Similar but not the same: normobaric and hyperbaric pulmonary oxygen toxicity, the role of nitric oxide

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Demchenko IT, Welty-Wolf KE, Allen BW, Piantadosi CA. Similar but not the same: normobaric and hyperbaric pulmonary oxygen toxicity, the role of nitric oxide. Am J Physiol Lung Cell Mol Physiol 293: L229–L238, 2007. First published April 6, 2007; doi:10.1152/ajplung.00450.2006.—Pulmonary manifestations of oxygen toxicity were studied and quantified in rats breathing >98% O2 at 1, 1.5, 2, 2.5, and 3 ATA to test our hypothesis that different patterns of pulmonary injury would emerge, reflecting a role for central nervous system (CNS) excitation by hyperbaric oxygen. At 1.5 atmosphere absolute (ATA) and below, the well-recognized pattern of diffuse pulmonary damage developed slowly with an extensive inflammatory response and destruction of the alveolar-capillary barrier leading to edema, impaired gas exchange, respiratory failure, and death; the severity of these effects increased with time over the 56-h period of observation. At higher inspired O2 pressures, 2–3 ATA, pulmonary injury was greatly accelerated but less inflammatory in character, and events in the brain were a prelude to a distinct lung pathology. The CNS-mediated component of this lung injury could be attenuated by selective inhibition of neuronal nitric oxide synthase (nNOS) or by unilateral transection of the vagus nerve. We propose that extrapulmonary, neurogenic events predominate in the pathogenesis of acute pulmonary oxygen toxicity in hyperbaric oxygenation, as nNOS activity drives lung injury by modulating the output of central autonomic pathways.

Central nervous system oxygen toxicity; hyperbaric oxygen

The mammalian lung is a primary target of the toxic effects of oxygen, however, at high oxygen partial pressures, acute manifestations of toxicity appear in the brain, including electrical discharges on the electroencephalogram, resulting in muscular jerks and convulsions. The central nervous system (CNS) effects appear relatively rapidly when inspired oxygen exceeds 2.0 atmosphere absolute (ATA), as reviewed by Balentine in 1982 (2) and Clark and Thom in 2003 (11). In normobaric conditions, direct pulmonary injury predominates, and relatively long exposures at inspired oxygen pressures ranging from 0.6 to 1.0 ATA cause diffuse damage to both alveolar epithelium and pulmonary capillary endothelium, resulting in interstitial and intra-alveolar edema, impaired gas exchange, and extensive infiltration by inflammatory cells (2, 3, 10, 12, 60). For example, adult rats continuously exposed to 100% O2 at 1 ATA begin to die of respiratory failure within 72 h (13, 14).

Hyperbaric oxygen (HBO2) at 2–4 ATA accelerates the development of gross and histological changes in the lungs, including pulmonary edema, congestion, and intra-alveolar hemorrhage (11), and pulmonary injury is more pronounced in animals that also exhibit the seizures of CNS O2 toxicity (2, 3). The mechanisms for this neurogenic accentuation of hyperoxic lung injury are unknown.

In normobaric hyperoxia, pulmonary injury has been attributed to the formation of reactive oxygen species (ROS) that overwhelm antioxidant defenses and oxidize lipids, nucleic acids, and proteins in lung tissue (26, 32, 37). The lung is uniquely vulnerable because it presents to the atmosphere a delicate, extensive region, which is the major interface between the external environment and the interior physiological milieu, and it is the only organ through which the total cardiac output must pass.

Since 1991, work in our laboratory has led to the development of the paradigm that O2-dependent changes in the activity of nitric oxide (NO) in the brain alter susceptibility to CNS O2 toxicity (45, 61). This hypothesis has been supported by experimental data showing that inhibition of nitric oxide synthase (NOS) prevents or attenuates HBO2-induced seizures (8, 9, 19, 20, 45) and that an increase in cerebral NO production always precedes the onset of O2 convulsions (18, 20, 21, 28, 51). The observations that HBO2 stimulates NO production in the brain and also accelerates lung damage suggest the hypothesis that NO pathways contribute to the severity of hyperbaric pulmonary oxygen toxicity.

Among the many investigations of pulmonary injury due to normobaric and hyperbaric oxygen, no single study has evaluated the pathological effects of both regimes of hyperoxia, side-by-side and under uniform experimental conditions. Therefore, the work reported here was designed to compare lung injury induced by oxygen under normobaric conditions with that occurring in hyperbaric conditions and to examine the contribution of NO to the acute pulmonary toxicity of hyperbaric hyperoxia.

Materials and methods

Animals and O2 exposures. Adult male Sprague-Dawley rats (Charles River Laboratories) weighing 283 ± 11 g were used in an experimental protocol approved by the Duke University Institutional Animal Care and Use Committee.

For normobaric experiments, rats were placed in a sealed acrylic chamber and continuously exposed to O2 with free access to food and water. The O2 concentration was monitored continuously (model 570 O2 analyzer, Servomex) and kept at or above 98%. The gas flow through the chamber was ∼12 l/min, sufficient to allow complete gas exchange every 5 min and keep CO2 levels below that in room air (∼0.5%). The temperature in the chamber was maintained between 22–24°C, and relative humidity between 60–70%.

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Hyperbaric experiments were conducted either in a small hyperbaric chamber (Bethlehem, Bethlehem, PA) or in a glove-bag apparatus inside a large pressure chamber (Duke Center for Hyperbaric Medicine and Environmental Physiology). The inspired oxygen concentration was maintained >99%, and CO₂ was limited to 0.1%. The temperature in the chamber was maintained between 22–24°C with relative humidity ~60%. Animals were continuously observed, particularly for signs of CNS O₂ toxicity.

**Experimental design.** In the first stage of experimentation, five groups of animals (n = 10–12, each group) were exposed to O₂ at 1, 1.5, 2, 2.5, or 3 ATA, and survival curves and 50% mortality (LD₅₀) were calculated for each exposure. In a second series of experiments, rats were exposed to O₂ at the same five pressures for times corresponding to ~80% of the LD₅₀ to assess histological and biochemical lung injury and keep mortality below 5%. Two rats were exposed to hyperbaric normoxia (7% O₂, 93% N₂) at 3 ATA to assess edema. The catalytic activity of LDH in cell-free BALF was measured by the amount of l-lactate oxidized at 30°C, per unit time, from reduction of NAD⁺. Total protein content was measured with the bicinchoninic acid assay using bovine serum albumin as a standard (55).

**Lung edema assessment.** As an index of edema, wet-dry weight ratio was assessed in freshly excised lungs. The lungs were weighed immediately on tared foil squares to determine wet weight and then placed in a vacuum oven at 60°F for at least 96 h or until a stable dry weight was obtained. No corrections were made for intravascular lung water.

**BALF assay.** Total cell counts (cells per milliliter of BALF) were made with a hemocytometer (Neubauer). After centrifugation (3,500 g for 5 min at 4°C), lactate dehydrogenase (LDH) activity and total protein content were measured in the supernatant. Total protein includes both plasma proteins and intracellular proteins and indicates damage to the blood-air barrier. The catalytic activity of LDH in cell-free BALF was measured by the amount of l-lactate oxidized at 30°C, per unit time, from reduction of NAD⁺. Total protein content was measured with the bicinchoninic acid assay using bovine serum albumin as a standard (55).

**Measurement of NO and peroxynitrite in BALF.** The oxidation products of NO, nitrite and nitrate (NOₓ), were determined by fluorometric assay (43, 44). Briefly, to convert nitrate to nitrite, deproteinized BALF (200 μl) was incubated with 10 μl of nitrate reductase (5 U/ml, Sigma) in the presence of 10 μl of FAD and 10 μl of NADPH. Then, excess NADPH was oxidized with 2 U/ml LDH. The samples were reacted in the dark for 10 min with 25 μl of 2,3-diaminonaphthalene (DAN) at room temperature. The pH of the mixture was adjusted to 11.5–12 with 1 N NaOH, and fluorescence was measured using a 96-well plate fluorescence spectrophotometer (Safire; Teca, Durham, NC). Nitrite concentration in the sample was calculated from the linear region (0.02–3.2 μM) of a standard curve constructed using sodium nitrite standards (Sigma) diluted in 0.9% NaCl.

**Peroxynitrite (ONOO⁻) reacts with tyrosine residues to yield 3-nitrotyrosine (3-NT), a stable marker of NO oxidation products.** We measured free and protein-bound 3-NT in BALF with HPLC with electrochemical detection (15). Protein was precipitated from BALF with ethanol and then hydrolyzed with pepsin, after which 3-NT was assayed using HPLC with a Shimadzu solvent delivery system (model 580; ESA, Chelmsford, MA) and a reverse-phase C18 column (5-μm bead size; 4.6 × 150 mm; mobile crystalline material). Isocratic elution was performed using a mobile phase of 50 mM acetic acid, 50% sodium hydroxide, and 15% methanol (pH 4.7). For the dual-channel electrochemical detector (Coulochem II, ESA), the potential for channel A was poised at +600 mV to quantify tyrosine and aminotyrosine as well as remove compounds oxidized at lower potentials (54). Channel B, downstream of channel A, was set to +800 mV to detect 3-NT. This arrangement optimizes detection of 3-NT in samples containing significant amounts of tyrosine. The retention time for 3-NT was about 7 min longer than for tyrosine, allowing a 1,000-fold enhancement of sensitivity to 3-NT by programming a variable gain into the detector. A guard cell set to +850 mV was interposed between the solvent delivery system and injector to remove electrochemically active compounds from the mobile phase. The retention time for 3-NT was confirmed by using authentic standards and by addition of 10 mM sodium hydrosulfite (dithionite) to 3-NT, resulting in reduction to aminotyrosine and changes in retention time and electrochemical characteristics. Data were expressed as 3-NT content in 20 μl of sample.

**Assessment of CNS toxicity.** All experimental animals were continuously visually monitored throughout all hyperoxic exposures for evidence of CNS toxicity. Signs included restlessness, rigidity, excessive cleaning movements, salivation, shaking, deep breathing movements, and convulsions.
Arterial blood gases and pH. Samples of arterial blood were drawn from the abdominal aorta, or from a femoral artery, before lung lavage, and blood gases and pH were measured with a blood gas/pH analyzer (model 1304; Instrumentation Laboratory, Lexington, MA).

Vagotomy. In one group of rats (n = 13), the left vagus nerve was transected at the cervical level. Using sterile procedure, an incision was made in the neck, and the vagus was carefully exposed by blunt dissection. The nerve was cut in two places so that a 1-mm section could be removed, and the wound was closed and sutured. Each of these animals was allowed to recover for 1 wk before exposure to hyperoxia. From eight of these animals, BALF was withdrawn from both lungs. In the remaining five animals, the contralateral bronchus was clamped to permit selective BAL of only the ipsilateral lung.

Statistical analysis. All values are expressed as means ± SE unless otherwise stated. Statistical evaluations were performed with commercially available software (Statview, Calabasas, CA) using one-way ANOVA and Scheffe’s post hoc test. A P value of <0.05 was considered significant.

RESULTS

Animal survival in hyperoxia. Figure 1 shows survival curves for rats exposed to normobaric O2 (1 ATA) or hyperbaric hyperoxia (>99% O2 at 2.5, 2.0, 2.5, and 3.0 ATA). Survival rate vs. time was plotted for each exposure (n = 10–12, each group). Inset: as inspired Po2 increases, exposure time required for 50% mortality (LD50) decreases exponentially.

Fig. 1. Survival analysis. Age-matched, freely moving rats were exposed to normobaric hyperoxia (>98% O2 at 1 atmosphere absolute (ATA)) or hyperbaric hyperoxia (>99% O2 at 1.5, 2.0, 2.5, and 3.0 ATA). Survival rate vs. time was plotted for each exposure (n = 10–12, each group). Inset: as inspired Po2 increases, exposure time required for 50% mortality (LD50) decreases exponentially.

Fig. 2. Pathology. Representative electron micrographs of rat lungs are shown from 4 levels of hyperoxia (magnification ×6,800). A: in 1 ATA O2, lethal lung injury occurred between 60–80 h, characterized by damage to capillary endothelium and alveolar epithelium, an increase in inflammatory cells in the interstitium ("a"), as well as edema in the interstitium ("b") and in the alveoli ("c"). Note the separation of endothelium from basement membrane ("d") and the ruffling of type I alveolar cell membranes ("e"). B: in 1.5 ATA O2, patterns of injury to endothelial and epithelial cells are comparable with those seen at 1 ATA. Note interstitial edema at (*). C: in 2.0 ATA O2, lung injury is lethal at a mean time of 14 h. Again, epithelial cell membranes are ruffled, and capillary endothelium is lifted from underlying basement membrane. Unlike exposures at 1 or 1.5 ATA, however, neither interstitial nor alveolar edema is prominent here. Clumping and margination now appear in endothelial cell nuclear chromatin (*). D: in 2.5 ATA O2, central nervous system toxicity was manifested in this animal, which resulted in more severe endothelial and epithelial injury, with some areas of alveolar septae almost completely denuded of type I epithelium (*). There is no infiltration of inflammatory cells, but endothelial cells are markedly swollen, and capillary lumina are packed with red blood cells to a greater extent than seen at the other exposures.
baric O₂ (1.5, 2, 2.5, and 3 ATA). The LD₅₀ decreased exponentially as inspired PO₂ increased, approximating the curve LD₅₀ = 60 (PO₂)⁻¹.⁹, R² = 0.975 (Fig. 1, inset). In normobaric hyperoxia, LD₅₀ occurred at ~69 h. In hyperbaric hyperoxia, LD₅₀ dropped to 24.2 h at 1.5 ATA, 14.2 h at 2.0 ATA, 11.2 h at 2.5 ATA, and 8.3 h at 3.0 ATA. Shorter exposure times (~80% of LD₅₀) were chosen for the remaining experiments: 56 h for 1 ATA, 20 h for 1.5 ATA, 10 h for 2 ATA, 8 h for 2.5, and 6 h for 3 ATA.

Animal behavior in hyperoxia. All rats exposed to 1 ATA O₂ for 56 h survived with no sign of seizures. Similarly, rats exposed to O₂ at 1.5 ATA for 20 h or at 2 ATA for 10 h showed motor impairment but no excitability or seizures. At 2.5 ATA, 2 of 14 (14%) animals convulsed during the 8-h exposure, and at 3 ATA, 6 of 16 animals (37%) did so between 4–6 h. Most rats exposed to O₂ at 3 ATA exhibited spasticity, rigidity, and kangaroo-like posturing, and two animals showed forelimb paresis.

Gross and microscopic pathology of the lung. After 56 h at 1 ATA O₂, lungs were grossly edematous and congested, and diffuse pulmonary hemorrhage was observed in some animals. Light microscopy disclosed accumulations of inflammatory cells in interstitial and alveolar spaces. Qualitative surveys of the electron micrographs typically revealed damage to capillary endothelium and alveolar epithelium, an increase in interstitial cells, and interstitial and alveolar edema (Fig. 2A). Endothelial injury was the most striking feature, with pyknotic nuclei and swelling of perinuclear cisternae, rough endoplasmatic reticula, and mitochondria. There was localized destruction of endothelial cell membranes, and some cells were lifted off the basement membrane. Capillary lumina were packed with red blood cells and platelets. Damage to epithelial cells was less prominent, consisting of a ruffling of type I cell membranes and a blunting of type II cell microvilli. Although damage was considerable, a few areas appeared to be relatively normal.

For rats exposed to 1.5 ATA O₂ for 20 h, changes seen on the electron micrographs were similar to those seen at 1 ATA with only quantitative differences. Thus endothelial cell separation from the basement membrane tended to be more widespread, there was more edema in type I epithelium (Fig. 2B), but far fewer inflammatory cells had accumulated within the interstitium. It was after exposure to higher pressures that qualitative differences began to appear. Therefore, following 10 h at 2.0 ATA O₂, nuclei of endothelial cells were now affected, manifested by clumping and margination of chromatin; this was in addition to further separation of endothelial cells from basement membrane (Fig. 2C). Overall, the damage became even less inflammatory in character, with reduced thickening of alveolar septa, decreased interstitial edema, and fewer inflammatory cells. In contrast to the injury pattern seen at 1 or 1.5 ATA, no areas of epithelium were unaffected. Because the patterns of injury seen at 1 and 1.5 ATA O₂ were similar, with important differences only appearing at 2 ATA and above, we omitted studies of some markers of pulmonary injury at 1.5 ATA.

Rats exposed to O₂ at 2.5 ATA for 8 h showed even more severe damage to type I epithelial cells, which in some places had sloughed off into the alveolar space, denuding the basement membrane (Fig. 2D). The interstitium was edematous, although with no abnormal increase in cellularity, and endothelial cells were markedly swollen. Capillary lumina were engorged with red blood cells to a greater extent than at any other exposure levels.

Lung-injury scores (Fig. 3) derived from semiquantitative analysis of light micrographs reveal that all three indices of pulmonary injury show an initial peak after 56 h at 1 ATA and progressively diminish as PO₂ is increased to 1.5 and then to 2 ATA. Compared with 1 ATA, alveolar debris is decreased sevenfold at 2 ATA, and alveolar edema is essentially undetectable. At 2.5 ATA, this trend is reversed: all three components of injury are now at least as great as after the much longer 1 ATA exposure. Multiple sections of inflation-fixed lungs from rats exposed to hyperbaric normoxia (7% O₂, balance N₂) at 3 ATA for 6 h showed neither gross abnormalities nor significant pathology by light microscopy (data not shown).

Pleural fluid volume and lung edema. During normobaric hyperoxia, pleural fluid volume increased progressively with duration of exposure, from 0.4 ± 0.07 ml after 24 h to 10.8 ± 1.8 ml after 56 h (Fig. 4). However, of the four levels of hyperbaric exposure studied, only 1.5 ATA produced significant accumulations of pleural fluid during the 20-h exposure, comparable to that produced at 1 ATA after 48 h.

Wet-dry ratio, an indicator of pulmonary edema, increased with duration of normobaric exposure to O₂ and, after 56 h, reached a maximum of 6.3, ~1.3× that in air-breathing controls (Fig. 4). For the HBO₂ exposures, wet-dry ratio increased significantly (with respect to air breathing controls) only at 1.5 ATA O₂.
Lung inflammation and injury. After 48–56 h of normobaric O₂, total inflammatory cell counts in BALF were significantly above control levels (Fig. 5). Rats exposed to 2.5 and 3 ATA O₂ also had significantly increased BALF cell counts compared with air controls but much lower than after normobaric exposure. BALF LDH increased progressively during normobaric O₂ exposure and, after 56 h, was 7.5-fold higher than during air breathing (Fig. 5). LDH activity in rats exposed to HBO₂ also increased significantly compared with air-breathing controls but with no statistically significant differences among those exposed to 2.0, 2.5, and 3.0 ATA O₂ (Fig. 6). Total protein concentration in BALF also increased in a time-dependent manner in normobaric hyperoxia. At 2.5 and 3 ATA (but not at 2 ATA), BALF protein was significantly higher than in air controls and not different from the pattern for BALF LDH, suggesting that a separate mechanism contributes to transepithelial protein leak in hyperbaric hyperoxia above 2 ATA O₂.

Blood gases. Hyperbaric oxygen exposures at 1.5 ATA (20 h, n = 8) and 2.0 ATA (10 h, n = 11) produced a characteristic hyperoxic hyperventilatory response (17) with significant decreases in PaCO₂ and increases in arterial pH compared with respective 0-h control values (all P < 0.05). The ratio of arterial PO₂ to alveolar PO₂ (arterial-alveolar ratio) decreased in animals after 20 h at 1.5 ATA O₂ (0.62 ± 0.07 vs. 0.74 ± 0.05; P < 0.01) but not in animals exposed to 2.0 ATA O₂ for 10 h. In contrast, animals breathing 2.0 ATA O₂ did not demonstrate either hyperventilation or a change in arterial-alveolar ratio by the end of the 6-h exposure compared with room-air controls.

Changes in NOx and 3-NT. Oxygen at 1 ATA increased NOx in BALF progressively over 56 h of exposure. After 48 h, total NOx had increased significantly, and after 56 h, the rats had risen sevenfold over that of air-breathing controls (Fig. 7). At 3 ATA, NOx levels in BALF were higher than in air-breathing controls but significantly less (P < 0.05) than seen after 56 h of normobaric hyperoxia. BALF 3-NT was below the limit of detection (0.1 pmol/20 μl) in control rats breathing air but was readily measured in rats breathing normobaric oxygen for 24 h, and it progressively increased with longer exposures (Fig. 7). Rats exposed to 3 ATA also showed increased 3-NT but less than in rats under normobaric hyperoxia for 56 h.

Lung injury in rats with convulsions. Rats exposed to 3 ATA for 6 h (n = 37) were divided into two groups based on the presence (n = 14) or absence (n = 23) of convulsions, and their lung injury endpoints were compared. Significant differences in wet-dry ratio as well as in BALF LDH and total protein content were observed between the two groups. The occurrence of convulsions correlated with a greater severity of acute edema, cellular damage, and disruption of the blood-alveolar barrier (Fig. 8).

NOS inhibition and lung injury. To evaluate the contribution of NO to lung injury, rats were treated with either the nonspecific NOS inhibitor, L-NAME, or the nNOS inhibitor, 7-NI. Either of these agents provided some protection to the lung from cell damage and protein leakage (Fig. 8).

Effects of vagotomy on lung injury. When unilateral, cervical vagotomy was performed 1 wk before exposure to 3 ATA O₂. Hypoxic pulmonary injury was diminished. Analysis of BALF from these animals showed that total protein and LDH were ~60% and 45%, respectively, of the values seen in animals without vagotomy (Fig. 9). Concentrations of BALF protein and LDH in the ipsilateral lung were below the corresponding aggregate values from both lungs, but only the protein data reached statistical significance.

DISCUSSION

Our results clearly demonstrate three novel points, which together provide a new perspective on the etiology of pulmonary injury in hyperbaric hyperoxia. First, oxygen exposures at hyperbaric levels that induce CNS toxicity are accompanied by a different pattern of lung histopathology than that seen in normobaric hyperoxia, and thus rats exposed to hyperbaric oxygen were most likely to manifest this pattern of pulmonary injury if they had also exhibited convulsions. Second, this...
pattern of injury is potentiated by NO derived predominately from nNOS. Finally, this injury requires intact autonomic innervation between lung and brain since unilateral transection of the vagus nerve at the cervical level mitigated the pulmonary injury.

Although the absolute values of indices of pulmonary injury seen at 2 ATA and above were significantly less than seen at 1 ATA after 56 h, the rates at which these signs appear are clearly increased in hyperbaric hyperoxia. Also, NOx levels after 6 h at 3 ATA were about twice those in air-breathing controls and approximated those found in rats exposed to 1 ATA O2 for 24 or 48 h, despite the much shorter exposure. The same is true for both inflammatory cells and LDH in BALF. Therefore, the underlying pathological processes in normobaric hyperoxia are accelerated by HBO2, additional processes must come into play, or both.

The idea that lung pathology in hyperoxia may be related to sympathetic excitation is supported by several early investigations, and endogenous epinephrine was among the agents implicated (4, 33, 34). Morphological changes in the lungs of animals exhibiting convulsions or seizures in hyperbaric oxygen have also been described (2, 36), but changes in pulmonary inflammation and biochemistry were not assessed. In addition, pulmonary edema and congestion have been reported in animals subsequent to seizures induced by hyperbaric oxygen, and those changes were prevented when seizures were inhibited, for example, by barbiturates or by GABA (5, 31, 60). Moreover, direct correlation between the numbers of repeated con-
nNOS-derived NO in accelerating oxygen seizures through in trans transmission in the brain (22, 23). Although we found in-
hibitors with drinking water (50), which introduces an un-
doses would have to be administered, for example by mixing
inhibitors is far shorter than the hyperoxic exposures, multiple
clinical and a physiological reason. Because the half-life of the
NOS inhibitors under normobaric conditions for both a tech-

It was shown very early that pressure per se is not likely to
be a significant factor in the pulmonary toxicity of hyperbaric
hyperoxia. In an elegant experiment described by Penrod in
1958 (48), the bronchi of cats were separately catheterized, and
oxygen was supplied to one lung and inert gas, not air, to the
other during exposure to 5 ATA for 7 h. The lung supplied with
inert gas was unchanged, while the lung exposed to oxygen
showed “considerable gross and microscopic damage” (48).
The primary and secondary effects of pressure on pulmonary
injury have also been examined in other ways as well. For
example, the question of whether lung damage in hyperbaric
oxygen could be caused or exacerbated by the increased
density of the breathing gas was studied by Bergé and Tysse-
both in 1991 (7). These investigators reported that no signif-
cant change in pulmonary blood flow occurred in rats breathing
normoxic gas at 5× the density of air (7).

The prevailing view has been that normobaric and hyper-
baric hyperoxia damage the lungs by the same mechanism:
production of ROS or RNS. And because the rate of production
of reactive species is more intense under hyperbaric conditions,
damage is accelerated. There is compelling evidence from
normobaric studies that ROS, RNS, and various cytokines
produced in the lung induce cellular damage by oxidation of
proteins, peroxidation of membrane lipids, and breakage of
DNA strands (29, 30, 38, 49, 53). There is also evidence that
NO specifically plays a role in the development of pulmonary
toxicity induced by normobaric oxygen (1, 16, 56, 57). Al-
though other studies have investigated changes in NO activity
in brain during hyperbaric exposure to oxygen, this work is the
first to evaluate RNS production in the lung under such
conditions.

It remains an open question as to what extent RNS are agents
of pulmonary injury in hyperoxia at 1 ATA. We did not use
NOS inhibitors under normobaric conditions for both a tech-
nical and a physiological reason. Because the half-life of the
inhibitors is far shorter than the hyperoxic exposures, multiple
doses would have to be administered, for example by mixing
inhibitors with drinking water (50), which introduces an un-
controllable variable. Also, NOS inhibition of more than a few
hours creates a shifting physiological state (for example by
varying kidney and cardiac function) that does not reach
equilibrium for weeks (50). It would be difficult to interpret
data from such an experiment. It may be possible to circumvent
this problem in future studies by using NOS-knockout mice.
Overall, our findings are difficult to reconcile with the idea
that the pathological processes in normobaric and hyperbaric
pulmonary injury are the same. Rats exposed to 2.5 or 3 ATA
without seizures show a pattern of lung damage different form
that seen at 1 ATA, whereas the injury pattern in rats with
seizures had similarities to those of rats exposed across the
entire range of O2 pressures. The most notable difference in the
lung injuries at O2 exposures of 2 ATA or above was the lack
of inflammatory cells in BALF and lung tissue. In addition,
only minor increases were found in BALF NO or ONOO−
in rats exposed to O2 at 3 ATA, much less than after 56 h of
normobaric oxygen (see Fig. 7). Despite this, composite lung
injury scores were the same after 8 h at 2.5 ATA as after 56 h
at 1 ATA. This was due in part to a shift in the character of
intra-alveolar cell debris seen after exposure to the higher
pressure. Although fewer intact inflammatory cells are
found in BALF after 2.5 ATA compared with 1 ATA (Fig. 3),
the light micrographs indicate that the total amount of intra-
alveolar debris is at least as great at 2.5 ATA, consistent with
more epithelial cell damage as well as with the presence of
broken cell components and proteinaceous exudates after this
insult.

Systemic inhibition of NOS protected against hyperbaric
lung injury and prevented seizures, suggesting a relationship
between the development of CNS and pulmonary O2 toxicity.
Moreover, the effects of nonselective (l-NAME) or selective
(7-NI) NOS inhibitors on pulmonary oxygen toxicity did not
differ significantly. These findings implicate NO in acute lung
injury and suggest that nNOS plays the prevalent role in the
development of pulmonary toxicity at 3 ATA O2. This new
finding is consistent with our previous work demonstrating
principal role for endothelial NOS (eNOS)-derived NO in
altering cerebral blood flow before CNS O2 toxicity and for
nNOS-derived NO in accelerating oxygen seizures through
amplification of the imbalance of excitatory-to-inhibitory neuro-
transmission in the brain (22, 23). Although we found in-
creases in NOx and OONO− in BALF after 56 h at 1 ATA O2,
it seems unlikely that suppression of NO production in the lung at 3 ATA accounts for the protective effects of selective inhibition of nNOS. This accords with the high lung injury scores in hyperbaric exposures, even though markers for RNS in the corresponding BALF samples were quite low. It is likely that the source of NO that potentiates pulmonary injury in normobaric conditions (and appears in BALF) is different from that responsible for the distinct character of hyperbaric pulmonary injury.

Because nNOS is expressed in lung, vasculature, muscle, and elsewhere, not just in neuronal tissue, specific inhibition of this NOS isoform does not establish an exclusive connection with the CNS. However, Vaughan et al. (59) have shown that “virtually all exhaled NO in the rabbit lung is produced by eNOS, which is present throughout the airways, alveoli, and vessels.” Therefore, the conspicuous effect we observed after selective inhibition of nNOS probably involves significant extrapulmonary sources of NO. Our findings, that seizures potentiate pulmonary injury and that vagotomy attenuates it, provide additional, strong evidence for CNS involvement. However, these findings, although striking, may not be unique to hyperbaric hypoxia.

Our observation that vagotomy prevented the pulmonary injury characteristic of hyperbaric hypoxia implicates a signal from the brain in the development of this pathology. The mechanisms or possible modes of lung injury could involve NO-mediated neuronal pathways and CNS-driven central hemodynamic alterations. Work by Grandpierre and colleagues in 1957 (27) has shown that vagal transection also mitigates pulmonary injury in normobaric hypoxia. Therefore, we do not exclude the possibility that events in the brain have a role in oxygen-induced lung injury in both normobaric and hyperbaric conditions. But the facts that the histopathologies are distinct and that seizures result in more severe pulmonary damage support our hypothesis that the CNS plays a more important role in pulmonary damage in hyperbaric hypoxia.

The biphasic response to increasing levels of hypoxia shown by the composite scores of lung injury (Fig. 3) suggests that at least two pathological processes are at work. The first involves time-dependent effects that are less fully developed as the duration of hyperoxic exposure decreases from 1 to 1.5 to 2 ATA. The second process supervenes as the oxygen pressure increases, for instance at 2.5 ATA, the lowest level of hypoxia at which convulsions from CNS toxicity were observed.

Although the specific mechanisms by which neuronal NO causes lung injury in hyperbaric hypoxia were not evaluated in the present study, the data imply that NO modulation of CNS-mediated adrenergic/cholinergic pathways leads to lung injury. Lung innervation is complex and includes not only autonomic adrenergic and cholinergic fibers, but also nonadrenergic noncholinergic (NANC) systems, which perform defensive, regulatory, and immunomodulatory functions (35, 40–42, 46, 52). In the brain, the inhibitory NANC system involving NO opposes cerebral vasoconstriction (58), but in the lung, nNOS does not seem to be important for normal function, since nNOS knockout mice have normal endothelium-dependent vasodilation (24).

Although epinephrine and acetylcholine are the major neurotransmitters involved in regulating pulmonary airway and vascular function, NANC neurotransmitters, including vasoactive intestinal peptide (VIP) and NO, also appear to be important. Immunohistochemical studies have shown the existence of nNOS in nerve terminals supplying lung vessels and lower airways (25, 39). A fruitful area for further study could be the role of CNS-mediated cardiovascular contributions to lung damage in hyperbaric hypoxia. In addition to its effects on the lung and brain, hyperbaric oxygen also increases systemic vascular resistance and reduces cardiac output. These effects could shift systemic blood to the pulmonary circulation, increasing pulmonary blood volume and pulmonary arterial or venous pressure. Although such hemodynamic alterations may be rapid and transient, structural disruption of pulmonary capillaries could cause protein-rich pulmonary edema and persistent damage to the blood-gas barrier.

In summary, we have demonstrated that as hypoxia increases from normobaric to hyperbaric levels, lung responses shift from direct inflammatory injury to CNS-mediated noninflammatory injury, and although the differences between normobaric and hyperbaric oxygen toxicity are qualitative and not absolute, the direct effect of oxygen on the lung predominates in normobaric hypoxia, whereas CNS effects prevail in hyperbaric conditions. As the balance between these two mechanisms shifts, the pattern of injury changes, but there is never a condition in which one or the other effect is totally lacking. The former develops slowly, and because the entire surface of the lung is directly exposed to the hyperoxic environment for many hours, the inflammatory response is diffuse with destruction of the alveolar-capillary barrier, edema, impaired gas exchange, respiratory failure, and death. However, at hyperbaric exposures, pulmonary damage develops more rapidly and is presaged by events in the brain. This more acute injury is driven by extrapulmonary mechanisms in which NO derived from nNOS may link elevated PO2 in brain to accelerated damage in the lung via central autonomic pathways.

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REFERENCES
HYPERBARIC PULMONARY O2 TOXICITY: ROLE OF NO


