Alveolar Type 1 Cells Express the α2 Na,K-ATPase, Which Contributes to Lung Liquid Clearance


Abstract—The alveolar epithelium is composed of alveolar type 1 (AT1) and alveolar type 2 (AT2) cells, which represent ≈95% and ≈5% of the alveolar surface area, respectively. Lung liquid clearance is driven by the osmotic gradient generated by the Na,K-ATPase. AT2 cells have been shown to express the α1 Na,K-ATPase. We postulated that AT1 cells, because of their larger surface area, should be important in the regulation of active Na⁺ transport. By immunofluorescence and electron microscopy, we determined that AT1 cells express both the α1 and α2 Na,K-ATPase isoforms. In isolated, ouabain-perfused rat lungs, the α2 Na,K-ATPase in AT1 cells mediated 60% of the basal lung liquid clearance. The β-adrenergic agonist isoproterenol increased lung liquid clearance by preferentially upregulating the α2 Na,K-ATPase protein abundance in the plasma membrane and activity in alveolar epithelial cells (AECs). Rat AECs and human A549 cells were infected with an adenovirus containing the rat Na,K-ATPase α2 gene (Adα2), which resulted in the overexpression of the α2 Na,K-ATPase protein and caused a 2-fold increase in Na,K-ATPase activity. Spontaneously breathing rats were also infected with Adα2, which increased α2 protein abundance and resulted in a ≈250% increase in lung liquid clearance. These studies provide the first evidence that α2 Na,K-ATPase in AT1 cells contributes to most of the active Na⁺ transport and lung liquid clearance, which can be further increased by stimulation of the β-adrenergic receptor or by adenovirus-mediated overexpression of the α2 Na,K-ATPase. (Circ Res. 2003;92: 1110–1116.)

Key Words: alveolar type 2 cells ■ alveolar type 1 cells ■ α1 and α2 Na,K-ATPase ■ alveolar fluid reabsorption

Active Na⁺ transport is critical for maintaining the alveolar space “dry” to ensure normal gas exchange. Sodium is actively transported across the alveolar epithelium; it enters via the apical Na⁺ channels and is actively extruded by basolaterally located Na,K-ATPase. The alveolar fluid is reabsorbed by the resulting osmotic gradient. The Na,K-ATPase is a heteromeric enzyme composed of an α isoform and a glycosylated β isoform. The α isoform is the catalytic component of the holoenzyme, containing the cation and nucleotide binding sites as well as the receptor site for the catalytic component of the holoenzyme, containing the cation and nucleotide binding sites as well as the receptor site for the cardiac glycoside ouabain. Four α isoforms have been identified (α1, α2, α3, and α4), each with a unique tissue distribution. The α1 and α2 Na,K-ATPase have been reported to be expressed in the lung. However, there are no previous reports on the precise cellular function or location of the α2 Na,K-ATPase in the lung.

In the human lung, the alveolar surface area is ≈100 m² and is composed of two morphologically distinct epithelial cells, type 1 and type 2. Alveolar type 2 (AT2) cells are small cuboidal cells that account for ≈5% of the alveolar surface area. AT2 cells terminally differentiate into alveolar type 1 (AT1) cells, which are elongated, flattened cells that cover ≈95% of the alveolar surface. Previously, it was thought that lung liquid clearance is effected by α1 Na,K-ATPase in AT2 cells, based on reports using ultrastructural immunocytochemistry and that AT1 cells lacked the proteins required for fluid and ion transport. However, two recent reports and the present study provide evidence to support a new paradigm that AT1 cells do indeed express the Na,K-ATPase and are capable of active Na⁺ transport and fluid reabsorption.

We provide evidence that both the α1 and α2 Na,K-ATPase contribute to active Na⁺ transport and alveolar fluid reabsorption under basal conditions, after catecholamine stimulation and adenovirus-mediated overexpression of the α2 Na,K-ATPase. The results of these studies demonstrate that (1) AT1 cells express the α1 and α2 Na,K-ATPase isoforms; (2) the α2 Na,K-ATPase is responsible for ≈60% of the basal alveolar fluid reabsorption in the lung; (3) catecholamine-mediated increases in alveolar fluid reabsorption are due mostly to the α2 Na,K-ATPase in the AT1 cells; and (4) adenovirus-mediated overexpression of the α2 Na,K-ATPase increases alveolar fluid reabsorption by ≈250%.
Materials and Methods

In Situ Hybridization

Methods were essentially as described previously. Complementary RNA probes were prepared as described previously.

Immunoelectron Microscopy

Expression of Na,K-ATPase α2 protein in alveolar type 1 was assessed by immunoelectron microscopy using the α2 Na,K-ATPase, McB2, antibody and goat anti-mouse antibody, conjugated to colloidal gold particles. Gold particles were counted on mitochondria, nuclei, and the cytoplasm/plasma membrane.

Isolation and Culture of Alveolar Epithelial Cells (AECs)

AT2 cells were isolated from pathogen-free male Sprague-Dawley rats (200 to 225 g), as previously described. Cells were cultured in DMEM containing 10% fetal bovine serum with 2 mmol/L l-glutamine, 40 μg/mL gentamicin, 100 U/mL penicillin, and 100 μg/mL streptomycin and placed in culture for 7 days before the start of all experimental conditions.

Na,K-ATPase Activity

Ouabain-sensitive $^{32}$P uptake was used to estimate the rate of K$^+$ transport by Na,K-ATPase in AECs. $^{32}$P influx was quantified in aliquots of the SDS extract by liquid scintillation counting.

Preparation of Basolateral Plasma Membranes

Basolateral membranes (BLMs) were purified as previously described. For Western blot analysis, equal amounts of protein from BLMs were resolved by 10% SDS-PAGE and analyzed by immunoblotting with Na,K-ATPase anti-α2 monoclonal antibody (McB2, generous gift from K. Sweadner) or anti-α1 (generous gift from M. Caplan) monoclonal antibody.

Adenoviruses

The pCMV vector, which contains the immediate-early promoter and enhancer from CMV, a full-length cDNA for Escherichia coli lacZ and the SV40 t intron polyadenylation signal, was used. The β-galactosidase cDNA was excised from pMRCMVβ-gal and replaced with full-length cDNA for the rat α2 Na,K-ATPase (pMRCMVO2) or no cDNA (pCMVNull). AECs or rats were infected as previously reported.

Isolated Perfused Lungs

The isolated perfused lung preparation used in our laboratory has been described in detail. Ten rat lungs were treated as control. Seventy rat lungs were perfused with 14 different concentrations of ouabain (10$^{-11}$ to 7.5×10$^{-4}$ mol/L) to determine the contribution of the α1 and α2 Na,K-ATPase to alveolar fluid reabsorption (AFR). Sixty-six rat lungs were perfused with 1 μmol/L isoproterenol in the presence or absence of ouabain (the same 14 concentrations of ouabain were used).

Figure 1. Representative photomicrograph of α2 Na,K-ATPase mRNA, detected by in situ hybridization, in normal rat lung tissue. Digoxigenin-labeled, antisense cRNA probe specific for the α2 isoform was hybridized under high-stringency conditions to sections of the alveolar epithelium (A). Sense cRNA probe was used to detect nonspecific hybridization (B). Hematoxylin and eosin staining of a serial section (C).

Data Analysis

Probability values were obtained by ANOVA followed by contrast analysis and considered significant below 0.05. An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Expression and Function of the α2 Na,K-ATPase

A strong hybridization signal corresponding to the α2 Na,K-ATPase mRNA was evident in the alveolar epithelium of normal rat lung tissue (Figure 1A). No signal was detected in sections hybridized with the sense probe (Figure 1B). Human lung, rat lung, and rat alveolar epithelial type 1–like (AT1-like) cells expressed both α1 and α2 Na,K-ATPase (Figure 2, lanes 3, 4, and 5, respectively) as determined by Western blot analysis. α1 Na,K-ATPase protein was detected in both rat brain and kidney tissue (Figure 2, lanes 1 and 2, respectively), but the α2 Na,K-ATPase was only detected in rat brain, as expected (Figure 2, lane 1), demonstrating the specificity of the Na,K-ATPase antibodies. Additionally, surfactant protein C (SP-C) and aquaporin 5 (AQ5) protein expression were determined in total cell lysates prepared from alveolar type 2 and AT1-like cells. As shown in Figure 2B, AT1-like cells express the AQ5, but not SP-C.

AT1 cells, but not AT2 cells, expressed the α2 Na,K-ATPase in normal rat and human lung tissue as determined by coimmunofluorescence (Figures 3A through 3I). AT2 cells were identified by staining with anti–SP-C antibody (Figures 3B and 3E). SP-C did not colocalize with α2 Na,K-ATPase (Figure 3C [rat] and Figure 3F [human]), indicating the AT1 cells, but not AT2 cells, express the α2 Na,K-ATPase. The α2 Na,K-ATPase protein was expressed in AT1 cells in normal rat lung tissue (Figure 3I) as determined by indirect immunofluorescence. T1α was used as a specific marker for AT1 cells (Figure 3H). Finally, the α1 Na,K-ATPase was ubiquitously expressed throughout the alveolar epithelium. As shown in Figures 3K and 3N, both human and rat lung tissue expressed the α1 Na,K-ATPase in both AT1 and AT2 cells. The α1 Na,K-ATPase colocalized with SP-C in AT2 cells (Figures 3M and 3P) as indicated by the yellow fluorescence.

The expression of the α2 Na,K-ATPase protein in AT1 cells was confirmed by immunoelectron microscopy. Figure 4A depicts the alveolus from which the AT1 cells in Figures 4B and 4C were magnified. The analysis of gold particle distribution demonstrated that immunogold labeling was...
specific to the cytoplasm/plasma membrane (Figure 4D). Labeling over mitochondria and nuclei was marginal, ie, equivalent to <6% of cytoplasm/plasma membrane labeling density, and was similar to nonspecific labeling.

In rodent tissue, it is possible to distinguish physiologically between the $\alpha_1$ and $\alpha_2$ Na,K-ATPase because of the large difference in their affinity/sensitivity for ouabain. The $\alpha_2$ Na,K-ATPase, but not the $\alpha_1$ Na,K-ATPase, is inhibited by 100 nmol/L ouabain,14,25 whereas >1 mmol/L ouabain is needed to inhibit the rat $\alpha_1$ Na,K-ATPase.14,25 We determined the contribution of the $\alpha_1$ and $\alpha_2$ Na,K-ATPases to AFR by perfusing, in different experiments, increasing concentrations of ouabain through the pulmonary circulation of the isolated rat lung model. As depicted in Figure 5A, the ouabain inhibition curve, obtained by least squares fit, of AFR was biphasic, indicating the presence of both $\alpha_1$ and $\alpha_2$ Na,K-ATPase. A class of low-affinity ouabain-insensitive $\alpha_1$ Na,K-ATPase constituted ~40% of AFR. A second class of high-affinity ouabain-sensitive $\alpha_2$ Na,K-ATPase constituted ~60% of AFR. The IC$_{50}$ values calculated from the data in Figure 5A are consistent with the $\alpha_1$ Na,K-ATPase (2.3$\times$10$^{-4}$ mol/L) and the $\alpha_2$ Na,K-ATPase (2.5$\times$10$^{-7}$ mol/L).

The accuracy of the two-site binding model (eg, expression of $\alpha_1$ and $\alpha_2$ Na,K-ATPase) result was validated using an F test, which quantifies the relationship between the relative increase in the sum of squares and the relative increase in the degrees of freedom. If the one-site model (eg, expression of either $\alpha_1$ or $\alpha_2$ Na,K-ATPase) were correct, we would expect to get an F ratio near 1.0. Our F ratio was 9.9, which suggests two possibilities: (1) The two-site model is correct. (2) The one-site model is correct, but random scatter allowed the two-site model to fit better. However, the probability value was 0.0002. Thus, if the one-site model is correct, there is only a 0.02% chance that we randomly obtained data that fits the two-site model better. Based on these analyses, we conclude that there are two ouabain-binding sites in the alveolar epithelium, $\alpha_1$ Na,K-ATPase and $\alpha_2$ Na,K-ATPase.

The passive movement of small solutes ([H]$\text{mannitol}$ and $^{22}\text{Na}$) and FITC-albumin across the epithelial barrier of the rat lung did not change when ouabain was perfused through the pulmonary circulation of the isolated rat lungs compared with control (data not shown), which validates the use of the model in assessing the role of $\alpha_1$ and $\alpha_2$ Na,K-ATPase in lung liquid clearance. The pulmonary circulation flow was measured periodically during the experiments and was similar (~14 mL/min) in all groups (data not shown).

Catecholamine-Mediated Increase in $\alpha_2$ Na,K-ATPase

We have previously reported that isoproterenol (ISO) increased AFR in rat lungs.3,22 In this report, we determined the contribution of the $\alpha_1$ and $\alpha_2$ Na,K-ATPase to the ISO-mediated increase in AFR by perfusing, in different experiments, increasing concentrations of ouabain through the pulmonary circulation of the isolated rat lung model. As depicted in Figure 5B, the ouabain inhibition curve of ISO-stimulated AFR was biphasic but was shifted to the left. Scatchard plot analysis of the data in Figures 5A and 5B demonstrated that ISO did not affect the binding affinity of ouabain to either the $\alpha_1$ nor $\alpha_2$ Na,K-ATPase (data not shown). The F test was 8.9, and the probability value was 0.0005 for the comparison of one- versus two-site model. These results indicate that ~80% of the increase in AFR was due to the high-affinity ouabain-sensitive $\alpha_2$ Na,K-ATPase (Figure 5B) located in AT1 cells (Figure 4).

AT1-like cells,14 which express both the $\alpha_1$ and $\alpha_2$ Na,K-ATPase (Figure 2), were treated with 1 mmol/L ISO for 15 minutes. ISO increased the Na,K-ATPase activity by ~4.0-fold (Figure 5C) as measured by $^{86}\text{Rb}^+$ uptake in the absence or presence of either 100 nmol/L ouabain to inhibit the $\alpha_2$ Na,K-ATPase or 5 mmol/L ouabain to inhibit the $\alpha_1$ Na,K-ATPase. The increase in Na,K-ATPase activity was mediated by both $\alpha_1$ and $\alpha_2$ Na,K-ATPase; however ~80% of this increase was due to the $\alpha_2$ Na,K-ATPase whereas ~20% was due to the $\alpha_1$ Na,K-ATPase. The increase in activity was associated with an increase in $\alpha_1$ and $\alpha_2$ Na,K-ATPase.
Na,K-ATPase protein abundance in the BLM of ISO-treated cells compared with control AT1-like cells (Figure 5D).

**Adenovirus-Mediated Overexpression and Function of the Rat α2 Na,K-ATPase**

Spontaneously breathing rats were infected with 10⁹ to 10¹⁰ pfu of Adα2; maximal clearance was noted with 2×10⁹ pfu (data not shown). Lung liquid clearance was increased ≈250% in rats infected with Adα2 compared with Adnull-infected or sham-infected rats (Figure 6A). As shown in Figure 6A, 10⁻³ mol/L ouabain (which inhibits only the α2 Na,K-ATPase) decreased AFR by ≈70% in Adα2-infected rat lungs, suggesting that the observed increase in clearance was due to the increased α2 Na,K-ATPase protein abundance.

Increased abundance of the α2 Na,K-ATPase protein was observed in the alveolar epithelium in Adα2-infected rat lungs but not in Adnull-infected control rat lungs (Figure 6B), as determined by indirect immunofluorescence and Western blot analysis. No change in the α1 Na,K-ATPase protein expression was observed in any of the rat lungs (data not shown).

Rat AT1-like cells and human A549 cells were infected with multiplicity of infection (MOI) of 1 to 25 of Adα2 for 24 hours. Both rat AT1-like cells and A549 cells infected with Adα2 (MOI 10) had a ≈2-fold increase in Na,K-ATPase activity compared with Adnull-infected cells (Figure 7). The increase in Na,K-ATPase activity was associated with an increase in α2 Na,K-ATPase protein abundance in the BLMs of Adα2-infected cells (Figure 7, top panel). There was no change in the α1 Na,K-ATPase protein abundance in either A549 cells or AT1-like cells (data not shown). The use of adenoviruses to overexpress the α2 Na,K-ATPase was not...
cytotoxic to the cells, given that there was no increase in K1 or LDH concentrations in the supernatant (data not shown).

Gene transfer efficiency and distribution were homogeneous in rat lungs and AECs by infection with Adβ-Gal, which produced lacZ expression in all regions of the lungs and cell monolayer (data not shown).

Discussion
It is well established that AFR is regulated by active Na+ transport mechanisms.3,5,17,22–24,26–28 Specifically, the Na,K-ATPase generates the electrochemical gradient for transcellular transport of sodium, with water following the osmotic gradient across the alveolar epithelium. These processes were thought to occur in alveolar type 2 cells, which account for, 5% of the alveolar surface area, whereas the alveolar type 1 cells, which account for, 95% of the alveolar surface area, were thought to lack the molecular machinery required for active Na+ transport.16,29 Our data and two very recent reports17,18 provide evidence that alveolar type 1 cells participate in active Na+ transport. In this report, we provide the first evidence that α2 Na,K-ATPase is expressed in alveolar type 1 cells and is an important contributor to lung liquid clearance. The importance of the α2 Na,K-ATPase is further emphasized by our studies where AFR was significantly increased in rat lungs that overexpressed the α2 Na,K-ATPase.

α2 Na,K-ATPase mRNA was detected in the alveolar epithelium by in situ hybridization (Figure 1), and α2 Na,K-ATPase protein was localized to AT1 cells in situ (Figures 3 and 4). AT1 cells were identified by electron microscopy or by immunofluorescent staining with antibodies against either T1α or aquaporin 5, AT1 cell-specific phenotype markers. The alveolar surface area in situ varies considerably; AT2 cells are ∼500 μm2, whereas the surface area of AT1 cells is ∼4500 μm2.15 Therefore, the density of Na,K-ATPase molecules per unit membrane surface area of an AT1 cell in situ is relatively low, as demonstrated by immunoelectron microscopy (Figure 4). Quantitative analysis of the number of α2 Na,K-ATPase molecules detected by immunoelectron microscopy was somewhat limited because some antibody sites were lost on exposure of the tissue to the fixative, despite that a comparatively gentle fixative was used.20 Additionally, this fixative did not preserve well the membrane structures. Therefore, we used semiquantitative analysis of gold particle distribution30 to determine in which regions of the cell that the α2 Na,K-ATPase protein was expressed (Figure 4E). As anticipated, the α2 Na,K-ATPase proteins were expressed mostly at the membrane/cytoplasm of AT1 cells (Figure 4). The pattern of Na,K-ATPase protein expression in lung appears similar to the expression patterns in the heart.31,32 The α1 isoform is uniformly distributed within the plasma membrane of most cells,31–33 whereas the α2 isoform localized to microdomains within the membrane.31,32

A recent report17 established that isolated rat AT1 and AT2 cells are capable of active Na+ transport and that AT1 cells transport more Na+ per microgram of protein than AT2 cells. The interpretation of these experiments is limited by their in vitro design. Therefore, we assessed the contribution of the α2 Na,K-ATPase in AT1 cells in situ and the contribution of ubiquitously expressed α1 Na,K-ATPase to AFR using an isolated, perfused rat lung model. In rodent tissues, the α1
and α2 Na,K-ATPase can be biochemically distinguished by their low and high affinity for ouabain, respectively. As shown in Figure 5A, we demonstrated that there are two distinct binding sites for ouabain in the alveolar epithelium: α1 Na,K-ATPase (low affinity, IC₅₀: 2.3 × 10⁻⁴ mol/L) and α2 Na,K-ATPase (high affinity, IC₅₀: 2.5 × 10⁻⁷ mol/L). Similar IC₅₀ values have been reported for the α1 and α2 Na,K-ATPases in rat AECs, cardiac muscle, adipocytes, and brain. 14,34,35

The α2 Na,K-ATPase accounts for ≈60% of the basal lung liquid clearance (Figure 5A). In accordance with recent reports, these data suggest that AT1 cells play an important role in maintaining the fluid balance in the lung. 17,18 It has been clearly established that catecholamines markedly increase alveolar fluid clearance in normal and injured lungs. 5,22–24,36 and that catecholamine-mediated increases in AFR are dependent on normal expression levels of Na,K-ATPase proteins. 37 A recent report demonstrated that in transgenic mice that overexpressed the β2-adrenergic receptor there was a 40% increase in AFR, which was associated with increased α2 Na,K-ATPase protein abundance, but not α1 Na,K-ATPase, in lung homogenates. 38 Our data provide evidence that β-adrenergic agonists increase AFR by regulating the α2 Na,K-ATPase in AT1 cells. As shown in Figure 5B, the ouabain inhibition curve of ISO-stimulated AFR was biphasic, indicating involvement of both α1 and α2 Na,K-

Figure 5. A, Ouabain inhibition curve of AFR in the isolated rat lung model. 10⁻¹¹ to 7.5 × 10⁻⁴ mol/L ouabain was perfused through the pulmonary circulation to assess the effect on AFR (mL/h). B, Ouabain inhibition curve of ISO-mediated increase in AFR in the isolated rat lung model. 10⁻¹¹ to 7.5 × 10⁻⁴ mol/L ouabain and 1 μmol/L ISO were perfused through the pulmonary circulation to assess the effect on AFR (mL/h). Data are presented as mean±SEM. Curve is nonlinear least square fit of data shown. C, ISO-mediated stimulation of Na,K-ATPase activity in AECs. Cells were treated with 1 μmol/L ISO for 15 minutes. ⁸⁶Rb⁺ uptake was measured in the presence or absence of 5 × 10⁻⁵ mol/L ouabain. Each bar represents the mean±SEM of 3 independent cell isolations. Determinations were performed in quadruplicate. *P<0.01. The open bars indicate the contribution to the activity by α1 Na,K-ATPase; the filled bars indicate the contribution to the activity by α2 Na,K-ATPase. D, Na,K-ATPase α1 subunit abundance in the BLM prepared from ISO-treated primary AECs. Cells were incubated with 1 μmol/L ISO for 15 minutes at room temperature. Equal amounts of protein (10 μg) were loaded in each lane. Representative Western blot of the α1 and α2 Na,K-ATPase protein abundance is shown, (n=3).
ATPase, but was clearly shifted to the left. These results suggest that 80% of the ISO-mediated increase in lung liquid clearance is regulated by the activity of the α2 Na,K-ATPase (Figure 5B). These results suggest that ISO preferentially regulates the α2 Na,K-ATPase, thus increasing the rate of lung liquid clearance across the AT1 cell.

To assess the mechanisms by which ISO increased AFR, we treated rat AT1-like cells, which express similar amounts of α1 and α2 Na,K-ATPase,39 with ISO. As shown in Figure 5D, these results suggest that ISO increases both α1 and α2 Na,K-ATPase protein abundance in the BLM of rat AT1-like cells. This increase in Na,K-ATPase protein abundance is not likely to represent increased de novo synthesis of Na,K-ATPase molecules, because the ISO effect occurred within 15 minutes, which concord with previous reports.4,21 The turnover rate of the Na,K-ATPase was then measured in the presence of ouabain at concentrations to selectively inhibit the α2 Na,K-ATPase. As shown in Figure 5C, both the α1 and α2 Na,K-ATPase contributed to the overall increase in activity; however, the majority of the increase was due to the α2 Na,K-ATPase (Figure 5). Under basal conditions, the α1 Na,K-ATPase is pumping at approximately one-half its maximum capacity,40 whereas the α2 Na,K-ATPase is pumping at one-twentieth of its $V_{\text{max}}$.25 Therefore, even similar amounts α1 and α2 Na,K-ATPase protein abundance, as previously reported in rat AT1-like cells, would not translate into equal pumping capacity.14 ISO increased the α1 Na,K-ATPase activity from 5.2 ± 1.0 to 12.0 ± 2.7 nmol K⁺/mg protein per minute, suggesting that the α1 Na,K-ATPase is functioning at $V_{\text{max}}$. In contrast, the α2 Na,K-ATPase activity increased 6-fold from 6.4 ± 1.5 to 36.8 ± 4.9 nmol K⁺/mg protein per minute, suggesting that its pumping capacity has not yet been maximized. These results suggest that both the α1 and α2 Na,K-ATPase can be regulated by β-adrenergic agonists, but that the α2 Na,K-ATPase regulation might be physiologically more significant.

We have previously reported that adenovirus-mediated transfer of Na,K-ATPase isoform genes increases Na,K-ATPase expression and function in human and rat lung epithelial cells and the alveolar epithelium of rats.24,36 Specifically, we demonstrated that the overexpression of the β1 Na,K-ATPase subunit gene, but not the α1 Na,K-ATPase, increases AFR by >100% in normal rats,41,42 mitigates oxidant-mediated decreases in active Na⁺ transport in rat fetal distal lung epithelial cells,43 and increases AFR and survival of rats exposed to 100% oxygen.44 In the present study, we explored whether overexpression of the α2 Na,K-ATPase in the alveolar epithelium could increase AFR. We instilled a surfactant-based vehicle to achieve a homogeneous delivery of our adenoviral vectors to the lung as previously reported and used the isolated rat lung model to assess AFR 7 days after adenoviral infection.24,36 As shown in Figure 6A, over-

**Figure 6.** A, AFR was increased by ~250% in rats infected with Adα2 compared with sham- and Adnull-infected rats during the first hour of the isolated perfused rat lung model. In the second hour, 10⁻⁵ mol/L ouabain perfused through the pulmonary circulation decreased alveolar fluid clearance by ~70%, to a level not different from sham- and Adnull-infected rat lungs. *P<0.01 compared with control or sham-infected rat lungs. Bars indicate mean ± SE, n = 6. B, top, Representative Western blot of the α2 Na,K-ATPase protein abundance in lung tissue from control, Adnull-, and Adα2-treated rats. B, bottom, Photomicrograph immunofluorescence localization of α2 Na,K-ATPase protein. Right, Adnull-infected rat lung tissue expressing α2 Na,K-ATPase. Left, Adα2-infected rat lung tissue expressing α2 Na,K-ATPase.
expression of the α2 Na,K-ATPase markedly increased the ability of the lung to reabsorb fluid. This increase in clearance was inhibited by $10^{-5}$ mol/L ouabain, a concentration that inhibits the α2 Na,K-ATPase, but not the rat α1 Na,K-ATPase. These findings are consistent with our in vitro data showing that the α2 Na,K-ATPase overexpression increases ouabain-inhibitable Na,K-ATPase activity in human A549 cells and rat AT1-like cells (Figure 7). The increases in α2 Na,K-ATPase protein after Adα2 infection and the absence of functional change after Adnull infection suggest that the observed increases are specific responses to adenoviral-mediated gene transfer. Thus, these results suggest that in rat AT1-like cells and human A549 cells, the rate-limiting Na,K-ATPase subunit is the α subunit and not the β1 subunit. Therefore, by overexpressing either α2 we can increase Na,K-ATPase activity.

Taken together, these studies demonstrate that (1) both α2 and α1 Na,K-ATPase are necessary for clearing fluid from the alveolar epithelium and (2) the α2 Na,K-ATPase, which is located in the alveolar epithelial type 1 cell, serves a specialized function, being responsible for ~60% of basal AFR and ~80% of the β-adrenergic–mediated increase in AFR. (3) Adenovirus-mediated overexpression of the α2 Na,K-ATPase markedly increases Na,K-ATPase activity and lung liquid clearance. These data provide evidence to support the newly developing hypothesis that the α2 Na,K-ATPase expressed within alveolar type 1 cells is the primary contributor to lung liquid clearance.

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References


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MATERIALS AND METHODS

*In situ hybridization:* Methods were essentially as described previously (1). Briefly, 5-μm paraffin-embedded tissue sections on 3-aminopropyltriethoxysilane (TES)-treated slides were treated for 30 min at 37°C with 1 μg/ml proteinase K, washed, and dipped in fresh 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min. After dehydration through a graded series of ethanol washes, the sections were dried and hybridized overnight at 55°C in 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1x Denhardt’s solution, 10% dextran sulfate, 0.5 mg/ml yeast tRNA, and 0.24 μg/ml cRNA probe. Complementary RNA probes were prepared as described previously (1). Before hybridization, limited alkaline hydrolysis of RNA probes was performed to reduce transcript length to 0.1 to 0.3 kb. Probed sections were covered with silanized glass coverslips and were incubated under mineral oil. After hybridization, the oil was removed by three consecutive 5-min chloroform washes and the sections were washed 3 times for 5 min each in 4x SSC. Sections were treated with RNase A (20 μg/ml) and RNase T1 (1 U/ml) in RNAse buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) for 30 min at 37°C. Slides were passed through 30 min washes of 37°C RNAse buffer, 2x SSC at room temperature, 0.1x SSC at 70°C, and 0.1x SSC at room temperature. Sections were dehydrated through a graded series of ethanol washes containing 300 mM ammonium acetate. After autoradiography, slides were counterstained with hematoxylin and eosin before photomicrography.

*Immunoelectron microscopy:* To reduce nonspecific binding grids were incubated for 30 min in TSA 4% (10 nM Tris base; 154 mM NaCl; 4% bovine albumin, pH 7.4) and then incubated overnight with the α2 Na,K-ATPase, McB2, antibody. One grid in each series was incubated
without a primary antibody or with a mouse monoclonal IgG against a parvovirus to determine nonspecific gold labeling. After washing by passage over 5 drops of TSA 4%, all grids were incubated for 45 min on a drop of goat anti-mouse antibody, conjugated to ~5 nm colloidal gold particles. Incubation was terminated by a final wash on 3 drops of TSA 4% and 2 drops of PBS-glutaraldehyde. To avoid possible dissociation of the immunogold label during negative staining, specimens were fixed for 5 min in 1% glutaraldehyde before the negative staining procedure. Fixation prior to the antibody incubation was not possible, because the epitope of the α2 Na,K-ATPase is sensitive to glutaraldehyde (2). Three to four lung samples per animal and two sections per tissue sample were analyzed by transmission electron microscopy. Grid squares were scanned from left to right, and top to bottom, and the first alveolar type 1 cell profile encountered in each grid square was photographed at x6,000. Overlapping pictures were taken for large cell profiles. One to three cell profiles were sampled per section.

Quantitation of labeling and subcellular morphometry. Gold particles were counted on mitochondria, nuclei, and the remaining cell components, which will be referred to here as the cytoplasm/plasma membrane. 250-point overlays were used on each micrograph and points on each of these cell compartments were tallied. The average of two counts was used for computations. Gold particles and points tallied on each compartment for all cell profiles obtained from one section were added. The cross-sectional surface area of the cell compartments was determined, given a mean area of 20 μm²/point, and labeling density (gold particles/μm²) values were calculated for each section. Nineteen cells were analyzed for the expression of Na,K-ATPase α2 isoform protein.

Isolation and culture of alveolar epithelial cells: AT2 cells were isolated from pathogen-free male Sprague-Dawley rats (200-225 g), as previously described (3). Briefly, the lungs were perfused via the pulmonary artery, lavaged and digested with elastase (30 U/ml). AT2 cells
were purified by differential adherence to IgG-pretreated dishes, and cell viability was assessed by trypan blue exclusion (>95%). Cells were suspended in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum with 2 mM L-glutamine, 40 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin and placed in culture for 7 days prior to the start of all experimental conditions. Cells were incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C.

**Na,K-ATPase activity:** Ouabain-sensitive $^{86}$Rb⁺ uptake was used to estimate the rate of K⁺ transport by Na,K-ATPase in alveolar epithelial cells. Briefly, cells were pre-incubated for 5 min at 37°C in a gyratory bath at 100 rpm in HEPES-buffered DMEM in the presence or absence of ouabain. This medium was then removed, and otherwise identical fresh medium containing 1 μCi/ml $^{86}$Rb⁺ was added. Following a 5 min incubation (37°C, 100 rpm), uptake was terminated by aspirating the assay medium and washing the plates in ice-cold MgCl₂. Plates were allowed to dry and cells were solubilized in 0.2% SDS. $^{86}$Rb⁺ influx was quantified in aliquots of the SDS extract by liquid scintillation counting. Protein was quantified in aliquots by the Bradford method (4).

**Preparation of basolateral plasma membranes:** Basolateral membranes (BLM) were purified as previously described (4), using a Percoll gradient. **Western blot analysis:** Equal amounts of protein from BLMs were resolved by 10% SDS-PAGE and analyzed by immunoblotting using specific Na,K-ATPase anti-α2 monoclonal antibody (McB2, generous gift from K. Sweedner) or anti-α1 (generous gift from M. Caplan) monoclonal antibody.

**Shuttle vector construction:** The expression cassette of pCMVβ (Clonetech, San Francisco, CA) was inserted into the XbaI site of pXC2, a pBR322 based plasmid containing the left end
(map unite 0-2 and 9.24-17.24) of the human adenovirus type 5 (a gift from F. Graham, McMaster University) to produce pMRCMVβ-gal. This expression vector contains the immediate early promoter and enhancer from CMV, a full length cDNA for *Escherichia coli lacZ* and the SV40 intron polyadenylation signal. The β-galactosidase cDNA was excised from pMRCMVβ-gal and replaced with full length cDNA for the rat α2 Na,K-ATPase to produce pMRCMVα2. A similar shuttle vector was produced that contained no cDNA (pCMVNull).

**Adenovirus construction:** A 40.3 kb plasmid containing a human type 5 adenovirus (dl309) genome (pJM17) without the E1a gene was cotransfected with the above described shuttle vectors into human embryonic kidney cells (ATCC 293; American Type Culture Collection, Rockville, MD). Homologous recombination, viral assembly, and replication were detected by the development of cytopathologic effect (CPE). Cells from plates showing CPE were collected and disrupted by six cycles of freezing and thawing. The crude viral lysate was expanded in 293 cells. After repeat development of CPE, PCR was used to confirm the presence of the appropriate cDNAs and cyomegalovirus (CMV) promoter in lysate. PCR positive cultures were plaque-purified three times in 293 cells before large scale amplification.

**Adenovirus propagation and purification:** Subconfluent 15-cm tissue culture plates were infected with three plaque-forming units (pfu) per cell. After development of CPE, the cells were harvested, concentrated, and disrupted with six cycles of freezing and thawing. The resultant cell lysate was cleared of cellular debris by centrifugation before purification through serial CsCl density gradient centrifugations. The resultant virus was dialyzed against 10 mM Tris HCl, pH 7.4/1 mM MgCl/10% glycerol to remove CsCl before storage in 10% glycerol at -70°C. Viral titers were ascertained by the enumeration of plaques produced by adenovirus in
293 cells grown under agarose. Viral purity was assessed by demonstrating the absence of detectable wild-type DNA by PCR and plaque production in A549 cells grown under agarose.

**Adenovirus infection protocol: Alveolar epithelial cells:** Cells were washed three time with DMEM/2% FBS (infection medium) before the application of adenovirus in 1-2ml of infection medium. Plates were intermittently rocked for 2 h after infection, 3-7 ml of DMEM/10% FBS with antibiotics was then added. **Rat lungs:** Rats were anesthetized with 40 mg/kg of pentobarbital intraperitoneally and orally intubated with a 14-g plastic catheter. Three experimental groups were studies: surfactant (n=4), AdNull (n=6), and Adα2 (n=7). A mixture of adenovirus in a 50% surfactant/50% dialysis buffer vehicle was administered in four aliquots of 200 μl. Rats were rotated 90° between instillations given at 5-min intervals. Immediately before instillation, a vehicle-forced exhalation was achieved by circumferential compression of the thorax. Compression was relinquished after endotracheal instillation of 200 μl of virus/vehicle followed by 500 μl of air. Rats were allowed to recover before extubation. Infected animals were maintained in separate isolator cages for seven days prior to conducting experimental protocols.

**Isolated perfused lungs:** The isolated perfused lung preparation used in our laboratory has been described in detail(5,6) (7) (8). Briefly, lungs were isolated from anesthetized rats (65 mg/kg Nembutal i.p.) following a 10-min ventilation with 100% O₂. The pulmonary artery and left atrial appendage were cannulated and perfused with a solution of 3% bovine serum albumin (BSA) in buffered physiological salt solution. Fluorescein-labeled (FITC-) albumin was added to the perfusate to monitor leakage of protein from the vascular space into the airways. The recirculating volume of the constant pressure perfusion system was 90 ml; arterial and venous pressures were set at 8 and 0 cm H₂O, respectively. The lungs were excised from the thoracic
cavity and placed in a "pleural" bath (100 ml) filled with the same BSA solution. The entire system was maintained at 37°C in a water bath. The lungs were then instilled via the tracheal catheter with 5 ml BSA containing Evans blue dye-labeled (EBD-) albumin, $^{22}$Na$^+$, and $^3$H-mannitol. Samples were taken from the instillate, perfusate, and bath solutions following a 10-min equilibration period and 60 min later. Absorbance at 620 nm (for EBD-albumin), fluorescence (excitation 487 nm; emission 520 nm; for FITC-albumin), and scintillation counting (for $^{22}$Na$^+$ and $^3$H-mannitol) were measured in centrifuged samples from each compartment. Ten rat lungs were treated as control and were not perfused with either ouabain nor isoproterenol. Seventy rat lungs were perfused with fourteen different concentrations of ouabain, ranging from $10^{-11}$ M to $7.5 \times 10^{-4}$ M, to determine the contribution of the $\alpha_1$ and $\alpha_2$ Na,K-ATPase to alveolar fluid reabsorption. Four to six rat lungs were perfused with each of the fourteen concentrations of ouabain. Sixty-six rat lungs were perfused with 1 µM isoproterenol in the presence or absence of ouabain (the same fourteen concentrations of ouabain were used as described above). Three to five rat lungs were perfused with each of the fourteen concentrations of ouabain.

*Calculations:* The derivation of all equations involved in the mathematical model of edema clearance has been previously described in detail (5,6). Concentration of EBD-albumin was used to estimate airspace volume. As virtually all EBD-albumin remains in the airspace, instillate volume (V) at a given time can be calculated from the increase in airspace protein concentration. The total unidirectional outflux of Na$^+$ from the alveolar space, a result of active transport and passive movement, was calculated from the rate of loss of $^{22}$Na$^+$ from the airspaces. Passive sodium flux was calculated by subtracting the active sodium flux, calculated from the rate of net fluid clearance, from the total. Similarly, the unidirectional volume flux of mannitol was calculated from the rate of loss of $^3$H-mannitol from the airspaces. Albumin flux from the pulmonary circulation into the alveolar space was determined from the fraction of FITC-albumin that appears
in the alveolar instillate during the experimental protocol. For comparison, fluxes are reported as volume fluxes (volume/time) by using the appropriate solute concentrations.

Data Analysis: When comparisons were made between two experimental groups an unpaired Student's t test was used. When multiple comparisons were made a one way analysis of variance and Duncan's means comparison test were used. Results were considered significant when p<0.05.


