Hyperoxic effects on alveolar sodium resorption and lung Na-K-ATPase

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Carter, Ethan P., O. Douglas Wangensteen, J ordan Dunitz, and David H. Ingbar. Hyperoxic effects on alveolar sodium resorption and lung Na-K-ATPase. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1191–L1202, 1997.—Active Na transport by the alveolar epithelium keeps alveoli relatively dry. Hyperoxia increases epithelial permeability, resulting in pulmonary edema. We sought to determine whether active Na resorption from the air spaces and Na-K-ATPase activity increased in rats exposed to >95% O2 for 60 h. The permeability × surface area products for unidirectional resorption of alveolar [14C]sucrose (PSsucrose) and 22Na+ (PSNa+) were measured in isolated, perfused rat lungs immediately after hyperoxia and after 3 and 7 days of recovery in room air. At 60 h of hyperoxia, the mean PSsucrose and PSNa+ increased from 6.71 ± 0.8 × 10−3 to 12.6 ± 1.6 × 10−5 cm/s (P < 0.029) and from 23.6 ± 1.1 × 10−5 to 31.0 ± 1.6 × 10−5 cm/s (P < 0.008), respectively. However, the values in individual rats ranged widely from no change to nearly a fourfold increase. Subgroup analysis revealed that benzamil- or amiloride-sensitive (transcellular) PSNa+ was significantly reduced in the exposed lungs with normal PSsucrose but was maintained in the lungs with high PSsucrose. By day 3 of recovery, mean Na+ and sucrose fluxes returned to values similar to control. Na-K-ATPase membrane hydrolytic maximal velocity (Vmax) activity fell significantly immediately after hyperoxic exposure but recovered to normal values by day 3 of recovery. The Na-K-ATPase β-subunit antigenic signal did not significantly change, whereas the α-subunit levels increased during recovery. In summary, there was a heterogeneous response of different rats to acute hyperoxia. Hyperoxia led to complex, nonparallel changes in Na+ pump antigenic protein, hydrolytic activity, and unidirectional active Na+ resorption. Active Na+ transport was differentially affected, depending on degree of injury, but permeability and transport normalized by day 3 of recovery.

acute lung injury; rat; amiloride; benzamil; sodium pump; pulmonary edema; sodium-potassium-adenosinetriphosphatase

Effective gas exchange requires that the alveoli remain essentially free of fluid. Although small amounts of fluid and solute leak into the alveoli, they are kept relatively dry. It had been thought that fluid was cleared from the air spaces primarily by passive oncotic and hydrostatic forces. It now is apparent that Na+ is actively transported from the air spaces to the pulmonary vasculature, establishing an osmotic gradient that removes fluid from the lungs (1, 7, 11, 21, 33, 34). Alveolar type II (ATII) epithelial cells actively transport Na+ in vitro (4, 11, 19). In the normal epithelium, Na+ enters these cells at the apical (alveolar) membrane, primarily through an amiloride-sensitive Na+ channel (9, 20, 32), but other potential pathways in-clude an Na+-glucose symporter (19, 34), an Na+-amino acid symporter, and an Na+/H+ antiporter (17). Intracellular Na+ then is pumped across the basolateral membrane by energy-dependent Na-K-ATPase (or Na+ pump). Transepithelial vectorial Na+ transport requires activity of both the Na+ channel and the Na+ pump.

In acute lung injury, the alveolar epithelium becomes damaged, leading to alveolar flooding, atelectasis, hemorrhage, and death. The accumulation of proteinaceous pulmonary edema fluid probably occurs because of leakage both between epithelial cells and across basement membranes denuded by epithelial cell necrosis. Surprisingly, humans with acute lung injury are capable of clearing edema fluid from the lung air spaces by active ion transport across the alveolar epithelial barrier (22). The efficiency of this clearance may affect the prognosis. In rat lungs damaged with Pseudomonas aeruginosa, catecholamines can accelerate fluid resorption (31), and augmented alveolar fluid resorption has been described in many animal models of lung injury (21). These results suggest that active Na+ resorption continues during and after alveolar flooding due to endothelial injury, but varied effects of acute epithelial injury on lung Na+ transport have been reported (29, 41).

Exposure of animals to high levels of O2 is a model for the study of the mechanisms of acute lung injury because it causes vascular endothelial and alveolar epithelial damage and pulmonary edema (25, 35, 36, 38). In a chronic hyperoxic model, after 7 days of exposure to 85% O2, ATII cells from the lungs of exposed rats had increased Na-K-ATPase expression and function (28) as well as increased Na+ channel expression and function (13, 40). Surprisingly, there was heterogeneity of the response of active (amiloride-inhibitable) alveolar Na+ resorption in different rats after this injury (39). In an acute hyperoxic model, exposure of rodents to 100% O2 resulted in vascular endothelial and alveolar epithelial damage, as evidenced in increased lung wet-to-dry weight measurements (25) and increased permeability to solutes such as sucrose and dextran 20 (30, 38). Although damage can be severe during acute exposure, in hamsters solute permeability returned to normal within 3 days after reintroduction to room air (30). Differing consequences for Na+ transport in an acute hyperoxic model of lung injury have been reported. Using large (280–360 g) rats, presumably with lesser antioxidant defenses, that were exposed to 100% O2 for 64 h, Olivera et al. (29) found that alveolar fluid clearance and Na-K-ATPase hydrolytic activity decreased at the end of exposure. Using smaller (200–300 g) rats exposed for 60 h, Zheng et al. (41)
recently reported that active alveolar Na\(^+\) resorption was normal. However, they excluded rats with severe injury from their analysis.

We used an established rat model with known morphometric changes (see Ref. 36), 60 h of exposure of 180- to 200-g Sprague-Dawley rats to \(>95\%\) \(O_2\), followed by recovery in room air (4) to investigate the effects on unidirectional alveolar Na\(^+\) resorption and Na\(^+\) pump. Previously, we reported that this exposure protocol led to early increases in peripheral lung Na-K-ATPase mRNA at 60 h of hyperoxia (25) before ATII cells proliferate (36). Shortly thereafter, during room air recovery, \(\alpha_1\)-protein levels increased. This suggested that hyperoxia may induce an early upregulation of Na-K-ATPase that serves as a homeostatic mechanism to deal with incipient pulmonary edema.

Recently, we reported that ATII cells isolated from rats exposed in vivo to \(>95\%\) \(O_2\) for 60 h had a complex series of changes in their Na-K-ATPase (3). The mRNA levels for both subunits in freshly isolated ATII cells were significantly elevated, but the \(\alpha_1\)-protein level was decreased, with a stable \(\beta_1\)-protein level. The Na-K-ATPase activity of the intact ATII cells was normal as measured by ouabain-inhibitable rubidium uptake, but the maximal velocity (\(V_{\text{max}}\)) of ATII cell membranes was significantly decreased. This suggested that there were multiple overlaid effects of hyperoxia on the Na-K-ATPase of these cells. However, because these isolated cells may represent a subpopulation of ATII cells after injury and because it is difficult to translate the functional transport properties of the isolated cells to the intact lung, we undertook complementary studies using isolated, perfused lungs and biochemical measurements on the peripheral lung.

The purposes of this investigation were to determine whether the previously observed increases in Na-K-ATPase \(\alpha_1\)- and \(\beta_1\)-mRNA and \(\alpha_1\)-antigenic protein in the peripheral lung are accompanied by increased unidirectional Na\(^+\) resorption from the alveolar space and increased peripheral lung membrane Na-K-ATPase activity and to compare the results of hyperoxia with the whole lung and previously described ATII cell changes (4). Because the \(\beta_1\)-subunit is rate limiting in the formation of functional Na-K-ATPase molecules in some systems (27) and, unlike the \(\alpha_1\)-subunit, changed in parallel with the Na\(^+\) pump function in the intact ATII cells, we also sought to determine whether the \(\beta_1\)-subunit antigenic protein increased during or after hyperoxia, as was reported with the \(\alpha_1\)-subunit (26).

**METHODS**

**Animals**

Male Sprague-Dawley specific pathogen-free rats (Harlan Sprague Dawley, Madison, WI) weighing 180–200 g were used in all experiments. Animals were housed in filter-top cages at 25°C with food and water ad libitum and a 12:12-h light-dark cycle.

**Hyperoxic Exposure Protocol**

Animals were exposed to hyperoxia at 1 atmosphere for 60 h in a chamber with ad libitum access to food and water as previously described (4, 38). The environment in the chamber was analyzed to ensure that the absolute values were maintained. The rats were housed in a 12-12-h light-dark cycle.

**Isolated, Perfused Lung Preparation**

The permeability \(\times \) surface area (PS) products of \(^{22}\text{Na}^+\) (PS\(_{\text{Na}}^+\)) and \(^{14}\text{C}\text{sucrose (PS}_{14}\text{sucrose})\) across the alveolar epithelium were determined in isolated, perfused lungs from four groups of animals: control rats (no hyperoxic exposure), immediately after 60 h of hyperoxic exposure, and on days 3 and 7 after recovery from hyperoxia. The lungs were perfused using Ringer solution containing \((in \text{mM}) 137 \text{NaCl, 2.68 KCl, 1.25 MgSO}_4, 1.82 \text{CaCl}_2, 5.55 \text{glucose, and 12.2 hydroyethylpiperazine-N}_2\text{-2-ethanesulfonic acid. The pH was adjusted to 7.4. The resulting osmolality was 280 ± 5 mosM. In addition, 0.5% bovine serum albumin and 1.0% Dextran 70 were added to the perfusate immediately before use. Our isolated lung preparation and single-pass nonrecirculating perfusion technique have been reported in detail previously (26, 30).** Briefly, the animals were anesthetized, the pulmonary artery was cannulated, and the heart and lungs were removed en bloc. This preparation was suspended from a force transducer and was perfused at a flow rate of 7.0–7.5 ml/min. The pulmonary artery pressure and lung weight were monitored throughout the experiment, and the temperature of the perfusate and the preparation was maintained at 37°C. Perfusion pressure was stable at 10–12 cmH\(_2\)O throughout all experiments. After 10–15 min of stabilization, a 3-ml volume of instillate solution was lavaged into and out of the lungs three times, at which point good mixing and uniform filling throughout the alveolar space should have occurred (29). Finally, 2.5 ml were left in the lungs; the remaining 0.5 ml was retained for measurement of initial alveolar solute concentrations. The instillate solution was similar to the vascular perfusate except it contained 1 \(\mu\text{Ci/3 ml}\) \(^{22}\text{Na}^+\), 2 \(\mu\text{Ci/3 ml}\) \(^{14}\text{C}\text{sucrose (PS}_{14}\text{sucrose})\), 1 mg/3 ml fluorescein isothiocyanate (FITC)-Dextran 150 (150-kDa molecular mass), and 5 mM sucrose, but it did not contain glucose. In some experiments, to determine whether Na\(^+\)-glucose cotransport was activated with hyperoxic injury, sucrose in the instillate was replaced with glucose. Uniformity of FITC-Dextran 150 (and the other test solutes) distribution was visually confirmed by the yellow coloration on the pleural surface of the lungs. After lavage, 0.2 ml of air was introduced into the lungs through the trachea to push the instillate beyond the anatomic dead space, and the trachea was temporarily sealed. Perfusate exiting the lungs passed through the left atrium and onto an umbilical tape wick that was placed over the heart and mediastinal tissue to direct the effluent and to ease the collection of these samples. Finally, a piece of cellophane was placed over the lungs to minimize heat loss and evaporation. Steady-state fluxes of tracers were reached by 15 min after lavage or introduction of inhibitor (see below). Experiments lasted 75–90 min. Vascular samples were collected every 5–15 min and were analyzed for test solute concentrations. At the end of the experiment, as much instillate as possible was drawn from the lungs through the trachea using a syringe to determine final alveolar solute concentrations.
PS measures both the endothelial and epithelial barrier properties but predominantly reflects epithelial permeability because endothelial PS Na\textsubscript{su} is \(-1,000\) times greater than the epithelial PS. We did not directly measure the surface area in this preparation because it is technically quite difficult and, as indicated in the discussion section, the morphological studies of this model by The et al. (36) showed a decrease in capillary surface area at 60 h of hyperoxia, which should lead to underestimation of the change in permeability.

The isotope concentrations in all samples were determined by scintillation counting. \(^{22}\)Na\textsuperscript{+} activity was determined with a Packard Auto-Gamma 5650 gamma counter, and \(^{14}\)C activity was determined with a Packard 4530 Tri-Carb liquid scintillation counter. Corrections were made for \(^{22}\)Na\textsuperscript{+} energy wash over into the \(^{14}\)C channel. In addition, all alveolar samples were analyzed for fluorescence emitted at 520 nm when FITC-Dextran 150 was excited at 490 nm in a fluorimeter (SPF-500C; SL Amino). Representative vascular samples also were checked for FITC-Dextran 150 fluorescence, and we confirmed that none entered the perfusate even in the injured lungs.

Calculation of PS\textsubscript{Na\textsuperscript{+}} and PS\textsubscript{su}crose

PS\textsubscript{Na\textsuperscript{+}} and PS\textsubscript{su}crose were calculated based on Fick’s principle of diffusion. The equation used and the assumptions made have been previously discussed (30)

\[
J_S = PS \times \Delta C
\]  

where \(J_S\) is net tracer flux (counts·min \(^{-1}\)·cm\(^{-2}\)·s\(^{-1}\)), \(PS\) is in cm/s, and \(\Delta C\) is the tracer concentration difference between the alveolar and vascular volumes (cpm/cm\(^3\)). Because the perfusate entering the pulmonary vasculature contained no isotopic tracers and the vascular concentrations remained very low, \(\Delta C\) equals the alveolar concentration (\(C_A\)). \(J_S\) was calculated from each vascular sample as the product of vascular tracer concentration (\(C_V\)) and perfusion flow rate (\(Q\)). PS then was calculated as follows

\[
PS = \frac{[C_V(Q)]}{C_A}
\]  

During the course of every experiment, the concentrations of the alveolar test solution changed; \(^{22}\)Na\textsuperscript{+} and \(^{14}\)C\textsuperscript{+}sucrose always decreased, and FITC-Dextran 150 either slightly increased or decreased. Therefore, for each vascular sample, the \(C_V\) values of labeled solutes were recalculated based on 1) the total solute that had appeared in the perfusate up to that time and 2) any changes in alveolar fluid volume determined from FITC-Dextran 150 concentration changes. The final alveolar solute concentrations calculated in this manner were always within 5.0% of those measured in the final alveolar fluid sample.

Inhibition of Transcellular Na\textsuperscript{+} Transport via Na\textsuperscript{+} Channels

To quantitate a major component of the transcellular Na\textsuperscript{+} transport, either amiloride or benzamil was added to the perfusate to inhibit alveolar epithelial influx of Na\textsuperscript{+} through the apical Na\textsuperscript{+} channels, which account for the majority of Na\textsuperscript{+} entry into ATII cells (16, 19, 20). The inhibitable fraction is referred to as the transcellular component, with the explicit recognition that other noninhibitable apical Na\textsuperscript{+} entry mechanisms also contribute to the transcellular Na\textsuperscript{+} movement and, in our analysis, are included in the “paracellular” pathway data.

In these experiments, the lungs initially were perfused as described above (without glucose and inhibitors present in the instillate) for 45 min. At that time, a sample of alveolar instillate (\(-60\) µl) was withdrawn for analysis, and the perfusate was switched to an identical one that also contained 0.1 or 1 mM amiloride or 1 µM benzamil. Multiple amiloride concentrations and the more selective Na\textsuperscript{+} channel blocker benzamil were used because of different affinity coefficients for type II cell amiloride binding reported in the literature and the potential for high-dose amiloride to affect other transporters. With permeability coefficients of \(-10^{-7}\) cm/s, these agents can readily diffuse from the vascular to the alveolar compartment where they have access to the apical Na\textsuperscript{+} channels (2). At 0.1 mM, \(>99\%\) of Na\textsuperscript{+} movement through apical epithelial Na\textsuperscript{+} channels is blocked (9). The 1 mM concentration of amiloride also should inhibit \(>99\%\) of Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity (17). After the inhibitor was added, the experiment was continued for another 45 min (90 min total). PS values were therefore obtained both before and after inhibitors were added. The reported PS values are comprised of measurements made at 35, 40, and 45 min for the preinhibition data and 80, 85, and 90 min for the inhibition data. The three PS values were averaged to comprise the datum for one lung; the mean of these data points are reported in the RESULTS section. The three serial PS measurements (preinhibition or during inhibition) were never \(>6.6\%\) different from the reported means for the control animals and 4% for the hyperoxic animals.

In preliminary experiments in which inhibitors were not added, PS\textsubscript{su}crose values were measured for up to 150 min. The values always were stable after the first 30 min. PS\textsubscript{su}crose values at 90 min were slightly lower than values at 45 min, but the decrease from the initial 30 min was always \(<20\%\) in every individual rat. Removal and reinstillation of alveolar fluid after 45 min did not affect this stability.

Measurement of Na\textsuperscript{+} Flux Across the Pleural Surface

The solutes in the perfusate leaving the lung could originate from the pulmonary veins, the lymphatics, or the pleural surface of the lung. To assess the contribution from Na\textsuperscript{+} movement via the lymphatics or across the pleural surface, 16 experiments also were performed in which the left atrium was cannulated with polyethylene tubing to collect the vascular perfusate, and a wick was placed down the dorsal midline of the preparation to collect any fluid that originated from the pleural surface or lymphatics. In control (\(n = 4\)), day 3 recovery (\(n = 4\)), and day 7 recovery (\(n = 4\)) rat lungs, fluid flow and Na\textsuperscript{+} flux from the pleural surface were not detectable. In four lungs from exposed rats with no recovery, the Na\textsuperscript{+} flux from the pleural surface wick was nonexistent for the first 15 min of the experiment but then increased and remained constant for 75 min, averaging 2.53 ± 0.3% of the total Na\textsuperscript{+} flux. This rate did not change significantly after administration of Na\textsuperscript{+} transport inhibitors. We considered this pleural contribution to total PS Na\textsuperscript{+}, to be minor and did not correct for it.

Assessment of Na-K-ATPase Activity in Peripheral Lung Membrane

Membrane preparation. The liberation of ouabain-sensitive P\textsubscript{i} by membrane Na-K-ATPase was measured spectrophotometrically, as previously described (4). Peripheral lung membrane was prepared as previously described (25). Briefly, in anesthetized rats, the lungs were perfused with Ringer solution (see above), the heart and lungs were removed en bloc, and the pulmonary circulation was perfused again with Ringer solution followed by 2–3 lavages to total lung capacity with Ringer solution. Heart, mediastinum, and central airways were trimmed away, and the remaining peripheral lung
was chopped with a razor blade and homogenized in glass duncers containing 10 mL/g tissue of 250 mM sucrose, 25 mM imidazole, 1 mM EDTA, and 0.1 vol of protease inhibitor solution (25 μg/mL antipain, 1 μg/mL aprotinin, 0.5 μg/mL EDTA, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, 0.1 μg/mL soybean trypsin inhibitor, and 200 μM phenylmethylsulfonyl fluoride). Tissue was sonicated (Vibra Cell, output 4; 4 10-s bursts) on ice and was centrifuged at 200 g for 20 min in a tabletop centrifuge to remove unbroken cells and nuclei. The supernatant was ultracentrifuged (Beckman LS-65 centrifuge with SW41TI rotor) at 280,000–300,000 g for 2.5 h, and the resulting supernatant and pellet were frozen at −70°C. At the same time, lung tissue was prepared from a complete set of samples (normoxic, hyperoxic, and days 1, 3, and 7 of recovery) and was ultracentrifuged.

Na-K-ATPase activity assay. The Na+ pump membrane phosphatase (or hydrolytic) activity was determined on lung membranes under Vmax conditions by measuring the ouabain-inhibitable generation of Pi from excess ATP. Activity assays were performed on sets of samples from each time point (control, 60 h hyperoxia, and days 3 and 7 of recovery) on the same day, which was 1 day after the membrane preparations. For each assay, the results were normalized to the control sample because of day-to-day variability in the absolute values obtained. All reagents and solutions for the assay were prepared in phosphate-free glassware. The membrane pellets were resuspended in sucrose-imidazole-EDTA buffer with protease inhibitors (as above), sonicated, and assayed for protein content (Pierce, Rockford, IL). One hundred microliters of sample were separated into aliquots in triplicate to two sets of borosilicate tubes: one set containing 1 mM ouabain and one without ouabain. Eight hundred microliters of reaction mixture (25 μg/mL antipain, 1 μg/mL aprotinin, 0.5 mg/mL N-ethylmaleimide (NEM)) were added to all tubes and were incubated for 1 min at 37°C. Total volume was 1 mL ATP (Sigma Chemical; final concentration 4.4 mM) was added to initiate the reaction. The reaction continued for 15 min at 37°C and was terminated by removal of the samples to an ice bath followed by the addition of 50 μL of 100% trichloroacetic acid. One milliliter of color reagent (5% FeSO4 and 1% ammonium molybdate in 10 N H2SO4) was added, and the samples were incubated at 37°C for 2 min, followed by centrifugation at 1,800 revolutions/min (800 g) for 10 min in a tabletop centrifuge to pellet precipitated protein. Samples were analyzed with a spectrophotometer (Beckman DU-640) for P, at an absorbance of 740 nm and were compared with a standard curve prepared with K2HPO4. The raw data were normalized to milligrams of protein, micrograms of DNA, and milligrams of wet lung weight.

Polyacrylamide Gel Electrophoresis and Western Blotting

To assess changes in the antigenic amounts of Na-K-ATPase, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed on peripheral whole lung ultracentrifuge membrane pellets prepared as described in Membrane preparation. Protein separation was performed with a Bio-Rad Mini-PROTEAN II dual-slab kit, and blotting was performed with a Bio-Rad mini Trans-Blot Electrophoretic transfer kit, as previously described (25). Twenty to thirty micrograms of total protein in a 4 x 10 vol of sample buffer [50% H2O, 10% glycerol, 12.5% 0.5 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 6.8), 20% of 10% (wt/vol) SDS, 5% β-mercaptoethanol, and 2.5% of 0.05% (wt/vol) bromphenol blue] were loaded per lane. Samples that were to be subsequently probed for the α1-subunit (97-kDa molecular mass) and β-subunit (46-kDa molecular mass) of Na-K-ATPase were separated through 7.5 and 12% acrylamide gels, respectively. After separation and electrophoretic transfer, the nitrocellulose membranes were incubated with polyclonal antisera to α1- or β-subunits (UBI, Lake Placid, NY; 1:500 in Tris-buffered saline with 0.05% Tween (TBST)), washed three times with TBST, incubated with anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Promega, Madison, WI) or horseradish peroxidase (Amersham, Arlington Heights, IL; 1:7,500 in TBST), and washed again in TBST. The antigenic sites were visualized with an alkaline phosphatase reaction (protoblot; Promega) on nitrocellulose or with enhanced chemiluminescence detection (Amersham) on Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). Molecular masses were determined with rainbow or biotin-labeled markers (Amersham). Signal intensity was quantified by densitometry. Relative changes in the antigenic signal were determined by normalizing the integrated optical density (IOD) of bands from exposed or recovering membranes to the IOD of the band from nonexposed membranes on the same blot. Each blot was exposed several times to be sure that the densitometric signal was in a linear range of the film’s response. In some experiments, the membrane proteins were treated with endoglycosidase F (Boehringer Mannheim; 1 U/30 μg of membrane protein for 2 h at 37°C) before SDS-PAGE and Western blotting.

Statistical Analysis

The PS products shown in Fig. 1 were not normally distributed. Therefore, we analyzed these data using the nonparametric Kruskal-Wallis analysis of variance (ANOVA) by rank for multiple groups, with P < 0.05 considered significant. To determine which pairwise comparisons were significantly different, the Mann-Whitney U test was used. The level of significance was calculated as follows: 0.05 (desired P value) ÷ 6 (total number of pairwise comparisons) = 0.008; therefore, P ≤ 0.008 was considered significant. In Table 1, multigroup comparisons were performed with an ANOVA. Significant pairwise comparisons were done with Scheffe’s post hoc multiple comparison test, with P ≤ 0.05 considered significant.

To analyze for subgroup differences in the PS products of the hyperoxic animals (Fig. 2), the 95% confidence interval (CI) for the control PSsurfase values was determined, and the hyperoxic animals were divided into two groups: 1) those with PSsurfase values above the upper 95% CI for the control animals; and 2) those with PSsurfase values below the upper 95% CI for the control animals. The PSenh values (total, inhibitor insensitive, and inhibitor sensitive) for the control, hyperoxic high PSsurfase, and hyperoxic normal PSsurfase then were analyzed using ANOVA. Significant correlations (P ≤ 0.05) in Fig. 3 were determined with a t distribution.

For the Na-K-ATPase activity assays, P; production for tissue assayed at the same time was expressed as a percentage of the enzyme activity in normoxic lungs in that assay and was analyzed using ANOVA. For the Western blot analysis, the relative change in antigenic signal with hyperoxic exposure (exposed membrane IOD/unexposed membrane IOD) was analyzed using ANOVA. Unless otherwise noted, all values are reported as means ± SE.

RESULTS

Animals

A total of 86 rats were used in the perfused lung experiments, and 43 were used in the Na-K-ATPase activity and Western blotting experiments. Of the 91
rats exposed to hyperoxia, one died during exposure from apparent O_2 toxicity. The average body weight of the control and experimental animals before O_2 exposure was 191.7 ± 4.3 g. Because age, and therefore animal size, determine the severity of hyperoxic injury, these weights are the same as animals previously used in biochemical and morphological experiments (25, 36). Immediately after 60 h of hyperoxia, the rats weighed an average of 168.6 ± 2.4 g. By day 3 postexposure, average body weight was 205.9 ± 5.5 g. At day 7 postexposure, body weight averaged 231.3 ± 4.5 g. The number of animals in each group and subgroup in the perfused lung experiments is shown in Fig. 1 and Table 1. The number of animals used per group in the Na^+ pump assays is indicated in Fig. 4.

PS_{sucrose}

The PS_{sucrose} was used as a measure of epithelial tight junction integrity because sucrose only crosses the alveolar epithelium between cells. The PS_{sucrose} data for each of the four groups are shown in Fig. 1A. The mean values ± SE for each group were 6.71 ± 0.80 × 10^{-5}, 12.6 ± 1.6 × 10^{-5}, 8.17 ± 0.58 × 10^{-5}, and 8.41 ± 1.4 × 10^{-5} cm^3/s for control, 60 h hyperoxia, and days 3 and 7 of recovery from exposure, respectively. After 60 h of hyperoxia, mean PS_{sucrose} nearly doubled compared with the other three groups. However, due to the wide range of observed values, some were not different from control, and others were as much as four times greater than control; this increase was not statistically significant after correction for multiple comparisons (P = 0.029). The PS_{sucrose} values at days 3 and 7 of recovery were not significantly different from the control value (P > 0.008).

A number of possible reasons for the variable acute response of sucrose flux to hyperoxia at 60 h were examined. The animals with no change in sucrose flux did not occur in particular grouped batches of rats, were not associated with a specific colony at the sole vendor, were not housed in different rooms at the vendor or our animal facilities, were not correlated with initial weight, and did not have gross or microscopic evidence of prior lung injury. Viral cultures of selected rat lungs obtained at different times from this vendor also did not reveal pathogens.

PS$_{Na^+}$

Total PS$_{Na^+}$, measured in the absence of inhibitors, is shown in Fig. 1B and again showed considerable range for the acutely injured animals. The mean values ± SE were 2.36 ± 0.11 × 10^{-4}, 3.10 ± 0.16 × 10^{-4}, 2.73 ± 0.15 × 10^{-4}, and 2.54 ± 0.16 × 10^{-4} cm^3/s for the control, 60 h of hyperoxia, and days 3 and 7 of recovery groups, respectively. The mean total PS$_{Na^+}$ after 60 h of hyperoxia was significantly higher compared with each of the other three groups (P < 0.008). As we observed for PS_{sucrose}, some animals had no increase in the total PS$_{Na^+}$ values compared with control. The mean PS$_{Na^+}$ values at days 3 and 7 of recovery were not significantly different from the control value (P > 0.008). When glucose was substituted for sucrose in the instillate, there was no change in the PS$_{Na^+}$ values for any of the groups (data not shown).

Inhibition of PS$_{Na^+}$ with Benzamil or Amiloride

The total PS$_{Na^+}$ in the absence of any inhibitors is comprised of Na^+ flux through both paracellular and transcellular pathways. Transcellular Na^+ transport was estimated at this time with the use of benzamil or amiloride to block the majority of apical Na^+ entry and thus inhibit transcellular Na^+ transport (19, 20). We assumed that the residual PS$_{Na^+}$ in the presence of inhibitors ("inhibitor-insensitive" PS$_{Na^+}$) was constituted by Na^+ movement through paracellular pathways. The difference between total PS$_{Na^+}$ and inhibitor-insensitive PS$_{Na^+}$ was the "inhibitor-sensitive" PS$_{Na^+}$, which thus reflected transcellular Na^+ movement. Benzamil is a highly specific competitive inhibitor of the apical Na^+ channels in alveolar epithelial cells (20).
Because others previously used amiloride to inhibit Na⁺ transport in isolated, perfused lungs (1, 7, 10), we conducted a series of experiments using two concentrations of amiloride, 1 and 0.1 mM.

Table 1 lists the PS₉Na⁺ values from the lungs of all rats in which benazamil or amiloride was added during the second half of the experiment. The total number of animals shown in Table 1 is less than that in Fig. 1 due to the fact that there were six experiments (5 control and 1 60 h) in which no Na⁺ transport inhibitors were used. The PS data in column 1 of Table 1 are the values plotted in Fig. 1, which are divided into groups based on the subsequently received inhibitor. As expected, the total (no inhibitor) PS₉Na⁺ values within a given group (i.e., control, 60 h of hyperoxia, etc.) were not different before inhibitor administration. In all lungs (unexposed and exposed), benazamil and both concentrations of amiloride significantly inhibited Na⁺ transport (P < 0.05) as reported in column 2 of Table 1. In the control lungs, the degree of inhibition was not different among the three groups (P > 0.05), and inhibitor-sensitive Na⁺ transport (transcellular) PS₉Na⁺ was increased compared with control rat lungs; and transcellular PS₉Na⁺ in normal PS₉sucrose group was significantly decreased compared with control values.

### Table 1. PS₉Na⁺ and pulmonary artery perfusion pressure in the absence and presence of inhibitors

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<th>Total (n)</th>
<th>Inhibitor insensitive (paracellular)</th>
<th>Inhibitor sensitive (transcellular)</th>
<th>Mean % inhibitor sensitive flux</th>
<th>Pulmonary Artery Pressure, cmH₂O</th>
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<td>Control</td>
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<td>24.5 ± 0.2</td>
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<td>12.3 ± 0.1*</td>
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<td></td>
<td>10.3 ± 0.25</td>
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<tr>
<td></td>
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<td>6</td>
<td>23.4 ± 0.2</td>
<td>123.2 ± 0.1*</td>
<td>10.3 ± 0.1*</td>
</tr>
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<td>12.3 ± 0.98</td>
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<tr>
<td></td>
<td>1.0 mM Amiloride</td>
<td>10</td>
<td>25.4 ± 0.2</td>
<td>12.9 ± 0.1*</td>
<td>12.5 ± 0.2*</td>
</tr>
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<td></td>
<td></td>
<td>11.3 ± 1.36</td>
</tr>
<tr>
<td>Mean</td>
<td>10</td>
<td>23.6 ± 0.1</td>
<td>12.8 ± 0.0*</td>
<td>11.7 ± 0.0*</td>
<td>49.6</td>
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<td>60-h Hyperoxia</td>
<td>19</td>
<td>30.4 ± 0.2</td>
<td>19.7 ± 0.2*</td>
<td>10.7 ± 0.1*</td>
<td>35.2</td>
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<td>Benzamil</td>
<td>12</td>
<td>31.0 ± 0.3</td>
<td>21.0 ± 0.2*</td>
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<tr>
<td></td>
<td>1.0 mM Amiloride</td>
<td>10</td>
<td>32.0 ± 0.3</td>
<td>19.1 ± 0.2*</td>
<td>12.9 ± 0.2*</td>
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<td>12.4 ± 0.66</td>
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<tr>
<td>Mean</td>
<td>10</td>
<td>31.0 ± 0.1</td>
<td>19.9 ± 0.1*</td>
<td>11.0 ± 0.0*</td>
<td>35.5</td>
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<td>3-Day recovery</td>
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<td>28.7 ± 0.2</td>
<td>17.3 ± 0.5*</td>
<td>11.4 ± 0.3*</td>
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<td>8.0 ± 3.35</td>
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<tr>
<td></td>
<td>1.0 mM Amiloride</td>
<td>4</td>
<td>26.0 ± 0.2</td>
<td>12.2 ± 0.3*</td>
<td>12.2 ± 0.3*</td>
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<td>13.0 ± 0.35</td>
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<tr>
<td>Mean</td>
<td>4</td>
<td>27.3 ± 0.1</td>
<td>14.7 ± 0.1*</td>
<td>12.6 ± 0.1*</td>
<td>46.2</td>
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<tr>
<td>7-Day recovery</td>
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<td>3</td>
<td>26.6 ± 0.3</td>
<td>15.1 ± 0.1*</td>
<td>11.5 ± 0.3*</td>
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<td>9.3 ± 0.35</td>
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<tr>
<td></td>
<td>1.0 mM Amiloride</td>
<td>4</td>
<td>24.4 ± 0.5</td>
<td>12.2 ± 0.2*</td>
<td>12.2 ± 0.4*</td>
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<tr>
<td>Mean</td>
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<td>13.5 ± 0.1*</td>
<td>11.9 ± 0.1*</td>
<td>46.9</td>
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</table>

Values are means ± SE; n is no. of animals. * Different from total permeability × surface area product for Na⁺ transport (PSNa⁺) in the same group, P < 0.05.
transport accounted for 44.0, 49.2, and 50.2% of total P$i$ in the presence of 0.1 mM amiloride, 1 mM amiloride, and 1 µM benzamid, respectively. After the determination that the three Na$^+$ transport inhibition conditions were not different within a given group (i.e., control, 60 h of hyperoxia, etc.), the inhibitor data within a given hyperoxic treatment group were pooled and compared between groups. The combined data are shown in Table 1, rows 4, 8, 11, and 14, for each group. The inhibitor-insensitive PNa$^+$ increased immediately after hyperoxia, indicating increased Na$^+$ moving through paracellular pathways, an observation supported by the P$sucrose$ data. The increased inhibitor-insensitive paracellular movement of Na$^+$ persisted into recovery. However, the mean inhibitor-sensitive PNa$^+$ at 60 h of hyperoxia was not significantly different from the unexposed control data or from the day 3 and day 7 recovery data. These mean data agree with the data of Zheng et al. (41) but differ from those of Olivera et al. (29) with much larger rats.

Finally, in columns 5 and 6 of Table 1, the perfusion pressures were not different between the control, hyperoxic, and recovering lungs and did not change significantly after inhibitor administration. Because Q values were essentially the same in all experiments (~7.0 ml/min), the lack of change in pulmonary artery pressure suggests that there were no significant differences in the lung volume or surface area being perfused between the different groups of animals or in the same lung with or without inhibitor.

Subgroup Analysis of PNa$^+$

Although the mean P$sucrose$ and transcellular Na$^+$ resorption were normal with hyperoxia, hyperoxia resulted in a wide spectrum of severity of epithelial injury, which suggested there were subgroups of response to hyperoxia. Therefore, we sought to determine whether Na$^+$ flux was differentially affected in the hyperoxic animals with P$sucrose$ values greater than control compared with those with P$sucrose$ values not different from control. To accomplish this, the data from hyperoxia-exposed animals were divided into two groups as described in Statistical Analysis. Nineteen of the hyperoxia-exposed lungs had P$sucrose$ values above the upper 95% CI of the control P$sucrose$ values, and twenty-three had P$sucrose$ values that were within the control P$sucrose$ 95% CI. The three Na$^+$ flux measurements (total, inhibitor insensitive paracellular, and inhibitor sensitive “transcellular”) were analyzed based on the subgroup assignment of the P$sucrose$ (Fig. 2). The hyperoxia-exposed lungs with high P$sucrose$ had significantly increased total PNa$^+$ and paracellular PNa$^+$, whereas the transcellular PNa$^+$ was not significantly altered from control values. However, the hyperoxia-exposed lungs with normal P$sucrose$ had significantly decreased transcellular PNa$^+$, whereas the total and paracellular PNa$^+$ were not different from control.

To further analyze the relationship between P$sucrose$ and transcellular Na$^+$ movement at 60 h of hyperoxia, the inhibitor-sensitive PNa$^+$ was plotted vs. the P$sucrose$ (Fig. 3). There was a strong statistically significant positive correlation between the sucrose flux and the transcellular Na$^+$ resorption (r = 0.89; P < 0.05). The results were similar with statistically significant positive correlations when each inhibitor and concentration results were similar with statistically significant positive correlations when each inhibitor and concentration was examined separately. These data are consistent with Fig. 2 because the rats with no increase in P$sucrose$ are in the bottom left corner of Fig. 3, with inhibitable PNa$^+$ below the control values, whereas the rats with elevated P$sucrose$ had increased inhibitable PNa$^+$ and constitute the top right data points.

Peripheral Lung Membrane Na-K-ATPase Activity

We measured the V$_{max}$ for Na$^+$ pump phosphatase activity in the peripheral lung membranes to determine whether our previously reported increases in mRNA at 60 h of hyperoxia and, by day 1 of recovery, α$_1$-antigenic protein (26) resulted in increased enzyme activity in the lung membrane. Na$^+$ pump V$_{max}$ activity significantly decreased (P < 0.05) immediately after hyperoxic exposure but began to recover by day 1 after exposure and was equal to or greater than control by day 3 of recovery (Fig. 4). Activity normalized to DNA content and wet lung weight showed a similar profile (data not shown). Activity in the cytosolic fraction was not detectable.

Western Blot Analysis

Given the decreased Na$^+$ pump phosphatase activity that was observed and the prior observation of stable α$_1$-antigenic protein at 60 h of hyperoxia (25), we...
examined whether the amount of the β1-subunit protein was altered by hyperoxia. The Western blots revealed immunostaining at the anticipated molecular masses for the α-subunit and several bands for the β1-subunit. Several β1-subunit bands were seen with both the UBI antibody and other anti-β1-monomonal and polyclonal antibodies (gifts of M. J. Caplan, Yale School of Medicine). Endoglycosidase F preincubation collapsed these bands into a lower molecular mass single band (data not shown). This suggested that there are several glycosylated forms of the β1-subunit in the lung.

Previously, we found that α1-subunit antigenic protein increased by day 1 of recovery after 60 h of 100% O2 or after 1 day of recovery, but levels of the β1-subunit were not measured in that study (25). In the present study, some lungs had IOD levels of the β-subunit protein that increased acutely or 1 day after exposure. However, mean IOD was not significantly increased (P > 0.05; Fig. 5). We reconfirmed that the amounts α1-protein levels increased by 1 day after exposure (data not shown).

**DISCUSSION**

The aim of the present study was to determine the effect of hyperoxic injury on 1) unidirectional transcellular and paracellular Na\(^+\) fluxes from the alveolar lumen to the vasculature in isolated, perfused rat lungs and 2) peripheral lung Na-K-ATPase activity and protein expression. We found that 60 h of hyperoxic exposure increased both the sucrose and total Na\(^+\) fluxes in approximately one-half of the lungs of 180- to 200-g rats. When the total Na\(^+\) flux increased, it was due to increased paracellular Na\(^+\) flux with no change in the mean transcellular (e.g., active or inhibitable) Na\(^+\) flux. PS\(_{\text{sucrose}}\) and total PS\(_{\text{Na}}\) returned to normal by day 3 of recovery in room air, indicating that alveolar epithelial integrity and normal Na\(^+\) transport were restored, even though prior morphometric studies indicate that this is the time of maximum type I cell loss, type II cell proliferation, and inflammation (38). Na-K-ATPase \(V_{\text{max}}\) activity in peripheral lung membranes decreased immediately after exposure but also returned to values similar to control by day 3 of recovery. The amounts of peripheral lung β1-antigenic protein were not significantly changed.

Three major observations from these experiments merit highlighting. First, the Na\(^+\) pump mRNA, antigenic protein, hydrolytic activity, and unidirectional Na\(^+\) resorption did not change in parallel at 60 h of hyperoxia. Second, there was significant heterogeneity in the degree of hyperoxic injury in individual rats. Third, the hyperoxia differentially affected active Na\(^+\) resorption depending on the severity of injury; with normal sucrose flux, the active Na\(^+\) flux was decreased while the active Na\(^+\) flux was maintained in rats with increased sucrose flux. This leads us to speculate that, in mildly injured animals, oxidation decreases transcellular Na\(^+\) resorption and maximal pump function. When injury is moderately severe, Na\(^+\) resorption and Na-K-ATPase activity of ATII cells are maintained through homeostatic mechanisms. When oxidative stress is severe, as in the larger rats studied by Olivera et al. (29), the damaging and inhibitory effects overwhelm the compensatory mechanisms and active transport decreases.

**Sucrose and Na\(^+\) Fluxes in Normal and Hyperoxic Lungs**

Total unidirectional Na\(^+\) flux in uninjured isolated, perfused rat lungs has been measured in several laboratories (1, 5, 6, 12, 33), and our control lung values are in a similar range. PS\(_{\text{sucrose}}\) was measured to determine paracellular permeability in each experimental group. Our control data are similar to values previously reported (1, 6, 10, 12), and other investigators also found that hyperoxia and other forms of lung injury increased alveolar PS\(_{\text{sucrose}}\) (26, 29, 30, 38, 41).
We were somewhat surprised by the degree of heterogeneity in PS responses, but this is not the first report describing the heterogeneous response of alveolar epithelial transport to hyperoxia. On review of our prior data, there was considerable variability in sucrose and albumin PS values in hamsters acutely exposed to hyperoxia (38). More recently, two distinct responses of alveolar fluid resorption were observed in rats after chronic (39) and acute hyperoxia (41); in the latter report there were variable responses in severity of injury, but the lungs with greater damage were excluded from analysis. There are three additional reasons why we believe our observed heterogeneity is a consequence of hyperoxic exposure and not due to technical artifacts inherent in the isolated lung preparation. First, neither $PS_{\text{sucrose}}$ nor $PS_{Na^+}$ heterogeneity was present in the unexposed lungs nor was any evident by day 3 of recovery in room air (Fig. 1). Second, we averaged three serial PS measurements to obtain mean PS values. The individual measurements were highly reproducible in both unexposed and exposed lungs and were not statistically different from one another within a set (i.e., preinhibition vs. inhibition), and they differed by <10% from the mean value. Third, the presence of heterogeneity withstood rigorous statistical subgroup analysis. Given these observations, we believe that the effect of hyperoxia on alveolar epithelium transport and fluid clearance mechanisms is truly heterogeneous, but the explanation is unknown at present.

Barrier function recovered rapidly in our model with normal $PS_{\text{sucrose}}$ and total $PS_{Na^+}$ at day 3 of recovery. The paracellular $PS_{Na^+}$ was still mildly increased at that time but was normal at day 7 of recovery. This recovery was more rapid than that observed by Olivera et al. (29), who studied older rats exposed for 64 h and presumably had more severe injury but was similar to what has been seen in hamsters (31) and rabbits (14). Direct comparisons between species should be made with caution because it is known that different species have considerable differences in their tolerance of hyperoxia.

Transcellular $Na^+$ transport, presumed to be active, previously has been estimated in isolated, perfused lungs in one of two ways: inhibition of $^{22}Na^+$ transport by amiloride or ouabain (1, 6, 12) or measurement of alveolar volume changes (1, 33). The latter method assumes that active $Na^+$ transport results in osmotic water clearance from the air spaces and is the only reason for transepithelial water movement. We used the former method because after hyperoxia there could be movement of water into or out of the alveolar space unrelated to active $Na^+$ transport. In our hands, inhibition of transcellular $Na^+$ transport with ouabain was unsuccessful because concentrations of ouabain that inhibited the $Na^+$ pump also led to massive weight gain of the isolated lung so that $PS_{Na^+}$ could not be reliably measured. Although some investigators have effectively reduced $Na^+$ flux in isolated, perfused lungs with ouabain, others also have had difficulty finding a dose that consistently inhibits transport while maintaining a viable preparation (1). In the presence of the apical $Na^+$ channel blockers benzamil and amiloride, Kim et al. (16) reported a strong correlation between the reduction in $^{22}Na^+$ permeability and the short-circuit current measured simultaneously across alveolar epithelial cell monolayers treated with amiloride or ouabain. This indicated that amiloride or benzamil effectively inhibit transepithelial active $Na^+$ transport through the inhibition of apical $Na^+$ channels, albeit inhibition may not be as complete as in the presence of ouabain. Because high concentrations of amiloride have undesirable, nonspecific side effects (i.e., inhibition of Na-K-ATPase), we used two concentrations of amiloride (0.1 and 1.0 mM) as well as benzamil to inhibit $Na^+$ transport through $Na^+$ channels. The inhibitory response was similar for all agents: either benzamil or amiloride inhibited 44–50% of total $Na^+$ transport in the control animals, similar to the 40–60% “active” $Na^+$ transport found in isolated lungs by other investigators using either vascular or air space amiloride (1, 12, 34), and consistent with the ~60% inhibition found in studies of $Na^+$ transport in ATII cells (11, 20). Although this strategy might have distinguished the presence of high or low amiloride affinity $Na^+$ channels (19, 20, 32), the results indicated that amiloride was acting specifically on $Na^+$ channels, but either two classes of amiloride-sensitive $Na^+$ channels were not present or this method was not sensitive enough to detect both classes of channels.

After hyperoxia, mean total and paracellular $Na^+$ flux increased, whereas transcellular $Na^+$ flux was unchanged (Table 1). However, subgroup analysis of our data indicated that hyperoxic lungs with normal $PS_{\text{sucrose}}$ had reduced transcellular $PS_{Na^+}$, whereas the hyperoxic lungs with high $PS_{\text{sucrose}}$ maintained control levels of transcellular $Na^+$ transport. There is conflicting information in the literature regarding the effects of exposure to 100% $O_2$ on $Na^+$ transport in the rat lung. Olivera et al. (29) studied paracellular solute flux, $Na^+$ transport, and fluid reabsorption in isolated, perfused rat lungs after exposure to 64 h of 100% $O_2$. Total unidirectional fluxes of $^{22}Na^+$ and $^{3}H$mannitol out of the air space increased, but it was concluded that active $Na^+$ transport decreased based on a decrease in alveolar water clearance. Thus their total $Na^+$ and saccharide flux data are consistent with ours, but the active $Na^+$ transport was decreased despite more severe injury. Zheng et al. (41) found little change in active $Na^+$ resorption after acute hyperoxic injury, but they excluded rats with severe injury. With this proviso, their results are very similar to the results reported herein.

In addition to $Na^+$ channels, three other potential apical $Na^+$ entry pathways could contribute to the transcellular $Na^+$ flux (19, 21, 34): 1) $Na^+$-glucose cotransporter, 2) $Na^+$-amino acid cotransporter, and 3) $Na^+/H^+$ antiporter. Because $Na^+$ channels transport $Na^+$ at a much higher rate than cotransporters (10⁶ to 10⁷ ions·s⁻¹·molecule⁻¹ vs. 10³ to 10⁴ ions·s⁻¹·molecule⁻¹, respectively), the primary apical entry pathway for $Na^+$ likely is the $Na^+$ channel. It is unlikely that the
cotransporters contributed significantly to alveolar-to-vascular Na$^+$ flux in our experiments because no added glucose or amino acids were present in the alveolar instillate. When the sucrose in the alveolar instillate was replaced with glucose, no change in Na$^+$ flux was observed in any of the four groups. This does not completely exclude some contribution from Na$^+$-glucose cotransport because specific inhibitors were not used and there could be back leakage of glucose into the alveolar space that then is taken up, but a major contribution is unlikely. The Na$^+$/H$^+$ antiporter is unlikely to have contributed because it is on the basolateral membrane of type II cells, the perfusion pH was 7.4, and it requires intracellular acidosis to become active. Furthermore, it should have been differentially inhibited at the higher amiloride concentration, but the values were not different from those observed with the more specific Na$^+$ channel inhibitor benzamil.

Limitations and Assumptions of the Isolated, Perfused Lung Model

Epithelial solute transport was expressed as the PS product. Although permeability and surface area were not individually measured, the differences in the PS products between the experimental groups likely reflects changes in alveolar epithelial permeability rather than surface area. After hyperoxia, PS$_{\text{sucrose}}$ in some lungs increased as high as four times that seen in control lungs. Although it is possible that differences in surface area could account for some of this increase, it is improbable that there was a fourfold increase in perfused surface area in these lungs. Two lines of evidence suggest that surface area was similar in all lungs: 1) alveolar filling was visualized to be complete in all lungs, and 2) pulmonary artery perfusion pressure in the face of constant flow was not different in the experiments with high PS values compared with those with low or normal PS values. If surface area did change as a result of hyperoxic injury, it most likely would decrease due to local endothelial damage; reduction or loss of blood flow to a given region of alveoli would increase pulmonary artery pressure, but this was not detected. Moreover, morphometric studies of this injury model and this age rat did not demonstrate increases in the alveolar capillary surface area (36). Finally, in the less severely injured animals, the PS products for sucrose and Na$^+$ did not change in parallel, which would be expected with changes in surface area. Therefore, our measured alterations in the PS products likely represent changes in permeability, but we may have underestimated the actual permeability increase in the injured lungs due to minor reductions in surface area.

Na-K-ATPase Activity and Protein Expression

Mounting evidence indicates that oxidants can alter the function of membrane proteins involved in Na$^+$ transport in several types of cells and tissues. Oxidants can either decrease (15, 29) or increase (8, 24) Na-K-ATPase activity. ATII cells isolated from rats exposed to 100% O$_2$ for 60 h (3) or 64 h (29) had decreased Na-K-ATPase hydrolytic $V_{\text{max}}$. However, ATII cells isolated from rats exposed to 85% O$_2$ for 7 days had both increased Na-K-ATPase activity (28) and Na$^+$ channel function (13). ATII cell Na$^+$ channel function and expression were further upregulated by subsequent in vivo exposure to 100% O$_2$ for 60 h after the initial exposure to 85% O$_2$ for 7 days (40). In the current studies, peripheral lung Na-K-ATPase $V_{\text{max}}$ activity decreased immediately after 100% O$_2$ exposure. In these lungs, the Na-K-ATPase activity data are similar to the isolated, perfused lung Na$^+$ transport data from exposed, no-recovery animals with normal PS$_{\text{sucrose}}$ (Fig. 2) that had diminished inhibitor-sensitive Na$^+$ resorption. By day 3 of recovery in room air, Na-K-ATPase activity increased to levels greater than control (Fig. 4) despite normal PS$_{\text{sucrose}}$ in the isolated, perfused lungs at this same time point (Fig. 1). The differences between the changes in Na$^+$ pump $V_{\text{max}}$ and the inhibitable PS$_{\text{sucrose}}$ are not inconsistent. The activity assay measured the Na-K-ATPase $V_{\text{max}}$, whereas the inhibitable PS$_{\text{sucrose}}$ is influenced by many factors including apical Na$^+$ entry and the percentage of maximal activity at which the pump is operating in situ. An increase in the maximal activity of Na-K-ATPase will not necessarily increase PS$_{\text{sucrose}}$ if 1) Na-K-ATPase does not operate at $V_{\text{max}}$ in vivo and/or 2) there are no corresponding increases in apical Na$^+$ entry.

There is precedence for our finding that Na$^+$ pump mRNA and enzyme activity respond differently to hyperoxia. During exposure of adult rats to >95% O$_2$, lung manganese-containing superoxide dismutase (Mn-SOD) activity fell ~50% despite a threefold increase in Mn-SOD mRNA concentration (5). The fall in Mn-SOD activity was due, at least partially, to oxidation of thiol moieties. The measurement of Na$^+$ pump activity in lung membranes may not accurately reflect the in vivo Na$^+$ pump activity in intact alveolar epithelial cells because the normal intracellular milieu was not present. Alternatively, changes in the $V_{\text{max}}$ of Na-K-ATPase may not be physiologically significant because in most cells Na-K-ATPase operates at less than maximal capacity.

Our results demonstrate differential changes in the levels of the antigenic $\alpha_1$- and $\beta$-subunit membrane proteins during hyperoxia, with increased $\alpha_1$-subunit and unchanged $\beta$-subunit protein early in recovery. Because the mRNA levels for both subunits are elevated, the protein differences could arise from differential effects of hyperoxia on either protein synthesis or degradation of the subunits. Both possibilities may occur with hyperoxia and oxidant stress (5, 18, 37). Our data do not allow us to determine whether either or both occurred in our system.

The changes in peripheral lung Na-K-ATPase reported here show considerable similarity to the Na-K-ATPase changes observed in ATII cells isolated from rats exposed to hyperoxia for 60 h (4). In the hyperoxic ATII cells, Na$^+$ pump mRNA levels were increased, the amount of $\beta_1$-subunit antigenic protein was unchanged, and the $V_{\text{max}}$ was decreased. In contrast, the amount of...
α₂-subunit was decreased in the ATII cells, whereas the Na⁺ pump activity of the intact ATII cells was unchanged. The Na⁺ pump activity of the intact ATII cells correlated best with the amounts of the β-subunit antigenic protein. The in vivo data presented here also support the idea that the β-subunit may be rate limiting in the formation of functional Na-K-ATPase molecules (27) because, early in recovery, Na⁺ transport and Na-K-ATPase activity were unchanged or were decreased while the α-subunit protein was increased (25) and β-subunit protein was unchanged.

In conclusion, the effects of acute hyperoxia on alveolar Na⁺ resorption and Na-K-ATPase are complex. The lack of parallel changes in Na⁺ pump mRNA, protein and enzyme activity, and active Na⁺ resorption likely represents the overlaying of multiple discrete effects of oxidant stress on the Na⁺ pump. This complexity is not surprising given the multiple ways that this enzyme is regulated and the variety of effects of oxidants on cell function (e.g., increased transcription of selected genes, oxidative inhibition of protein function, altered protein synthesis). A working model that is compatible with all current data is that oxidants have a biphasic effect on active Na⁺ transport. In mildly injured animals, oxidation decreases transcellular Na⁺ resorption and maximal pump function. As yet undefined homeostatic mechanisms maintain active Na⁺ resorption and lung Na-K-ATPase activity with moderate oxidant stress. When this stress is severe, as in the injury model of Olivera et al. (29), the damaging and inhibitory effects overwhelm the compensatory mechanisms. This speculative model requires further testing, and future studies need to define the triggers and mechanisms for homeostasis.

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