Continuous enteral nutrition attenuates pulmonary edema in rats exposed to 100% oxygen

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Factor, Phillip, Karen Ridge, John Alverdy, and Jacob I. Sznaider. Continuous enteral nutrition attenuates pulmonary edema in rats exposed to 100% oxygen. J Appl Physiol 89: 1759–1765, 2000.—Adult rats exposed to hyperoxia develop anorexia, weight loss, and a lung injury characterized by pulmonary edema and decreased lung liquid clearance. We hypothesized that maintenance of nutrition during hyperoxia could attenuate hyperoxia-induced pulmonary edema. To test this hypothesis, we enterally fed adult male Sprague-Dawley rats via gastrostomy tubes and exposed them to oxygen (inspired O2 fraction >0.95) for 64 h. In contrast to controls, enterally fed hyperoxic animals did not lose weight and had smaller pleural effusions and wet-to-dry weight ratios (a measure of lung edema) that were not different from room air controls. Enterally fed rats exposed to hyperoxia had increased levels of mRNA for the Na+-K+-ATPase α1- and β-subunits and glutathione peroxidase. These findings suggest that maintenance of nutrition during an oxidative lung injury reduces lung edema, perhaps by allowing for continued expression and function of protective proteins such as the Na+-K+-ATPase.

sodium-potassium-adenosine 5′-triphosphatase; glutathione peroxidase; hyperoxia

Adult rats exposed to toxic levels of normobaric oxygen (inspired O2 fraction >95% for 60–66 h) develop a lung injury that is characterized by increased alveolar permeability and lung edema (11, 31). In the alveolar epithelium, edema clearance is effected by the active transport of Na+ out of the alveolar air space. Current data support the hypothesis that Na+ transport across the alveolar epithelium, and hence edema clearance, is dependent on the function of epithelial Na+ channels and Na+-K+-ATPases (30, 35, 39, 40). During hyperoxia Na+-K+-ATPase is inhibited, resulting in decreased alveolar edema clearance (3).

Exposure of cells to oxidants results in a dose-dependent uncoupling of normal cellular metabolic pathways (5, 15). In experimental models, low doses of oxygen-derived free radicals increase expression of antioxidant enzymes and increase active Na+ transport (17, 22). Higher doses destabilize cell membranes, increase membrane permeability, oxidize cytosolic and membrane-bound proteins, and interfere with alveolar epithelial Na+ transport (1, 18, 28). These alterations of cellular function impact on cellular defense mechanisms and membrane integrity, contributing to pulmonary edema accumulation. It has been reported that augmentation of antioxidant defense mechanisms attenuates hyperoxic lung injury. Such protection has been observed after pretreatment with endotoxin, interleukin-1, and sublethal levels of O2 (85% for 7 days) (4, 11, 16). Endotracheal instillation or adenoviral-mediated overexpression of antioxidants have also been shown to ameliorate the effects of hyperoxia (12).

We reasoned that decreased availability of the metabolic precursors necessary for cellular function may contribute to hyperoxic lung injury in rats. Thus we inserted feeding gastrostomy tubes into adult rats and tested whether continuous enteral nutrition could affect hyperoxic lung injury by assessing changes in total lung water, total body weight, pleural effusions, and Na+-K+-ATPase and glutathione peroxidase (GSH-Px) mRNA expression after 64 h of normobaric hyperoxia.

METHODS

Experimental design. A total of 66 rats were studied. The rats with gastrostomy tubes were randomized to four groups: hyperoxia/fed (n = 12), hyperoxia/starved (n = 12), room air/fed (n = 6), and room air/starved (n = 6). These four groups were compared with 30 control rats: hyperoxia/sham operated (n = 12), room air/sham operated (n = 6), room air/unoperated (n = 6), and hyperoxia/unoperated (n = 6). The use of animals for this study was approved by the Michael Reese Hospital Institutional Animal Care and Use Committee.

Gastrostomy tube placement. Adult male Sprague-Dawley rats (300–320 g; Harlan Sprague Dawley, Indianapolis, IN) were anesthetized with intraperitoneal ketamine HCl (30 mg/kg; Aveco, Fort Dodge, IA) and pentobarbital sodium (5 mg/kg; Abbott, North Chicago, IL). Abdominal fur was clipped, and the skin was disinfected with isopropyl alcohol and iodine. After induction of anesthesia, a 2-cm midline laparotomy incision was made. A sterile fenestrated Silastic catheter (0.030-in ID, Dow Corning, Midland, MI) was ad-
vanced through the anterior gastric wall into the duodenum to the level of the ligament of Treitz. The catheter was passed through the abdominal musculature and tunneled beneath the skin before exiting on the dorsum of the neck. The catheter was advanced through a stainless steel button and sheath (Harvard Apparatus, South Natick, MA). After closure of the incisions, the catheter and sheath were affixed to a swivel device (Harvard Apparatus) that allowed free movement of the animals within their cages and simultaneous continuous administration of fluids through the catheter (see Fig. 1). Enteral feeding was begun immediately after recovery from anesthesia. Feeding was rapidly advanced to 10 ml·kg⁻¹·h⁻¹. Sham-operated rats were exposed to identical surgical and anesthetic conditions with the exception that the catheter, button, and sheath were not placed. Animals requiring >35 min of surgery were excluded from the study due to presumed excessive surgical stress. All surgery was performed by a single individual (P. Factor). All animals received 7 ml of 0.9% NaCl subcutaneously immediately after surgery to replace intraoperative and postoperative volume losses. All animals were allowed 3 days recovery before exposure to hyperoxia.

**Enteral feeding.** All gastrostomy tube animals received 10 ml·kg⁻¹·h⁻¹ of Vivonex T.E.N. diluted per manufacturer’s recommendations (Norwich Eaton Pharmaceuticals, Norwich, NY) beginning immediately after recovery from anesthesia (2, 3). This enteral nutrition formula provides 20.6 g/100 ml (g%) carbohydrate, 3.8 g% protein, and 0.2 g% fat (caloric distribution = 82.2, 15.3, and 2.5%, respectively). Starved/gastrostomy tube animals were switched from enteral feedings to the same weight-adjusted volume of water at the initiation of hyperoxic exposure. The gastrostomy tube animals were not provided with access to rat chow or water (other than that delivered via their gastrostomies). Sham-operated and unoperated control animals were allowed rat chow (Purina rodent laboratory chow 5020, Ralston Product, St. Louis, MO) and water ad libitum throughout the recovery and injury periods.

**Hyperoxic protocol.** The injury animals were exposed to hyperoxia in an environmental chamber (Kirschner-Collison, Aberdeen, MD) for 64 h. The inspired O₂ fraction was maintained, without interruption, at >0.95 for the duration of the experiment. Barometric pressure within the chamber remained <3 mmHg above the pressure in the room in which the chamber was kept. All animals were marked for identification purposes and weighed immediately before oxygen exposure. Chamber oxygen concentration, volume of enteral formula administered, temperature, and humidity were monitored continuously.

**Animal euthanasia protocol.** The animals were weighed before administration of 1,000 units heparin sodium and 50 mg of pentobarbital sodium intraperitoneally. After anesthesia was documented a midline laparotomy and thoracotomy were performed. The presence of pericardial fat pads was documented in each animal. The volume of pleural fluid was measured in three animals from each group. Animals were exsanguinated by laceration of the inferior vena cava and descending aorta. The trachea was cannulated before en bloc removal of the lungs and mediastinum. The right upper lobe hilum was ligated, and the right upper lobe removed for the prelavage wet-to-dry weight ratio. The lungs were then repeatedly lavaged with 7 ml of PBS to remove alveolar cells that might confound subsequent Northern blot analyses. The pulmonary vasculature was flushed by cannulation of the pulmonary artery via the right ventricle and perfused with 40–60 ml of PBS. The mediastinum and large airways were dissected away from the lungs and discarded. The left lung was immediately immersed in liquid nitrogen and stored at −70°C for RNA isolation.

**Wet-to-dry weight determinations.** The right upper lobe of each animal was used for prelavage wet-to-dry weight indexes. Specimens were weighed immediately after removal and placed in a heated vacuum chamber (Speedvac, Savant Instruments, Farmingdale, NY) until repeated weighings demonstrated no change in dry weight.

**RNA isolation.** RNA was isolated by using a modified single-step phenol-chloroform extraction protocol (10, 34). Briefly, 0.8–1.0 g of frozen whole lung was homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) for 30–60 s in 2.5 ml of homogenization solution [5.2 M guanidine, 25 mM trisodium citrate, 0.5% sodium sarcosyl, 0.1% of 30% antifoam A (Sigma Chemical), and 0.1 M β-mercaptoethanol, pH 6.5] at room temperature. RNA was extracted from the homogenate by addition of 0.625 ml of 1 M sodium acetate (pH 4.0), 3.125 ml phenol (Tris equilibrated to pH 7.4), and 0.625 ml of a 24:1 mixture of chloroform and isoamyl alcohol. Specimens were shaken and placed on ice for 20 min before centrifugation at 15,000 g for 20 min at 4°C. The aqueous phase was transferred to new tubes and precipitated at −20°C for 2 h by the addition of 1 vol of isopropanol. The resulting pellet was dissolved in 2 ml of 7.5 guanidine HCl, 5 mM dithiothreitol, and 25 mM trisodium citrate (pH 7.0) at 68°C for 15 min. A second precipitation was performed by addition of 50 µl of 1 M acetic acid (0.025 vol) and 1 ml of absolute ethanol (0.5 vol). The RNA was precipitated at −20°C for >2 h. After pelleting of the RNA by centrifugation, the RNA pellets were washed with 1 vol of 7.5 M guanidine HCl-1 M acetic acid-ethanol (1:0.025:0.5) followed by three washes with cold 80% ethanol. The resulting pellets were solubilized in a low-salt buffer (10 mM Tris·HCl, 1 mM EDTA, 0.05% SDS, and 1 µg/ml protease K). Total RNA was quantified spectrophotometrically at 260 and 280 nm. All specimens had ratios of optical density at 260 nm to optical density at 280 nm between 1.90 and 2.10. RNA integrity was assessed on the basis of the presence of ribosomal RNA bands in agarose gels stained with ethidium bromide.

Fig. 1. An example of a rat in a metabolic cage. Visible in the photograph is a metal swivel attached to the base of the neck. Silastic tubes were protected by a metal sheath that was attached to the swivel.
Northern blot analysis. Ten micrograms of total RNA dissolved in loading buffer (60 mM MOPS, 0.1 M sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, 50% formamide, 5% glycerol, and 0.05 bromphenol blue, pH 7.0) were separated electrophoretically in 1.0% agarose-1 MOPS-2.2 M formaldehyde gels. After electrophoresis, the gel was stained with ethidium bromide and electrophoretically transferred to solid support (Nytran, Schleicher and Schuell, Keene, NH) in electroblotting buffer (10 mM Tris-HCl, pH 7.4, and 0.5 mM EDTA). The RNA was crosslinked to the membranes by exposure to ultraviolet light (302 nm for 5 min) and baking at 80°C for 2 h. Each membrane contained four serial dilutions of total RNA harvested from the lungs of age- and weight-matched, uninjured control rats. These dilutions were used to allow comparison of densitometric values between blots and for correction for nonlinear hybridization within a blot. Hybridization of the blots for assessment of steady-state mRNA levels was achieved by using a 1.0-kb rat cDNA probe for the mRNAs for Na""-K""-ATPase α1- and β1-subunits (the generous gift of Dr. Janet Emmanuel, Yale University, New Haven, CT). Radiolabeled anti-sense ribo-probe was transcribed using [32P]CTP (10 mCi/ml; ICN Pharmaceuticals) by using a commercial end-labeling kit (Promega) that was 5' end labeled with [32P]ATP (10 mCi/ml, ICN Pharmaceuticals, Irvine, CA) and SP6 RNA polymerase (Promega, Madison, WI). Nylon membranes were prehybridized for 3 h at 57°C in a solution consisting of 50% formamide, 1% SDS, 0.1% sodium chloride-sodium citrate (SSC) containing 1% Blotto [0.2% sodium azide and 10% nonfat dried milk (Carnation, Los Angeles, CA)], sheared salmon sperm DNA (250 μg/ml), and yeast tRNA (250 μg/ml). Membranes were hybridized for 16 h in the same solution after addition of the radiolabeled probe (10⁹ disintegrations·min⁻¹·μg⁻¹). After hybridization the membranes were sequentially washed in 2× SSC-1% SDS (3 × 30 min) at 68°C followed by 1% SDS-0.2% SSC at 68°C (3 × 30 min).

Corrections for variations in lane loading were based on ethidium bromide staining and subsequent hybridization of all membranes with a human oligonucleotide probe complementary to 18S ribosomal RNA (Synthetic Genetics, San Diego, CA) that was 5' end labeled with [32P]ATP (10 mCi/ml, ICN Pharmaceuticals) by using a commercial end-labeling kit (Promega). Membranes were then hybridized at 65°C as above except for the absence of formamide and dextran sulfate from the hybridization solutions. Wash conditions were as described above but at 55 and 35°C.

After the membranes were stripped, they were probed for the expression of GSH-Px. A commercially available oligolabeling kit (Promega) was used to generate a 1.5-kb cDNA probe corresponding to the 19-kDa monomer of GSH-Px (N. Q. Li and C. Reddy, Pennsylvania State University, University Park, PA). Membranes were prehybridized and hybridized as above at 42°C. Washes were performed at 60°C in 0.1× SSC-1% SDS.

After exposure of the blots to X-ray film (XAR5, Kodak, Rochester, NY), the resulting autoradiograms were analyzed by using a linear scanning transmittance densitometer (model GS300, Hoeffer Scientific Instruments, San Francisco, CA). Transmittance values were integrated by using LTI integration software (Lakeshore Technologies, Chicago, IL). Interlane variations in RNA content were corrected to the 18S ribosomal signal. Expression between blots was standardized by comparing Na""-K""-ATPase and GSH-Px steady-state mRNA expression. Normalized expression of Na""-K""-ATPase α1- and β1-subunit mRNA was elevated approximately equal to three- and two-fold respectively (Fig. 5) in the hyperoxic/fed group. Steady-state levels of β1-subunit mRNA in the hyperoxic/starved, hyperoxic/sham, and hyperoxic/unoperated groups were not different from unoperated room air controls. Stable-state levels of mRNA (Fig. 6) were elevated by nearly 100% in the hyperoxic/fed group. Levels of GSH-Px mRNA were not different from untreated room air controls group in the other experimental groups.

RESULTS

Appearance, pleural effusions, and urine output. All hyperoxic rats were noted to be tachypneic and to have bilateral pleural effusions at the end of the 64 h of exposure to 100% O₂. The volume of the effusions was less in the hyperoxic/fed rats than in the other hyperoxic controls (Fig. 2). No pleural effusions were noted in the room-air animals. Pericardial fat pads were noted to be present in the hyperoxic/fed, room air/fed, room air/sham, and unoperated control groups but were absent in the other groups.

Weight change. As shown in Fig. 3, enterally fed room air and hyperoxic rats maintained their weight compared with unoperated/room-air rats. Hyperoxic/starved and hyperoxic/sham and hyperoxic/unoperated animals all lost weight.

Wet-to-dry weight ratios (total lung water). Enteral feeding of hyperoxic rats had wet-to-dry weight indexes that were not different from room air controls and were significantly less than the hyperoxic/starved, hyperoxic/sham, and unoperated hyperoxic controls (Fig. 4). All room-air animals had normal wet-to-dry weight indexes.

Na""-K""-ATPase and GSH-Px steady-state mRNA expression. The specific activity of the Na""-K""-ATPase cRNA probe was 10⁹ and 10¹² disintegrations·min⁻¹·μg⁻¹ for the 18S and GSH-Px probes, respectively. Lanes containing RNA extracted from rat kidney and liver were used as positive and negative controls, respectively. All blots were hybridized simultaneously.

Statistical analysis. Statistical significance was assessed by using STATPAK statistical software. Unpaired t-tests assuming unequal variances between groups (Bonferroni correction) were used to assess significance by comparing specific groups with unoperated room-air controls.
DISCUSSION

Rats exposed to 100% O₂ cease their usual consumption of food and water. This typically occurs within the first 24 h and is followed by the development of generalized lethargy and weight loss. Their high baseline metabolic rate combined with malnutrition and stress-induced elevations in metabolic rate rapidly lead to depletion of nutritional stores. After 60–66 h of hyperoxia most rats appear moribund and have large bilateral pleural effusions and increased extravascular lung water. This experimental lung injury causes epithelial and endothelial cytotoxicity, pulmonary edema, and a high mortality rate (11). We hypothesized that nutrition could provide protection from hyperoxia by allowing the lung to upregulate mechanisms that protect against lung injury. To test this hypothesis, we inserted gastrostomy tubes into adult male rats and continuously fed them while exposing them to hyperoxia for 64 h. We then measured changes in body weight and total lung water. We also measured steady-state mRNA levels of a key enzyme required for lung edema clearance, Na⁺-K⁺-ATPase, and an important antioxidant enzyme, GSH-Px. Our results show that enterally fed hyperoxic rats did not lose weight and did not have increased lung water. These findings were associated with maintenance of pericardial fat pads, smaller pleural effusions, and increased steady-state levels of Na⁺-K⁺-ATPase and GSH-Px mRNA.

Nici et al. (29) reported that 180- to 200-g rats exposed to acute hyperoxia (100% O₂ × 60 h) have three- to fourfold elevations of both α₁- and β₁-subunit message that is associated with increased whole lung α₁-subunit protein expression. Carter et al. (8) subsequently reported that active Na⁺ transport in hyperoxic rats correlated with the degree of lung injury and alveolar permeability. This group, which used younger rats (which better tolerate hyperoxia) and a shorter duration of hyperoxia than that used in the present study, concluded that hyperoxia variably affects active alveolar Na⁺ transport and liquid reabsorption and that Na⁺-K⁺-ATPase subunit mRNA, protein, and function do not respond in parallel fashion in their model. Conversely, Borok and colleagues (7) reported that hyperoxia reduces Na⁺-K⁺-ATPase mRNA levels and function in alveolar type II cells isolated from adult rats. They also observed that increasing β₁-subunit expression (with keratinocyte growth factor) restores Na⁺-K⁺-ATPase function in their in vitro hyperoxia model. Our group previously showed that Na⁺-K⁺-ATPase expression parallels function in two rat models of hyperoxia (31, 32). In these studies, it was observed that Na⁺-K⁺-ATPase function and lung liquid clearance were reduced by >50% when rats were exposed to acute hyperoxia (95% O₂ × 64 h). Conversely, clearance and Na⁺-K⁺-ATPase protein abundance both increased when rats were exposed to sub-
acute hyperoxia (85% O₂ × 7 days). In both of these studies, Na⁺-K⁺-ATPase expression paralleled lung liquid clearance. Although we did not measure Na⁺-K⁺-ATPase protein expression or lung liquid clearance in the present study, the prior work suggests that the increased steady-state levels of α₁- and β₁-subunit mRNA may correlate with alveolar Na⁺-K⁺-ATPase protein abundance and lung liquid clearance in this model.

The effects of hyperoxia and other oxidants on Na⁺-K⁺-ATPases are complex and variable and have been studied in numerous models. In most studies, oxidants inhibit sodium pump function in a dose-dependent fashion via direct oxidation of the Na⁺-K⁺-ATPase or surrounding membrane lipids, reduction of substrate availability, or alteration of ion gradient driving forces (6, 17, 21, 22). Similar changes in epithelial Na⁺-channel function have also been reported (41). We believe that the increased lung water seen in rats exposed to 100% O₂ is due to increased alveolar permeability and decreased transepithelial Na⁺ transport. On the basis of prior work by our laboratory, the ill appearance of rats, and the presence, albeit reduced, of pleural effusions, it is reasonable to conclude that the hyperoxic/fed rats experienced a lung injury that should have increased alveolar permeability (31, 32). Thus the finding of normal lung water and increased Na⁺-K⁺-ATPase mRNAs in the hyperoxic/fed rats supports the reasoning that enteral nutrition maintains or improves alveolar permeability and/or Na⁺ transport in this model.

The impact of fasting and dietary deficiency on hyperoxia has been investigated previously. Smith and co-workers (36) have reported that fasting potentiates hyperoxic lung injury and increases mortality in mice. They postulated that these findings are due to reduced lung glutathione levels. Similar findings have been reported by Deneke et al. (14), who demonstrated that inhibition of glutathione synthesis with diethylmaleate is associated with decreased survival and increased pulmonary edema after exposure to hyperoxia. These investigators also reported that protein restriction contributes to hyperoxic lung injury via reductions of lung glutathione levels (13). In a subsequent study, another group reported that dietary supplementation with sulfur-containing amino acids (precursors of glutathione) ameliorated hyperoxic lung injury and improved survival in rats (9). Similar findings have been reported in murine models of oxidant-induced hepatic injury (38). Langley and Kelly (27) have reported that the survival of starved preterm guinea pig pups exposed to hyperoxia is 40% less at 72 h compared with fed controls. This group has also reported that adult, but not neonatal, guinea pigs are unable to

![Figure 5](http://jap.physiology.org/)  
Fig. 5. Steady-state levels of mRNA for the α₁- and β₁-subunits of the Na⁺-K⁺-ATPase, relative to room-air controls, were elevated 2- to 3-fold. All other groups demonstrated levels of expression not different from room-air controls with the exception of the sham-operated rats, which had α₁-subunit mRNA levels that were slightly reduced. Values are means ± SD of the density of signal measured by transmittance densitometry and corrected for lane loading for 6 animals/group. *P < 0.05.

![Figure 6](http://jap.physiology.org/)  
Fig. 6. Glutathione peroxidase steady-state mRNA levels were elevated only in hyperoxic/injured animals (n = 6). Hyperoxic/starved rats (n = 6) express levels of this message that were not different from room-air controls. In contrast, glutathione peroxidase message is reduced (P < 0.05) in room-air/starved animals (n = 6). Values are means ± SD of densitometric values corrected for lane loading. *P < 0.05.
maintain glutathione synthesis and lung and liver glutathione levels when starved (26). A more recent study reported that parenteral feeding improves survival of hyperoxic guinea pig pups, in part due to improve lung glutathione synthesis (9). These studies suggest that nutrition has a significant impact on the pathophysiology of hyperoxic lung injury. We did not test what component of Vivonex was responsible for our findings. However, on the basis of the work of our group and that of other investigators, we speculate that increased glutathione administration could have contributed to our results (2, 3). The choice of the elemental enteral formula Vivonex was on the basis of its relatively high content of glutamine, a precursor of glutathione (2, 3). It has been previously reported that glutamine supplementation can affect glutathione synthetase function and glutathione levels in lung and intestine (20, 24, 25).

The methodology we have used to provide enteral nutrition has been previously shown to provide adequate nourishment for rats (2, 3, 37). Our observation that the enteral nutrition preserved mediastinal fat pads and total body weight suggests that our animals received adequate nutrition during hyperoxia.

It has been previously reported that tumor necrosis factor-α and interleukin-1 are elevated by surgical stress, which could confer tolerance to hyperoxia, probably via antioxidant pathways. The significant levels of lung water noted in the starved and sham-operated hyperoxic controls make it unlikely that the surgical procedure of gastrostomy tube placement induced tolerance to hyperoxia.

In contrast to Na+/K+-ATPase mRNA, it has been shown that GSH-Px message synthesis can continue in the absence of changes in protein levels and function in the setting of malnutrition in erythrocytes and liver (19, 33). Given that toxic oxygen metabolites interfere with DNA and RNA synthesis and repair, the increased steady-state levels of mRNA seen in the fed hyperoxic animals suggest that enteral nutrition allows for maintenance of nucleic acid metabolism and not necessarily antioxidant function (23). Consequently, it is difficult to propose a cause-and-effect relationship between enteral nutrition and increased GSH-Px function.

In summary, our findings suggest that enteral nutrition confers protection against hyperoxia by allowing continuation of intracellular anabolic functions. Our data provide support for the hypothesis that nutritional therapy using a glutathione-rich formula provides protection from hyperoxia in rats by attenuating pulmonary edema, possibly via upregulation of alveolar Na+/K+-ATPases and fluid reabsorption.

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