Alveolar epithelial fluid clearance persists in the presence of moderate left atrial hypertension in sheep

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Campbell, Andre R., Hans G. Folkesson, Yves Berthiaume, Jolanta Gutkowska, Sotashi Suzuki, and Michael A. Matthay. Alveolar epithelial fluid clearance persists in the presence of moderate left atrial hypertension in sheep. J. Appl. Physiol. 86(1): 139–151, 1999.—The effect of moderate left atrial (LA) hypertension on alveolar liquid clearance (ALC) was investigated in anesthetized, ventilated sheep, surgically prepared to measure lung lymph flow as well as hemodynamics. To simulate alveolar edema, 3–4 ml/kg of isosmolar 5% albumin in Ringer lactate were instilled into each lower lobe, and ALC was measured. After 4 h of LA hypertension (24 cmH2O), ALC was similar to that in control sheep (31 ± 3% with LA hypertension vs. 34 ± 10% with normal LA pressure). Because plasma epinephrine levels were moderately elevated in the presence of LA hypertension, ALC was then studied in the presence of LA hypertension following bilateral adrenalectomy. Without endogenous release of epinephrine, ALC was significantly reduced compared with normal LA pressure (20 ± 7% compared with 34 ± 10%, P < 0.05). Thus endogenous catecholamines caused a submaximal stimulation of ALC in the presence of LA hypertension. Exogenous administration of aerosolized β2-agonist therapy with salmeterol increased ALC in the presence of normal LA pressure but had no stimulatory effect in the presence of moderate LA hypertension. Therefore, we tested the hypothesis that endogenous release of atrial natriuretic factor (ANF) may downregulate alveolar epithelial Na+ and fluid transport in the presence of LA hypertension. There was a modest twofold increase in plasma ANF levels after LA hypertension. Additional in vitro studies demonstrated that, in the presence of β2-agonist stimulation, ANF decreased Na+ pump activity (Na+-K+-ATPase) in isolated rat alveolar epithelial type II cells. ANF may downregulate vectorial Na+ and fluid transport stimulated by endogenous or exogenous β2-adrenergic agonist stimulation in the presence of LA hypertension. In summary, ALC continues even in the presence of moderate LA hypertension. Aerosolized β2-adrenergic agonist therapy significantly increased ALC, but only when LA pressure was normal.

pulmonary edema; heart failure; β-adrenergic therapy; lung lymph flow; atrial natriuretic factor; alveolar epithelial type II cells; salmeterol

CLINICAL PULMONARY EDEMA in the presence of heart failure primarily occurs because of elevated left atrial pressure (1, 34, 38). However, the relationship of elevated left atrial pressure to the resolution of alveolar edema is not well understood. Since work from several investigators has demonstrated that alveolar fluid transport depends on active Na+ transport across the alveolar epithelium (2, 9, 12, 18, 20, 23, 24, 26, 40), it is possible that active fluid transport protects against alveolar flooding in the presence of elevated lung microvascular pressures. With the exception of one study in newborn lambs (29), the effect of left atrial hypertension on lung liquid clearance has not been studied.

Therefore, the first objective was to determine the effect of moderate left atrial hypertension on alveolar fluid clearance in anesthetized, ventilated sheep. Because the initial experiments demonstrated that alveolar fluid clearance persisted at a normal rate despite moderate left atrial hypertension, the second objective was to investigate the potential role of endogenous epinephrine release in maintaining alveolar fluid clearance at normal levels during moderate left atrial hypertension. Two lines of evidence supported a role for endogenous epinephrine in sustaining normal alveolar fluid clearance in the setting of moderately elevated left atrial pressure. First, plasma epinephrine levels were increased during left atrial hypertension. Second, bilateral adrenalectomies reduced alveolar fluid clearance in the presence of left atrial hypertension by 30%. The third objective was to evaluate the potential effect of aerosolized β2-agonist therapy as a method for stimulating alveolar fluid clearance to a maximal level in the presence of left atrial hypertension. However, aerosolized salmeterol (a lipophilic β-adrenergic agonist) did not increase alveolar fluid clearance in the presence of left atrial hypertension, whereas aerosolized salmeterol did increase alveolar fluid clearance in sheep with normal left atrial pressure. Therefore, the fourth objective was to test the hypothesis that release of atrial natriuretic factor (ANF) in the presence of left atrial hypertension may blunt the normal response of the alveolar epithelium to β2-adrenergic agonist stimulation. This hypothesis was suggested by prior in vivo (25) and in vitro (37) studies in which ANF reduced alveolar epithelial Na+ transport. In the sheep experiments in these studies, there was a modest twofold increase in plasma ANF after left atrial hypertension. In addition, in vitro studies with rat alveolar epithelial type II cells indicated that ANF inhibited Na+ pump activity in the presence of β2-adrenergic stimulation.

The in vivo experiments were done in anesthetized, ventilated sheep so that systemic and pulmonary hemodynamics could be measured at the same time that alveolar and lung liquid clearance was measured. Measurement of lung lymph flow and the lymph-to-plasma protein concentration ratio made it possible to determine the effect of increased left atrial pressures on lung vascular filtration as well as to estimate absorption of
protein-free alveolar fluid into the lung lymph, as we have done before (3, 20).

METHODS

Sheep Preparation and General Experimental Protocol

Thirty yearling sheep (28 ± 4 kg) were anesthetized with intravenous thiopental sodium. A tracheotomy was done via a midline incision in the neck. After insertion of the tracheotomy tube, the sheep were ventilated with a constant-volume piston pump (Harvard Apparatus, Dover, MA) with an inspired O2 fraction of 1.0 and a tidal volume of 13–15 ml/kg body wt. Anesthesia was maintained by including 1% halothane in the inspired O2. A catheter was inserted into the carotid artery to measure arterial blood gases, systemic blood pressure, and to obtain blood samples. Positive end-expiratory pressure (3 cmH2O) was maintained throughout the experiment, and airway pressures were monitored. The respiratory rate was adjusted to maintain the arterial Pco2 between 30 and 40 Torr. Pancuronium bromide (0.3 mg·kg body wt·h−1, Pavulon, Organon, West Orange, NJ) was given for neuromuscular blockade. The protocol was approved by the Committee on Animal Research at the University of California, San Francisco.

The sheep were surgically prepared for collection of lung lymph as described previously (3, 20). The surgical preparation of the sheep usually required 2 h. A Foley catheter (20 Fr., Bard Urological Division, Covington, GA) was inserted into the left atrium to approximate the pathological condition of left atrial hypertension. The Foley catheter was inserted and secured in the left atrium, as we have done before (15). In all experiments, after the surgical preparation, there was a 2-h baseline of stable heart rate, systemic blood pressure, pulmonary vascular pressures, and arterial blood gases. One hour into the baseline, the left atrial pressure was raised by inflation of the left atrial Foley catheter to reach a left atrial pressure of either 18 or 24 cmH2O. A catheter was placed in the left atrium and recorded the left atrial pressure continuously. At the end of the baseline period, the test solution was instilled into both lungs.

Fiber-optic bronchoscopy was used to instill the test solution (3 ml/kg) directly into both the right lower and left lower lobes of the sheep lung. In some sheep, either aerosolized salmeterol (5 mg) or saline was delivered over a 1-h period after fluid instillation. Aerosolization was done with a Whisper Jet nebulizer system (Marquest, model 123014) in the inspiratory limb of the ventilatory circuit with a driving flow rate of 10 l/min. Lymph samples were taken at 15-min intervals throughout the study, and blood was sampled hourly.

At the end of the 4-h experimental period, the sheep was exsanguinated. A median sternotomy was done to excise the lungs. Alveolar fluid samples from the distal air spaces of each lung were obtained by gently guiding a catheter to a wedged position and aspirating fluid, as we have done before in the sheep studies (3, 20). The gravimetric lung water in the lungs was then determined.

In selected experiments, serum samples for measurements of epinephrine levels were obtained once the sheep had stabilized in the baseline period and at 2 and 4 h after fluid instillation. Salmeterol levels were measured in the plasma, alveolar fluid, and lung lymph in selected sheep experiments. In two sheep, ANF was measured in the plasma at baseline and 2 and 4 h after elevation of left atrial pressure to 24 cmH2O.

Preparation of the Instillate

A solution of 5% albumin was prepared by using BSA (Sigma Chemical, St. Louis, MO) dissolved in Ringer lactate. Evans blue dye (Aldrich, Milwaukee, WI) was added to the solution to follow the location of the instillate in both lower lobes at postmortem examination.

Specific Experimental Protocols

Group I: Left atrial hypertension (24 cmH2O; n = 5) vs. normal left atrial pressure (n = 4). Left atrial pressure was increased to 24 cmH2O. After the baseline period, 3 ml/kg of the 5% albumin solution were instilled with a bronchoscope into each lower lung lobe. After instillation, 5 ml of 0.9% saline were nebulized over 60 min (nebulization was done as a control for the experiment in groups III and IV). Then, the sheep were processed as described in Sheep Preparation and General Experimental Protocol. The same protocol was followed in the four control sheep with no increase of left atrial pressure.

Group II: Left atrial hypertension (24 cmH2O) in the presence of bilateral adrenalectomy (n = 4). After the surgical preparation, the bilateral adrenalectomies were done through posterior incisions. Left atrial pressure was increased to 24 cmH2O. After the baseline period, 3 ml/kg of 5% albumin solution were instilled into both lower lung lobes as previously described. Then, 5 ml of 0.9% saline were nebulized over 60 min. Neosynephrine (phenylephrine HCl injection, Elkins-Sinn, Cherry Hill, NJ), an α-adrenergic agonist, was used at a dose of 100 µg/min to support systemic blood pressure after the bilateral adrenalectomies. To provide controls for the bilateral adrenalectomies and neosynephrine infusion, three sheep with normal left atrial pressure underwent bilateral adrenalectomies with neosynephrine in infusion. Alveolar fluid clearance in these three sheep was 30 ± 6%, similar to control sheep.

Group III: Left atrial hypertension (18 and 24 cmH2O) with aerosolized or instilled salmeterol (n = 12). Initially, left atrial pressure was increased to 24 cmH2O. Then, 3 ml/kg of 5% albumin solution were instilled into each lower lung lobe. Salmeterol (5 mg in 5 ml 0.9% saline) was nebulized over 60 min. Initially, studies were done only with a left atrial pressure of 24 cmH2O (n = 4). Because there was no effect of aerosolized salmeterol, left atrial pressure was reduced to a lower level, i.e., 18 cmH2O (n = 5). Because again there was no effect, in additional sheep with left atrial pressure of 24 cmH2O, a solution of 10−6 M salmeterol was dissolved in the instillate (n = 3). All sheep were processed as described in Sheep Preparation and General Experimental Protocol.

Group IV: Normal left atrial pressures with aerosolized salmeterol (n = 4). After the baseline period, 3 ml/kg of the 5% albumin solution were instilled into each lower lobe as previously described. Salmeterol, 5 mg in 5 ml of 0.9% saline, was nebulized over 60 min. Then, the sheep were processed as described in Sheep Preparation and General Experimental Protocol.

Measurements

Hemodynamics, airway pressures, arterial blood gases, and protein concentration. Systemic blood and airway pressures were continuously monitored by using calibrated pressure transducers (Pd23 ID, Gould, Oxnard, CA) and recorded continuously on a Grass polygraph (Grass model 7 polygraph, Grass Instruments, Quincy, MA). Arterial blood gases were measured every 15 min before instillation and hourly after instillation of the albumin solution. Samples of blood, instillate, and the final alveolar fluid from the air spaces were collected to measure total protein concentration and 131I-labeled albumin counts in selected experiments.

Radioactivity studies to measure flux of intravascular protein into the air spaces of the lung. Although the elevated hydrostatic pressure increases transvascular fluid filtration without altering barrier permeability to protein (as indicated in the lymph to plasma protein concentration ratios in Fig 1),
we wanted to verify that the integrity of the alveolar epithelium was maintained in the presence of moderately elevated left atrial pressure. Therefore, in three control sheep and in three sheep with left atrial hypertension (24 cmH2O), we injected 10 µCi iv of 131I-labeled human serum albumin (131I-albumin; Frost Laboratories, Montreal, Canada) during the baseline period.

The quantity [counts·min⁻¹ (cpm)·g⁻¹] of 131I-albumin in the alveolar fluid was measured after 4 h. There were no differences in the counts of 131I-albumin in the alveolar fluids of sheep with left atrial hypertension compared with the counts in the alveolar fluids from sheep with normal left atrial pressure. Also, the total counts, expressed as a ratio of alveolar to plasma 131I-albumin at 4 h (39), indicated that there was <1 ml of plasma in the air spaces of the lung.

Trichloroacetic acid precipitation was carried out on the instillates and on selected samples from each experiment; it was established that the tracer 131I was always >98% bound to the protein.

Measurement of alveolar liquid clearance. As in previous studies (3, 20), alveolar liquid clearance was estimated by measuring the increase in the final alveolar protein concentration, compared with the initial alveolar (instilled) protein concentration (raw data shown in Figs. 1A, 2A, 3A, and 4A).

Alveolar liquid clearance (ALC) was calculated as

\[ ALC = \frac{V_f (V_i - F_{wi})}{V_i (V_i - F_{wi})} \times 100 \]  

(1)

where \( V_i \) is the volume of the instilled fluid (ml), \( F_{wi} \) is the water fraction of the instilled fluid, and \( V_f \) is the volume of the final alveolar fluid (ml). \( V_i \) was estimated as \( V_i = \frac{V_f (V_i - F_{wi})}{TP} \), where TP refers to the total protein concentration of the initial (Tp) and of the final alveolar fluid (Tp).

Lung liquid clearance (excess lung water measurement). To determine the extravascular water in the lungs of the sheep, standard methods were used, as in our prior studies (3, 20). The excess water (E) in the lungs was calculated by using the following equation

\[ E = \left( \frac{W_i - D_i}{W_i - D_i} \right) (D_i - P) \]  

(2)

where lung liquid clearance was determined by first calculating the excess lung water in the instilled lungs; \( W_i \) is the weight of the instilled lungs; \( D_i \) is the dry weight of the instilled lungs, and \( P \) is the weight of the protein instilled with the instillate. \( W_i \) and \( D_i \) are the reference data from control lungs, as in our prior studies (3, 19), or from studies with left atrial hypertension with a left atrial pressure of 24 cmH2O (10, 16). Thus lung liquid clearance was the difference between the instilled volume and the excess lung water remaining in the lungs 4 h after the 5% albumin instillation, divided by the instilled volume.

Determination of plasma concentration of epinephrine. Plasma epinephrine was measured by HPLC by a laboratory technician blinded to the conditions of the experiments. Plasma was taken from the animal at the baseline and at 2 and 4 h. One milliliter of blood was collected in a heparinized tube, as we have done before (28). Blood samples were immediately centrifuged at 3,000 rpm for 5 min at +4°C; 0.5 ml of plasma was transferred to an Eppendorf tube and quickly frozen to -70°C in acetone and dry ice. Samples were stored at -70°C until analyzed. Plasma samples were spiked with an internal standard and absorbed on activated alumina at alkaline pH. Epinephrine was eluted by 0.1 M perchloric acid, analyzed by reverse-phase HPLC using a C8 column, and measured by the amperometric method with the use of an electrochemical detector. Correlation coefficient and detection limit of this method were 0.96 and 10 pg/ml, respectively. Salmeterol measurements were done courtesy of Glaxo Ware, Herfordshire, UK. Measurements were done by HPLC with fluorescence detection after sample preparation by solid-phase extraction, as previously described (7).

Determination of plasma concentrations of ANF. A radioimmunoassay (13) was used to measure concentrations of ANF in duplicate in plasma samples after prior extraction on Sep-Pak cartridges.

In Vitro Studies With Isolated Rat Alveolar Epithelial Type II Cells

Cell isolation. Rat alveolar type II cells were isolated from male Sprague-Dawley rats weighing 175–250 g by enzymatic digestion with elastase and then purified by differential adhesion technique in rat IgG-coated plastic dishes, as we have previously described (35). The cells were cultured in DMEM containing 10% fetal bovine serum and 40 µg/ml of gentamicin in plastic culture flasks and kept in a 5% humidified 5% CO2 incubator at 37°C. The culture media were changed every 2 days, and the experiments were done on cells kept in cultures for 5 days.

Na⁺-K⁺-ATPase activity. Activity of the Na⁺-K⁺-ATPase was quantified by a radiometric monitoring of ouabain-sensitive ATP hydrolysis at maximal velocity (Vmax), as optimized previously (35). In brief, a crude cell homogenate was obtained by sonicating the cells. ATP hydrolysis was analyzed by monitoring P2 release with the use of [γ-32P]ATP (ICN Biochemicals, Montreal, Quebec, Canada) as a tracer. Na⁺-K⁺-ATPase activity was calculated as the difference between the slopes of the regression lines of P2 release obtained in the presence and absence of 2 mM ouabain (Sigma Chemical). The data were standardized to cellular protein content determined by the method of Bradford (35).

Binding assays. The binding assays were done on alveolar type II cell by using a technique previously described (21). Briefly, the harvested alveolar type II cells were solubilized in a 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 3 mM MgCl₂, and 1 mM EDTA, using a poltron (setting 10, 3 × 20 s, separated by short period for cooling). The homogenates were centrifuged at 15,000 g at 4°C for 20 min, and then the microsomal fraction was obtained by centrifugation of supernatant at 100,000 g for 90 min at 4°C. The pellet was resuspended in 50 mM Tris-HCl buffer, and aliquots were frozen at -80°C. To proceed to the binding assay, the microsomal fraction was thawed and diluted in 50 mM Tris-HCl buffer, pH 7.4 (to give membrane concentration of 75 µg/100 µl), containing 0.1% bacitracin, 0.4% BSA, 5 mM MgCl₂, and 0.5 mM phenylmethylsulfonyl fluoride. Labeled 125I-ANF (20,000 cpm/100 µl) and unlabeled ANF, C-ANF and C-type natriuretic peptide (CNP) (10⁻¹² to 10⁻⁷ M) were prepared also in Tris-HCl buffer. To discriminate between guanylyl cyclase and clearance receptors, C-ANF was used. C-ANF-(102–121) is a 5-amino acid ring-deleted ANF analog des[Gln¹¹⁶, Ser¹¹⁷, Gly¹¹⁸, Leu¹¹⁹, Gly¹²⁰], ANF-(120–121) (Peninsula Laboratories, Belmont, CA) that possesses high specificity and affinity for ANF clearance receptors. To determine which one of guanylyl cyclase receptors is present, we used CNP, which is considered a natural ligand for guanylyl cyclase and clearance receptors. After the use of three unlabeled peptides it was possible to determine the predominant receptors of the alveolar type II cells. Binding kinetics was determined by incubating 75 µg of membrane protein with 125I-labeled ANF and unlabeled peptides (10⁻¹² to 10⁻⁶ M) in a volume of 0.2 ml for 90 min at room temperature. The reaction was stopped by the addition of 3 ml of ice-cold Tris-HCl, pH 7.4. Bound radioactivity was separated by filtration, and then both free and bound radioactivities were counted.

Measurement of cGMP. The cultured cells were incubated with increasing concentrations of rat ANF for 90 min at 37°C in the presence of 500 µM 3-isobutyl-1-methylxanthine. After
90 min, the supernatant was collected and stored in a glass tube containing EDTA at −40°C. cGMP was measured in the supernatant by radioimmunoassay as described previously (13).

Statistics

Data are means ± SD. The experimental interventions were compared with controls by an unpaired t-test (for example, left atrial hypertension compared with controls with normal left atrial pressure). One-way ANOVA was used for comparison of these different periods for hemodynamics in experimental groups, as in Table 1.

RESULTS

Alveolar and Lung Liquid Clearance With Left Atrial Hypertension

Alveolar liquid clearance in the presence of left atrial hypertension was similar (34 ± 10%) to that with
normal left atrial pressures (31 ± 3%) (Fig. 1A). Also, lung liquid clearance was the same in the sheep with or without left atrial hypertension (Fig. 1B). Plasma epinephrine levels were increased in sheep with left atrial hypertension. At 2 h after instillation, plasma epinephrine with left atrial hypertension was 1,080 pg/ml (median), with a range of 295–1,089 pg/ml (n = 4), compared with 422 pg/ml (median), with range of 386–458 pg/ml in sheep with normal left atrial pressures at 2 h (n = 2). These 2-h differences did not quite reach statistical significance, but at 4 h the plasma epinephrine level was 1,304 pg/ml (median), with a range of 1,083–1,633 pg/ml in sheep with left atrial hypertension, compared with 280 pg/ml (median), with a range of 247–314 pg/ml in sheep with normal left atrial pressure (P < 0.05).

Alveolar and Lung Liquid Clearance With Left Atrial Hypertension After Bilateral Adrenalectomy

Bilateral adrenalectomies were done to determine whether endogenous release of epinephrine was an important mechanism that sustained alveolar liquid clearance at normal levels during moderate left atrial hypertension. Alveolar liquid clearance (Fig. 2A) and lung liquid clearance (Fig. 2B) were significantly lower in sheep that underwent bilateral adrenalectomies before left atrial hypertension than in sheep with left atrial hypertension alone. Plasma epinephrine levels were negligible in sheep with adrenalectomies.

Alveolar and Lung Liquid Clearance With and Without Left Atrial Hypertension in the Presence of Aerosolized Salmeterol

Because clearance of excess alveolar liquid was normal in sheep with left atrial hypertension (Fig. 1), an effort was made to augment the rate of clearance with aerosolized or instilled salmeterol, a potent β-agonist, in the presence of left atrial hypertension. However, salmeterol did not increase alveolar liquid clearance (Fig. 3A) or lung liquid clearance (Fig. 3B) when left atrial pressure was elevated. However, aerosolized salmeterol significantly increased alveolar liquid clearance (Fig. 4A) and lung liquid clearance (Fig. 4B) in sheep with normal left atrial pressure.

Hemodynamic Measurements With and Without Left Atrial Hypertension

The pulmonary arterial pressures increased, as expected, in the sheep with left atrial hypertension, although cardiac output was not significantly decreased (Table 1). Aerosolized salmeterol did not affect pulmonary artery or systemic blood pressures or cardiac output (Table 1).

Measurements of Lung Lymph Flow With and Without Left Atrial Hypertension

As expected, lung lymph flow markedly increased in sheep with left atrial hypertension, compared with the control sheep with normal left atrial pressure (Fig. 1C). Also, the lymph-to-plasma protein concentration ratio declined in sheep with left atrial hypertension compared with control sheep with normal left atrial pressure (Fig. 1D). After bilateral adrenalectomies, the volume of lymph flow was unchanged. However, the lymph-to-plasma protein concentration ratio was lower in the sheep with left atrial hypertension with intact adrenal glands (Fig. 2D) than in the sheep with their adrenal glands removed. This finding is consistent with transport of more protein-free alveolar fluid into the lung interstitium in the sheep with intact adrenal glands than in sheep that had undergone adrenalectomies, as shown in Fig. 2A. The lung lymph flow and the lymph-to-plasma protein concentration ratio did not change in the sheep with left atrial hypertension given salmeterol compared with sheep with left atrial hypertension alone (Fig. 3, C and D), a finding that is consistent with no effect on alveolar fluid clearance in this condition. However, the lymph-to-plasma protein concentration ratio declined more between 90 and 150 min in the salmeterol-treated sheep with normal left atrial pressures (Fig. 4D) than in control sheep with normal left atrial pressure, probably because more protein-free fluid was transported into the lung interstitium in the salmeterol-treated sheep (Fig. 4A).

Salmeterol Levels

To measure the delivery of aerosolized salmeterol to the lung, salmeterol levels were measured after 4 h in the alveolar fluid, lung lymph, and plasma in sheep with both normal and elevated left atrial pressure (Fig. 5). Markedly higher levels of salmeterol were found in the alveolar fluid than in the other compartments. These concentrations were equal to ~10⁻⁶ M of salmeterol in the alveolar fluid after 4 h.

ANF Studies

In two sheep, baseline plasma ANF levels were 93 and 92 fmol/ml. After 2 h of left atrial hypertension (24
cmH2O), the plasma levels increased to 99 and 193 fmol/ml, respectively; the plasma levels after 4 h were 200 and 158 fmol/ml, respectively. Thus, by 4 h, plasma ANF levels had approximately doubled following left atrial hypertension in these two sheep. In view of this increase in plasma ANF levels, in vitro studies were carried out to test the hypothesis that ANF might downregulate the alveolar epithelial Na+ transport.

The binding assay that was carried out on membrane preparations of the alveolar type II cells suggests that C-ANF does not compete with 125I-ANF, since only very
high concentrations partially displaced $^{125}$I-ANF from membrane receptors, thus suggesting that clearance receptors are absent on alveolar type II cells (Fig. 6). To discriminate between both subtypes of guanylyl cyclase receptors that could be present, we evaluated the displacement of $^{125}$ANF by CNP, considered a natural ligand for guanylyl cyclase receptors of subtype B. The results indicated that only a very high concentration ($10^{-7}$ M) of CNP could inhibit the binding (Fig. 6). Overall, these results suggest that the guanylyl
cyclase receptor of subtype A is the main ANF receptor on these cells. To determine whether this receptor was functional, the release of cGMP was measured after ANF stimulation. As shown in Fig. 7, there was a gradual increase in cGMP release with ANF stimulation.

The impact of ANF on basal and stimulated Na\(^+\)-K\(^+\)-ATPase activity was then determined. Although ANF (10\(^{-7}\) M) did not inhibit basal Na\(^+\)-K\(^+\)-ATPase activity, it did inhibit the increase in activity induced by terbutaline (10\(^{-2}\) M) (Fig. 8). This effect could be reproduced by incubating the...
cells with dibutyryl-cGMP (10^{-3} \text{ M}) (data not shown).

DISCUSSION

Although several mechanisms that regulate fluid movement across the lung endothelial barrier in the presence of elevated lung vascular pressure have been identified (4, 34, 38), there is very little information regarding the regulation of lung fluid balance across the alveolar epithelial barrier in the presence of elevated lung vascular pressure. The experiments in this study were designed to determine the effect of acute elevations of left atrial pressure on net alveolar fluid clearance in sheep. Left atrial pressure was increased by 10.2 \pm 0.3 \text{ cmH}_2\text{O} so that the effect of moderate left atrial pressure could be studied under steady-state conditions over 4 h in anesthetized, ventilated sheep. Lung lymph flow was measured to provide a quantitative index of lung vascular filtration as well as to provide an index of alveolar fluid reabsorption on the basis of a decline in the lymph-to-plasma protein ratio, as we have reported in prior studies (3, 20, 32). The first objective was to determine the effect of moderate left atrial hypertension on alveolar fluid clearance. Interestingly, the results indicated that the net alveolar fluid clearance was maintained at a normal level in the presence of moderate elevations of left atrial pressure. This was a remarkable finding, since lung lymph flow
was increased nearly threefold over baseline levels (Fig. 1), indicating a marked increase in the transvascular fluid filtration in the lung. In addition, since lung liquid clearance remained at a normal level, the removal of the excess fluid transported to the interstitial space from the alveolar space was also maintained.

In the only previous work by Raj and Bland (29) on elevated pulmonary vascular pressure and lung liquid clearance, newborn lambs were used to assess the impact of a hydrostatic stress. These investigators found that elevated left atrial pressure decreased the clearance of instilled isotonic saline (6 ml/kg) in the first 2 h after instillation, although at 6 h there was no difference. In contrast, our experiments utilized a 5% albumin in Ringer lactate solution as the instillate, so that we could measure both alveolar and lung liquid clearance. Their 6-h results in the newborn lamb are similar to our 4-h data in the adult sheep in terms of no change in the lung liquid clearance in the presence of left atrial hypertension.

To determine the mechanism responsible for maintenance of normal alveolar fluid clearance in the presence of moderate left atrial hypertension, we tested the hypothesis that endogenous release of epinephrine might be responsible in part for sustaining alveolar fluid clearance. There were two lines of evidence that supported a role for endogenous epinephrine in sustaining alveolar fluid clearance in the setting of moderate left atrial hypertension. First, plasma epinephrine levels were elevated during left atrial hypertension to approximately two- to threefold normal levels. Second, bilateral adrenalectomies reduced alveolar fluid clearance in the newborn lamb by 30% (Fig. 2). Thus endogenous release of epinephrine appears to account for 30% of the alveolar fluid clearance that occurred in the presence of moderate left atrial hypertension. It is remarkable that alveolar fluid clearance persisted at 70% of normal rates in the sheep with elevated left atrial pressures (Fig. 1).

Interestingly, aerosolized salmeterol, a potent lipophilic β2-adrenergic agonist, did not increase alveolar fluid clearance in the sheep with left atrial hypertension, even when the pressure was increased to only 18 cmH2O (Fig. 3). In contrast, aerosolized salmeterol markedly increased alveolar and lung liquid clearance when left atrial pressure was normal (Fig. 4). It is possible that the failure to respond to exogenous salmeterol in the presence of left atrial hypertension reflected maximal stimulation from the modest increase in endogenous epinephrine levels. This interpretation is, however, unlikely, since recent work indicates that progressively higher levels of epinephrine in dogs produce a dose-dependent increase in alveolar liquid clearance (17). Second, the response to endogenous catecholamines was submaximal, so, theoretically, additional β2-adrenergic stimulation should have increased alveolar fluid clearance. An alternative explanation is that the effect of salmeterol was inhibited by a circulating endogenous factor released by left atrial hypertension.

It is well known that left atrial hypertension leads to ANF release (22), and it has been shown recently that ANF can inhibit both unstimulated and stimulated Na+ transport in the isolated perfused rat lung (25) as well as across cultured alveolar epithelial type II cells (37). To test the hypothesis that the failure of salmeterol to stimulate alveolar liquid clearance in these sheep could be related to ANF secretion, we first measured the level of circulating ANF in the sheep with left atrial hypertension. Plasma ANF increased by ~100% in two sheep after left atrial hypertension. Although the increase in plasma ANF was less than the increase seen in animals developing heart failure secondary to a cardiomyopathy (14), the magnitude of cardiac dysfunction was also less in our sheep. In view of the elevated plasma ANF levels, we thought it was reasonable to examine the effect of ANF on β-adrenergic stimulation of Na+ transport in a well-established in vitro model of cultured rat alveolar type II cells. To evaluate this issue, we first determined whether there were functional ANF receptors on rat alveolar type II cells. In the intact lung, the most predominant receptor for ANF is the clearance receptor (27); however, in alveolar type II cells, the guanylyl-cyclase-transducing receptors are exclusively present (Fig. 6). In fact, like Tharaux et al. (37), we found that the guanylyl cyclase receptor of subtype A is the predominant receptor on alveolar type II cells. These receptors are functional, since ANF stimulation caused a significant increase in cGMP release (Fig. 7). We then determined whether ANF could inhibit β2-adrenergic stimulation of Na+ transport in alveolar type II cells. To address this question, we measured the activity of Na+-K+-ATPase, since it is an essential component of transcellular Na+ transport and it can be activated by β-adrenergic agonists (35). Interestingly, as in the work of Tharaux et al. (37), ANF by itself did not inhibit Na+-K+-ATPase. However, ANF inhibited the stimulating effect of terbutaline on Na+-K+-ATPase (Fig. 8). Thus this in vitro data support the possibility that the lack of effect of β2-agonist stimulation with salmeterol in sheep (Fig. 4) could be secondary to the presence of elevated levels of ANF in the lung. These results would then also support the hypothesis raised by two previous investigators suggesting that ANF may inhibit Na+ absorption in the lung (25, 37).

However, this inhibitory action of ANF in our studies or in the experiments by Tharaux et al. (37) was achieved with concentrations of ANF at 10−7 M. This concentration exceeded the levels of ANF measured in the plasma in our sheep studies. Therefore, although the in vitro results are consistent with a possible role of ANF, they are not conclusive. The need to use larger quantities of ANF in vitro could be explained by a rapid degradation of the peptide when it is applied to the cell. This would mean that a greater concentration of ANF is necessary to obtain significant receptor stimulation in vitro. This is possible, since ANF is metabolized by the neutral endopeptidase 24–11 (16), an enzyme that has been shown to be present in the membrane of alveolar type II cells (11). However, another possibility is that...
the effect of ANF in sheep in vivo is determined by tissue concentrations of ANF, and, of course, our plasma assays do not accurately assess tissue or receptor concentrations. The plasma data simply demonstrate that there is more ANF released with left atrial hypertension. Interestingly, it has been shown in hamsters with a cardiomyopathy that concentrations of ANF in lung tissue increase with the evolution of heart failure (14). So, local tissue concentration of ANF may be higher and, therefore, could be responsible for the inability of salmeterol to stimulate clearance. In summary, the in vitro data suggest that an elevation of ANF may prevent the stimulatory effect of β-adrenergic agonist therapy on alveolar liquid clearance during moderate left atrial hypertension.

A basic issue in our experiments was to explain how alveolar fluid could be removed from the air spaces and the interstitium of the lung in the presence of markedly elevated lung vascular filtration. First, there are several mechanisms that protect against alveolar flooding in the presence of moderate left atrial hypertension. There is a pressure gradient in the lung interstitium from the alveolar to the extra-alveolar interstitium, even in the presence of edema (5). Therefore, a significant fraction of the microvascular filtrate moves to the extra-alveolar compartment, where increases in lung lymph flow can facilitate the removal of some of the excess interstitial fluid (15, 34). Also, some of the interstitial edema fluid flows across the low-resistance visceral pleural mesothelium into the pleural space (6). Furthermore, it is well known that the alveolar epithelial barrier is very tight, resisting the movement of both low- and high-molecular-weight molecules (19, 20, 33, 36). Finally, based on the results of this study, the active ion-transport properties of the alveolar epithelial barrier provide an additional mechanism for removing alveolar fluid. Evidently, net alveolar epithelial fluid clearance can be maintained, as illustrated in these experiments, in the presence of a marked increase in lung vascular filtration with moderate elevations of left atrial pressure. Obviously, when left atrial pressure is increased to markedly higher levels (1, 38), progressive interstitial edema will result in a rise in interstitial pressure to a critical level that breaks the epithelial barrier at the distal airway and/or alveolar level and will result in bulk flow of interstitial edema fluid into the distal air spaces (8, 34). However, under conditions of moderate left atrial hypertension, overt alveolar flooding does not seem to occur (8). The data in this study support the critical role of alveolar epithelial fluid transport in preventing accumulation of edema fluid in the air spaces of the lung in the presence of moderate elevation of left atrial pressure. It is possible that some alveolar flooding occurs at moderate levels of left atrial hypertension, but it is likely, based on the data in this study, that mild alveolar flooding can be efficiently and rapidly removed across the alveolar barrier.

As already discussed, an important objective of this study was to determine whether aerosolized salmeterol, a long-acting lipophilic β-adrenergic agonist, could increase alveolar fluid clearance in the presence of elevated left atrial pressure. There was no effect of aerosolized salmeterol in the presence of either 18 or 24 cmH2O left atrial hypertension. However, the data indicate a substantial increase in alveolar fluid clearance in the presence of aerosolized salmeterol in sheep with normal left atrial pressure (Fig. 4). Delivery of salmeterol to the distal air spaces of the lung was reasonably efficient, with measured levels of salmeterol in the alveolar fluid compartment at a mean level of ~700 ng/ml. Interestingly, this level was equivalent to a concentration of 10−6 M, a level that is similar to the concentration needed for maximal alveolar clearance in the ex vivo human lung with salmeterol (31). The studies with aerosolized salmeterol demonstrated the expected decline in the lymph-to-plasma protein ratio (Fig. 4) that is associated with the transport of more protein-free alveolar fluid from the air spaces into the lung interstitium, reflected by a decrease in the lymph-to-plasma protein ratio, as we reported before (3). The results of these studies in sheep predict that aerosolized β2-adrenergic agonist therapy is likely to be ineffective until left atrial pressure is lowered to a normal range. Thus it appears that exogenous β2-adrenergic therapy may be beneficial, but only when left atrial pressure is normalized.

There are some limitations to these studies. First, although the studies were done in anesthetized sheep, endogenous release of epinephrine was not prevented. Second, positive-pressure ventilation maintained lung volume at a higher functional residual capacity than would probably occur in the setting of spontaneous ventilation. At lower lung volumes, interstitial edema might be cleared less efficiently, a factor that could lower the threshold for alveolar flooding and/or slow the rate of alveolar fluid clearance. Although steady-state conditions were achieved over 4 h, longer-term studies of fluid clearance (12–24 h) were not done. Thus the rate of clearance that we measured over 4 h might not be sustained for longer time periods. Finally, halothane (2%) itself may cause a modest reduction in the rate of alveolar liquid clearance, at least in rats (30), although the levels of halothane used in this study were low (1%). Also, the basal rates of alveolar fluid clearance in these anesthetized sheep were similar to our published data for alveolar liquid clearance over 4 h in unanesthetized, spontaneously ventilating sheep (19).

In summary, alveolar epithelial fluid transport is maintained at normal levels in anesthetized, ventilated sheep for 4 h in the presence of moderate left atrial hypertension (24 cmH2O). The normal alveolar fluid clearance was sustained, in part, by endogenous release of epinephrine in the presence of left atrial hypertension. Even without epinephrine stimulation, however, alveolar fluid clearance continued at 70% of normal levels in the presence of moderate left atrial hypertension. Aerosolized β2-adrenergic therapy was only effective in accelerating the rate of alveolar fluid clearance in the presence of normal left atrial pressure. The inability of exogenous β2-adrenergic therapy to
stimulate alveolar fluid clearance in the presence of left atrial hypertension may have been related to the release of ANF, a factor that has been shown to downregulate vectorial Na⁺ transport across the alveolar epithelium both in vivo and in vitro. On balance, these in vivo studies in sheep support the conclusion that alveolar epithelial fluid transport is an important mechanism that prevents or minimizes the accumulation of alveolar edema fluid in the presence of moderate left atrial hypertension.

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