basal conditions (43).

Our $K_m$ value is consistent with the NO-dependent increase in fetal pulmonary blood flow when pulmonary arterial $P_O_2$ was increased from 25 to 55 mmHg (42) and with the decrease in NO synthesis during hypoxia (mean $P_O_2$ in perfuse of 25 mmHg) in isolated perfused rabbit lung (22). However, it does not explain the decreased ACh-induced relaxations we observed during HBO exposure, suggesting a more complex relation between oxygen concentration and eNOS-derived NO in the intact cellular environment during HBO exposure.

**Norepinephrine Contraction During HBO Exposure**

We observed a hyporeactivity to high concentrations of norepinephrine during HBO exposure in aortic rings incubated with PEG-SOD and PEG-catalase. This hyporeactivity was abolished by the NOS inhibitor L-NNA, but not by endothelial denudation, indicating an activation of nonendothelial NO production during HBO exposure.

It is possible that norepinephrine activates $\beta_2$-adrenergic receptors on nNOS-containing perivascular nerves, resulting in the release of NO (37) during HBO exposure. However, because the experiments were carried out in the presence of 1 $\mu$M propranolol to eliminate any effect of norepinephrine on $\beta$-adrenoceptors, another more likely explanation may exist. Cheah et al. (8) reported an electrical field stimulation/Ni$^{2+}$/Mg$^{2+}$-induced vasorelaxation in precontracted endothelium-denuded rat aortic rings and vasorelaxation mediated through activation of nNOS in the vascular smooth muscle cells. Data from other studies also indicate the existence of nNOS in vascular smooth muscle cells (6, 12). Therefore, it is tempting to speculate that the HBO-induced NO-dependent hyporeactivity to norepinephrine in our study is due to synthesis of nNOS-derived NO in vascular smooth muscle cells. nNOS has also been found to be abundant in rat arterial interstitial mast cells, representing another possible nonendothelial source of vascular NO production (23). The assumption that the norepinephrine hyporeactivity during HBO exposure in our study is due to stimulation of nNOS in vascular smooth muscle cells and/or interstitial mast cells is consistent with previous in vivo findings in rats (41). However, further investigations are required to explore this issue.

It is not likely that the norepinephrine hyporeactivity during HBO exposure is due to iNOS-derived NO, because iNOS, once synthesized, is permanently active (26) and, thus, would be expected to produce NO also at smaller concentrations of norepinephrine/low levels of contraction. Also, the duration of HBO exposure in our experiments was well below 3 h, which is considered necessary for induction of iNOS activity by gene transcription and posttranscriptional alterations (29).

**Vasomotor Responses and eNOS Activity After In Vivo HBO Exposure**

In our study, endothelium-intact and endothelium-denuded aortic rings from rats killed 0 and 17 h after in vivo HBO exposure did not show significant changes in reactivity to ACh or norepinephrine compared with rings from untreated control rats. Nor did endothelial cell eNOS activity change in rats killed 0 or 17 h after exposure. These results support the findings that the changes in perivascular and vascular NO activity and NO bioavailability during HBO (41, present study) occur only during, and not after, exposure. However, our findings are inconsistent with the reported increase in eNOS protein in human umbilical vein endothelial cells 8–24 h after in vitro HBO exposure at 2.5 ATA for 90 min (7) and the reported increase in eNOS mRNA and protein in bovine (25) and fetal ovine (5) endothelial cells after in vitro exposure for 24 h at 95% normobaric oxygen. However, in our study, we focused on eNOS-functional activity and NO release, and an increase in total eNOS protein and/or mRNA is possible without a parallel increase in enzymatic activity (17, 24), which could explain the inconsistency. Rat aortic ring ACh-induced relaxation has previously been found to be abolished after 6 h of in vivo normobaric (28) or hyperbaric (33) oxygen exposure. A unifying explanation encompassing the different findings could be that damage or impairment of endothelial function and eNOS mRNA/protein occurs during prolonged hyperoxic exposures, which may lead to compensatory eNOS induction without a parallel increase in total eNOS activity.

Some investigators have shown an increase in rat aortic ring ACh-induced relaxation after 1 h of in vivo exposure to >95% normobaric oxygen, and they found a parallel increase in relaxation to SNP (38). In our study, we found a similar increase in the potency of SNP in aortic rings from rats killed 17 h after HBO exposure, and it is possible that the response is caused by increased sensitivity of guanylate cyclase to activation by NO.

The present findings may contribute to an understanding of the basic physiological effects of hyperoxia and clinical HBO therapy. However, responses of rat and human endothelial cells to HBO exposure might not be comparable, although rat and
human circulatory physiology are very similar. Also, extrapolation of our findings in vitro to the physiological responses in vivo should be made with some caution, because additional factors that could not be assessed in our model, such as blood flow and shear stress (34), also influence NO synthesis and bioavailability.

In conclusion, we have demonstrated an immediate and reversible decrease in endothelial NO bioavailability during in vitro HBO exposure, possibly due to autoxidation of NO. In addition, we indirectly showed activation of nonendothelial vascular NO production during in vitro HBO exposure. We found no evidence of altered eNOS activity and/or vascular NO bioavailability after in vivo HBO exposure.

ACKNOWLEDGMENTS

The technical assistance of Jens Romlund, Kurt Juel Soerensen, Peter Engholm (deceased), and Yngve Damgaard and the help and advice of Joop Madsen, Martin Lauritzen, and Charly Garbarsch are gratefully acknowledged.

GRANTS

This work was supported by The Danish Armed Forces, Knud og Dagny Gad Andresens Fond and the AGA Medical Research Fund. A portion of this work was supported by National Institutes of Health Grant AT-00428.

REFERENCES


