DeCREASED EXPRESSION OF BOTH THE $\alpha_1$- AND $\alpha_2$-SUBUNITs OF THE Na-K-ATPase REDUCES MAXIMAL ALVEOLAR EPITHELIAL FLUID CLEARANCE

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Looney, Mark R., Claudio Sartori, Santanu Chakraborty, Paul F. James, Jerry B. Lingrel, and Michael A. Matthay. Decreased expression of both the $\alpha_1$- and $\alpha_2$-subunits of the Na-K-ATPase reduces maximal alveolar epithelial fluid clearance. Am J Physiol Lung Cell Mol Physiol 289:L104–L110, 2005.—Impaired epithelial sodium channel function predisposes to delayed resorption of pulmonary edema and more severe experimental lung injury, whereas even a small fraction of the normal Na-K-ATPase activity is thought to be sufficient to maintain normal ion transport. However, direct proof is lacking. Therefore, we studied baseline and cAMP-stimulated alveolar fluid clearance (AFC) in mice with a 50% decrease in lung protein expression of the $\alpha_1$- and/or $\alpha_2$-subunit of the Na-K-ATPase. There was no difference in basal and stimulated AFC in $\alpha_1^{+/+}$ or $\alpha_2^{+/+}$ mice compared with wild-type littermates. Also, the compound heterozygous mice ($\alpha_1^{+/+}/\alpha_2^{+/+}$) had normal basal AFC. However, the combined $\alpha_1^{+/+}/\alpha_2^{+/+}$ mice showed a significant decrease in cAMP-stimulated AFC compared with wild-type littermates (11.1 ± 1.0 vs. 14.9 ± 1.8%/30 min, $P < 0.001$). When exposed to 96 h of >95% hyperoxia, the decrease in stimulated AFC in the $\alpha_1^{+/+}/\alpha_2^{+/+}$ mice was not associated with more lung edema compared with wild-type littermates (lung wet-to-dry weight ratio 6.6 ± 0.9 vs. 5.9 ± 1.1, respectively; $P =$ not significant). Thus a 50% decrease in protein expression of the $\alpha_1$- or $\alpha_2$-subunits of the Na-K-ATPase does not impair basal or stimulated AFC. However, a 50% protein reduction in both the $\alpha_1$- and $\alpha_2$-subunits of the Na-K-ATPase produces a submaximal stimulated AFC, suggesting a synergistic role for $\alpha_1$- and $\alpha_2$-subunits in cAMP-dependent alveolar epithelial fluid clearance.

PULMONARY EDEMA RESULTS FROM an imbalance between forces that cause alveolar flooding and the biological mechanisms responsible for the clearance of alveolar fluid from the distal air spaces into the pulmonary interstitium, where the edema fluid can be removed by the lung lymphatics and the pulmonary circulation. Vectorial sodium transport across the alveolar epithelium creates the osmotic gradient leading to water reabsorption from the air spaces of the lung. Sodium enters the apical membranes of alveolar epithelial cells primarily through amiloride-sensitive sodium channels (predominantly epithelial sodium channels [ENaC]) and is then transported across the basolateral membrane into the interstitium by the ouabain-inhibitable Na-K-ATPase (13, 17, 18, 26, 29).

Recovery from pulmonary edema depends on preserved active salt and water transport from the distal air spaces of the lung (18, 19, 31), and it is well known that stimulated activity of the ENaC and the Na-K-ATPase increases vectorial salt and water transport across the alveolar epithelium and can reduce susceptibility to experimental acute lung injury (17, 18, 27, 29). Furthermore, impaired ENaC function causes a predisposition to delayed resorption of pulmonary edema and more severe experimental lung injury (6, 22), whereas, in parallel to what is observed in other organs, even a small fraction of the normal Na-K-ATPase activity is thought to be sufficient to maintain normal ion transport (16, 32). However, this possibility has not been experimentally tested in the lungs.

In alveolar epithelial cells, there are two $\alpha$-subunits and one $\beta$-subunit of the Na-K-ATPase expressed. The $\alpha$-subunits ($\alpha_1$ and $\alpha_2$) have catalytic function and are ouabain inhibitable, whereas the $\beta$-subunit appears to have chaperone function (29). We reasoned that if the Na-K-ATPase plays a limiting role in vectorial ion transport in the lung then impaired expression of the $\alpha$-subunits in the lung might reduce basal and cAMP-stimulated alveolar fluid clearance (AFC) and cause a predisposition to more severe pulmonary edema in the setting of experimental lung injury. To test this hypothesis, we first measured basal and cAMP-stimulated AFC in mice with a 50% reduced protein expression of either the $\alpha_1$-subunit or the $\alpha_2$-subunit. We then measured basal and cAMP-stimulated AFC in the presence of a combined reduction of both the $\alpha_1$- and $\alpha_2$-subunits of Na-K-ATPase in the lung. Finally, we tested whether the mice with reduced expression of one or both $\alpha$-subunits would develop more severe pulmonary edema in a standard model of severe hyperoxia lung injury.

MATERIALS AND METHODS

Animals and Genotypes

We used 12- to 15-wk-old mice for all studies. Heterozygous mice containing only one copy of either the Na-K-ATPase $\alpha_1$-isoform gene ($\alpha_1^{+/+}$) or one copy of the Na-K-ATPase $\alpha_2$-isoform gene ($\alpha_2^{+/+}$) were generated by gene-targeting methods as described previously (14). We generated compound heterozygous mice ($\alpha_1^{+/+}/\alpha_2^{+/+}$) containing only one copy of each of the $\alpha_1$- and $\alpha_2$-Na-K-ATPase subunit genes by breeding $\alpha_1^{+/+}$ mice to $\alpha_2^{+/+}$ mice. It was necessary to use mice heterozygous for the Na-K-ATPase $\alpha$-isoform since homozygous deletion of either the $\alpha_1$- or the $\alpha_2$-isoform is incompatible with life (2, 21). Genomic DNA was extracted from mice tails and

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used to identify genotypes by Southern blot and/or PCR analysis as described (14). The mice were housed in air-filtered, temperature-controlled units (20 ± 2°C) and had food and water ad libitum. All procedures were approved by the University of California, San Francisco committee on animal research. All measurements were carried out by an investigator who was blinded to the mouse genotype.

**Preparation of Crude Microsomes**

For each micropsamal preparation, 10 whole lungs from each genotype (α1+/−, α2+/−, α1+/−/α2+/−, and the corresponding wild types) were homogenized on ice with two 15-s bursts of a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA) in 7 ml of homogenization buffer [250 mM sucrose, 30 mM imidazole (pH 7.5), 1 mM EDTA]. The homogenates were centrifuged at 4,000 g for 15 min at 4°C, and the supernatants were saved. The pellets were then homogenized again in 5 ml of homogenization buffer and centrifuged at 4,000 g for 15 min at 4°C, and the supernatants were combined. The combined supernatants were then centrifuged at 100,000 g for 60 min at 4°C. The pellets were resuspended in 10 ml of 30 mM imidazole (pH 7.5)/1 mM EDTA buffer and centrifuged at 100,000 g for 60 min. The resulting pellets were resuspended in a small volume (200–500 μl) of imidazole-EDTA buffer. Protein concentrations were estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Microsomal preparations were stored at −80°C until used for Western blot analysis.

**Quantification of Na-K-ATPase Isoform Levels**

Western blot analysis was used to quantify protein levels of the Na-K-ATPase α1-isoforms in whole microsomes. Microsomal proteins were separated by SDS-PAGE as described (14). Protein samples were incubated for 30 min at 37°C in sample buffer containing 50 mM Tris (pH 6.9), 5% SDS, 1% 2-mercaptoethanol (BME), and 10% glycerol and then electophoresed through 10% polyacrylamide gels. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) overnight at 4°C. Membranes were blocked with 1% Blocking Reagent (Roche Diagnostics, Indianapolis, IN) in TBS [5 mM Tris (pH 7.4), 150 mM NaCl] for a minimum of 2 h at room temperature or overnight at 4°C. The membranes were then incubated in TBST (TBS with 0.5% Tween 20) containing individual primary antibodies overnight at 4°C as follows: α1-isoform-specific monoclonal α1F, 1:1,000 (University of Iowa Developmental Hybridoma Bank, Iowa City, IA); affinity purified α1-isoform-specific polyclonal antisera α1ZR2, 0.5 μg/ml (see below for details concerning generation and specificity determination). The membranes were incubated for 2 h at room temperature in TBST containing the appropriate secondary antibodies as follows: blots incubated with the α1-isoform-specific monoclonal α1F were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:40,000, whereas blots incubated with the affinity purified α1-isoform-specific polyclonal antisera α1ZR2–2 were incubated with peroxidase-conjugated goat anti-rabbit IgG (Calbiochem, La Jolla, CA) at a dilution of 1:20,000. Na-K-ATPase α1-immunoreactivity was visualized after incubation of the membranes with enhanced chemiluminescent substrate (Amersham Biosciences, Piscataway, NJ) and then scanning on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) in blue fluorescence mode. Na-K-ATPase α2-immunoreactivity was visualized using Kodak BioMax MR X-ray film and the enhanced chemiluminescence system (Amersham Life Science) following the manufacturer’s recommendations. Signal intensities were quantified with ImageQuant 5.0 software (Molecular Dynamics). Multiple protein amounts were analyzed on each blot, and multiple exposures of the blots visualized with the ECL substrate were obtained to ensure linearity of signal intensities.

**Polyclonal Antibody Production**

A polyclonal antibody to the mouse Na-K-ATPase α2-isoform, designated α2ZR2, was produced in rabbits at Zymed Laboratories. A 15-aminoc acid peptide corresponding to amino acids 7–21 (REY-SPATAAENGGG) of the deduced mouse cDNA sequence was used as an antigen for polyclonal antibody production. Sera from injected rabbits were affinity-purified with a SulfoLink Kit column (Pierce) containing a 6% cross-linked agarose support conjugated to the antigenic peptide. The concentration of purified α2ZR2 antibody was quantified spectrophotometrically at 280 nm.

**Determination of α2 (α2ZR2)-Antibody Specificity**

Specificity of α2ZR2 for the mouse Na-K-ATPase α2-isoform was determined by mouse multissue Western blot. Microsome preparation, SDS-PAGE, and Western blotting were performed as described previously (14, 33).

**Cell microsomes.** In brief, cells [wild-type mouse L cells (MLC) and HeLa cells and HeLa cells expressing the mouse α1- and mouse α2-isoforms of Na-K-ATPase] were harvested from near-confluent 150-mm culture dishes by scraping in 0.25 M sucrose. The harvested cells were then pelleted by centrifugation, and cell pellets were stored in homogenization buffer (0.25 M sucrose, 1 mM EDTA, and 30 mM imidazole) at −80°C until used. Microsomal proteins were prepared as follows: the frozen cell pellets were thawed, and the homogenization buffer was removed. Seven milliliters of cell lysis buffer (1 mM NaHCO3, 2 mM CaCl2, 5 mM MgCl2) were added to the pellets, and the cells were resuspended by vortexing and then placed on ice for 30 min. The cells were homogenized with a glass/double homogenizer and then centrifuged at 6,800 g for 15 min at 4°C. The supernatant was transferred to a polyallomer centrifuge tube (Beckman) and placed on ice. The pellet was resuspended in 5 ml of lysis buffer and then rehomogenized and centrifuged as above, and the supernatants were combined. The combined supernatants were centrifuged for 1 h at 27,000 g at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 ml of imidazole/EDTA buffer (30 mM imidazole/1 mM EDTA, pH 7.5) and centrifuged as above for 1 h at 27,000 g at 4°C. The supernatant was discarded, and the pellet was resuspended in 100 μl of imidazole/EDTA buffer and stored at −80°C until used for Western blotting.

**Western blot.** Protein concentrations of the microsomal preparations were estimated with the Pierce BCA protein assay kit. Microsomal proteins (25 μg from cultured cells, 45 μg from heart and lung, and 10 μg from brain and kidney) were incubated for 1 h at 37°C in 2× Western sample buffer [50 mM Tris (pH 6.8), 5% SDS, 1% BME, 10% glycerol, and bromphenol blue]. Samples were electrophoretically separated through a 10% polyacrylamide gel and then electrophoretically transferred to PVDF membrane (Millipore) overnight at 4°C in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). The next day, the membranes were blocked for 2 h in phosphate-buffered saline (PBS) containing 5% nonfat dry milk (NFDM) at room temperature. The blots were then incubated overnight at 4°C with the following primary antibody dilutions in PBS containing 5% NFDM: 1:1,000 of the α1-isoform-specific monoclonal antibody α1F; 0.5 μg/ml of the α1-isoform-specific polyclonal antibody α1ZR2; and 1:1,000 of α1-isoform-specific monoclonal antibody XVIP9-G10 (Affinity Bioreagents). The immunospecificity of α1F and the XVIP9-G10 have been previously demonstrated (1, 30), and their utility for detecting individual mouse isoforms has been documented (14). After incubation with the primary antibody, the blots were washed three times for 15 min each with PBS/1% NFDM containing 0.1% Tween 20 and then incubated with either horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG secondary antibody (Calbiochem) in PBS/5% NFDM for 2–2.5 h. The blots were then washed six times with PBS/1% NFDM/0.1% Tween 20 for 5 min each. The specific immunoreactive proteins were detected using an enhanced chemiluminescent substrate for detection of
HRP (Pierce) and exposing the blots to scientific imaging films. The films were developed with a Kodak M35AX-OMAT processor.

**AFC Measurements**

**Preparation of instillate.** The instillate consisted of 5 g/100 ml of BSA (Sigma Chemical) in Ringer lactate adjusted to 330 mosmol/kgH2O with NaCl to be isosmolar with mouse plasma and 0.1 μCi of 131I-labeled albumin (Merck-Frosst, Montreal, Canada) as the alveolar protein tracer. The measurement of AFC was done with an in situ preparation, as we have previously published (9, 10). For measurements of cAMP-stimulated AFC, terbutaline (10^-3 M, Sigma Chemical) was added to the instillate (10, 23, 25).

**General protocol.** The mice were killed by an overdose of pento-barbital sodium (200 mg/kg ip). The trachea was dissected and cannulated with a 20-gauge, trimmed Angiocath plastic needle (Becton Dickinson, Sandy, UT). The lungs were inflated with 5 cmH2O of continuous positive airway pressure and oxygenated with 100% oxygen throughout the experiment. The body temperature was maintained at 37–38°C with a heating blanket and heat lamp. In all studies, 13 ml/kg of the instillate was delivered over 30 s into both lungs through the tracheal cannula. After 30 min, an alveolar fluid sample (0.05–0.10 ml) was aspirated with a 1-ml syringe directly connected to the 20-gauge Angiocath. The aspirate was weighed, and the radioactivity in the instilled sample/radioactivity in the final sample (% of instilled fluid volume) by measuring the increase in tracer-radioactivity in the instilled sample/radioactivity in the final sample × 100.

AFC is expressed as a percentage of the initial volume of instillate that was cleared from the distal air spaces during the 30 min (10).

**Hyperoxia-Induced Pulmonary Edema**

We induced hyperoxic lung injury by exposing mice to >95% oxygen in a sealed Plexiglas chamber at normal atmospheric pressure. An oxygen sensor was used to confirm the chamber oxygen concentration, and CO2 concentrations were found to be <0.005% with an oxygen flow rate of 2 l/min. Lungs were excised after 96 h of hyperoxic exposure.

**Wet-to-Dry Lung Weight Ratios**

The gravimetric method was used to determine lung wet-to-dry weight ratios as previously published (10). For these experiments, at the end of the 96-h exposure to hyperoxia, the lungs were removed, and the wet weight was recorded. The lungs were then placed in a 37°C incubator for 24 h, at which time the dry weight was recorded. Measurements of the hemoglobin concentration in the lung homogenate allowed for the calculation of bloodless lung wet-to-dry weight ratios (25).

**Specific Protocols**

**Protocol A.** We compared baseline and cAMP (terbutaline, 10^-3 M)-stimulated AFC, in mice heterozygous for the α1 (group 1), the α2 (group 2), and both the α1- and α2-subunits (group 3) of Na-K-ATPase to their respective wild-type littermate controls.

**Protocol B.** We compared wet/dry lung weight ratio after 96 h of hyperoxia in mice heterozygous for the α1 (group 1), the α2 (group 2), and both the α1- and α2-subunits (group 3) of Na-K-ATPase to their respective wild-type littermate controls.

**Statistical Analysis**

The data are summarized as means ± SD or SE. ANOVA and paired and unpaired t-tests were used for comparisons as appropriate. We regarded a P value of <0.05 as statistically significant.

**RESULTS**

Using the antibody α2ZR2, we detected an ~110-kDa protein only in cells transfected with the mouse Na-K-ATPase α2-isoform cDNA (Fig. 1). No proteins were detected with α2ZR2 in untransfected HeLa cells, which express only the human α1-isoform, or in untransfected MLC, which express only the mouse α1-isoform (Fig. 1). Furthermore, using α2ZR2 we detected no proteins in HeLa cells transfected with the mouse Na-K-ATPase α3-isoform cDNA, which express both the human α1-isoform and the mouse α3-isoform (Fig. 1). These results demonstrate that α2ZR2 recognizes the mouse Na-K-ATPase α2, but not the mouse α1, the human α1, or the mouse α3-isoform and therefore establishes α2ZR2 as a Na-K-ATPase α2-specific antibody. In addition, precomplexing the α2ZR2 antibody with a 200-fold molar excess of the antigenic peptide before probing the Western blots eliminated the immunoreactive bands seen in HeLa cells expressing the Na-K-ATPase α2-isoform and in mouse heart, brain, and lung, again confirming the specificity of the α2ZR2 antibody (data not shown).

The Na-K-ATPase α2-specific antibody α2ZR2 was used in conjunction with the α1-specific antibody α1F (1, 30) and the α3-specific antibody XVI9-G10 (1) to probe a mouse multi-organ Western blot to determine the Na-K-ATPase α3-isoform content of mouse lungs. As expected, α1F detected the presence of the Na-K-ATPase α1-isoform in all organs examined including the lung (Fig. 1). In control tissues, α2ZR2 detected expression of the Na-K-ATPase α2-isoform in mouse heart and brain, two organs known to express this isoform (14), but not in mouse kidney, an organ known to contain only the mouse α1-isoform (14). Expression of the Na-K-ATPase α3-isoform was also clearly detected in the mouse lung with α2ZR2 (Fig. 1). Using XVI9-G10, we detected expression of the Na-K-ATPase α3-isoform only in the brain, an organ known to express the α1-, α2-, and α3-isoforms (14) (Fig. 1). These

![Fig. 1. Western blot analyses of Na-K-ATPase α-isoform expression in transfected cells lines and mouse tissues.](http://ajplung.physiology.org/)

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results demonstrate that the mouse lung contains both the Na-K-ATPase \( \alpha_1 \)- and \( \alpha_2 \)-isoforms but not the Na-K-ATPase \( \alpha_3 \)-isoform.

Having established that the mouse lung expresses both the Na-K-ATPase \( \alpha_1 \)- and \( \alpha_2 \)-isoforms, and since the Na-K-ATPase is known to be important for AFC, we studied heterozygous Na-K-ATPase \( \alpha_1 \)- and \( \alpha_2 \)-isoform knockout mice to examine the contributions of individual Na-K-ATPase isoforms to AFC.

The quantitative analysis of the Na-K-ATPase \( \alpha_1 \)- and \( \alpha_2 \)-protein levels in the lung demonstrated that lung \( \alpha_1 \)-subunit protein expression was reduced by \( \sim 50\% \) in the \( \alpha_1^{+/+} \) and the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice (Fig. 2), and the \( \alpha_2 \)-protein expression was also reduced by half in the \( \alpha_2^{+/+} \) mice and the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice (Fig. 3).

Basal AFC was not different in wild-type littermate mice compared with the \( \alpha_1^{+/+}, \alpha_2^{+/+} \), or the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice (Fig. 4). There was a trend for the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice to have lower basal AFC, but this was not different from their littermate controls.

cAMP-stimulated AFC was not impaired in mice that were heterozygous for either the \( \alpha_1 \)- or the \( \alpha_2 \)-subunit alone (Fig. 5). However, the combined \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice demonstrated a significantly decreased cAMP-stimulated fluid clearance compared with their wild-type littermates (11.1 ± 1.0 vs. 14.9 ± 1.8%/30 min, \( P < 0.001 \)) (Fig. 5). This submaximal response with cAMP stimulation in \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice was statistically greater than the basal fluid clearance in \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice (\( P < 0.05 \), Fig. 4).

We then exposed the \( \alpha_1^{+/+}, \alpha_2^{+/+} \), and the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice to hyperoxia (>95% oxygen) for 96 h. The \( \alpha_1^{+/+} \) and \( \alpha_2^{+/+} \) mice had no difference in the lung wet-to-dry weight ratio compared with their wild-type littermates (Fig. 6). Similarly, the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice had no difference in the lung wet-to-dry weight ratio compared with wild-type littermates (Fig. 6).

DISCUSSION

The major findings of this study are that 1) a 50% reduction of either the \( \alpha_1 \)- or \( \alpha_2 \)-subunit protein expression of the Na-K-ATPase in total lungs of WT and genetically modified mice with deficiency of 1 allele of the \( \alpha_1 \)- and/or \( \alpha_2 \)-subunit of the Na-K-ATPase. Quantification of signal intensity revealed a 50% decrease in \( \alpha_2 \)-protein expression in the \( \alpha_2^{+/+} \) and \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice compared with WT controls. Mice heterozygous for the \( \alpha_2 \)-isoform (\( \alpha_2^{+/+} \)) had no decrease in \( \alpha_2 \)-protein expression compared with wild-type controls. Data are presented as means ± SE. *\( P < 0.05 \) \( \alpha_2^{+/+} \) and \( \alpha_1^{+/+}/\alpha_2^{+/+} \) compared with \( \alpha_2^{+/+} \) and WT mice.

\( \alpha_2^{+/+} \) mice had no difference in the lung wet-to-dry weight ratio compared with their wild-type littermates (Fig. 6). Similarly, the \( \alpha_1^{+/+}/\alpha_2^{+/+} \) mice had no difference in the lung wet-to-dry weight ratio compared with wild-type littermates (Fig. 6).
Na-K-ATPase in the lung does not impair basal or cAMP-stimulated AFC, 2) a combined 50% reduction of both α1- and α2-subunit protein expression does not impair basal fluid clearance, but 3) the combined 50% reduction in both α1- and α2-subunits significantly decreases maximal cAMP-dependent fluid clearance, and 4) the decrease in maximal fluid clearance in the compound (α1/α2) heterozygous mice did not result in a greater amount of excess lung water in a 96-h model of hyperoxia.

The role of the Na-K-ATPase in lung liquid clearance, and specifically the differential roles of the α- and β-subunits, has recently been more clearly elucidated. Although originally thought to be significantly present in only alveolar type II cells (12, 28), Na-K-ATPase has also been localized to alveolar type I cells (5, 15). Immunocytochemical colocalization studies and immunogold electron microscopy have revealed the cellular distribution of the α- and β-subunits of Na-K-ATPase in the alveolar epithelium (15, 24). Alveolar type II cells contain α1- and β1-subunits, whereas alveolar type I cells contain the α1-, α2-, and β1-subunits. Furthermore, the α2-subunit, and thus the alveolar type I cell, has emerged as an important subunit in the alveolar epithelium, being responsible for ~60% of basal and 80% of cAMP-stimulated AFC in an isolated, perfused rat lung model (24).

Transgenic studies have also been important in defining the role of the α- and β-subunits of the Na-K-ATPase in lung liquid clearance at baseline conditions and in models of lung injury (7, 8). Adenoviral delivery of the α2-subunit of the Na-K-ATPase to the lungs of rats increases AFC by >300% compared with hyperoxic control rats when exposed to 64 h of 100% oxygen (7). Remarkably, the adenovirus-β1-treated rats had no mortality after 14 days of 100% oxygen. However, adenoviral delivery of the α1-subunit did not change AFC or affect survival with hyperoxia. Preliminary work on adenoviral delivery of the α2-subunit has revealed an increase in basal AFC (24), but experiments in an injury model, such as hyperoxia, have not been published. Targeted expression of the β2-adrenergic receptor in the alveolar type II cells of mice has also yielded important information about the roles of the Na-K-ATPase subunits. Mice overexpressing the β2-adrenergic receptor in alveolar type II cells had a significant increase in basal AFC, which was associated with an increase in total lung α2-, but not α1-, protein expression, suggesting an important role for the α2-subunit (20).

The activity of Na-K-ATPase under normal conditions works at a low percentage of its maximum pumping capacity (11, 16). In fact, the α1-subunit pumps at ~1/2 of its maximal capacity (32), and the α2-subunit pumps at 1/20 of its maximum (16). Therefore, the Na-K-ATPase appears to have an important reserve capacity thought to be sufficient to maintain normal ion transport even in pathological conditions associated with downregulation of its expression/activity. Expression and activity of the Na-K-ATPase can be upregulated in the short-term by recruiting new units to the basolateral membrane from a cytoplasmic pool and by increasing the activity of the subunits already present in the membrane (29). What is not known in the lung, however, is the critical mass of Na-K-ATPase units needed to maintain vectorial sodium transport at baseline or with cAMP stimulation or in states of lung injury. In these experiments we tested the effect of decreasing protein expression by 50% of the catalytic α1- and/or α2-subunits.

The results indicate that there is a significant Na-K-ATPase reserve in the lungs of mice since the depletion of 50% of the α1-protein or the α2-protein does not affect basal or cAMP-stimulated fluid clearance. The interesting finding of a submaximal response of the compound (α1/α2) heterozygous mice...
to cAMP stimulation points to a synergistic role of the \( \alpha_1 \)- and \( \alpha_2 \)-subunits in AFC since impaired expression of one subunit alone did not result in any quantifiable impairment. In our model of in situ AFC, the addition of the cAMP agonist terbutaline tests the short-term (30 min) regulation of the Na-K-ATPase. In important work from Sznaider and colleagues (4, 29), \( \beta_2 \)-adrenergic agonists have been shown to regulate Na-K-ATPase activity in the short term by primarily recruiting subunits to the basolateral membrane from a pre-existing cytoplasmic pool. The inability of terbutaline to maximally stimulate AFC in the compound heterozygous mice could point to a critically low cytoplasmic pool of \( \alpha_1 \)- and \( \alpha_2 \)-subunits in these 50% protein-deficient mice. In other words, while the compound heterozygous mice were able to increase AFC in response to terbutaline, the submaximal response could indicate that the Na-K-ATPase is functioning at near maximal capacity without the preformed cytoplasmic reserve subunits needed to augment AFC in response to \( \beta_2 \)-adrenergic agonists. Also, the submaximal response in the compound heterozygous mice suggests an important role for the \( \alpha_2 \)-subunit and therefore provides more circumstantial evidence for the likely importance of alveolar type I cells in vectorial fluid transport in the lung.

The submaximal response to terbutaline in the compound heterozygous mice was not associated with more lung edema formation in a severe model of hyperoxia. Although there was a trend for an increased lung wet-to-dry weight ratio in the compound heterozygous mice compared with wild-type littersmates, no statistically significant difference could be found even though large numbers of mice were studied (\( n = 12 \) for both groups). We speculate that by using 96 h of hyperoxic exposure that the oxidative damage to the alveolar epithelium may be so advanced that the capacity for alveolar epithelial fluid clearance is markedly diminished. Adding support to this hypothesis is a recent study of AFC in mice that could explain the lack of a difference in lung water in the compound heterozygous mice and wild-type littersmates with hyperoxia. Paine et al. (23) found that there was no net AFC in wild-type mice exposed to 80 h of hyperoxia. Therefore, there is likely to be minimal AFC with 96 h of hyperoxia in the wild-type, \( \alpha_1 \)-, \( \alpha_2 \)-, or \( \alpha_1/\alpha_2 \)-heterozygous mice, and thus a statistically significant difference in lung edema with only a submaximal difference in cAMP-stimulated AFC may be difficult to establish.

Previous studies done by our research group on the role of CFTR in AFC in mice could also potentially explain why no difference was found in the lung wet-to-dry weight ratios in the wild-type and compound heterozygous mice. In mice that have no functioning CFTR (ΔF508 mice), we found that basal AFC was normal, but there was no increase in cAMP-stimulated AFC (9). When these ΔF508 mice were subjected to acute intravascular volume overload, there was only a modest increase in the lung wet-to-dry weight ratio compared with controls. Therefore, experimental acute lung injury utilizing mice (\( \alpha_1^{-1}/\alpha_2^{-1} \)) that can significantly increase cAMP-stimulated AFC, albeit submaximally, may be unlikely to detect a significant difference in the net quantity of pulmonary edema in the lung.

A potential limitation of our model is that the reduction of the Na-K-ATPase protein expression is not limited to the lung. Because the Na-K-ATPase is a ubiquitous protein, this may have induced other systemic consequences (in particular renal and cardiovascular) that may have had potentially confounding effects. However, our AFC measurements were performed in situ, ex vivo conditions during which these possible confounding effects should not play a significant role (3, 10). Another potential limitation of our model is that we quantified the \( \alpha_1 \)- and \( \alpha_2 \)-isoform protein content in whole lung homogenates from the \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_1/\alpha_2 \)-compound heterozygote mice, which may not reflect the isoform densities on the basolateral cell surface of alveolar epithelial cells. It is possible that the \( \beta_1 \)-subunit of the Na-K-ATPase could traffic more \( \alpha \)-subunits to the basolateral cell surface in the \( \alpha \)-isoform heterozygote mice, which could potentially maintain the \( \alpha \)-isoform content of the basolateral membrane at normal levels. Future experiments analyzing the basolateral distribution of the Na-K-ATPase \( \alpha \)-isoforms in these knockout mice will be important for determining whether the lack of a phenotype observed in the \( \alpha_1 \)- and \( \alpha_2 \)-heterozygous mice is due to the ability of the alveolar epithelial cells to maintain normal basolateral Na-K-ATPase content in the face of a 50% decrease in either the Na-K-ATPase \( \alpha_1 \)- or \( \alpha_2 \)-isoform. However, it is clear, based on the phenotype of the \( \alpha_1/\alpha_2 \)-compound heterozygous mice described in this study, that a loss of 50% of both the \( \alpha_1 \)- and \( \alpha_2 \)-isoforms cannot be completely compensated for by the alveolar epithelial cells.

In conclusion, mice that are 50% protein deficient in either the \( \alpha_1 \)- or \( \alpha_2 \)-subunits of the Na-K-ATPase have normal basal and stimulated AFC and no increase in lung edema formation with hyperoxia. Compound heterozygous mice that are 50% protein deficient in both the \( \alpha_1 \)- and \( \alpha_2 \)-subunits of the Na-K-ATPase have preserved basal fluid clearance but exhibit only submaximal cAMP-stimulated AFC. These results suggest a synergistic role for the \( \alpha_1 \)- and \( \alpha_2 \)-subunits in lung liquid clearance and focus more attention on the alveolar type I cell and its role in lung fluid balance.

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