Endothelin-1 Impairs Alveolar Epithelial Function via Endothelial ET_B Receptor

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Rationale: Endothelin-1 (ET-1) is increased in patients with high-altitude pulmonary edema and acute respiratory distress syndrome, and these patients have decreased alveolar fluid reabsorption (AFR).

Objectives: To determine whether ET-1 impairs AFR via activation of endothelial cells and nitric oxide (NO) generation.

Methods: Isolated perfused rat lung, transgenic rats deficient in ET_B receptors, cocubation of lung human microvascular endothelial cells (HMVEC-L) with rat alveolar epithelial type II cells or A549 cells, ouabain-sensitive ⁸⁶Rb⁺ uptake.

Measurements and Main Results: The ET-1-induced decrease in AFR was prevented by blocking the endothelin receptor ET_B, but not ET_A. Endothelial-epithelial cell interaction is required, as direct exposure of alveolar epithelial cells (AECs) to ET-1 did not affect Na,K-ATPase function or protein abundance at the plasma membrane, whereas cocubation of HMVEC-L and AECs with ET-1 decreased Na,K-ATPase activity and protein abundance at the plasma membrane. Exposing transgenic rats deficient in ET_B receptors in the pulmonary vasculature (ET-B^{-/-}) to ET-1 did not decrease AFR or Na,K-ATPase protein abundance at the plasma membrane of AECs. Exposing HMVEC-L to ET-1 led to increased NO, and the ET-1-induced down-regulation of Na,K-ATPase was prevented by the NO synthase inhibitor L-NAME, but not by a guanylate cyclase inhibitor.

Conclusions: We provide the first evidence that ET-1, via an endothelial-epithelial interaction, leads to decreased AFR by a mechanism involving activation of endothelial ET_B receptors and NO generation leading to alveolar epithelial Na,K-ATPase down-regulation in a cGMP-independent manner.

Keywords: endothelium; lung injury; sodium-potassium-exchanging ATPase; acute respiratory distress syndrome

Alveolar fluid reabsorption (AFR) is regulated by active Na⁺ transport, whereby Na⁺ enters alveolar epithelial cells (AECs) via the apical Na⁺ channels and exits via the basolateral Na,K-ATPase (1, 2). During acute lung injury epithelial and endothelial dysfunction occurs (2–4), leading to edema formation and impairment of the mechanisms responsible for AFR (2–4).

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although it is well known that alveolar epithelial injury decreases alveolar fluid reabsorption (AFR), there has been a paucity of data regarding the role of the endothelium in affecting the alveolar epithelial function and thus the regulation of AFR.

What This Study Adds to the Field

In this study, we show that there is cross-talk between the pulmonary endothelium and alveolar epithelium, specifically that endothelin-1 (ET-1) activates the endothelial ET_B receptor that generates nitric oxide and thus causes alveolar epithelial dysfunction.

In models of lung injury a decrease in the number of Na,K-ATPase molecules at the plasma membrane, via endocytosis and subsequent protein degradation, results in inhibition of Na⁺ transport and thus AFR (5–8). Therefore, regulation of the Na,K-ATPase represents an important mechanism by which to modulate alveolar epithelial function (6, 9).

Endothelin-1 (ET-1), a potent vasoactive peptide released during injurious stimuli (10, 11), has been reported to increase pulmonary microvascular pressure and lung edema formation via ET_A and ET_B receptors (12–14). ET-1 is increased in serum and bronchoalveolar lavage of patients with acute lung injury, suggesting lung endothelial-epithelial dysfunction (13, 15–17). The endothelium as a source of oxidative injury releases nitric oxide (NO) via endothelial ET_B receptor activation (11, 18, 19). Several reports have indicated that NO down-regulates active sodium transport in AECs via a cGMP-mediated inhibition of epithelial cation channels (20–22). Also, it has been reported that NO decreases sodium absorption across AEC monolayers by inhibiting both amiloride Na⁺ channels and Na,K-ATPase through a cGMP-independent mechanism (21). Accordingly, we hypothesized that ET-1 decreases active sodium transport in AECs and consequently reduces AFR.

In the present study, we provide the first evidence that endothelial-epithelial interaction is necessary for ET-1 to impair alveolar epithelial function by down-regulating Na,K-ATPase in AECs via the activation of endothelial ET_B receptors and NO generation in a cGMP-independent pathway leading to alveolar epithelial dysfunction.

Some of the results of these studies have been previously reported in the form of an abstract (23, 24).

Figure 1. Endothelin-1 (ET-1) decreases alveolar fluid reabsorption (AFR) in isolated perfused rat lungs with no changes in permeability. (A) Isolated rat lungs were perfused for 1 hour with vehicle (control, CT) or ET-1 (10^{-11} – 10^{-6} M) and AFR was measured as described in METHODS ($n = 6$). (B) Passive movement of $^{22}\text{Na}^+$ (solid columns) and ^3H -mannitol (open columns) was measured as described in METHODS ($n = 6$). Differences among groups were not statistically significant. (C) Fluorescein isothiocyanate (FITC) flux was measured as described in METHODS ($n = 6$). Differences among groups were not statistically significant. Graphs represent means $\pm$ SEM. $^{**}P < 0.01$.

NHS-SS-biotin and streptavidin beads were purchased from Pierce Biochemical (Rockford, IL). Endothelin B receptor antibody was from Novus Biologicals (Littleton, CO). Endothelin-1 antibody was from Alexis Biochemicals as marketed by Axxora (San Diego, CA). Spermine NONOate was from Cayman Chemical (Ann Arbor, MI). *N*^ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), amiloride hydrochloride, cyclic guanosine monophosphate analog 8-Br-cGMP (8-bromoguanosine-3',5'-cyclic monophosphate), and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) were from Sigma-Aldrich (St. Louis, MO). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was from Calbiochem (San Diego, CA). All other reagents were commercial products of the highest grade available.

Animals

One hundred and twenty-five pathogen-free male Sprague-Dawley rats weighing 250–310 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were studied. In addition, two groups of rats, male and female, were studied: wild-type control [dopamine β -hydroxylase [DBH] transgene^{-/-}, ET_B receptor^{+/+}] (12 rats) and transgenic (*sl/sl*) [DBH transgene^{+/+}, ET_B receptor (*sl/sl*)] (10 rats). These animals were provided by M. Yanagisawa (Southwestern Medical School, University of Texas Southwestern Medical Center, Dallas, Texas). The genotype of each animal was confirmed by polymerase chain reaction of genomic DNA, performed according to standard techniques as previously described (25). All animals were provided food and water *ad libitum* and maintained on a 12:12 hour light/dark cycle. This study was conducted in accordance with both local institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health).

Alveolar Epithelial Type II Cells and Human Endothelial Lung Microvascular Cells

Rat alveolar type II (ATII) cells were isolated from the lungs of Sprague-Dawley rats weighing 200–225 g, as previously described (26). ATII and human alveolar epithelial cells (A549 cells [CCL-185; American Type Culture Collection, Manassas, VA]) were grown in

METHODS

Materials

Na,K-ATPase α_1 subunit monoclonal antibody (clone 464.6) was purchased from Upstate Biotechnology (Lake Placid, NY). EZ-Link

Figure 2. Endothelin-1 (ET-1) decreases alveolar fluid reabsorption (AFR) in isolated perfused rat lungs via a decrease in Na,K-ATPase activity. (A) Isolated rat lungs were perfused for 1 hour with vehicle (control, CT) or 10^{-7} M ET-1 in the presence or absence of 10^{-4} M amiloride instilled into the airspace. AFR was measured as described in METHODS ($n = 5$). (B)

Isolated rat lungs were perfused for 1 hour with vehicle (CT) or 10^{-7} M ET-1 in the presence or absence of 5×10^{-4} M ouabain (Ouab). AFR was measured as described in METHODS ($n = 6$). Graphs represent means $\pm$ SEM. $^{***}P < 0.001$.

TABLE 1. OLIGONUCLEOTIDE PRIMERS FOR AMPLIFICATION BY POLYMERASE CHAIN REACTION

	Primer Sequences (5' → 3')	GenBank ID	Expected Size (bp)
ET _A	ETA-5': CGTCTTCTGCTTGGTGTCA ETA-3': GCAACAGAGGCATGACTGAA	NM_012550	238
ET _B	ETB-5': TGCACACCTTTCCGCAAGCACG ETB-3': AGCTGGTCCCTTCATACAGAAGGC	AF074963	919
PPT	ET-1-5': GAAAGTGTATCTATCAGCAGC ET-1-3': GGAACACCTCAACCTCTCTTGG	M64711	334
GAPDH	GAPDH-5': ACCACAGTCCATGCCATCAC GAPDH-3': TCCACCACCTGTTGCTGTA	BC064681	452

Definition of abbreviations: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; ET_A and ET_B = endothelin receptors A and B, respectively; PPT = prepro-endothelin.

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Human endothelial lung microvascular cells (HMVEC-L) were purchased from Lonza (Walkersville, MD). HMVEC-L were plated, grown, and maintained as specified by the supplier, using endothelial cell medium (EGM-2 BulletKit; Lonza, Walkersville, MD) containing the following supplements: human epidermal growth factor, hydrocortisone, GA-1000 (gentamicin, amphotericin-B), fetal bovine serum, vascular endothelial growth factor, human fibroblast growth factor (basic), long R3 insulin-like growth factor-I, ascorbic acid, and heparin. All cells were incubated in a humidified atmosphere of 5% CO₂–95% air at 37°C. ATII cells isolated from pathogen-free male Sprague-Dawley rats were cocultured with HMVEC-L and endothelial cell medium for various time intervals, 30 minutes and 6 hours. A549 cells were cocultured with HMVEC-L and endothelial cell medium for 30 minutes. All AECs cocultured with HMVEC-L were then switched to DMEM without serum 30 minutes before treatment.

Isolated Lung Experiments

Briefly, rats were anesthetized with pentobarbital (50 mg/kg body weight), tracheotomized, and mechanically ventilated at a rate of 35 rpm, tidal volume of 5 ml/kg, positive end-expiratory pressure of 0 cm H₂O, and 100% oxygen for 5 minutes. The chest was opened via a median sternotomy, after which 400 U of heparin sodium was injected into the right ventricle. After exsanguination, the heart and lungs were removed *en bloc*. The pulmonary artery and left atrium were catheterized and perfused continuously with a solution of 3% bovine serum albumin in buffered physiological salt solution (135.5 mM Na⁺, 119.1 mM Cl⁻, 25 mM HCO₃⁻, 4.1 mM K⁺, 2.8 mM Mg⁺, 2.5 mM Ca²⁺, 0.8 mM SO₄²⁻, 8.3 mM glucose). Trace amounts of fluorescein

isothiocyanate (FITC)-conjugated albumin was also added to the perfusate. The recirculating volume of the constant pressure perfusion system was 90 ml; arterial and venous pressures were set at 12 and 0 cm H₂O, respectively. The vascular pressures were recorded every 10 seconds with a multichannel recorder (Cyber Sense Inc., Nicholasville, KY). The lungs were immersed in a "pleural" bath (100 ml) filled with the same bovine serum albumin solution. The entire system was maintained at 37°C in a water bath. Perfusate pH was maintained at 7.40 by bubbling with a gas mixture of 95% O₂–5% CO₂. The lungs were then instilled via the tracheal cannula in two sequential phases with a total volume of 5 ml of the bovine serum albumin solution containing Evans blue dye-conjugated albumin (0.1 mg/ml), ²²Na⁺ (0.02 µCi/ml), and [³H]mannitol (0.12 µCi/ml). Samples were taken from the instillate, perfusate, and bath solutions after an equilibration time of 10 minutes from the instillation and again 60 minutes later. To ensure homogeneous sampling of the instillate, a volume of 2 ml was aspirated and reintroduced into the airspaces three times before removing each sample. All samples were centrifuged at 3,000 × *g* for 10 minutes. Absorbance analysis of the supernatant or Evans blue dye-albumin was performed at 620 nm in a Hitachi model U2000 spectrometer (Hitachi, San Jose, CA). Analysis of FITC-albumin (excitation, 487 nm; emission, 520 nm) was performed in a model LS-3B fluorometer (PerkinElmer, Oakbrook, IL) (27). Additional detail on the method for performing these measurements is provided in the online supplement.

Cell Surface Labeling

Cells were labeled for 20 minutes with EZ-Link Sulfo-NHS-SS-biotin (1 mg/ml) and pulled down (captured and purified) with streptavidin as previously described (6, 7). Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted with an antibody specific for Na,K-ATPase α₁ subunit.

Determination of Na,K-ATPase Activity

Na,K-ATPase activity was determined by ouabain-sensitive ⁸⁶Rb⁺ uptake (Amersham Biosciences, Arlington Heights, IL) as previously described (6, 7, 29, 30). Protein was quantified in aliquots by the Bradford method (31).

Determination of NO from Endothelial and Alveolar Epithelial Cells through DAF-2 DA Imaging

Epithelial and endothelial cells were cultured on 35-mm glass coverslips and were washed twice with phosphate-buffered saline before a 15- or 30-minute incubation in 1 or 12 µM DAF-2 DA at 37°C in serum-free and phenol and red-free medium (Cellgro; Mediatech, Herndon, VA). Additional detail on the method for making these measurements is provided in the online supplement.

Figure 3. Direct incubation of endothelin-1 (ET-1) on rat alveolar type II (ATII) cells does not decrease Na,K-ATPase activity or protein abundance at the plasma membrane. (A) ATII cells were incubated for 30 minutes with vehicle (control, CT) or with 10⁻⁷ M ET-1. Na,K-ATPase activity was determined in ATII cells by ouabain-sensitive ⁸⁶Rb⁺ uptake (*n* = 6). Differences among groups were not statistically significant. (B) ATII cells were incubated with vehicle (CT) or with 10⁻⁷ M ET-1 for various time intervals. Na,K-ATPase α₁ subunit at the plasma membrane was determined by biotin–streptavidin pulldown and Western blot. A representative Western blot of Na,K-ATPase α₁ subunit is shown (*n* = 3). Differences among groups were not statistically significant. Graphs represent means ± SEM.

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Figure 4. Lung human microvascular endothelial cells (HMVEC-L) are required for the endothelin-1 (ET-1) decrease in Na,K-ATPase activity and fluid reabsorption in alveolar epithelial cells (AECs). (A) HMVEC-L were coincubated with alveolar type II (ATII) cells for 30 minutes and 6 hours, followed by treatment for 30 minutes with vehicle (control, CT) or with 10^{-7} M ET-1. Na,K-ATPase activity was determined by ouabain-sensitive $^{86}\text{Rb}^+$ uptake in ATII cells ($n = 4$). (B) HMVEC-L were coincubated with ATII cells for 30 minutes, followed by treatment for 30 minutes with vehicle (CT) or with 10^{-7} M ET-1. Na,K-ATPase α_1 subunit at the plasma membrane was determined by biotin-streptavidin pull-down and Western blot. A representative Western blot of Na,K-ATPase α_1 subunit is shown ($n = 3$). (C) A549 cells were treated for 30 minutes with vehicle (CT)

or with 10^{-7} M ET-1 and coincubated with HMVEC-L for 30 minutes followed by treatment with vehicle (CT) or with 10^{-7} M ET-1 for 30 minutes. Na,K-ATPase activity was determined by ouabain-sensitive $^{86}\text{Rb}^+$ uptake in A549 cells ($n = 3$). (D) ET-1 (10^{-7} M) was perfused for 1 hour or instilled into the airspace of isolated rat lungs and AFR was measured as described in METHODS ($n = 4$). Graphs represent means $\pm$ SEM. ** $P < 0.01$; *** $P < 0.001$.

Figure 5. Endothelin-1 (ET-1) decreases alveolar fluid reabsorption via activation of the endothelial ET_B receptor. (A) Isolated rat lungs were perfused for 1 hour with vehicle (CT), 10^{-7} M ET-1, 10^{-6} M PD 142893 (PD), PD + ET-1, 10^{-6} M BQ-123 (BQ), BQ + ET-1, 10^{-6} M IRL-1620, or BQ + IRL-1620 and alveolar fluid reabsorption (AFR) was measured as described in METHODS ($n = 5$). (B) Passive movement of $^{22}\text{Na}^+$ (solid columns) and [^3H]mannitol (open columns) was measured as previously described (5) ($n = 6$). Differences among groups were not statistically significant. Graphs represent means $\pm$ SEM. ** $P < 0.01$.

Figure 6. Endothelin-1 (ET-1) decreases alveolar fluid reabsorption and Na,K-ATPase α_1 subunit abundance at the basolateral membrane in rat lungs, via activation of endothelial ET_B receptor. (A) Isolated rat lungs of wild-type (WT) and ET_B receptor transgenic rats were perfused for 1 hour with vehicle (control, CT) or 10^{-7} M ET-1 and alveolar fluid reabsorption (AFR) was measured as described in METHODS ($n = 8$). (B) Passive movement of $^{22}\text{Na}^+$ (solid columns) and [^3H]mannitol (open columns) was measured as previously described (5) ($n = 6$). Differences among groups were not statistically significant. (C) Basolateral membranes were purified from the peripheral lung tissue of rat lungs (28) treated as in (A), and Na,K-ATPase protein abundance was assessed by Western blot. A representative Western blot of Na,K-ATPase α_1 subunit is shown ($n = 3$). Graphs represent means $\pm$ SEM. ** $P < 0.01$; *** $P < 0.001$.

Reverse Transcription-Polymerase Chain Reaction

Additional detail on the method for making these measurements is provided in the online supplement.

Statistical Analysis

Data are reported as means $\pm$ SEM. Statistical analysis was done by one-way analysis of variance and Tukey correction. When two groups were compared analysis was done by Student *t* test. Results were considered significant when $P < 0.05$.

RESULTS

ET-1 Decreases Alveolar Fluid Reabsorption in Isolated Rat Perfused Lungs

ET-1 decreased AFR by about 40% when perfused for 60 minutes in isolated rat lungs, without affecting the passive fluxes of small or large solutes or FITC flux, indicating that there were no significant changes in epithelial barrier permeability (Figures 1A, 1B, and 1C). Because epithelial Na⁺ channels and Na,K-ATPase contribute to AFR, we set out to determine whether ET-1 had an effect on either epithelial Na⁺ channels or Na,K-ATPase in this *ex vivo* model. As shown in Figure 2A, amiloride or ET-1 decreased AFR by about 50% and the combination of both caused complete inhibition of AFR, suggesting that ET-1 does not act on the amiloride-sensitive Na⁺ channels. Conversely, the addition of ET-1 and ouabain alone or in combination resulted in a 50% decrease in AFR without evidence of an additive effect (Figure 2B).

Endothelial Cells Are Required for the ET-1-mediated Down-regulation of Alveolar Epithelial Na,K-ATPase and Decreased Alveolar Fluid Reabsorption

To determine whether expression of ET receptors was present in alveolar epithelial type I (ATI), ATII, and ATII Day 7 cells, we designed oligonucleotide primers for these receptors and prepro-endothelin (Table 1). As shown in Figure E1A in the online supplement, there was a strong signal for ET_B receptor and prepro-endothelin mRNA in all three cell types. Furthermore, Western blot analysis showed the presence of ET_B receptor in total cell lysate and plasma membrane of isolated ATII cells (Figure E1B). In addition, immunohistochemistry experiments were performed in rat lungs, where the ATII cell marker LB 180 and the ET_B receptor signal colocalized (Figure E1C).

To determine whether ET-1 had a direct effect on Na,K-ATPase activity and protein membrane abundance in AECs, rat ATII cells were incubated with ET-1 for 15, 30, and 60 minutes. ET-1 did not decrease Na,K-ATPase activity (Figure 3A) or Na,K-ATPase α_1 protein abundance at the plasma membrane (Figure 3B). To determine whether activation of endothelial cells by ET-1 is required for regulation of Na,K-ATPase function in the alveolar epithelium we coincubated HMVEC-L with polarized ATII cells for 30 minutes and 6 hours, followed by treatment with ET-1 (10^{-7} M) for 30 minutes. This resulted in a decrease in Na,K-ATPase activity and α_1 subunit protein abundance at the plasma membrane in ATII cells (Figures 4A and 4B). In addition, we observed that the results obtained for rat ATII cells were reproduced when human A549 cells were coincubated with HMVEC-L and then treated with ET-1 (10^{-7} M) for 30 minutes, as Na,K-ATPase activity decreased approximately 50% (Figure 4C). Furthermore, as depicted in Figure 4D, ET-1 decreased AFR only when added to the vascular space.

ET-1 Decreases Alveolar Fluid Reabsorption via Activation of Endothelial ET_B Receptor

To determine the type of ET receptor involved in the ET-1-induced decrease in AFR, we conducted experiments with a specific endothelin receptor agonist and antagonists. As shown in Figure 5A, isolated rat lungs perfused with the nonselective ET receptor antagonist PD 142893 prevented the ET-1-induced decrease in AFR. Conversely, perfusion with the selective ET_A antagonist BQ-123 did not prevent the ET-1-mediated decrease in AFR. When the specific ET_B receptor agonist IRL-1620 was perfused through the pulmonary circulation, in the presence and absence of the ET_A antagonist BQ-123, there was, in both cases, a significant decrease in AFR, comparable to that seen with ET-

Figure 7. Endothelin-1 (ET-1) increases nitric oxide (NO) generation in human microvascular endothelial cells (HMVEC-L) but not in alveolar type II (ATII) cells. (A) NO was determined by immunofluorescence as described in METHODS. HMVEC-L were pretreated with vehicle (Basal) or with 100 μ M L-NAME for 30 minutes followed by 15 minutes of equilibration to assess baseline rates of fluorescence changes, followed by treatment with ET-1 (10^{-7} M) for 5 minutes ($n = 3$). (B) NO was determined by immunofluorescence as described in METHODS. After 15 minutes of equilibration to assess baseline rates of fluorescence changes, ET-1 (10^{-7} M) or spermine NONOate (1 mM) was added for 5 minutes ($n = 3$). Graphs represent means $\pm$ SEM. $^{**}P < 0.01$; $^{***}P < 0.001$.

1, with no effect on the passive fluxes of small or large solutes (Figure 5B). To further determine the role of the ET_B receptor, we studied whether ET-1 decreased AFR in a transgenic rat model that does not express ET_B receptors in the lung endothelium and has minimally functional ET_B receptors in the lung parenchyma (32). ET-1 decreased AFR in wild-type rats, but not in transgenic rats, without affecting the passive fluxes of small or large solutes (Figures 6A and 6B). Moreover, the decrease in Na,K-ATPase α_1 subunit protein abundance at the basolateral membranes observed in wild-type rats was not observed in transgenic rats (Figure 6C).

ET-1 Decreases Alveolar Fluid Reabsorption via Endothelial Cell NO Generation

It has been described previously that ET_B activation generates NO in endothelial cells (11, 18, 19). Thus, we investigated

whether the ET-1-induced NO production was responsible for the ET-1-induced decrease in AFR. In our system, HMVEC-L respond to ET-1 by increasing NO production, which was blocked by preincubation with the nitric oxide synthase inhibitor *N*^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), 100 μ M for 30 minutes (Figure 7A). This dose was based on previous reports that showed nitric oxide synthase inhibition as well as restoration of AFR (22, 33). As shown in Figure 7B, when ATII cells were treated with ET-1 there was no increase in NO production. As a positive control for the NO measurement, ATII cells were incubated in the presence of the NO donor spermine NONOate (1 mM). Furthermore, when ATII and A549 cells were treated with spermine NONOate at different concentrations (10–250 μ M for 30 min), they had a decrease in Na,K-ATPase activity (Figures 8A and 8B). In addition, pretreatment of HMVEC-L with L-NAME prevented the ET-1-mediated decrease in Na,K-ATPase activity and

Figure 8. Endothelin-1 (ET-1) decreases alveolar fluid reabsorption via nitric oxide generation from endothelial cells. (A) alveolar type II (ATII) cells were incubated for 30 minutes with vehicle (control, CT) or with 50, 100, and 250 μ M spermine NONOate. Na,K-ATPase activity was determined in ATII cells by ouabain-sensitive $^{86}\text{Rb}^+$ uptake ($n = 3$). (B) A549 cells were incubated for 30 minutes with vehicle (CT) or with 10 μ M spermine NONOate. Na,K-ATPase activity was determined in A549 cells by ouabain-sensitive $^{86}\text{Rb}^+$ uptake ($n = 3$). (C) Human microvascular endothelial cells (HMVEC-L) were treated for 30 minutes with vehicle (CT) or with 10^{-7} M ET-1, in the presence and absence of 100 μ M L-NAME, and coincubated with ATII cells. Na,K-ATPase activity was determined in ATII cells by ouabain-sensitive $^{86}\text{Rb}^+$ uptake ($n = 3$). (D) HMVEC-L were treated for 30 minutes with vehicle (CT) or 10^{-7} M ET-1 in the presence and absence of 100 μ M L-NAME and coincubated with ATII cells. Na,K-ATPase α_1 subunit at the plasma membrane was determined by biotin-streptavidin pulldown and Western blot. A representative Western blot of Na,K-ATPase α_1 subunit is shown ($n = 4$). (E) Isolated rat lungs were perfused for 1 hour with vehicle (CT) or with 10^{-7} M ET-1 in the presence and absence of 100 μ M L-NAME, and alveolar fluid reabsorption was measured as described in METHODS ($n = 5$). Graphs represent means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

protein abundance in ATII cells (Figures 8C and 8D). Moreover, as shown in Figure 8E, pretreatment of rat lungs with L-NAME, 100 μ M for 30 minutes, prevented the ET-1-induced decrease in AFR.

ET-1 Decreases Alveolar Fluid Reabsorption via a cGMP-independent Pathway

Classically, it has been described that NO acts via activation of soluble guanylate cyclase (22, 34, 35). To determine whether the decrease in AFR is via a cGMP-dependent pathway, we pretreated isolated rat lungs with 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a highly selective inhibitor of soluble guanylate cyclase. Figure 9A shows that when ODQ, 15 μ M, was instilled into the airspace, it did not block the effect of the ET-1-induced decrease in AFR. Moreover, pretreatment of ATII cells with ODQ, 15 μ M for 30 minutes, did not prevent the ET-1-induced decrease in Na,K-ATPase activity and protein abundance at the plasma membrane (Figures 9B and 9C) in the coculture system. In addition, the cGMP agonist 8-Br-cGMP (100 μ M), when added exogenously to ATII cells

for 30 minutes, did not decrease Na,K-ATPase activity (Figure 9D).

DISCUSSION

It is widely accepted that during lung injury AFR is decreased as the result of epithelial injury leading to the down-regulation of alveolar epithelial Na,K-ATPase (36, 37). However, the interactions of endothelial and epithelial cells in the regulation of alveolar epithelium function have not been elucidated.

ET-1 increases pulmonary microvascular pressure, causing edema formation, and several studies have suggested that ET-1 receptor inhibition has a protective effect in models of acute lung injury (12, 38–40). ET-1 is a potent vasoactive peptide released by endothelial and epithelial cells during stimuli such as shear stress, hypoxia, and inflammation (11, 18, 19). Levels of ET-1 are increased in patients with acute respiratory distress syndrome (ARDS) and high-altitude pulmonary edema (13, 16, 17, 41–43). We demonstrated that ET-1 decreased AFR by 50% in isolated perfused rat lungs without having any significant effect on the epithelial barrier (*see* Figure 1). In addition, the

Figure 9. Endothelin-1 (ET-1) decreases alveolar fluid reabsorption (AFR) via a cGMP-independent pathway. (A) Isolated rat lungs were perfused for 1 hour with vehicle (control, CT) or 10^{-7} M ET-1 in the presence and absence of $15 \mu\text{M}$ 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) instilled into the airspace. AFR was measured as described in METHODS ($n = 4$). (B) Human microvascular endothelial cells (HMVEC-L) were treated for 30 minutes with vehicle (CT) or 10^{-7} M ET-1 in the presence or absence of $15 \mu\text{M}$ ODQ and coincubated with alveolar type II (ATII) cells. Na,K-ATPase α_1 subunit at the plasma membrane was determined by biotin-streptavidin pulldown and Western blot. A representative Western blot of Na,K-ATPase

α_1 subunit is shown ($n = 3$). (C) HMVEC-L were treated as in (B) and coincubated with ATII cells. Na,K-ATPase activity was determined in ATII cells by ouabain-sensitive $^{86}\text{Rb}^+$ uptake ($n = 3$). (D) ATII cells were incubated for 30 minutes with vehicle (CT) or $100 \mu\text{M}$ 8-Br-cGMP. Na,K-ATPase activity was determined by ouabain-sensitive $^{86}\text{Rb}^+$ uptake ($n = 3$). Graphs represent means $\pm$ SEM. Differences among groups were not statistically significant. * $P < 0.05$; ** $P < 0.01$.

ET-1-induced decrease in AFR was further reduced when amiloride was present in the airspace. Conversely, when ouabain was perfused via the pulmonary circulation there was no further reduction in AFR, suggesting that the ET-1-induced decrease in AFR occurs via an effect on Na,K-ATPase (see Figure 2).

ET-1 has multiple effects depending on which receptor is activated. Jain and colleagues reported the presence of these receptors in ATII cells (14). Although ET_B receptors are expressed in ATII cells (see Figure E1), ET-1-induced down-regulation of sodium transport in AECs requires the activation of endothelial cells as evidenced by the lack of response of ATII cells when they were directly incubated with ET-1 (see Figure 3). Conversely, when ATII cells were coincubated with HMVEC-L in the presence of ET-1, Na,K-ATPase activity and protein abundance at the plasma membrane were decreased. Furthermore, this effect remained even after coincubating HMVEC-L and ATII cells for a longer time period (see Figures 4A and 4B). In addition, the ET-1-induced decrease in Na,K-ATPase activity occurred in A549 cells coincubated with HMVEC-L, demonstrating that the ET-1-induced Na,K-ATPase down-regulation is independent of the cell species, as rat (ATII) and human (A549) alveolar epithelial cells responded similarly (see Figure 4C). Consistent with these *in vitro* findings, ET-1 decreased AFR only when added to the vascular space, suggesting that ET-1 decreases AFR by down-regulating the Na,K-ATPase function in AECs via its effects on endothelial cells. Consequently, we hypothesized that endothelial ET_B receptor is involved in the ET-1-induced decrease in AFR. ET_B receptor in smooth muscle has a vasoconstrictor effect, whereas in endothelial cells it causes the release of NO, leading to vasodilation (11, 18, 19). To determine the type of ET receptor involved in the ET-1-induced decrease in AFR, we performed

studies using a specific endothelin receptor agonist and antagonists, showing that the ET_B receptor is responsible for the ET-1-induced decrease in AFR (see Figure 5). To further elucidate the role of the ET_B receptor in the ET-1-induced decrease in AFR we used an ET_B transgenic rat model (25, 32). The spotting lethal rat strain has a nonfunctional ET_B receptor leading to intestinal aganglionsis, intestinal obstruction, and death shortly after birth (44). Gariepy and colleagues rescued the spotting lethal rat by transgenic expression of the ET_B gene, using the DBH promoter. Rats carrying the transgene were crossed with spotting lethal rats, which resulted in animals that express ET_B only under the transcriptional control of the DBH promoter. These animals lack expression of the ET_B receptor except in adrenergic tissue (25). Ivy and colleagues characterized ET_B receptor expression in these animal lungs, finding no mRNA signal in the pulmonary arteries and virtually nonfunctional ET_B receptors in the lung parenchyma (32). Lungs isolated from these transgenic rats did not have an ET-1-induced decrease in AFR or a down-regulation of Na,K-ATPase protein abundance at the basolateral membrane (see Figure 6), further demonstrating that ET_B receptors are required for the ET-1-induced decrease in AFR.

As it has been previously described, NO decreases amiloride-sensitive sodium transport in AECs and inhibits AFR (20–22, 45–47). Guo and colleagues showed inhibition of Na,K-ATPase in ATII cells after exposure to propylamine propylamine (PAPA) NONOate (21). Kaestle and colleagues showed that acute elevation of hydrostatic pressures in isolated perfused rat lungs caused an increase in endothelial NO levels leading to a decrease in AFR (22). Conversely, mechanisms associated with a decrease in AFR in noncardiogenic pulmonary edema such as ARDS or high-altitude pulmonary edema cannot be explained by an increase in hydrostatic pressure

Figure 10. Endothelin-1 binds to the ET_B receptor in endothelial cells, releasing nitric oxide, which leads to the down-regulation of Na,K-ATPase function in alveolar type II (ATII) cells.

causing an increase in NO release. In addition, patients with congestive heart failure have increased levels of ET-1 (48–51) and ET-1 is released from endothelial cells when exposed to stretch or shear stress (11, 52). Here, we provide evidence that ET-1, which is increased in cardiogenic and noncardiogenic pulmonary edema, increases endothelial NO levels, which are responsible for the decrease in AFR (*see* Figures 7 and 8). Although our results are consistent with previous reports that NO decreases active sodium transport (20–22, 45), the ET-1-mediated effect appears not to inhibit the amiloride-sensitive sodium channels but the Na,K-ATPase. We speculate that the difference in the effects could be explained by the endothelial source of NO, which probably interacts with the basolateral AEC domains where Na,K-ATPase is located, as opposed to the epithelial apical amiloride-sensitive sodium channels (1, 2, 4, 9). Also, the ET-1-mediated decrease in active sodium transport occurs via a cGMP-independent pathway, as opposed to most reports, which have shown it to be a cGMP-dependent pathway (20–22, 45). Our results are in agreement with the initial description by Goodman and colleagues, in which the exogenous addition of 8-Br-cGMP did not alter dome formation in AECs (53), and with Guo and colleagues, who reported that when ATII cells were exposed to various concentrations of NO donors there was no increase in cGMP levels and 8-Br-cGMP added to the bathing solution of ATII cells did not alter their short circuit currents (21). Several mechanisms have been proposed to explain the decrease in epithelial sodium transport via a cGMP-independent mechanism, including reactive nitrogen species promoting the endocytosis of Na,K-ATPase, post-translational modifications of Na,K-ATPase by oxidizing and/or

nitrosylating key thiol residues, or modifying structural proteins such as actin (21, 54).

In summary we provide the first evidence that ET-1 decreases the ability of the lungs to clear alveolar fluid. Furthermore, the endothelial activation by ET_B receptors and NO generation leads to Na,K-ATPase down-regulation and decreased AFR via a cGMP-independent mechanism in the alveolar epithelium (Figure 10). These findings have potential clinical implications, as patients with ARDS have higher levels of ET-1 (17, 41, 42), which could be pharmacologically modulated with endothelin receptor antagonists.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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