Chloride transport-driven alveolar fluid secretion is a major contributor to cardiogenic lung edema

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Alveolar fluid clearance driven by active epithelial Na^+ and secondary Cl^- absorption counteracts edema formation in the intact lung. Recently, we showed that impairment of alveolar fluid clearance because of inhibition of epithelial Na^+ channels (ENaCs) promotes cardiogenic lung edema. Concomitantly, we observed a reversal of alveolar fluid clearance, suggesting that reversed transepithelial ion transport may promote lung edema by driving active alveolar fluid secretion. We, therefore, hypothesized that alveolar ion and fluid secretion may constitute a pathomechanism in lung edema and aimed to identify underlying molecular pathways. In isolated perfused lungs, alveolar fluid clearance and secretion were determined by a double-indicator dilution technique. Transepithelial Cl^- secretion and alveolar Cl^- influx were quantified by radionuclide tracing and alveolar Cl^- imaging, respectively. Elevated hydrostatic pressure induced ouabain-sensitive alveolar fluid secretion that coincided with transepithelial Cl^- secretion and alveolar Cl^- influx. Inhibition of either cystic fibrosis transmembrane conductance regulator (CFTR) or Na^+-K^+-Cl^- cotransporters (NKCC) blocked alveolar fluid secretion, and lungs of $CFTR^{-/-}$ mice were protected from hydrostatic edema. Inhibition of ENaC by amiloride reproduced alveolar fluid and Cl^- secretion that were again CFTR-, NKCC-, and Na^+-K^+-ATPase–dependent. Our findings show a reversal of transepithelial Cl^- and fluid flux from absorptive to secretory mode at hydrostatic stress. Alveolar Cl^- and fluid secretion are triggered by ENaC inhibition and mediated by NKCC and CFTR. Our results characterize an innovative mechanism of cardiogenic edema formation and identify NKCC1 as a unique therapeutic target in cardiogenic lung edema.

epithelial Cl^- transport | pulmonary edema

Traditionally, the formation of cardiogenic pulmonary edema has been attributed to passive fluid filtration across an intact alveolocapillary barrier along an increased hydrostatic pressure gradient. However, recent studies show that cardiogenic edema is critically regulated by active signaling processes. Activation of mechanosensitive endothelial ion channels increases lung vascular permeability (1), whereas alveolar epithelial cells lose their physiological ability to clear the distal airspaces from excess fluid by their capacity to actively transport ions across the epithelial barrier (2–4).

In the intact lung, the predominant force driving alveolar fluid clearance is an active transepithelial Na^+ transport from the alveolar into the interstitial space. A major portion of the apical Na^+ entry is mediated by the amiloride-inhibitable epithelial Na^+ channel (ENaC), with basolateral Na^+ extrusion through the Na^+-K^+-ATPase (5). Cl^- and water are considered to follow paracellularly for electroneutrality and osmotic balance. In cardiogenic lung edema, the physiological protection against alveolar flooding provided by an intact alveolar fluid clearance is largely attenuated (3, 4). Previously, we have outlined the signaling events at the alveolocapillary barrier that underlie this inhibition of alveolar fluid clearance by showing that hydrostatic stress increases endothelial NO production in lung capillaries (6), which in turn, blocks alveolar Na^+ and liquid absorption by a cGMP-dependent inhibition of epithelial ENaC (2).

Unexpectedly, however, we observed that increased hydrostatic pressure not only blocks alveolar fluid clearance but reverses transepithelial fluid transport, resulting in effective alveolar fluid secretion that accounts for up to 70% of the total alveolar fluid influx at elevated hydrostatic pressure (2). This effect is not explicable by impaired alveolar fluid clearance and/or passive fluid leakage, and thus, it points to a previously unrecognized and potentially therapeutically exploitable pathomechanism in cardiogenic lung edema, namely alveolar fluid secretion driven by active transepithelial ion transport.

Here, we aimed to analyze alveolar fluid secretion and its underlying cellular mechanisms in cardiogenic lung edema. We considered the Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) as a putative key ion channel in this scenario, because it permits bidirectional permeation of anions under physiologically relevant conditions (7). Hence, the direction of Cl^- flux by CFTR may reverse depending on actual electrochemical gradients, thus turning an absorptive into a secretory epithelium or vice versa. This notion is supported by reports describing CFTR as both an absorptive and secretory channel in the regulation of alveolar fluid homeostasis (8, 9). By a combination of indicator dilution, imaging, and radioactive tracer techniques for the measurement of alveolar ion and fluid fluxes in the isolated lung, we show a critical role for CFTR-mediated Cl^- secretion in cardiogenic lung edema

Significance

This study describes a novel mechanism for the formation of cardiogenic lung edema, a potentially fatal complication of left heart disease that was previously attributed to passive fluid filtration across an intact alveolo-capillary barrier. Instead, we demonstrate that a major part of cardiogenic edema results from active epithelial secretion of Cl^- and secondary fluid flux into the alveolar space. Transepithelial Cl^- secretion is triggered by inhibition of epithelial Na^+ uptake and mediated via cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1), providing a mechanistic explanation for extrarenal effects of furosemide in lung edema.

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and identify the Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) as a therapeutic target in this pathology.

Results

Hydrostatic Stress Induces Active Alveolar Fluid Secretion. The net fluid shift into the alveolar space that occurs at elevated lung hydrostatic pressures and gives rise to the formation of cardiogenic lung edema consists of two independent components, namely the pressure-dependent increase in fluid filtration, which is described by the Starling equation, and changes in alveolar fluid transport. Differentiation between the two by use of our previously established double-indicator dilution technique (2) in isolated perfused rat lungs revealed that only a relatively small fraction of the alveolus-directed net fluid shift that occurs during an increase of left atrial pressure (P_{LA}) from 5 to 15 cmH_2O results from an increase in fluid filtration (Fig. 1*A*). In contrast, the transient receptor potential vanilloid 4 (TRPV4) agonist 4αPDD, which causes pulmonary edema by increasing lung vascular permeability (1), induced a marked rise in fluid filtration, attesting to the sensitivity of the double-indicator technique to detect bulk fluid filtration into the alveolar space (Fig. 1*A*). Rather than by filtration, P_{LA} elevation increased net fluid shift into the alveolus by an inhibition of alveolar fluid clearance. In fact, alveolar fluid transport reversed when P_{LA} exceeded 10 cmH_2O, indicating that the epithelium switched from an absorptive to a secretory mode. The fact that both absorptive and secretory alveolar fluid transports, as seen at low (5 cmH_2O) and elevated (15 cmH_2O) P_{LA}, respectively, were inhibitable by the Na^+-K^+-ATPase inhibitor ouabain (Fig. 1*B*) identified alveolar fluid secretion as an active process likely driven by transepithelial ion transport.

Alveolar Fluid Secretion Is Driven by Transepithelial Cl^- Transport. Because Cl^- transport facilitates fluid secretion in various epithelial organs, including the alveolus (8), we next tested its contribution to alveolar fluid secretion at hydrostatic stress. To this end, we replaced Cl^- in the alveolar instillate or lung perfusate, respectively, with iso-osmolar NO_3^- in our experiments. Lack of Cl^- in the alveolar instillate attenuated alveolar fluid clearance at baseline P_{LA} of 5 cmH_2O, but it had no significant effect on alveolar fluid secretion at elevated P_{LA} of 15 cmH_2O (Fig. 2*A*). This finding is essentially in line with the proposed facilitation of alveolar fluid clearance by Cl^- uptake under physiological conditions (10). Conversely, Cl^--free lung perfusion selectively inhibited secretory fluid transport at elevated P_{LA} (Fig. 2*B*), indicating that alveolar fluid secretion is driven by a reversed transepithelial Cl^- transport.

To substantiate the hypothesis that alveoli may actively secrete Cl^- at increased hydrostatic pressure, we adapted our radionuclide technique for tracing of transepithelial $^{22}Na^+$ transport (11) to the study of transepithelial $^{36}Cl^-$ fluxes using [^{3}H]-mannitol as control for paracellular solute flux. Elevation of P_{LA} resulted in a marked $^{36}Cl^-$ flux from the vascular compartment into the distal airspaces (Fig. 2*C*) that was not paralleled by a similar increase in [^{3}H]-mannitol flux (Fig. 2*D*), indicating that it resulted from Cl^- secretion rather than paracellular ion flux. The interpretation of alveolar Cl^- flux as an active secretion is corroborated by its inhibition at 4 °C (Fig. 2 *C* and *D*). $^{36}Cl^-$ shifts were paralleled by respective changes in lung wet-to-dry weight ratio, attesting to their putative relevance for cardiogenic edema formation (Fig. 2*E*).

Role of CFTR in Alveolar Fluid Secretion. Depending on the actual electrochemical gradient, CFTR expressed in the apical plasma membrane can mediate both Cl^- absorption and secretion by alveolar epithelial cells (10). We, therefore, tested whether alveolar secretion of Cl^- and fluid at hydrostatic stress requires CFTR. At baseline P_{LA} of 5 cmH_2O, CFTR inhibition by the nonspecific blocker glibenclamide or the specific inhibitor $CFTR_{inh}$-172 markedly reduced absorptive alveolar fluid transport (Fig. 3*A*), suggesting a contribution of CFTR to basal alveolar fluid clearance under these experimental conditions. Conversely, at elevated P_{LA} (15 cmH_2O), CFTR inhibition completely prevented alveolar fluid secretion in double-indicator experiments. Alveolar fluid secretion was equally blocked by the cGMP-dependent protein kinase II (cGKII) inhibitor 8-(4-Chlorophenylthio)guanosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer (Rp-8pCPT-cGMP), suggesting a role for the NO/cGMP signaling axis in CFTR activation under these conditions. This notion was further substantiated by immunoprecipitation analyses in whole rat lungs showing that hydrostatic stress increases CFTR phosphorylation that is blocked by the nonspecific NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (Fig. S1). $CFTR_{inh}$-172 also largely blocked the alveolar influx of ^{36}Cl at elevated P_{LA} (Fig. 3*B*). Paracellular solute

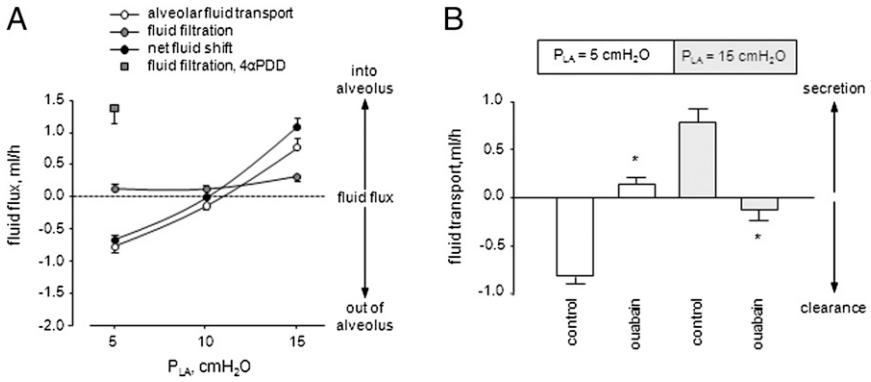

Fig. 1. Hydrostatic stress induces active alveolar fluid secretion. (*A*) Alveolar fluid transport, fluid filtration, and net fluid shifts were determined by double-indicator dilution technique in isolated rat lungs perfused at P_{LA} of 5, 10, or 15 cmH_2O, respectively, for 60 min. Alveolar fluid transport and fluid filtration are given as vectorial fluxes into the alveolar space, with positive values representing alveolar fluid influx and negative values representing alveolar fluid efflux. With higher P_{LA}, net fluid shift representing the numerical sum of both alveolar fluid transport and fluid filtration increased to positive values, indicating alveolar edema formation. Notably, this effect was largely attributable to a reversal of alveolar fluid transport, whereas fluid filtration increased only moderately over the studied pressure range. TRPV4 activation by 4αPDD (10 μmol/L) served as positive control for fluid filtration at baseline P_{LA}. All data are mean ± SEM from $n = 7$ experiments each. (*B*) Alveolar fluid transport was determined by double-indicator dilution technique in isolated rat lungs perfused at baseline P_{LA} (5 cmH_2O; open bars) or hydrostatic stress (15 cmH_2O; gray bars) in the absence (control) or presence of the Na^+-K^+-ATPase inhibitor ouabain (100 μmol/L). Ouabain blocked both alveolar fluid clearance (fluid transport < 0 mL/h) at baseline P_{LA} and alveolar fluid secretion (fluid transport > 0 mL/h) at elevated P_{LA}. All data are mean ± SEM from $n = 7$ experiments each. $^*P < 0.05$ vs. control.

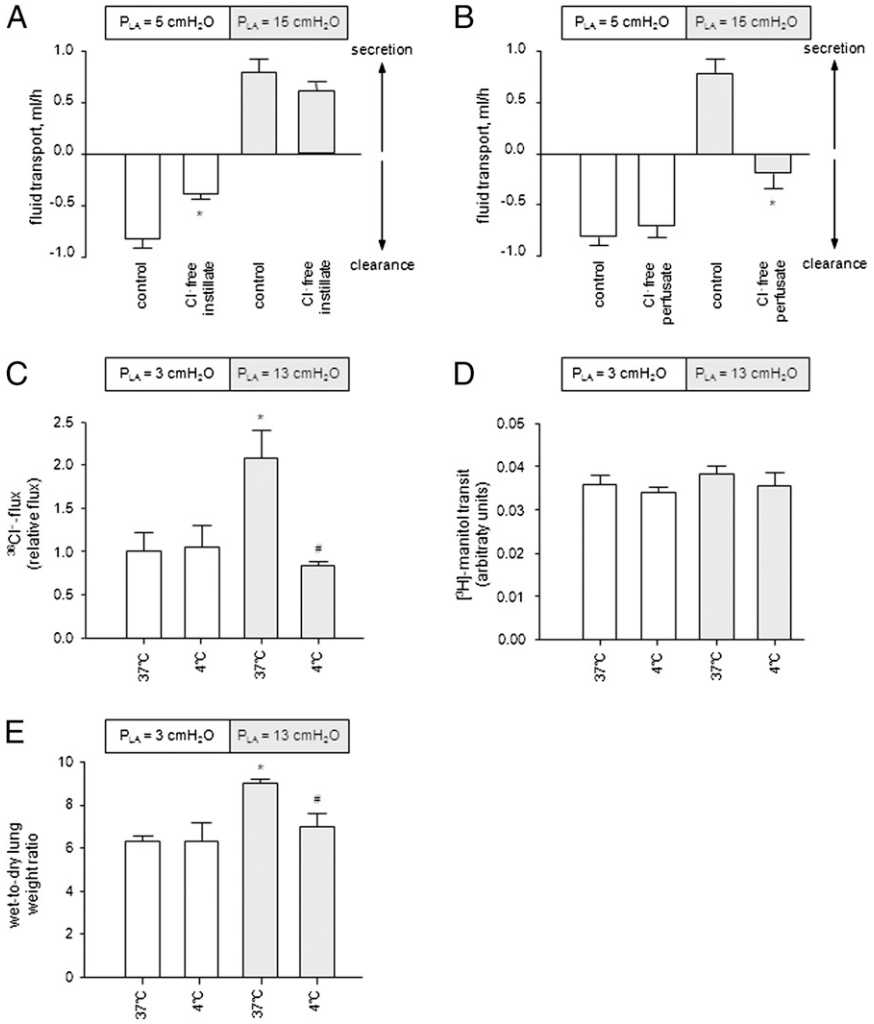

Fig. 2. Alveolar fluid secretion is driven by transepithelial Cl^- transport. (*A* and *B*) Alveolar fluid transport was determined by double-indicator dilution technique in isolated rat lungs at baseline P_{LA} (5 cmH_2O; open bars) or hydrostatic stress (15 cmH_2O; gray bars). Fluid transport was assessed in lungs (*A*) instilled with Cl^--rich (control) or -free instillate and (*B*) perfused with Cl^--rich (control) or -free perfusate. Whereas Cl^--free instillate attenuated alveolar fluid clearance at baseline P_{LA}, Cl^--free perfusate blocked alveolar fluid secretion at hydrostatic stress. All data are mean ± SEM from $n = 5$ experiments each. $^*P <$ 0.05 vs. control. (*C*) Transepithelial $^{36}Cl^-$ flux into the alveolar compartment was determined by radionuclide tracer technique in isolated rabbit lungs perfused at baseline P_{LA} (3 cmH_2O; open bars) or hydrostatic stress (13.5 cmH_2O; gray bars) at 37 °C or 4 °C. $^{36}Cl^-$ flux is expressed in relative units normalized to baseline P_{LA} at 37 °C. Hydrostatic stress approximately doubled transepithelial $^{36}Cl^-$ flux into the alveolar compartment, but this effect was blocked at 4 °C. (*D*) Parallel assessed [^{3}H]-mannitol flux across the alveolar barrier as a marker of paracellular transport increased only modestly with hydrostatic stress without reaching significance, and it was not regulated by temperature. (*E*) Changes in wet-to-dry weight ratio as an indicator of edema formation paralleled pressure-induced, temperature-dependent changes in transepithelial $^{36}Cl^-$ flux in isolated rabbit lungs. All data are mean ± SEM from $n = 8$ (37 °C) or 3 (4 °C) experiments each. $^*P < 0.05$ vs. P_{LA} = 3 cmH_2O; $^{\#}P < 0.05$ vs. 37 °C.

flux, as assessed by [^{3}H]-mannitol tracing, was not affected by CFTR inhibition. The sensitivity to $CFTR_{inh}$-172 was equally evident for the pressure-induced increase in lung wet-to-dry weight ratio (Fig. 3*C*), suggesting an edematogenic role for CFTR at hydrostatic stress. To test this hypothesis, we compared wet-to-dry lung weight ratios in isolated lungs of CFTR KO and corresponding WT mice after 25 min of perfusion at basal (2 cmH_2O) or elevated (6 cmH_2O) P_{LA}. In WT lungs, hydrostatic stress caused a marked increase in lung water content; however, this effect was attenuated by >2/3 in CFTR KO mice (Fig. 3*D*).

Role of NKCC1 in Alveolar Fluid Secretion. A potential candidate to allow for corresponding basolateral Cl^- influx that is required for transepithelial Cl^- secretion is the electrically neutral, secondary active NKCC1. At basal P_{LA}, inhibition of NKCCs by either furosemide or bumetanide partially attenuated absorptive alveolar fluid transport in isolated perfused rat lungs (Fig. 4*A*). This finding was unexpected in that the basolaterally expressed NKCC1 is not considered to contribute directly to alveolar ion and fluid absorption. Of greater relevance for the present hypothesis of Cl^--driven alveolar fluid secretion, both furosemide and bumetanide completely blocked secretory alveolar fluid transport at elevated P_{LA} and restored alveolar fluid clearance in part (Fig. 4*A*). The role of NKCC in secretory Cl^- and fluid flux was substantiated in experiments showing that the increase in $^{36}Cl^-$ flux (Fig. 4*B*) and lung wet-to-dry weight ratio (Fig. 4*C*) at elevated compared with control P_{LA} is, for the most part, sensitive to furosemide. To consolidate the emerging relevance of a basolateral Na^+-K^+-Cl^- cotransport for alveolar fluid secretion, we perfused isolated rat lungs with Na^+-free perfusate. Na^+-free perfusion did not alter alveolar fluid clearance at basal P_{LA}, but it completely blocked secretory fluid transport at elevated pressure (Fig. 4*D*). These findings suggest that alveolar fluid secretion is driven by a transepithelial Cl^-

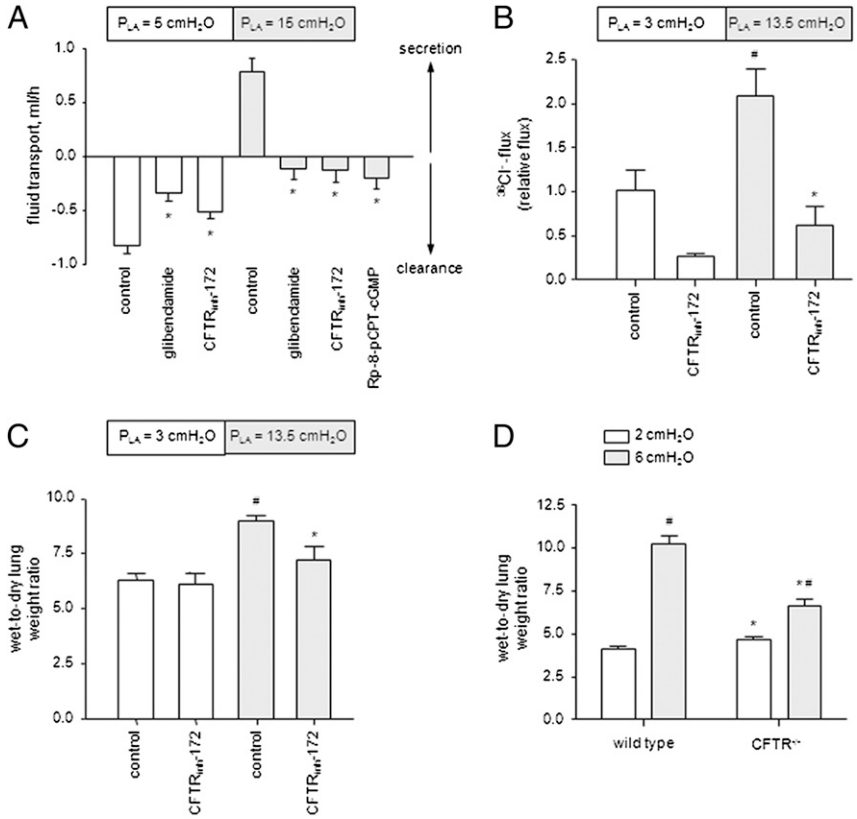

Fig. 3. Role of CFTR in alveolar fluid secretion. (*A*) Alveolar fluid transport was determined by double-indicator dilution technique in isolated rat lungs at baseline P_{LA} (5 cmH_2O; open bars) or hydrostatic stress (15 cmH_2O; gray bars). Fluid transport was assessed in control lungs (control), with the CFTR inhibitors glibenclamide (200 μmol/L) or $CFTR_{inh}$-172 (10 μmol/L), or the cGKII inhibitor Rp-8-pCPT-cGMP (10 μmol/L) in the alveolar instillate. Both CFTR and cGKII inhibitors blocked alveolar fluid secretion at elevated P_{LA}. All data are mean ± SEM from n = 5 experiments each. *$P < 0.05$ vs. control. (*B* and *C*) Transepithelial $^{36}Cl^-$ flux into the alveolar compartment as measured by (*B*) radionuclide tracer technique and (*C*) wet-to-dry lung weight ratio was determined in isolated rabbit lungs perfused at baseline P_{LA} (3 cmH_2O; open bars) or hydrostatic stress (13.5 cmH_2O; gray bars) in the absence (control) or presence of $CFTR_{inh}$-172 (10 μmol/L) in the alveolar instillate. CFTR inhibition significantly reduced transepithelial $^{36}Cl^-$ flux and wet-to-dry lung weight ratio at elevated P_{LA}. All data are mean ± SEM from n = 3–8. *$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. P_{LA} = 3 cmH_2O. (*D*) Wet-to-dry lung weight ratio of WT and CFTR KO mice was determined after 25 min isolated lung perfusion at baseline P_{LA} (2 cmH_2O; open bars) or hydrostatic stress (6 cmH_2O; gray bars). The increase in wet-to-dry weight ratio in response to P_{LA} elevation was significantly attenuated in CFTR KO mice. All data are mean ± SEM from n = 6 experiments each. *$P < 0.05$ vs. WT; $^{\#}P < 0.05$ vs. P_{LA} = 2 cmH_2O.

transport that is facilitated by basolateral couptake of Cl^- with Na^+ by NKCC and apical Cl^- extrusion by CFTR.

Inhibition of Apical Na^+ Entry Replicates CFTR- and NKCC-Dependent Alveolar Fluid and Cl^- Secretion. A putative mechanism by which increased hydrostatic pressure may induce transepithelial Cl^- and thus, fluid secretion is predicated on our previous finding that apical Na^+ uptake by ENaC is inhibited by increased endothelial-derived NO formation at hydrostatic stress (2). Inhibition of apical Na^+ entry in the presence of an active basolateral Na^+-K^+-ATPase may generate a concentration gradient for basolateral Na^+ uptake with Cl^- by NKCC and concomitantly generate an electrochemical gradient that promotes the apical secretion of Cl^-. To probe this hypothesis, we blocked apical Na^+ uptake from the distal airways in rat lungs by either amiloride or replacement of Na^+ with *N*-methyl-D-glucamine in the alveolar instillate. Both interventions reversed basal fluid clearance and induced a marked alveolar fluid secretion at physiological P_{LA} levels (Fig. 5 *A* and *B*). Analogous to our findings in hydrostatic stress, alveolar fluid secretion, as induced by inhibition of Na^+ entry, was sensitive to inhibition of CFTR, NKCC, or Na^+-K^+-ATPase, which was shown by its inhibitability by glibenclamide, $CFTR_{inh}$-172, ouabain, or furosemide. To test whether inhibition of apical Na^+ entry not only replicates the characteristic features of alveolar fluid secretion but may actually trigger transepithelial Cl^- secretion, we directly imaged alveolar Cl^- influx by real-time fluorescence microscopy (12). To this end, we instilled alveoli with a Cl^--free Ringer solution that contained the Cl^--sensitive dye lucigenin. As Cl^- enters the alveolar space, lucigenin fluorescence is quenched (Fig. 5*C*), and alveolar Cl^- influx can be calculated from the fluorescence decay (12). Lung perfusion with 30 mmol/L Cl^- resulted in a gradual decay of alveolar fluorescence, indicating a basal Cl^- flux from the vascular into the alveolar space (Fig. 5*D*). Alveolar Cl^- influx was largely abrogated when lungs were perfused with Cl^--free buffer. Inhibition of ENaC by amiloride stimulated a marked increase in alveolar Cl^- influx, consolidating the concept that alveolar Cl^- (and thus, fluid) secretion is inducible by inhibition of apical Na^+ entry. Consistent with the proposed model of transepithelial Cl^- transport, amiloride-induced alveolar Cl^- influx was again blocked by inhibitors of either NKCC or CFTR.

NKCC and CFTR Inhibitors Attenuate Acute Cardiogenic Edema in Vivo. The evolving relevance of alveolar Cl^- secretion identifies the involved transporters as putative therapeutic targets for the treatment of acute cardiogenic lung edema. Although NKCC inhibition by, for example, furosemide has long been recognized as the first line of treatment in pulmonary edema, its effectiveness has been largely attributed to its diuretic and venodilatory effects (13).

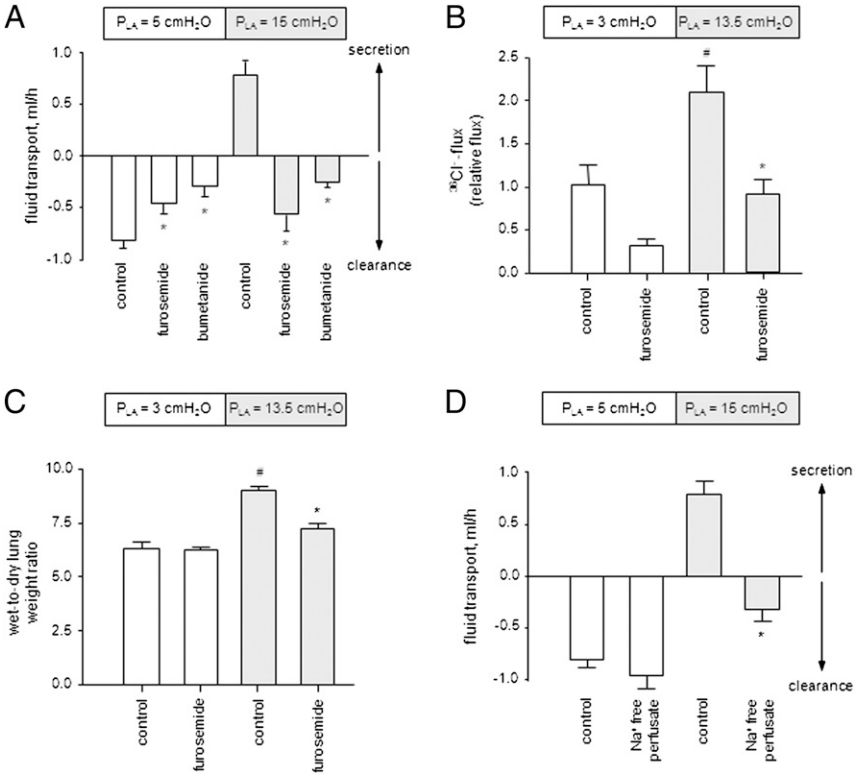

Fig. 4. Role of NKCC1 in alveolar fluid secretion. (*A*) Alveolar fluid transport was determined by double-indicator dilution technique in isolated rat lungs at baseline P_{LA} (5 cmH_2O; open bars) or hydrostatic stress (15 cmH_2O; gray bars). Fluid transport was assessed in control lungs (control) or with the NKCC inhibitors furosemide (500 μmol/L) or bumetanide (10 μmol/L) in the lung perfusate. Both furosemide and bumetanide blocked alveolar fluid secretion at elevated P_{LA}. All data are mean ± SEM from $n = 5$ experiments each. $^*P < 0.05$ vs. control. (*B* and *C*) Transepithelial $^{36}Cl^-$ flux into the alveolar compartment as measured by (*B*) radionuclide tracer technique and (*C*) wet-to-dry lung weight ratio were determined in isolated rabbit lungs perfused at baseline P_{LA} (3 cmH_2O; open bars) or hydrostatic stress (13.5 cmH_2O; gray bars) in the absence (control) or presence of furosemide (500 μmol/L) in the lung perfusate. NKCC inhibition significantly reduced transepithelial $^{36}Cl^-$ flux and wet-to-dry lung weight ratio at elevated P_{LA}. All data are mean ± SEM from $n = 3$–10. $^*P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. $P_{LA} = 3$ cmH_2O. (*D*) Alveolar fluid transport was determined by double-indicator dilution technique at baseline P_{LA} (5 cmH_2O; open bars) or hydrostatic stress (15 cmH_2O; gray bars) in isolated rat lungs perfused with Na^+-rich (control) or -free perfusate, which prevented alveolar fluid secretion at elevated P_{LA}. All data are mean ± SEM from $n = 5$ experiments each. $^*P < 0.05$ vs. control.

To probe the hypothesis that pulmonary NKCC may be critical for the formation of lung edema, we tested the effect of inhaled furosemide in a rat model of cardiogenic pulmonary edema subsequent to acute myocardial infarction. Compared with sham-operated control rats, left anterior descending coronary artery (LAD) occlusion for 60 min resulted in considerable cardiogenic edema evident as marked increase in lung wet-to-dry weight ratio and concomitant arterial hypoxemia and hypotension (Fig. 6 *A*–*C*). Inhalation of furosemide attenuated lung edema and improved both oxygenation and arterial blood pressure, suggesting that lung-specific inhibition of NKCC may suffice to effectively protect from cardiogenic lung edema. Analogously, cardiogenic lung edema in mice after LAD occlusion and a concomitant i.v. volume load of 20 μL/g saline was markedly reduced by the CFTR inhibitor Gly-H101, attesting to the in vivo relevance of the reported alveolar Cl^- and fluid secretion pathway.

Discussion

Here, we propose a fundamentally unique concept for the pathogenesis of cardiogenic pulmonary edema. We identify active alveolar fluid secretion driven by a transepithelial Cl^- transport as a mechanism of impaired alveolar fluid homeostasis and show its critical relevance in cardiogenic edema formation. Using a combination of indicator dilution, in situ imaging, and radionuclide tracing techniques, we (*i*) show that transepithelial alveolar Cl^- and fluid flux reverse from an absorptive to a secretory mode in lung hydrostatic stress, (*ii*) identify basolateral NKCC and apical CFTR as important Cl^- entry and exit channels mediating transepithelial Cl^- secretion, and (*iii*) propose inhibition of amiloride-sensitive Na^+ uptake in the presence of an active Na^+-K^+-ATPase as a plausible cause for reversed transepithelial ion and fluid fluxes (Fig. 7). The described mechanism implicates lung epithelial NKCC1 as a target for the treatment of lung edema and provides a singular mechanistic explanation for the rapid and diuresis-independent action of furosemide in lung edema.

Alveolar Fluid Secretion. We present alveolar fluid secretion as a unique critical mechanism of cardiogenic edema formation that results from active transepithelial ion transport, because it can be blocked by inhibition of Na^+-K^+-ATPase, CFTR, and NKCC. Thus far, alveolar fluid shifts driven by transepithelial ion transport have been largely described in the context of alveolar fluid clearance as active mechanisms that both clear the lung from amniotic fluid at birth and protect from edema in the adult lung. Alveolar fluid clearance is driven by epithelial Na^+ absorption, an active mechanism mediated largely by apical Na^+ channels and the basolateral Na^+-K^+-ATPase (5). Cl^- must follow for electroneutrality, and failure to do so will limit transepithelial Na^+ flux and thus, alveolar fluid clearance. Although epithelial ion transport was originally considered as a characteristic of alveolar type II cells, recent data show that the majority of channels and transporters involved is equally expressed in type I cells (14), although the quantitative contribution to alveolar fluid clearance remains to be determined.

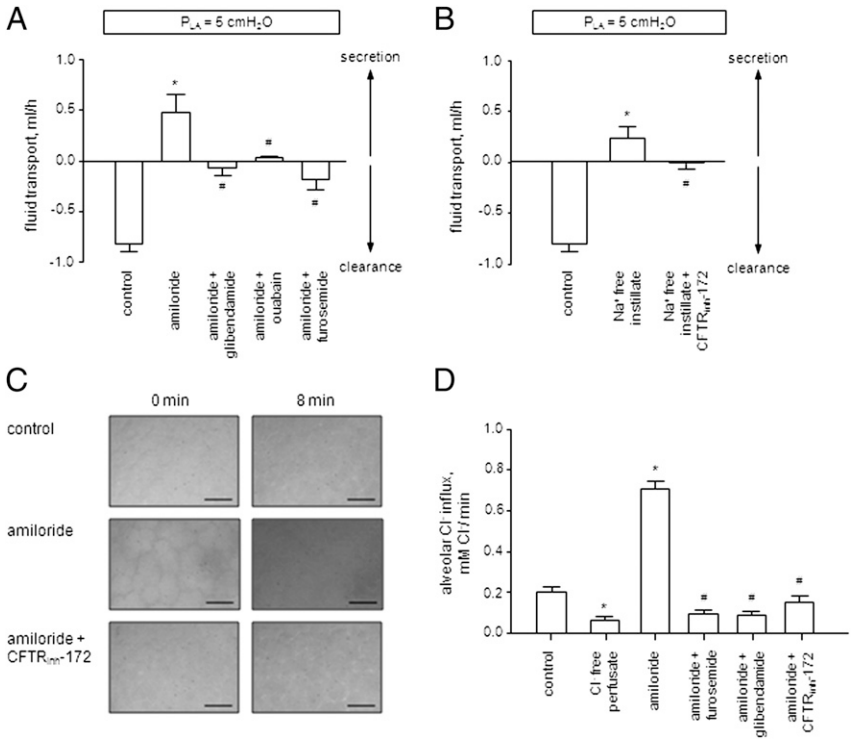

Fig. 5. Inhibition of apical Na^+ entry replicates CFTR- and NKCC-dependent alveolar fluid and Cl^- secretion. (*A*) Alveolar fluid transport was determined by double-indicator dilution technique in isolated rat lungs in the absence (control) or presence of the ENaC inhibitor amiloride (10 μmol/L) alone or combined with glibenclamide (200 μmol/L) in the alveolar instillate or ouabain (100 μmol/L) or furosemide (500 μmol/L) in the lung perfusate. Amiloride induced alveolar fluid secretion that was blocked by inhibitors of CFTR, Na^+-K^+-ATPase, or NKCC, respectively. All data are mean ± SEM from $n = 5$ experiments each. $^*P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. amiloride. (*B*) Alveolar fluid transport was determined by double-indicator dilution technique in isolated rat lungs instilled with Na^+-rich (control) or -free instillate combined with $CFTR_{inh}$-172 (10 μmol/L). Na^+-free instillate induced alveolar fluid secretion that was blocked by $CFTR_{inh}$-172. All data are mean ± SEM from $n = 5$ experiments each. $^*P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. Na^+-free instillate. (*C*) Alveolar Cl^- influx was determined by real-time imaging of lucigenin-instilled alveoli of isolated perfused rat lungs. Representative gray-scale images show subpleural alveoli immediately after and 8 min after lucigenin instillation. In control lungs perfused with 30 mmol/L Cl^- (*Top*), fluorescence decay over time reflects progressive Cl^- influx into the alveolar space. Amiloride (*Middle*; 10 μmol/L) in the alveolar instillate accelerated fluorescence decay and thus, Cl^- influx, but its effect was blocked by concomitant instillation of $CFTR_{inh}$-172 (*Bottom*; 10 μmol/L). (Scale bar: 100 μm.) (*D*) Alveolar Cl^- influx was calculated from alveolar lucigenin decay in control lungs perfused with 30 mmol/L Cl^-, Cl^--free perfused lungs, and amiloride-instilled lungs (10 μmol/L) in the absence or presence of furosemide (500 μmol/L), glibenclamide (200 μmol/L), or $CFTR_{inh}$-172 (10 μmol/L). Amiloride induced alveolar Cl^- influx that was blocked by inhibition of either NKCC or CFTR, respectively. All data are mean ± SEM from $n = 3$ experiments each. $^*P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. amiloride.

Impaired alveolar fluid clearance has been documented in patients with both cardiogenic (4) and permeability-type (15) lung edema, where it is considered to contribute to alveolar fluid accumulation. In previous work, we showed the inhibition of alveolar fluid clearance with increasing levels of hydrostatic stress and identified an NO-mediated inhibition of apical ENaC channels as the underlying mechanism (2). Unexpectedly, however, we also detected a reversal of alveolar fluid shifts with moderate left atrial hypertension that could not be explained by a respective increase in fluid filtration. Reversal of alveolar fluid clearance to fluid influx with increasing hydrostatic stress had previously been reported by Saldías et al. (3), but it was, at that time, attributed to alveolocapillary stress failure. In the present experiments, we can exclude a significant contribution of capillary barrier failure, because (*i*) increased NaF influx reflecting barrier leakage in double-indicator dilution experiments did not account for the bulk of alveolar fluid entry, (*ii*) mannitol did not cross the alveolar barrier in parallel with fluid in radionuclide tracing experiments, and (*iii*) alveolar fluid influx at hydrostatic stress was inhibitable by ouabain, identifying it as the result of an active ion transport process.

Role of CFTR and NKCC1 in Alveolar Fluid Secretion. CFTR, which is expressed in the apical membrane of both alveolar types I (14) and II (10) cells, may facilitate both absorption or secretion of Cl^- depending on the actual electrochemical gradient. Under conditions of stimulated fluid absorption, CFTR constitutes a rate-limiting factor for alveolar fluid clearance (9, 16). Here, CFTR mediated alveolar fluid clearance at physiological pressures, which was shown by reduced absorptive fluid transport and an increase in wet-to-dry weight ratio in glibenclamide- and $CFTR_{inh}$-172–treated isolated rat lungs or isolated lungs of CFTR KO mice. This finding seems to be in contrast to a previous work by Fang et al. (17), which did not observe a relevant role of CFTR for unstimulated alveolar fluid clearance in cystic fibrosis ΔF508 mice that mimic the most common genetic mutation in human cystic fibrosis disease. At this stage, potential reasons for the discrepant findings regarding the role of CFTR in basal fluid absorption remain speculative, but they may entail sufficient residual function of the ΔF508 CFTR protein (18) or up-regulation of alternative Cl^- absorption mechanisms in mutant mice, because their basal fluid absorption can be blocked by nonspecific Cl^- channel blockade (17). Notably, the notion that CFTR may contribute to basal fluid absorption is supported by data showing that transgenic overexpression of human CFTR increases basal alveolar fluid clearance in rats that can be blocked by glibenclamide (9).

Hydrostatic stress induced an alveolus-directed (i.e., secretory) transepithelial Cl^- transport that was blocked by pharmacological inhibitors of CFTR. CFTR inhibition or genetic deficiency likewise blocked alveolar fluid secretion and cardiogenic lung edema

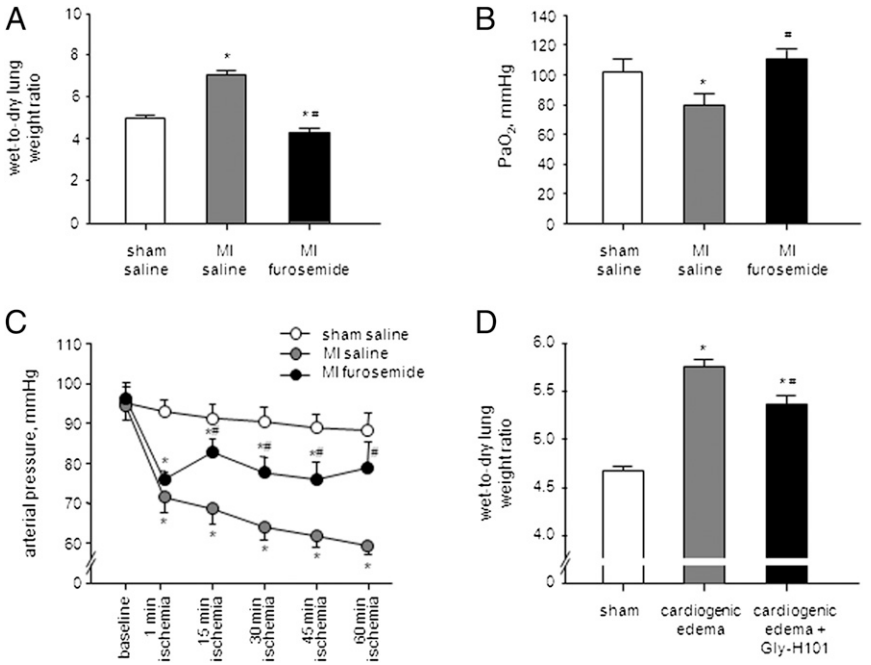

Fig. 6. NKCC and CFTR inhibitors attenuate acute cardiogenic edema in vivo. In a rat model of acute myocardial infarction, animals inhaled either saline (MI saline) or furosemide (10 mg/mL; MI furosemide) continuously from the time of LAD occlusion. Saline-inhaling sham-operated rats without LAD occlusion served as control (sham saline). (*A*) Wet-to-dry lung weight ratio and (*B*) arterial PO_2 (PaO_2) were determined 60 min after LAD occlusion, and (*C*) systemic arterial pressure was monitored every 15 min. Furosemide inhalation prevented the increase in wet-to-dry lung weight ratio and concomitant systemic hypoxemia and hypotension in acute myocardial infarction. All data are mean ± SEM from $n = 7$ experiments each. $^{*}P < 0.05$ vs. sham saline; $^{\#}P < 0.05$ vs. MI saline. (*D*) In mice, cardiogenic lung edema was induced by LAD occlusion and concomitant saline (20 μL/g) infusion; 90 min after LAD occlusion, wet-to-dry lung weight ratio was lower in mice treated with an i.p. bolus of the CFTR inhibitor Gly-H101 (6 mg/kg 30 min before LAD occlusion) compared with vehicle control. Data are mean± SEM from $n = 5$ experiments each. $^{*}P < 0.05$ vs. sham; $^{\#}P < 0.05$ vs. cardiogenic edema.

in isolated mouse, rat, or rabbit lungs. Alveolar fluid secretion was likewise blocked by inhibition of cGKII, which mediates the NO/cGMP-dependent activation of CFTR (19), suggesting that pressure-induced endothelial NO production (6) may assist CFTR-mediated Cl^- secretion at hydrostatic stress. The notion of a critical role for transepithelial Cl^- transport in alveolar fluid secretion is further supported by the fact that alveolar fluid secretion was prevented in Cl^--free perfused lungs. Notably, substitution of Cl^- for NO_3^- only partially inhibited basal alveolar fluid clearance, but it completely blocked alveolar fluid secretion at hydrostatic stress; this finding is in line with the fact that CFTR will allow for basal NO_3^- uptake but with approximately one-half the conductance compared with Cl^- (20), whereas NO_3^- cannot replace Cl^- for anion entry by NKCC1 in alveolar fluid secretion. We considered that epithelial Na^+ absorption and Cl^- secretion can coexist in the intact alveolus (21), in which case inhibition of apical Na^+ uptake may simply unmask Cl^- secretion. However, this notion is refuted by the fact that Cl^--free instillate or CFTR inhibition attenuated rather than increased basal alveolar fluid clearance. Taken together, these findings show a reversal of CFTR-mediated transepithelial Cl^- flux, which causes a subsequent switch from alveolar fluid clearance to fluid secretion at hydrostatic stress.

Apical Cl^- extrusion by CFTR requires a concomitant basolateral Cl^- entry mechanism to allow for sustained alveolar Cl^- and

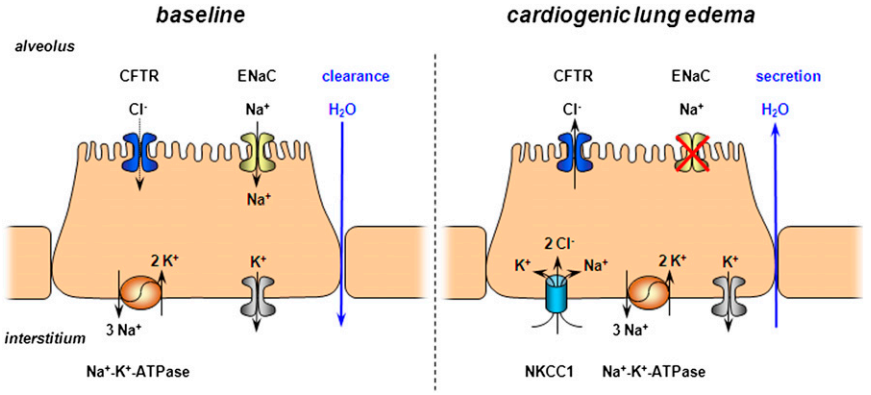

Fig. 7. Proposed model: At baseline (*left*), alveolar fluid clearance is driven by apical Na^+ entry into the alveolar epithelium via ENaC and its basolateral extrusion by Na^+-K^+-ATPase; Cl^- will follow for electroneutrality. In cardiogenic lung edema (right), ENaC is inhibited, which generates a gradient for Na^+ influx via basolateral NKCC1. Cl^- enters in co-transport with Na^+, and now exits the cell along an electrochemical gradient via apical CFTR, thereby driving active fluid secretion into the alveolar space that promotes the formation of lung edema.

thus, fluid secretion in cardiogenic edema. Here, we identify NKCC1 as a corresponding basolateral Cl^- entry channel based on the findings that the NKCC inhibitors furosemide and bumetanide attenuated alveolar Cl^- influx at hydrostatic stress in radionuclide tracing and alveolar Cl^- imaging experiments and blocked alveolar fluid secretion in double-indicator dilution studies. Although furosemide and bumetanide do not differentiate between the two different NKCC channels, only NKCC1 is expressed in alveolar epithelial cells (22), whereas NKCC2 expression is confined to the kidney (23). The identification of a critical relevance for NKCC1 in alveolar fluid secretion and cardiogenic edema formation provides an intriguing explanation for the consistent (pre)clinical observation that the initial improvement of respiratory function by the unspecific NKCC blocker and loop diuretic furosemide in lung edema precedes the onset of diuresis and thus, cannot be explained by its renal effects (24, 25). Unexpectedly, inhibition of NKCCs by either furosemide or bumetanide also attenuated, in part, absorptive alveolar fluid transport at baseline pressures. Although this finding contrasts with the classic view of a unidirectional cotransporter at the basolateral membrane, it is in agreement with previous reports that bumetanide inhibits alveolar fluid clearance in guinea pig (26) and CFTR-overexpressing rat lungs (9). NKCC expression in the apical membrane (26) or bidirectional Cl^- transport through NKCC1 (27) may facilitate this effect, but the exact mechanism remains to be elucidated.

Regulation of Transepithelial Cl^- Flux and Fluid Secretion. Because transepithelial Na^+ and Cl^- transport has been proposed to be functionally and potentially even structurally coupled, we considered that Cl^- transport may be regulated by ENaC activity and transepithelial Na^+ flux. Although it has become wildly recognized that CFTR negatively regulates ENaC function and expression (28), ENaC may also impact on Cl^- flux through CFTR. Airway-specific overexpression of ENaC produces a cystic fibrosis-like phenotype in mice (29), and mutations in amiloride-sensitive epithelial sodium channels are associated with a cystic fibrosis-like disease in patients (30), suggesting that CFTR secretory activity may be inversely regulated by Na^+ absorption by ENaC. Hence, we considered that the reversal of transepithelial Cl^- flux may have been caused by an inhibition of ENaC activity in the presence of an active Na^+-K^+-ATPase. This concept was particularly appealing, because we had previously shown ENaC activity to be blocked at hydrostatic stress (2); also, transepithelial Cl^- and alveolar fluid secretion were blocked at 4 °C or by ouabain. Calculation of epithelial membrane potentials based on published ionic concentrations of alveolar type II cells (31) and a basal conductance ratio for $g_{Na}{}^+$ over $g_K{}^+$ of 2.8 (32) predicts an inward Cl^- current at baseline that will reverse when Na^+ conductance is partially (50%) or fully blocked (Fig. S2). Similarly, in mathematical models, the decrease in Na^+ conductance caused by ENaC inhibition results in membrane hyperpolarization that is alleviated when CFTR conductance is high, implicating ENaC inhibition in stimulation of Cl^- efflux by CFTR (33). Probably of even greater importance, inhibition of ENaC in the presence of an intact Na^+-K^+-ATPase will generate an electrochemical gradient for Na^+ influx through other amiloride-insensitive ion channels or transporters, such as the basolateral NKCC1. For each Na^+ cation, NKCC1 will symport 2 Cl^- anions into the cell, thus generating both a chemical and—because of K^+ efflux by basolateral K^+ channels—an electrical gradient for subsequent apical Cl^- efflux by CFTR (34). In the present study, alveolar Cl^- and fluid secretion could be effectively mimicked by both pharmacological inhibition of ENaC and alveolar instillation of an Na^+-free solution. A similar precedent for the stimulation of Cl^- secretion by ENaC inhibition was recently shown in cortical collecting ducts (35). Effects of alveolar Na^+ uptake inhibition shared the same mechanistic characteristics that we had established for cardiogenic lung edema, in that alveolar Cl^- influx and alveolar fluid secretion depended on CFTR, NKCC, and Na^+-K^+-ATPase. These findings support the concept that alveolar fluid secretion is a secondary consequence of impaired alveolar Na^+ uptake. Remarkably, the proposed scenario shares an inverse analogy with the physiological transition from the fetal to the neonatal lung, where a rapid increase in ENaC abundance and activity at the apical membrane reverses Cl^--driven alveolar fluid secretion into Na^+-driven absorption (36).

It is noteworthy that, whereas amiloride is consistently found to inhibit alveolar fluid clearance to a similar extent as ouabain in isolated lungs, which commonly lack appropriate substrates for sodium cotransporters (17), amiloride-sensitive Na^+ uptake accounts for only 50% of alveolar fluid clearance in vivo (5). Amiloride-containing diuretics can occasionally cause pulmonary edema in the absence of other cardiogenic or inflammatory triggers, but this adverse effect is rare (37). In vivo, the mechanisms regulating epithelial Na^+ and Cl^- transport can, thus, be expected to be more complex. However, our finding that both CFTR inhibition and inhaled furosemide attenuated cardiogenic lung edema in mice and rats shows that the overall concept of Cl^--driven alveolar fluid secretion similarly applies to the in vivo situation.

In hydrostatic stress, epithelial Na^+ uptake becomes impaired as a result of pressure-induced endothelial NO production (6) and subsequent NO-dependent inhibition of Na^+ absorption (2, 38). Notably, endothelial NO production may simultaneously activate CFTR, which was indicated by the fact that alveolar fluid secretion was associated with NO-dependent CFTR phosphorylation and blocked by cGKII inhibition. This notion is in line with previous data from airway epithelial cells showing that NO stimulates Cl^- secretion through cGKII-dependent phosphorylation of CFTR (39). Taken together, these findings implicate a key dual role for NO as a critical trigger of alveolar fluid secretion in cardiogenic lung edema, in that hydrostatic stress stimulates endothelial NO production, which will simultaneously block epithelial Na^+ absorption and facilitate Cl^- secretion, thus reversing the Na^+-driven clearance of alveolar fluid into a Cl^--driven fluid secretion.

Clinical Implications. Cardiogenic pulmonary edema is a frequent complication of acute left heart failure most commonly caused by ischemia with or without myocardial infarction, exacerbation of chronic systolic or diastolic heart failure, and dysfunction of the mitral or aortic valve (40). Interstitial pulmonary edema and alveolar flooding impair lung mechanics and gas exchange, thus causing dyspnea and tachypnea, and they ultimately result in an in-hospital mortality rate of ~15% (41). The identification of a transepithelial Cl^- transport by NKCC1 and CFTR that drives alveolar fluid secretion gives rise to a revised mechanistic concept for cardiogenic edema formation and identifies therapeutic targets for innovative treatment strategies. This evolving concept may not be uniquely restricted to cardiogenic pulmonary edema, but it could also apply to other forms of hydrostatic lung edema. Although inhibition of CFTR does not seem a realistic option given the clinical severity of cystic fibrosis disease, NKCC1 may present a promising target for pharmacological interventions. Inhaled furosemide has been used acutely in a series of small clinical trials without detectable adverse effects (42), but it has not been used for chronic treatment, where prolonged inhibition of Cl^- secretion in airway epithelia may potentially evoke cystic fibrosis-like symptoms. Specific NKCC1 inhibitors are currently not available for clinical or experimental use; however, targeted NKCC1 inhibition may present a particularly attractive strategy in those forms of hydrostatic lung edema where unspecific NKCC inhibition by furosemide is contraindicated or associated with an increased risk of hypovolemia [e.g., in neurogenic lung edema after subarachnoid hemorrhage (43) or high-altitude lung edema (44)].

Materials and Methods

A detailed method section is provided in *SI Materials and Methods*. In brief, alveolar fluid transport and alveolar Cl^- influx were measured in isolated

perfused lungs of male Sprague–Dawley rats by double-indicator dilution technique (2) and in situ fluorescence microscopy (12), respectively. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, and the study was approved by the animal care and use committee of the local government authorities. For alveolar fluid transport measurements, the high-molecular mass tracer Texas Red Dextran (70 kDa), which cannot cross the intact alveolocapillary barrier, was instilled into the distal air space, whereas the low-molecular mass tracer Na^+-fluorescein (360 Da), which reflects paracellular fluid filtration because of its rapid convective transport, was added to lung perfusate. Spectrophotometric assessment of tracer concentrations in alveolar instillate and lung perfusate allowed for differentiation between fluid filtration and active alveolar fluid transport in a two-compartmental double-indicator dilution model (2). Active alveolar fluid transport was differentiated based on the direction of the osmotically driven fluid shift into alveolar fluid clearance for alveolar fluid transport < 0 mL/h and alveolar fluid secretion for values > 0 mL/h. Alveolar Cl^- influx was determined by real-time in situ fluorescence imaging in isolated perfused rat lungs instilled with the Cl^--sensitive dye lucigenin as increase in alveolar Cl^- concentration in an initially Cl^--free alveolar instillate during lung perfusion with 30 mmol/L Cl^- solution (12). Transepithelial Cl^- transport was determined by radionuclide tracing in isolated perfused rabbit lungs (11). In brief, ~6 μCi [^{3}H]-mannitol was deposited by nebulization in the distal airspace, whereas 0.7 μCi $^{36}Cl^-$ was applied to the perfusate. Transit of [^{3}H]-mannitol as an indicator of barrier permeability to passive solvent flux was followed by repetitive perfusate sampling, whereas $^{36}Cl^-$ flux into the alveolar space was assessed from bronchoalveolar lavage fluid by scintillation counting. Edema formation was assessed as increase in wet-to-dry lung weight ratio in isolated perfused lungs of CFTR KO (*CFTR^{tm1HGU}*) mice and corresponding WT littermates (2).

Hydrostatic stress was induced in isolated perfused lungs by elevation of the venous outflow reservoir until P_{LA} increased to 6 (mice), 13.5 (rabbits), or 15 (rats) cmH_2O. For pharmacological interventions, the Na^+-K^+-ATPase inhibitor ouabain (100 μmol/L), the NKCC inhibitors furosemide (500 μmol/L) or bumetanide (10 μmol/L), or the TRPV4 activator 4αPDD (10 μmol/L) was added to perfusate. CFTR inhibitors glibenclamide (200 μmol/L) or $CFTR_{inh}$-172 (10 μmol/L), the ENaC inhibitor amiloride (10 μmol/L), or the cGKII inhibitor Rp-8-pCPT-cGMP (10 μmol/L) was added to alveolar instillate. For Na^+- or Cl^--free conditions, Na^+ or Cl^- was replaced by equal amounts of *N*-methyl-D-glucamine or NO_3^- in alveolar instillate or lung perfusate (12).

To address the role of alveolar NKCC in cardiogenic edema in vivo, acute myocardial infarction was induced in Sprague–Dawley rats by ligation of the LAD, and nebulized furosemide or saline was inhaled from the time of LAD occlusion for 60 min. In C57BL/6 mice, the CFTR inhibitor Gly-H101 (6 mg/kg) or solvent was administered i.p. 30 min before the induction of cardiogenic edema by LAD occlusion and concomitant i.v. volume load.

All data are given as mean ± SEM. Groups were compared by Student's *t* test or ANOVA, and statistical significance was assumed at $P < 0.05$.

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