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IL-8 inhibits cAMP-stimulated alveolar epithelial fluid transport *via* a GRK2/PI3K-dependent mechanism

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ABSTRACT Patients with acute lung injury (ALI) who retain maximal alveolar fluid clearance (AFC) have better clinical outcomes. Experimental and small clinical studies have shown that β_2 -adrenergic receptor (β_2 AR) agonists enhance AFC *via* a cAMP-dependent mechanism. However, two multicenter phase 3 clinical trials failed to show that β_2 AR agonists provide a survival advantage in patients with ALI. We hypothesized that IL-8, an important mediator of ALI, directly antagonizes the alveolar epithelial response to β_2 AR agonists. Short-circuit current and whole-cell patch-clamping experiments revealed that IL-8 or its rat analog CINC-1 decreases by 50% β_2 AR agonist-stimulated vectorial Cl^- and net fluid transport across rat and human alveolar epithelial type II cells *via* a reduction in the cystic fibrosis transmembrane conductance regulator activity and biosynthesis. This reduction was mediated by heterologous β_2 AR desensitization and down-regulation (50%) *via* the G-protein-coupled receptor kinase 2 (GRK2)/PI3K signaling pathway. Inhibition of CINC-1 restored β_2 AR agonist-stimulated AFC in an experimental model of ALI in rats. Finally, consistent with the experimental results, high pulmonary edema fluid levels of IL-8 (>4000 pg/ml) were associated with impaired AFC in patients with ALI. These results demonstrate a novel role for IL-8 in inhibiting β_2 AR agonist-stimulated alveolar epithelial

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Key Words: β_2 adrenergic receptor • acute lung injury

ACUTE LUNG INJURY (ALI) is a clinical syndrome manifested by the rapid onset of respiratory failure associated with high mortality (1). ALI is characterized by increased permeability of the alveolar-capillary barrier, decreased surfactant function, and impaired AFC (alveolar fluid clearance) (2). Notably, patients with ALI who retain maximal AFC have better clinical outcomes (3). The mechanisms responsible for the inhibition of the alveolar epithelial fluid transport in patients with ALI are not well understood. A multicenter observational study reported that increased pulmonary edema is the most important predictor of mortality in ICU patients (4). Therefore, significant effort has been undertaken to pharmacologically up-regulate AFC to reverse the progression of lung injury, but without success (5).

The use of β_2 -adrenergic receptor (β_2 AR) agonists may stimulate AFC in patients with ALI. Indeed, β_2 AR agonists have been shown to enhance alveolar epithelial fluid transport *via* a cAMP-dependent mechanism under physiological conditions (6–9) and in experimental models of lung injury (10–12), as well as in one prospective study of extravascular lung water in patients with ALI (13).

Abbreviations: 8-CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate, acetoxymethyl ester; [¹²⁵I]-ICYP, (–)-[¹²⁵I]iodocyanopindolol; β_2 AR, β_2 -adrenergic receptor; AFC, alveolar fluid clearance; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; ATE, alveolar epithelial type II; B_{max} , maximal number of ICYP binding sites; CFTR, cystic fibrosis transmembrane conductance regulator; CINC-1, cytokine-induced neutrophil chemoattractant 1; CXCR2, C-X-C chemokine receptor type 2; EPI, epinephrine; GRK2, G-protein-coupled receptor kinase 2; KC, keratinocyte-derived chemokine; K_D , equilibrium dissociation constant; PI3K, phosphatidylinositol-3-kinase; PIK-90, phosphatidylinositol-3-kinase inhibitor 90; PKA, protein kinase A; PKC, protein kinase C; RSV, respiratory syncytial virus; PIK-90, phosphatidylinositol-3-kinase inhibitor 90

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However, two recent phase III multicenter trials of β_2 -adrenergic agonists by the U.S. National Heart, Lung, and Blood Institute (NHLBI) acute respiratory distress syndrome (ARDS) network group in the United States (14) and by the Medical Research Council (15) in the UK were stopped for futility.

Although the reasons for the lack of success of these phase III clinical trials are unclear, one possible mechanism could be agonist- and non-agonist-specific desensitization of the β_2 AR. For example, a recent study suggested that the decreased alveolar fluid clearance observed in respiratory syncytial virus (RSV)-infected mice was mediated by insensitivity to β_2 AR agonists (16), an effect that could be attenuated by antibodies against the neutrophil keratinocyte-derived chemokine (KC), a mouse analog of IL-8. Interestingly, IL-8 has been shown to be the predominant neutrophil chemokine present in the distal airspaces of patients with ALI and is a predictor of mortality in these patients (17–21). However, whether IL-8 directly inhibits β_2 AR agonist-stimulated alveolar ion and fluid transport is still unknown.

Since recent experimental evidence has demonstrated convincingly in mice and in humans that β_2 AR agonist-dependent stimulation of alveolar epithelial fluid transport is dependent on the activity of the cystic fibrosis transmembrane conductance regulator (CFTR; refs. 22–25), the first objective of our study was to determine whether IL-8 and/or cytokine-induced neutrophil chemoattractant 1 (CINC-1), the rat analog of IL-8 [that is also called chemokine (C-X-C motif) ligand 1 (Cxcl1) in the new U.S. National Center for Biotechnology Information (NCBI) database], would inhibit β_2 AR agonist-stimulated CFTR-dependent alveolar epithelial fluid transport. Desensitization of the β_2 AR has been shown to depend on the binding of G-protein-coupled receptor kinase 2 (GRK2) [that is also called adrenergic β -receptor kinase 1 (ADRBK1) in the new NCBI database] to activated phosphatidylinositol-3-kinase (PI3K) and on the translocation of the GRK2/PI3K complex to the plasma membrane (26, 27). Thus, the second objective was to test the role of PI3K in mediating the inhibitory effects of IL-8/CINC-1-dependent inhibition of the β_2 AR agonist-stimulated CFTR-dependent alveolar epithelial fluid transport. The results show that IL-8/CINC-1 inhibits β_2 AR agonist-stimulated alveolar fluid transport *via* a GRK2/PI3K-dependent mechanisms.

MATERIALS AND METHODS

Reagents

All cell culture media were prepared by the University of California, San Francisco (UCSF) Cell Culture Facility or in the J.-F.P. laboratory at the University of Alabama at Birmingham (UAB), using deionized water and analytical grade reagents. (–)-[¹²⁵I]iodocyanopindolol ([¹²⁵I]-ICYP) was purchased from Perkin Elmer (Waltham, MA, USA). 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphate, acetoxymethyl ester (8-CPT-cAMP) was purchased from Calbiochem (San

Diego, CA, USA). The CFTR inhibitor, CFTR_{inh}-172, was a kind gift from Alan S. Verkman (UCSF). The PI3K inhibitor, PI3K inhibitor 90 (PIK-90), is a potent and cell-permeable inhibitor of p110 α , p110 β , p110 γ , and p110 δ with IC₅₀ of 11, 350, 18, and 58 nM, respectively (28). The GRK2 inhibitor was purchased from EMD Biosciences (San Diego, CA, USA). The cell-permeable inhibitor of the protein kinase C ζ (PKC ζ) isoform (myristoylated PKC ζ inhibitory peptide) and the cell-impermeable inhibitor of the PKC ζ isoform (unmyristoylated PKC ζ inhibitory peptide) were obtained from EMD Biosciences (Gibbstown, NJ, USA). IL-8 and CINC-1 ELISA were purchased from R&D (Minneapolis, MN, USA). Antibodies and phosphoantibodies for the β_2 AR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies and phosphoantibodies for Akt and GRK2 were purchased from Calbiochem (San Diego, CA, USA). Antibody 217 for CFTR was purchased from the University of North Carolina Department of Biochemistry/Biophysics and Cystic Fibrosis Center (Chapel Hill, NC, USA). Goat anti-mouse and goat anti-rabbit IRDye-conjugated secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA). The CFTR-luc plasmids were a kind gift from G. Stanley McKnight (University of Washington, Seattle, WA, USA). Cationic liposomes (FuGene6) were obtained from Roche Biochemicals (Indianapolis, IN, USA). Protein concentration of cell lysates, pulmonary edema fluid, and plasma from patients with ALI was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA).

Cell culture

Following approval from the Committee on Animal Research (UCSF), or the Institutional Animal Care and Use Committee (UAB), rat alveolar epithelial type II (ATII) cells were isolated, as described previously (29, 30). Human ATII cells were isolated using a modification of methods previously described (31) from human lungs that were not used by the California Transplant Donor Network (32). Cell viability after exposure to different experimental conditions was measured by the Alamar Blue assay (33).

In vitro experiments

Short-circuit current studies were performed on freshly isolated rat or human ATII cells, as we have previously reported using established methods (34). Fluid transport across primary rat ATII cell monolayers was measured as described previously (35). Transepithelial resistance ($\kappa\Omega/\text{cm}^2$) was measured using the Millicell-ERS (Millipore, Bedford, MA, USA). The effect of CINC-1 (0.1–10 ng/ml for 24 h) or its vehicle on bioelectric properties of ATII cell monolayers was evaluated on d 4 in culture. Active protein kinase A (PKA) was measured in triplicate by a commercially available solid-phase ELISA from Assays Designs (Ann Arbor, MI, USA). Isolation of plasma membrane-enriched fractions was performed, as we have previously reported (34). Western blot for calreticulin (an ER protein marker) was performed to ensure purity of plasma membrane proteins, as we have previously done (34). Western blot analyses from frozen lungs and cell homogenates were performed as described previously (36). Quantification was performed with the LI-COR Biosciences analysis software. Saturation binding experiments were performed on plasma membrane-enriched fractions from primary rat ATII cells, as we have previously reported (34). For CFTR promoter reporter studies, rat ATII cells were transiently transfected with a CFTR-promoter reporter vector (37) containing the luciferase gene subcloned downstream of the CFTR

promoter [CFTR(wt)-luc; ref. 34]. Real-time RT-PCR primers and probes (**Table 1**) were designed using Primer Express software [PE-Applied Biosystems (PE-ABI), Warrington, UK]. Quantitative real-time RT-PCR was performed as described previously (38). IL-8 levels in the pulmonary edema fluid from patients with ALI and CINC-1 levels in cell culture supernatant from A2II cell monolayers and from rat lung homogenates were measured by an ELISA purchased from R&D Systems (Minneapolis, MN, USA) following the manufacturer's instructions. cAMP levels were measured in triplicate by a commercially available ELISA from R&D Systems.

Whole-cell patch-clamping studies

Freshly isolated rat A2II cells were plated on glass coverslips. Whole-cell current recordings were obtained using standard amphotericin B perforated patch methodology at room temperature from cells seeded on glass coverslips and mounted in a flow-through chamber on the stage of a Leica DM IRB inverted microscope (Leica Microsystems, Heidelberg, Germany). Cellular responses were confirmed using conventional whole-cell voltage-clamp methodology. Bath solution exchange was achieved using a pinch-valve control system converging on an 8-1 manifold. Tips of borosilicate recording pipettes (5–7 mΩ) were backfilled with pipette solution [150 mM KCl and 10 mM HEPES, pH 7.2 (Tris/HCl)] and then with the same solution containing ~0.2 mg/ml amphotericin B. Currents were obtained using an Axopatch 200B patch-clamp amplifier [Axon Instruments (AI); Molecular Devices, Sunnyvale, CA, USA] with voltage commands, and data acquisition was controlled by Clampex software (pClamp 8; AI) and digitized (Digidata 1321A interface; AI) at a sampling frequency of 2 kHz. Current-voltage relationships were obtained using a pulse protocol in which cells were stepped from a -40-mV holding potential from -100 to +80 mV in 20-mV increments for 250 ms. Mean currents were obtained from the average of 3 sweeps during the 200- to 250-ms period of each sweep using Clampfit software (AI). Bath solutions contained (in mM) 140 NaCl, 4.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 glucose, and 10 HEPES, pH 7.4 (NaCl/HCl). Appropriate vehicle controls were performed. Cells were preincubated with CINC-1 for ≥1 h prior to and throughout the entire duration of the experiment.

Rat studies

The protocol for measurement of AFC after hemorrhagic shock in rats was approved by the UCSF Committee on Animal Research, and was performed as described previously (34, 36). Briefly, male Sprague-Dawley rats weighing 300–350 g were anesthetized with pentobarbital sodium (60 mg/kg i.p.), and anesthesia was maintained with 30 mg/kg of

pentobarbital sodium every hour. An endotracheal tube (14-gauge) was inserted through a tracheotomy. Catheters (PE-50) were inserted into both carotid arteries to monitor systemic arterial pressure, obtain blood samples, and withdraw blood for induction of prolonged hemorrhagic shock. The rats were ventilated with a constant-volume pump (Harvard Apparatus, Holliston, MA, USA) with an inspired oxygen fraction of 1.0, peak airway pressures of 8–12 cmH₂O, supplemented with positive end-expiratory pressure of 3 cmH₂O. The respiratory rate was adjusted to maintain the PaCO₂ between 35 and 40 mmHg during the baseline period. After the surgery, heart rate and systemic blood pressure were allowed to stabilize for 60 min. Hemorrhagic shock was induced by withdrawing blood from the carotid artery to maintain a mean systemic arterial pressure of 40–45 mm Hg for 60 min. This corresponded to the removal of 9–12 ml of blood. After 1 h of hemorrhagic shock, the rats were resuscitated with intravascular 4% albumin solution in 0.9% NaCl over 30 min to maintain a central venous pressure < 8 mmHg. The volume of 4% albumin solution administered was twice the amount of blood withdrawn. At 5 h after the onset of the hemorrhagic shock, the rats were exsanguinated, and AFC was determined in the absence of ventilation or blood flow by measuring the increase in protein tracer concentration (¹²⁵I-labeled albumin, 1 μCi) in the lungs over a 30-min period. For these experiments, we instilled intratracheally 12 ml/kg of warmed 5% albumin in 0.9% NaCl solution labeled with ¹²⁵I-albumin, aspirated and reinstalled the solution 3 times and applied continuous positive airway pressure (CPAP: 8 cmH₂O, 100% F_IO₂) to prevent alveolar collapse and kept the animals at 37°C body core temperature. The instillate, an initial sample (after aspiration and reinstallation), and a sample after 30 min were obtained and analyzed. In some experiments, rats were pretreated with a CINC-1 antibody or its isotype control antibody that were administered i.p. 30 min before the onset of hemorrhage.

Human studies

Patients with ALI or ARDS as defined by the North American European Consensus Conference definitions (2) were identified from the adult Intensive Care Unit of Moffitt-Long Hospital, UCSF, and San Francisco General Hospital, San Francisco, CA. Additional inclusion criteria included acute respiratory failure requiring mechanical ventilation and aspirable pulmonary edema fluid within 1 h of tracheal intubation and repeated within 4 h. Patients who had undergone cardiopulmonary bypass were excluded. The initial edema fluid-to-plasma protein ratio was >0.65, consistent with increased permeability pulmonary edema. The Committee for Human Research (UCSF) approved the study.

TABLE 1. *Primer sequences*

Designation	Sequence	GenBank accession no.
Rat		AB017801
GAPDH		
Forward	5'-CTGCCAAGTATGATGACATCAAGAA-3'	
Reverse	5'-AGCCAGGATGCCCTTTAGT-3'	
Probe	5'-TCGGCCGCTGCTTACCA-3'	
Rat		M89906
CFTR		
Forward	5'-GCTCTGCCTCGCAGCAAC-3'	
Reverse	5'-CAGACTGTCTTCTTCTGCCTGGA-3'	
Probe	5'-TGATCACCGCCGGGCCA-3'	

Rate of net AFC

The rate of AFC was calculated as described previously (3). Undiluted pulmonary edema fluid and plasma were collected simultaneously. All samples were centrifuged at 3000 *g* for 10 min, and supernatants were stored at -70°C . If the sample volume was insufficient for measurement with the Bio-Rad protein assay kit (less than 1% of samples), then refractometry was used. The rate of AFC was calculated by comparing the final edema fluid protein concentration to the initial edema fluid concentration using the equation $\text{AFC} (\%) = (C_f - C_i)/C_i$, where C_i and C_f are initial and final edema fluid protein concentration, respectively. All measurements of AFC were expressed as percentage per hour by adjusting for the time interval between the initial and final edema fluid sample. For some analyses, patients were grouped into two groups, those with mean levels of IL-8 in the edema fluid > 4000 pg/ml and those with mean levels of IL-8 < 4000 pg/ml.

Statistics

All the data are summarized as means \pm SE. One-way ANOVA followed by a Dunnett's test was used to compare ≥ 3 experimental groups and a Student's *t* test to compare 2 experimental groups. A value of $P < 0.05$ was considered statistically significant. Saturation binding experiments were analyzed by nonlinear regression. The maximal number of ICYP binding sites (B_{max}) and the equilibrium dissociation constant (K_D) were calculated from saturation binding curves by nonlinear least squares curve fittings for one binding site. The goodness of the fit was determined by the *F*-ratio test. The correlation between the AFC and the levels of IL-8 in pulmonary edema fluid of patients with ALI was assessed by the Spearman correlation coefficient.

RESULTS

CINC-1 decreases $\beta_2\text{AR}$ agonist-stimulated AFC across primary rat ATII cell monolayers and in an *in vivo* model of hemorrhagic shock in rats

CINC-1, the rat homologue of IL-8, inhibited epinephrine (EPI)-dependent fluid absorption across primary

rat ATII monolayers (Fig. 1A). Hemorrhagic shock was associated with elevated levels of CINC-1 (Fig. 1B). Inhibition of CINC-1 signaling by a blocking antibody restored the EPI-dependent increase in AFC after hemorrhagic shock in response to $\beta_2\text{-AR}$ agonist stimulation (Fig. 1C).

CINC-1 decreases $\beta_2\text{AR}$ agonist-stimulated CFTR-specific Cl^- transport across primary rat and human ATII cell monolayers

The EPI-stimulated vectorial Cl^- transport across the apical membrane of rat ATII cells was measured in Ussing chambers in the presence of a Cl^- gradient after permeabilization of the basolateral membrane with nystatin (40 μM). EPI-stimulated vectorial Cl^- transport was completely inhibited in the presence of the CFTR inhibitor, CFTR_{inh}-172 (Fig. 2A). Similarly, the effect of EPI or terbutaline, a specific $\beta_2\text{-AR}$ agonist, on Cl^- transport was also inhibited by CINC-1 (Fig. 2B–D). Further, IL-8 caused a time- and dose-dependent inhibition of the EPI-stimulated vectorial Cl^- transport through the apical membrane of human ATII cells (Fig. 3A, B) without affecting their viability (IL-8: $98 \pm 2\%$ vs. control: $99 \pm 1\%$, NS). We then tested the effect of CINC-1 on whole-cell currents in rat ATII cells. As shown in Fig. 4A, current voltage relationships recorded under basal conditions exhibited slight outward rectification with a reversal potential approximately -12 mV. EPI perfusion induced a significant increase in outward currents consistent with a greater electrochemical gradient at these potentials. A slight leftward shift in reversal potential (~ -8 mV) was observed. After maximal stimulation was achieved, cells were perfused with the CFTR inhibitor (CFTR(inh)-172, 10 μM), which led to complete inhibition of the EPI-stimulated currents. Thus, EPI induces Cl^- currents consistent with activation of CFTR channels. Next ATII cells were pre-incubated with CINC-1 for a minimum of 30 min.

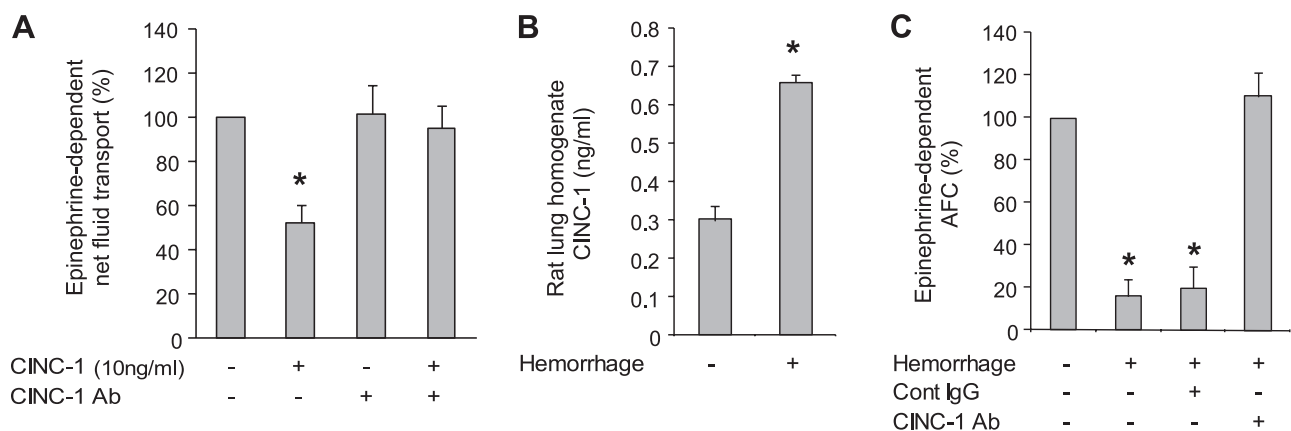


Figure 1. CINC-1 decreases $\beta_2\text{AR}$ agonist-stimulated alveolar net fluid transport across primary rat ATII cell monolayers and in an *in vivo* model of hemorrhagic shock in rats. A) CINC-1 decreases EPI-dependent net fluid transport across polarized rat ATII cell monolayers (control: $4.0 \pm 0.7\%$ of instilled, EPI (20 μM) treated: $7.5 \pm 1.4\%$; $n=9$); $*P < 0.05$ from controls. B) CINC-1 is elevated in lung homogenates after hemorrhagic shock in rats ($n=6$). C) CINC-1 blockade restores the EPI-stimulated AFC after hemorrhagic shock in rats (AFC control $7.0 \pm 1.2\%/30$ min, EPI-treated $13.7 \pm 1.1\%/30$ min; $n=6$). Results are means \pm SE. $*P < 0.05$ vs. controls.

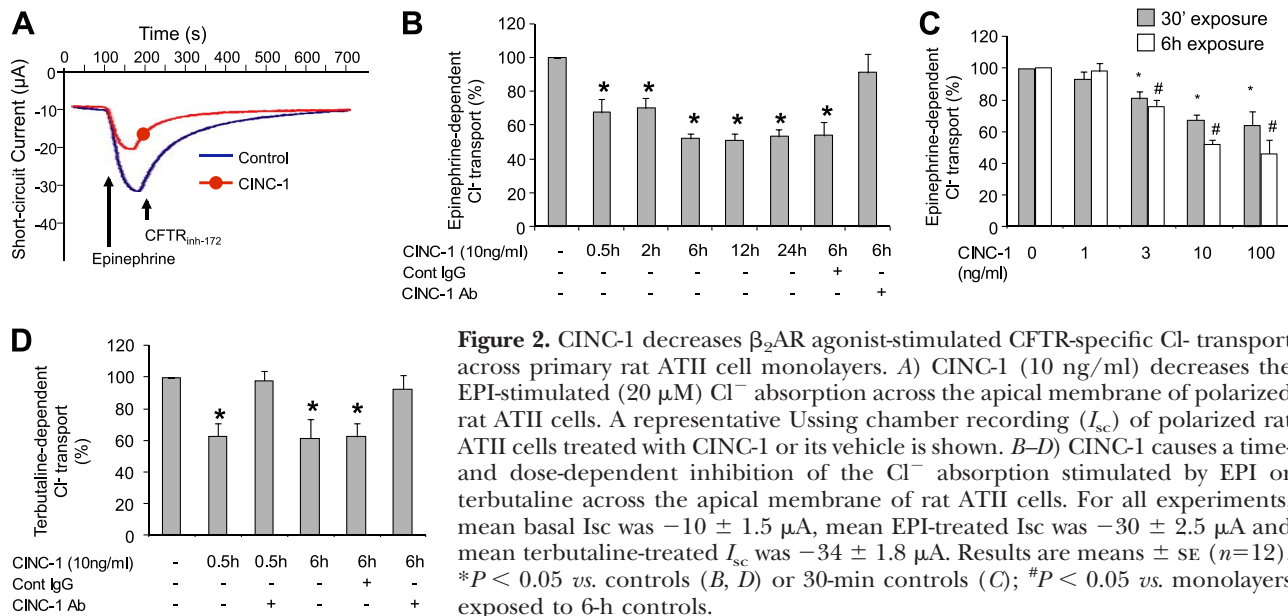


Figure 2. CINC-1 decreases β_2 AR agonist-stimulated CFTR-specific Cl⁻ transport across primary rat ATII cell monolayers. A) CINC-1 (10 ng/ml) decreases the EPI-stimulated (20 μ M) Cl⁻ absorption across the apical membrane of polarized rat ATII cells. A representative Ussing chamber recording (I_{sc}) of polarized rat ATII cells treated with CINC-1 or its vehicle is shown. B–D) CINC-1 causes a time- and dose-dependent inhibition of the Cl⁻ absorption stimulated by EPI or terbutaline across the apical membrane of rat ATII cells. For all experiments, mean basal I_{sc} was $-10 \pm 1.5 \mu$ A, mean EPI-treated I_{sc} was $-30 \pm 2.5 \mu$ A and mean terbutaline-treated I_{sc} was $-34 \pm 1.8 \mu$ A. Results are means \pm SE ($n=12$). * $P < 0.05$ vs. controls (B, D) or 30-min controls (C); # $P < 0.05$ vs. monolayers exposed to 6-h controls.

Currents derived at -60 mV were normalized to control currents (Fig. 4B). Epinephrine induced a 36% increase in outward currents, which returned to control levels after exposure to CFTR(inh)-172. Following preincubation with CINC-1, EPI induced a small ($\sim 9\%$) but significant decrease in whole-cell currents. Short exposure to CINC-1 (~ 5 min) had no significant effect on basal currents under these ex-

perimental conditions. Thus, preincubation with CINC-1 completely abolished the outward β_2 AR agonist-induced currents in rat ATII cells. These data confirm at the single-cell level, our findings in monolayer studies are consistent with a direct effect of CINC-1 on transcellular Cl⁻ transport.

Short exposure to CINC-1 decreases β_2 AR agonist-stimulated Cl⁻ transport across primary rat ATII cell monolayers via a PI3K/GRK2-dependent mechanism

CINC-1 significantly inhibited EPI-induced cAMP accumulation and PKA activity in rat ATII cell monolayers (Fig. 5A), an effect inhibited when cell monolayers were exposed to a cell-permeable cAMP analog (CPT-cAMP) (Fig. 5B, C). Because activation of PI3K has been shown to play an important role in homologous desensitization and down-regulation of β_2 AR in cardiomyocytes (27), we pretreated ATII cell monolayers with PIK-90, an inhibitor of PI3K. CINC-1 alone induced the phosphorylation of Akt in rat ATII cell monolayers (Fig. 6A), but pretreatment with PIK-90 prevented the inhibitory effect of CINC-1 on alveolar Cl⁻ transport across the apical membrane of rat ATII cells (Fig. 6B). Finally, PIK-90 completely restored the EPI-dependent increase in AFC after hemorrhagic shock (Fig. 6C). As PI3K activity has been shown to be required for the insulin-induced translocation of GRK2 to the plasma membrane (39), we next tested the effect of CINC-1 on GRK2 activity. CINC-1 induced the translocation of GRK2 to the cell membrane of ATII cell monolayers (Fig. 7A) and the subsequent phosphorylation of the β_2 AR without the presence of a β_2 AR agonist (Fig. 7B). We further validated the role of GRK2 in the CINC-1-dependent decrease of β_2 AR agonist-stimulated apical Cl⁻ transport by inhibiting GRK2. Inhibition of GRK2 or of its upstream kinase PKC ζ (40) preserved the β_2 AR agonist-dependent Cl⁻ transport in the presence of CINC-1 (Fig. 7C, D). Finally, a short

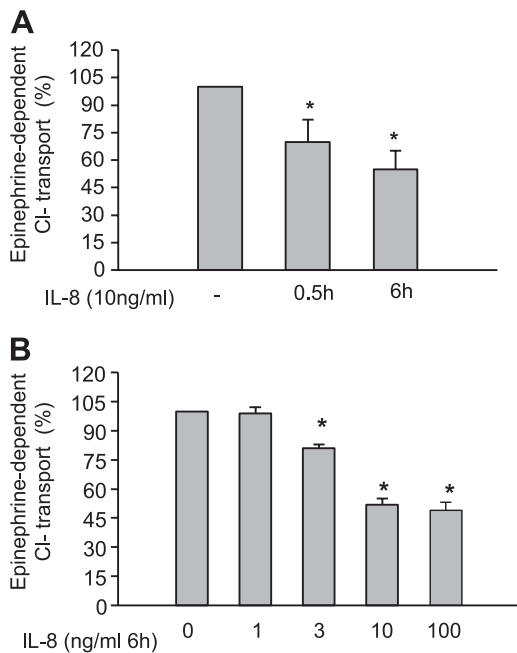


Figure 3. IL-8 decreases β_2 AR agonist-stimulated CFTR-specific Cl⁻ transport across human ATII cell monolayers. IL-8 causes a time-dependent (A) and dose-dependent (B) inhibition of the EPI-stimulated CFTR-specific Cl⁻ absorption across the apical membrane of human ATII cells. For all experiments, mean basal I_{sc} was $-8 \pm 1.9 \mu$ A, mean EPI-treated I_{sc} was $-25 \pm 2.3 \mu$ A. Results are means \pm SE ($n=12$). * $P < 0.05$ vs. controls.

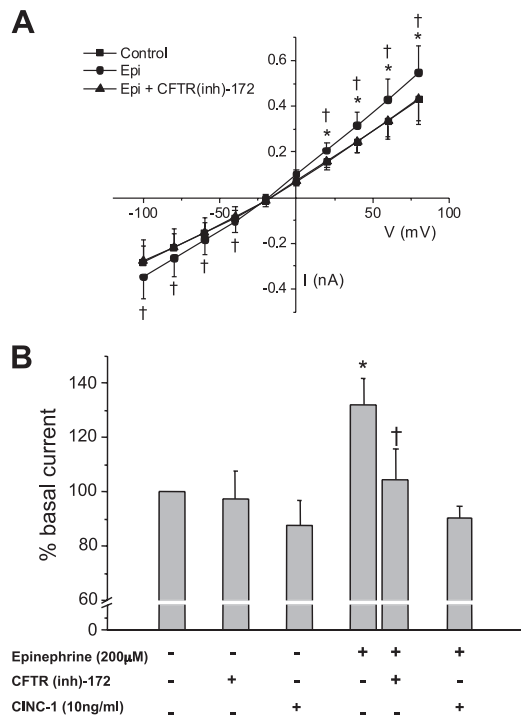


Figure 4. CINC-1 abolishes β_2 AR agonist-induced whole-cell currents in rat ATII cells. *A*) Current-voltage relationship from 7 whole-cell patch-clamp recordings using amphotericin B perforated-patch methodology. Cells were held at -40 mV and stepped from -100 to $+80$ mV in 20-mV increments for a duration of 100 ms. Data plotted are derived from the final 20 ms of recording at each potential. Currents were recorded under basal currents (solid squares), after perfusion with 200 μ M epinephrine (solid circles) and following perfusion with 10 μ M CFTR(inh)-172 in the continued presence of epinephrine (solid triangles). *B*) Using the same protocol as *A*, currents recorded at $+60$ mV were normalized to basal currents and expressed as percent of control following application of test reagent. Data taken from *A* are shown in bars 4 and 5 ($n=7$). In similar experiments, cells were preincubated with CINC-1 (10 ng/ml) for a minimum of 30 min prior to application of epinephrine (200 μ M; bar 6). In this series of experiments, CINC-1 was present throughout the entire experiment ($n=7$). Bar 2 shows the effect of CFTR(inh)-172 ($n=2$); bar 3 shows the effect of acute exposure (<5 min) to CINC-1 ($n=4$) on basal currents. Results are means \pm SE. * $P < 0.05$ vs. basal current; † $P < 0.05$ vs. epinephrine alone (paired Student's *t* test).

exposure to CINC-1 did not cause a decrease of the β_2 AR density at the cell membrane of ATII cells measured by saturation binding experiments (Fig. 7E), indicating that a short exposure to CINC-1 causes a desensitization of the β_2 AR on plasma membrane of ATII cells.

Prolonged exposure to CINC-1 inhibits the β_2 AR-dependent activation of the PKA and CFTR promoter activity, gene expression, and function via a heterologous down-regulation of the β_2 AR at the cell membrane

Prolonged exposure to β AR agonists has been reported to cause the endocytosis of the (β_2 AR leading to the

inhibition of its cell signaling (27). Thus, we determined whether prolonged exposure to CINC-1 would decrease β_2 AR expression in the plasma membrane by performing saturation binding experiments. The dissociation constant K_D of [125 I]-ICYP was not affected after treatment with CINC-1 (10 ng/ml, 6 h). However, we found that B_{max} was significantly decreased in CINC-1-treated cells (Fig. 8A), indicating that prolonged exposure to CINC-1 causes a decrease of β_2 AR expression at the cell membrane of ATII cells. Further, this down-regulation of the β_2 AR expression at the cell membrane was associated with an inhibition of the EPI-stimulated PKA activity (Fig. 8B) that was restored by treating these cell monolayers with CPT-cAMP. However, there was no effect of CINC-1 on the baseline β_2 AR RNA expression in rat ATII cells (data not shown). In addition, the prolonged exposure to CINC-1 inhibited the EPI-stimulated Cl^- transport across the apical membrane of ATII cell monolayers, but had no effect on CPT-cAMP stimulated Cl^- transport across the apical membrane of ATII cell monolayers, indicating that the CINC-1 prolonged inhibitory effect on Cl^- transport is not downstream of cAMP generation (Fig. 8C). cAMP plays a critical role in activating CFTR promoter and mRNA expression in lung epithelial cells (41). Thus, we hypothesized that prolonged exposure to CINC-1 would result in a reduction in the PKA-dependent CFTR promoter activity and mRNA expression. Exposure to CINC-1 (6 h) significantly inhibited CFTR promoter activity and mRNA expression, which were prevented by pretreatment with CPT-cAMP (Fig. 8D, E). CFTR protein at the plasma membrane was also decreased after exposure to CINC-1, (Fig. 8F).

Pulmonary edema fluid levels of IL-8 are inversely correlated with the rate of AFC in patients with ALI

To confirm that the inhibitory effect of IL-8 on AFC may be important in patients with ALI, the last series of experiments was designed to determine the relationship between the rate of AFC and pulmonary fluid levels of IL-8 in 18 patients with ALI. From this cohort of 18 patients, 9 patients developed ALI from sepsis and 9 patients had ALI due to other causes. We found that the rate of AFC was inversely related to the levels of IL-8 in the undiluted pulmonary edema fluid obtained at the time of endotracheal intubation (Fig. 9A). Furthermore, the rate of AFC was also significantly lower in patients who had a pulmonary edema fluid concentration of IL-8 above the median level of IL-8 measured in this cohort of patients (4,000 pg/ml; Fig. 9B). Finally, the median edema fluid IL-8 level was in the same range as the concentrations used in the experimental models described above. Taken together, these clinical results suggest that IL-8 may play an important role in reducing alveolar epithelial fluid transport in patients with ALI.

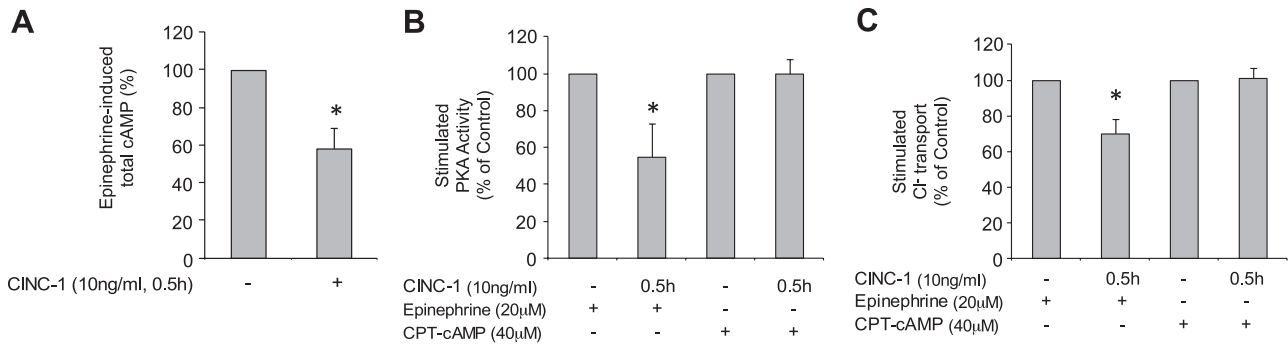


Figure 5. Short exposure (30 min) to CINC-1 inhibits the β_2 AR-dependent activation of the cAMP/PKA in rat ATII cells. *A, B*) Short exposure to CINC-1 significantly decreases EPI-dependent cAMP (*A*) and PKA (*B*) activation in rat ATII cells, but does not affect the CPT-cAMP-dependent PKA activation. cAMP content: control 4.7 ± 1.5 pmol/ml, EPI-treated 13.3 ± 1.9 pmol/ml; PKA activity: control 1.7 ± 0.4 ng active PKA/ μ g protein, EPI-treated 4.2 ± 0.7 ng active PKA/ μ g protein. *C*) Pretreatment with CPT-cAMP prevents CINC-1-dependent decrease in β_2 AR agonist-stimulated alveolar Cl^- transport across rat ATII cells. Mean basal I_{sc} was -10 ± 2.4 μ A, and mean EPI-treated I_{sc} was -28 ± 2.5 μ A. For all experiments, results are means \pm SE ($n=12$). * $P < 0.05$ vs. controls.

DISCUSSION

ALI is characterized by an increase in lung vascular permeability that is associated with a leak of fluid into the lung interstitium and the alveolar space. Recovery from ALI requires the removal of fluid from the alveolar space. One of the major mechanisms that drives the removal of edema fluid from the airspace is the active transport of sodium and chloride ions across alveolar type I and type II cells, producing an osmotic gradient for the reabsorption of water (42). However, there is an impairment of the AFC in more than 80% of patients with ALI. Notably, the impairment of the AFC is associated with increased morbidity and mortality in these patients (3). The mechanisms that are responsible for the impairment of AFC in patients with ALI are not fully understood. Previously published studies have reported that some inflammatory mediators, such as IL-1 β , TGF- β , and reactive oxygen species, inhibit basal vectorial alveolar epithelial fluid transport (35,

38, 43, 44). In addition, ALI is also associated with apoptosis and necrosis of the alveolar epithelium that may prevent the removal of edema fluid from the airspace (45, 46). Thus, there is a strong rationale for the identification of mechanisms that stimulate vectorial fluid transport across the alveolar epithelium in patients with ALI.

There is a large body of experimental and some clinical evidence that the cAMP-mediated stimulation of AFC by β_2 AR agonists is a major mechanism in the resolution of pulmonary edema (6, 8, 12, 13, 42, 47). The U.S. National Institutes of Health NHLBI ARDS Network recently performed a placebo-controlled phase III clinical trial with the aerosolized β_2 -adrenergic agonist albuterol in patients with ALI. Unfortunately, albuterol did not reduce ventilator-free days or mortality in patients with ALI (14). Another recent phase III multicenter trial conducted by the UK Medical Research Council (BALTI-2 study; ref. 15) that examined the effect of a β_2 AR agonist given

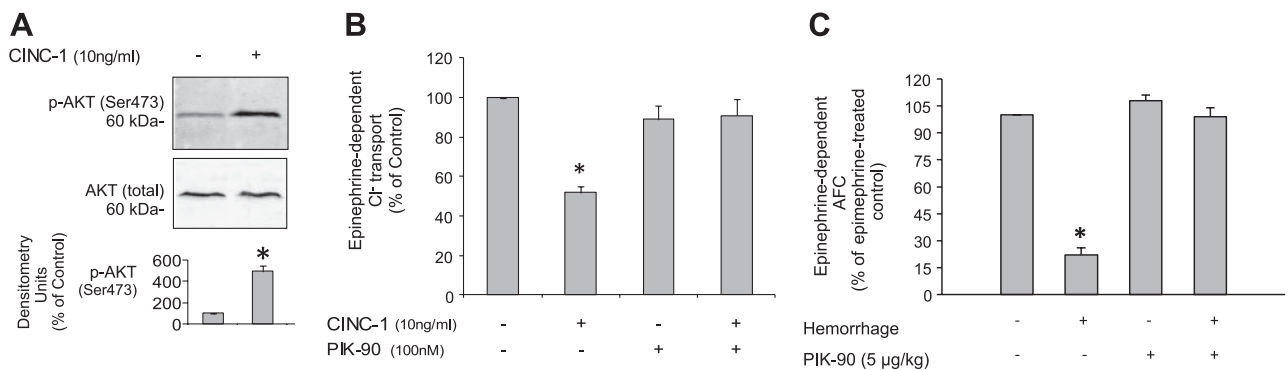


Figure 6. Short exposure (30 min) to CINC-1 decreases β_2 AR agonist-stimulated Cl^- transport across primary rat ATII cell monolayers *via* a PI3K-dependent mechanism. *A*) Short exposure to CINC-1 induces phosphorylation of Akt in rat ATII cells ($n=4$). *B*) PI3K inhibition prevents CINC-1-dependent decrease in β_2 AR agonist-stimulated alveolar Cl^- transport across rat ATII cells. Mean basal I_{sc} was -9 ± 2.1 μ A, and mean EPI-treated I_{sc} was -29 ± 2.3 μ A ($n=12$). *C*) PI3K inhibition restores the EPI-stimulated AFC in rats after hemorrhagic shock. Rats were pretreated intraperitoneally with PIK-90 (5 mg/kg) or with its vehicle 30 min prior to hemorrhage or sham surgery and fluid resuscitation (AFC: control $7.1 \pm 1.4\%$ /30 min, EPI-treated $14.9 \pm 1.5\%$ /30 min; $n=6$). Results are expressed as means \pm SE. * $P < 0.05$ vs. controls.

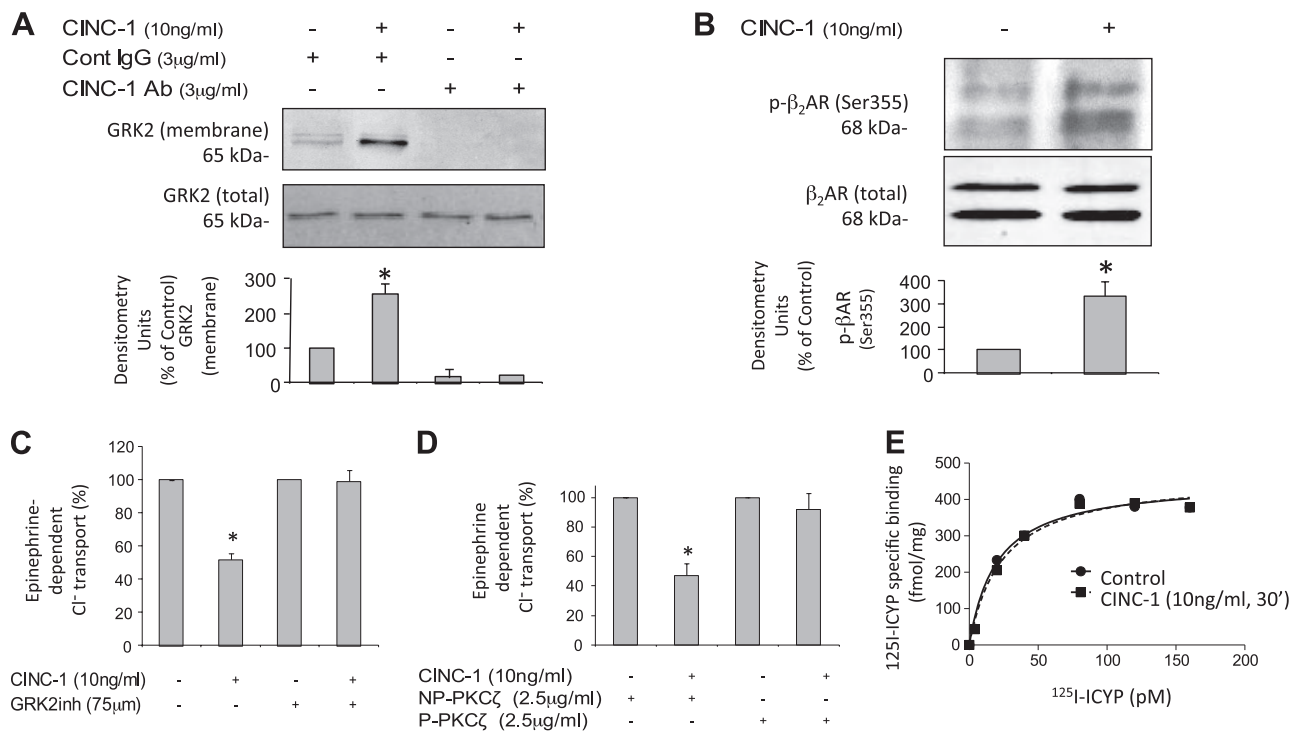


Figure 7. Short exposure to CINC-1 decreases β_2 AR agonist-stimulated Cl^- transport across rat ATII cells *via* a GRK2-dependent mechanism. *A*) Short exposure (5 min) to CINC-1 induces translocation of GRK2 protein to the plasma membrane in polarized rat primary ATII cells without exposure to a β_2 AR agonist. *B*) Short exposure (5 min) to CINC-1 induces phosphorylation of the β_2 AR in rat ATII cells. *C*) Pretreatment with a GRK2 inhibitor (75 μM) prevents the CINC-1-mediated inhibition of the EPI-stimulated Cl^- transport across rat ATII cells. *D*) Pretreatment with a membrane-permeable PKC ζ inhibitor (P-PKC ζ 2.5 $\mu\text{g}/\text{ml}$), but not with a nonmembrane-permeable PKC ζ inhibitor (NP-PKC- ζ 2.5 $\mu\text{g}/\text{ml}$), prevents the CINC-1-induced decrease of the EPI-stimulated Cl^- transport across the apical membrane of rat ATII cells. *E*) Short exposure (30 min) to CINC-1 does not affect β_2 AR density at the cell membrane measured by saturation binding experiments ($[^{125}\text{I}]\text{-ICYP}$; control $B_{\text{max}} = 533.5 \pm 19.08$ fmol/mg; $n = 6$). ATII cell membranes were incubated with increasing concentrations of ICYP for 90 min at 37°C. For the experiments reported in *C* and *D*, mean basal I_{sc} was -10 ± 2.1 μA , and mean EPI-treated I_{sc} was -28 ± 2.6 μA . For all experiments, results are means \pm SE ($n = 12$). For Western blot experiments, densitometry analysis results are means \pm SE ($n = 4$). * $P < 0.05$ vs. controls.

intravenously (salbutamol 15 $\mu\text{g}/\text{kg}$ ideal body weight/h) on lung function and outcome in patients with ALI also reported negative results. Inadequate aerosol delivery of albuterol in the ALTA study, alveolar epithelial damage, and agonist-induced down-regulation of the β_2 AR, may in part explain these negative results (48, 49).

Another mechanism could involve the inhibition of β_2 AR-mediated AFC stimulation by inflammatory mediators released during the acute-phase of ALI. Among these mediators, IL-8 has been shown to be a critical mediator of ALI in humans. Indeed, BAL fluid and pulmonary edema fluid levels of IL-8 are predictors of mortality in patients with ALI (17–21). Furthermore, blocking IL-8 significantly attenuates lung injury caused by smoke inhalation, acid aspiration or ischemia-reperfusion injury (50–52). In a recent study, the impairment of AFC observed with RSV lung infection in mice was caused by a lack of response to β_2 AR agonists that was mediated in a paracrine fashion by KC, the murine homologue of IL-8 and reversed by inhibition of either KC or its receptor, C-X-C chemokine receptor type 2 (CXCR2) (16). The results of the present study demonstrate a new role for IL-8/CINC-1 for inhibiting both

in vitro and *in vivo* β_2 AR signaling and its stimulation of vectorial fluid transport across the alveolar epithelium. Our results provide a potential explanation for the inhibitory effect of RSV on the cAMP-stimulated AFC. Our clinical data also provide one explanation for the negative results of the phase III clinical trials of β_2 AR agonist therapy in patients with ALI, because it demonstrates an inverse correlation between the pulmonary edema fluid level of IL-8 and the rate of AFC in patients with ALI.

Our *in vitro* experiments indicate that IL-8/CINC-1 inhibits β_2 AR signaling in the alveolar epithelium *via* heterologous desensitization followed by an internalization of the β_2 AR from the plasma membrane without affecting its mRNA expression level (data not shown). Previous studies have shown that the PI3K pathway is implicated in the homologous desensitization of the β_2 AR in cardiomyocytes. In particular, PI3K interacts with GRK2 *via* its phosphoinositide-kinase homology (PIK) domain and enhances the translocation of GRK2 at the cell membrane (27), an effect that we observed in the present study. Furthermore, lipid and protein kinase activities of PI3K are involved in the G α_i - and β -arrestin-mediated endocytic process of the β_2 AR that

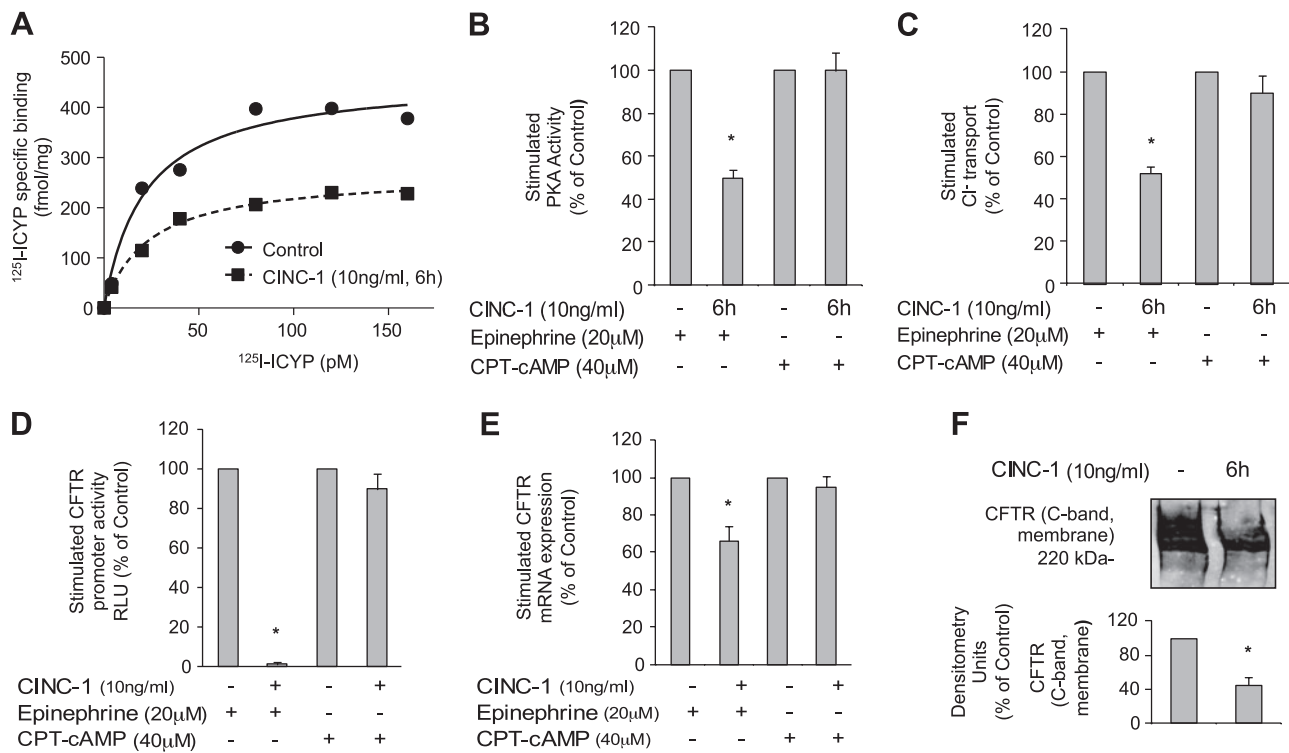


Figure 8. Prolonged exposure (6 h) to CINC-1 inhibits the β_2 AR-dependent activation of the PKA and CFTR promoter activity, gene expression, and function, *via* a down-regulation of the β_2 AR at the cell membrane. *A*) Prolonged exposure to CINC-1 decreases β_2 AR cell membrane density, measured by saturation binding experiments ($[^{125}\text{I}]\text{-ICYP}$; control $B_{\text{max}}=511.2\pm 15.26$ fmol/mg, $K_D=24.32\pm 3.09$ pM; CINC-1-treated $B_{\text{max}}=297.7\pm 13.5$ fmol/mg, $K_D=19.15\pm 4.13$ pM; $n=6$). *B*) Prolonged exposure to CINC-1 inhibits the EPI-dependent PKA activation in rat ATII cells. CINC-1 does not affect the CPT-cAMP-dependent PKA activation in polarized rat primary ATII cells (control 2.1 ± 0.6 ng active PKA/ μg protein, EPI-treated 4.5 ± 1.1). *C*) Prolonged exposure to CINC-1 decrease the EPI-stimulated Cl^- transport across of rat ATII cells; Mean basal I_{sc} was -9 ± 2.3 μA , and mean EPI-treated I_{sc} was -28 ± 2.7 μA . *D*) Prolonged exposure to CINC-1 inhibits the EPI- but not the CPT-cAMP-stimulated CFTR promoter activity in rat ATII cells. Control luciferase activity = 85.2 ± 3.5 (RLU/ μg protein). *E*) Prolonged exposure to CINC-1 decreases the EPI- but not the CPT-cAMP-stimulated CFTR mRNA expression in rat ATII cells. *F*) Prolonged exposure to CINC-1 decreases the EPI-dependent CFTR protein expression at the plasma membrane of polarized rat primary ATII cells. Results are means \pm SE ($n=12$). For Western blot experiments, one representative experiment is shown, 3 additional experiments gave comparable results; densitometry analysis results are means \pm SE ($n=4$). * $P < 0.05$ vs. monolayers exposed to CINC-1 vehicle (*B-E*) or controls (*F*).

is associated with a homologous down-regulation of this receptor from the cell membrane (53, 54). In the current studies, IL-8/CINC-1 activated both PI3K and GRK2 signaling and the inhibitory effect of IL-8/CINC-1 on β_2 AR signaling in alveolar epithelial cells was reversed by pretreatment with a PI3K inhibitor or an inhibitor of GRK2 or of its upstream kinase, PKC ζ . PI3K may also regulate the c-AMP-dependent activation of PKA *via* its activation of the catalytic activity of phosphodiesterase (PDE) type 4, a critical enzyme that together with the adenylyl cyclase, controls the c-AMP activity at the cell membrane. A recent study reported that β_2 AR-coupled PI3K decreases c-AMP activity in myocytes by a mechanism that involves activation of PDE4, as PI3K inhibition decreases PDE4 activity and increases c-AMP levels (54). Further studies will be required to determine the importance of these mechanisms in modulating β_2 AR signaling in alveolar epithelial cells.

Finally, our results show that IL-8/CINC-1 causes a rapid inhibition of the β_2 AR agonist-stimulated CFTR-dependent Cl^- and fluid transport in rat and human

primary cultures of ATII cells. These results are important because CFTR plays an important role in the β_2 AR agonist-dependent stimulation of alveolar epithelial fluid transport (22–25). Interestingly, results from our *in vivo* experimental model of hemorrhagic shock in rats (6 h) demonstrated that IL-8/CINC-1 inhibited >80–90% of the β_2 AR agonist-dependent stimulation of transepithelial fluid transport, a value much greater than the IL-8/CINC-1-mediated inhibition of chloride transport in ATII cell monolayers (40–50%). Previous studies have shown that both the chloride channel CFTR and the sodium channel ENaC present in the alveolar epithelium are under β -adrenergic control (41). Thus, our results suggest that exposure to IL-8/CINC-1 inhibits both the chloride channel CFTR and the sodium channel ENaC, explaining why we observed a near-complete inhibition of β_2 AR agonist-dependent stimulation of alveolar epithelial fluid transport in these experiments.

In summary, we found that IL-8, a critical mediator of ALI, inhibits β_2 AR agonist-stimulated fluid trans-

port across rat and human alveolar epithelia *via* a reduction of CFTR activity and biosynthesis. This reduction is mediated by a PI3K-dependent desensitization and down-regulation of the β_2 AR from the cell membrane that is associated with an inhibition of cAMP generation normally observed in response to β_2 AR agonist stimulation (Fig. 10). Consistent with our *in vitro* results, we found that the PI3K pathway blockade by inhibitors that have already entered clinical trials for other applications (55), restored physiological rates of β_2 AR agonist-stimulated AFC, overcoming inhibition by IL-8/CINC-1 in an experimental model of ALI induced by hemorrhagic shock in rats. This therapeutic approach that could be directed at the alveolar epithelial PI3K pathway may be preferable for patients with ALI rather than a direct inhibition of IL-8 that could adversely affect neutrophil-mediated bacterial clearance in acute lung injury due to infections (56, 57). Finally, consistent with the experimental results, high pulmonary edema fluid levels of IL-8 were associated with impaired AFC in patients with ALI. [FJ]

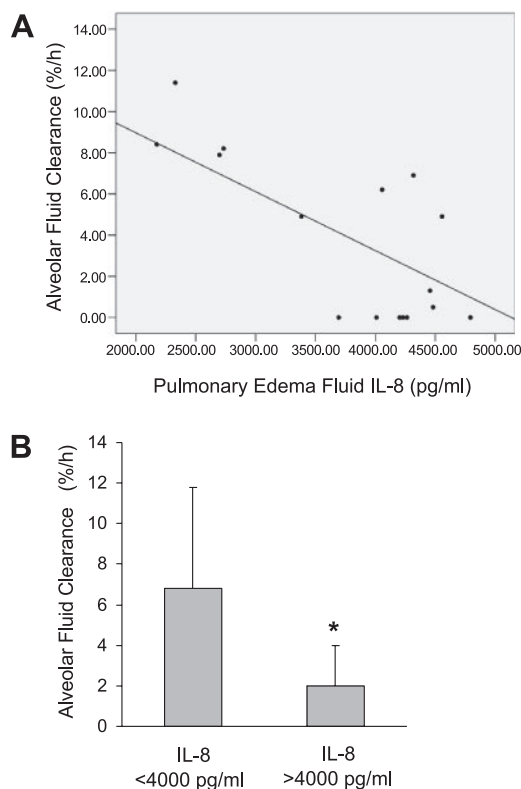


Figure 9. Pulmonary edema fluid levels of IL-8 are inversely associated with the rate of AFC in patients with ALI. *A*) There is an inverse correlation between rates of AFC and pulmonary edema fluid levels of IL-8 in patients with ALI ($n=18$; $P<0.05$). *B*) AFC is significantly lower in patients with pulmonary edema fluid levels of IL-8 above the median IL-8 level measured in the 18 patients with ALI included in the study (>4000 pg/ml). Results are expressed as means \pm SE. * $P<0.05$.

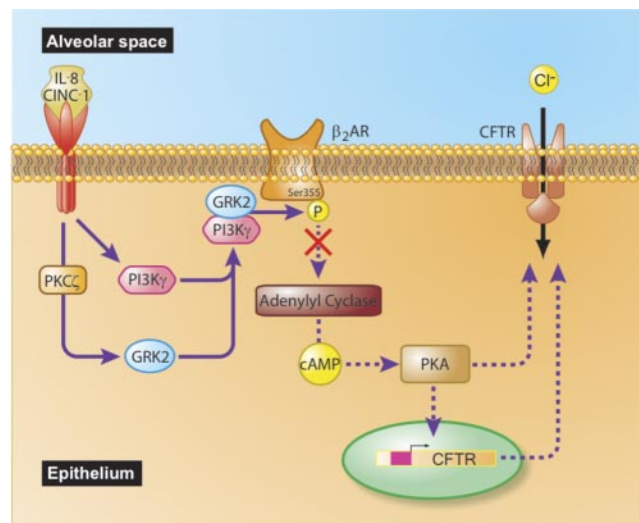


Figure 10. Schematic of the mechanisms by which IL-8 inhibits the signaling pathway of the β_2 AR in ATII cells. IL-8/CINC-1 causes the translocation of GRK2 and PI3K to the cell membrane. This protein complex causes phosphorylation at the Ser355 heterologous desensitization and down-regulation of the β_2 AR in ATII cells. IL-8/CINC then prevents the activation of cAMP/PKA pathway that up-regulates the vectorial fluid transport across the alveolar epithelium *via* phosphorylation and increased expression of CFTR at the plasma membrane of ATII cells. Solid lines indicate the pathways stimulated by IL-8/CINC-1; dashed lines indicate the pathways inhibited by these mediators.

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REFERENCES

- Rubinfeld, G. D., Caldwell, E., Peabody, E., Weaver, J., Martin, D. P., Neff, M., Stern, E. J., and Hudson, L. D. (2005) Incidence and outcomes of acute lung injury. *N. Engl. J. Med.* **353**, 1685–1693
- Ware, L. B., and Matthay, M. A. (2000) The acute respiratory distress syndrome. *N. Engl. J. Med.* **342**, 1334–1349
- Ware, L. B., and Matthay, M. A. (2001) Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* **163**, 1376–1383
- Craig, T. R., Duffy, M. J., Shyamsundar, M., McDowell, C., McLaughlin, B., Elborn, J. S., and McAuley, D. F. (2010) Extravascular lung water indexed to predicted body weight is a novel predictor of intensive care unit mortality in patients with acute lung injury. *Crit. Care Med.* **38**, 114–120
- Calfee, C. S., and Matthay, M. A. (2007) Nonventilatory treatments for acute lung injury and ARDS. *Chest* **131**, 913–920
- Berthiaume, Y., Staub, N. C., and Matthay, M. A. (1987) β -adrenergic agonists increase lung liquid clearance in anesthetized sheep. *J. Clin. Invest.* **79**, 335–343
- Berthiaume, Y. (1991) Effect of exogenous cAMP and aminophylline on alveolar and lung liquid clearance in anesthetized sheep. *J. Appl. Physiol.* **70**, 2490–2497

8. Sakuma, T., Folkesson, H. G., Suzuki, S., Okaniwa, G., Fujimura, S., and Matthay, M. A. (1997) Beta-adrenergic agonist stimulated alveolar fluid clearance in ex vivo human and rat lungs. *Am. J. Respir. Crit. Care Med.* **155**, 506–512
9. Sakuma, T., Tuchihiro, C., Ishigaki, M., Osanai, K., Nambu, Y., Toga, H., Takahashi, K., Ohya, N., Kurihara, T., and Matthay, M. A. (2001) Denopamine, a β_1 -adrenergic agonist, increases alveolar fluid clearance in ex vivo rat and guinea pig lungs. *J. Appl. Physiol.* **90**, 10–16
10. Su, X., Robriquet, L., Folkesson, H. G., and Matthay, M. A. (2006) Protective effect of endogenous β -adrenergic tone on lung fluid balance in acute bacterial pneumonia in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L769–L776
11. McAuley, D. F., Frank, J. A., Fang, X., and Matthay, M. A. (2004) Clinically relevant concentrations of β_2 -adrenergic agonists stimulate maximal cyclic adenosine monophosphate-dependent airspace fluid clearance and decrease pulmonary edema in experimental acid-induced lung injury. *Crit. Care Med.* **32**, 1470–1476
12. Pittet, J. F., Wiener-Kronish, J. P., McElroy, M. C., Folkesson, H. G., and Matthay, M. A. (1994) Stimulation of lung epithelial liquid clearance by endogenous release of catecholamines in septic shock in anesthetized rats. *J. Clin. Invest.* **94**, 663–671
13. Perkins, G. D., McAuley, D. F., Thickett, D. R., and Gao, F. (2006) The β -agonist lung injury trial (BALTI): a randomized placebo-controlled clinical trial. *Am. J. Respir. Crit. Care Med.* **173**, 281–287
14. National Heart Lung and Blood Institute Acute Respiratory Distress Syndrome (ARDS) Clinical Trials Network, Matthay, M. A., Brower, R. G., Carson, S., Douglas, I. S., Eisner, M., Hite, D., Holets, S., Kallet, R. H., Liu, K. D., MacIntyre, N., Moss, M., Schoenfeld, D., Steingrub, J., and Thompson, B. T. (2011) Randomized, placebo-controlled clinical trial of an aerosolized β_2 -agonist for treatment of acute lung injury. *Am. J. Respir. Crit. Care Med.* **184**, 561–568
15. Gao Smith, F., Perkins, G. D., Gates, S., Young, D., McAuley, D. F., Tunnicliffe, W., Khan, Z., and Lamb, S. E. (2012) Effect of intravenous β_2 agonist treatment on clinical outcomes in acute respiratory distress syndrome (BALTI-2): a multicentre, randomised controlled trial. *Lancet* **379**, 229–235
16. Davis, I. C., Xu, A., Gao, Z., Hickman-Davis, J. M., Factor, P., Sullender, W. M., and Matalon, S. (2007) Respiratory syncytial virus induces insensitivity to beta-adrenergic agonists in mouse lung epithelium in vivo. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **293**, L281–L289
17. Kunkel, S. L., Standiford, T., Kasahara, K., and Strieter, R. M. (1991) Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp. Lung Res.* **17**, 17–23
18. Donnelly, S. C., Strieter, R. M., Kunkel, S. L., Walz, A., Robertson, C. R., Carter, D. C., Grant, I. S., Pollok, A. J., and Haslett, C. (1993) Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* **341**, 643–647
19. Kurdowska, A., Miller, E. J., Noble, J. M., Baughman, R. P., Matthay, M. A., Brelsford, W. G., and Cohen, A. B. (1996) Anti-IL-8 autoantibodies in alveolar fluid from patients with the adult respiratory distress syndrome. *J. Immunol.* **157**, 2699–2706
20. Miller, E. J., Cohen, A. B., Nagao, S., Griffith, D., Maunder, R. J., Martin, T. R., Weiner-Kronish, J. P., Sticherling, M., Christophers, E., and Matthay, M. A. (1992) Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am. Rev. Respir. Dis.* **146**, 427–432
21. Amat, M., Barcons, M., Mancebo, J., Mateo, J., Oliver, A., Mayoral, J. F., Fontcuberta, J., and Vila, L. (2000) Evolution of leukotriene B₄, peptide leukotrienes, and interleukin-8 plasma concentrations in patients at risk of acute respiratory distress syndrome and with acute respiratory distress syndrome: mortality prognostic study. *Crit. Care Med.* **28**, 57–62
22. Nielsen, V. G., Duvall, M. D., Baird, M. S., and Matalon, S. (1998) cAMP activation of chloride and fluid secretion across the rabbit alveolar epithelium. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **275**, L1127–L1133
23. Jiang, X., Ingbar, D. H., and O'Grady, S. M. (2001) Adrenergic regulation of ion transport across adult alveolar epithelial cells: effects on Cl⁻ channel activation and transport function in cultures with an apical air interface. *J. Membr. Biol.* **181**, 195–204
24. Fang, X., Fukuda, N., Barbry, P., Sartori, C., Verkman, A. S., and Matthay, M. A. (2002) Novel role for CFTR in fluid absorption from the distal airspaces of the lung. *J. Gen. Physiol.* **119**, 199–208
25. Fang, X., Song, Y., Hirsch, J., Galiotta, L. J., Pedemonte, N., Zemans, R. L., Dolganov, G., Verkman, A. S., and Matthay, M. A. (2006) Contribution of CFTR to apical-basolateral fluid transport in cultured human alveolar epithelial type II cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **290**, L242–L249
26. Naga Prasad, S. V., Barak, L. S., Rapacciuolo, A., Caron, M. G., and Rockman, H. A. (2001) Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase I. A role in receptor sequestration. *J. Biol. Chem.* **276**, 18953–18959
27. Naga Prasad, S. V., Laporte, S. A., Chamberlain, D., Caron, M. G., Barak, L., and Rockman, H. A. (2002) Phosphoinositide 3-kinase regulates β_2 -adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. *J. Cell Biol.* **158**, 563–575
28. Van Keymeulen, A., Wong, K., Knight, Z. A., Govaerts, C., Hahn, K. M., Shokat, K. M., and Bourne, H. R. (2006) To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front. *J. Cell Biol.* **174**, 437–445
29. Dobbs, L. G., Gonzalez, R., and Williams, M. C. (1986) An improved method for isolating type II cells in high yield and purity. *Am. Rev. Respir. Dis.* **134**, 141–145
30. Dobbs, L. G. (1990) Isolation and culture of alveolar type II cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **258**, L134–L147
31. Fang, X., Song, Y., Zemans, R., Hirsch, J., and Matthay, M. A. (2004) Fluid transport across cultured rat alveolar epithelial cells: a novel in vitro system. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L104–L110
32. Ware, L. B., Wang, Y., Fang, X., Warnock, M., Sakuma, T., Hall, T. S., and Matthay, M. (2002) Assessment of lungs rejected for transplantation and implications for donor selection. *Lancet* **360**, 619–620
33. Tiballi, R., He, X., Zarins, L., Revankar, S., and Kauffman, C. (1995) Use of a colorimetric system for yeast susceptibility testing. *J. Clin. Microbiol.* **33**, 915–917
34. Roux, J., Carles, M., Koh, H., Goolaerts, A., Ganter, M. T., Chesebro, B. B., Howard, M., Houseman, B. T., Finkbeiner, W., Shokat, K. M., Paquet, A. C., Matthay, M. A., and Pittet, J. F. (2010) Transforming growth factor β_1 inhibits cystic fibrosis transmembrane conductance regulator-dependent cAMP-stimulated alveolar epithelial fluid transport via a phosphatidylinositol 3-kinase-dependent mechanism. *J. Biol. Chem.* **285**, 4278–4290
35. Frank, J., Roux, J., Kawakatsu, H., Su, G., Dagenais, A., Berthiaume, Y., Howard, M., Canessa, C. M., Fang, X., Sheppard, D., Matthay, M. A., and Pittet, J. F. (2003) Transforming growth factor-beta1 decreases expression of the epithelial sodium channel α ENaC and alveolar epithelial vectorial sodium and fluid transport via an ERK1/2-dependent mechanism. *J. Biol. Chem.* **278**, 43939–43950
36. Pittet, J. F., Lu, L. N., Morris, D. G., Modelska, K., Welch, W. J., Carey, H. V., Roux, J., and Matthay, M. A. (2001) Reactive nitrogen species inhibit alveolar epithelial fluid transport after hemorrhagic shock in rats. *J. Immunol.* **166**, 6301–6310
37. McDonald, R. A., Matthews, R. P., Idzerda, R. L., and McKnight, G. S. (1995) Basal expression of the cystic fibrosis transmembrane conductance regulator gene is dependent on protein kinase A activity. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7560–7564
38. Roux, J., Kawakatsu, H., Gartland, B., Pespeni, M., Sheppard, D., Matthay, M. A., Canessa, C. M., and Pittet, J. F. (2005) Interleukin-1 β decreases expression of the epithelial sodium channel alpha-subunit in alveolar epithelial cells via a p38 MAPK-dependent signaling pathway. *J. Biol. Chem.* **280**, 18579–18589
39. Bandy, A. A., Fazili, F. R., and Lokhandwala, M. F. (2007) Insulin causes renal dopamine D1 receptor desensitization via GRK2-mediated receptor phosphorylation involving phosphatidylinositol 3-kinase and protein kinase C. *Am. J. Physiol. Renal Physiol.* **293**, F877–F884
40. Winstel, R., Freund, S., Krasel, C., Hoppe, E., and Lohse, M. J. (1996) Protein kinase cross-talk: membrane targeting of the β -adrenergic receptor kinase by protein kinase C. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2105–2109

41. Mutlu, G. M., and Factor, P. (2008) Alveolar epithelial β 2-adrenergic receptors. *Am. J. Respir. Cell. Mol. Biol.* **38**, 127–134
42. Matthay, M. A., Folkesson, H. G., and Clerici, C. (2002) Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol. Rev.* **82**, 569–600
43. Song, W., and Matalon, S. (2007) Modulation of alveolar fluid clearance by reactive oxygen-nitrogen intermediates. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **293**, L855–L858
44. Lee, J. W., Fang, X., Dolganov, G., Fremont, R. D., Bastarache, J. A., Ware, L. B., and Matthay, M. A. (2007) Acute lung injury edema fluid decreases net fluid transport across human alveolar epithelial type II cells. *J. Biol. Chem.* **282**, 24109–24119
45. Albertine, K. H., Soulier, M. F., Wang, Z., Ishizaka, A., Hashimoto, S., Zimmerman, G. A., Matthay, M. A., and Ware, L. B. (2002) Fas and fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. *Am. J. Pathol.* **161**, 1783–1796
46. Bachofen, M., and Weibel, E. R. (1977) Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. *Am. Rev. Respir. Dis.* **116**, 589–615
47. Sakuma, T., Gu, X., Wang, Z., Maeda, S., Sugita, M., Sagawa, M., Osanai, K., Toga, H., Ware, L. B., Folkesson, G., and Matthay, M. A. (2006) Stimulation of alveolar epithelial fluid clearance in human lungs by exogenous epinephrine. *Crit. Care Med.* **34**, 676–681
48. Maron, M. B., Folkesson, H. G., Stader, S. M., and Hodnichak, C. M. (2006) Impaired alveolar liquid clearance after 48-h isoproterenol infusion spontaneously recovers by 96 h of continuous infusion. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **291**, L252–L256
49. Sartori, C., Fang, X., McGraw, D. W., Koch, P., Snider, M. E., Folkesson, H. G., and Matthay, M. A. (2002) Selected contribution: long-term effects of beta(2)-adrenergic receptor stimulation on alveolar fluid clearance in mice. *J. Appl. Physiol.* **93**, 1875–1880
50. Laffon, M., Pittet, J. F., Modelska, K., Matthay, M. A., and Young, D. M. (1999) Interleukin-8 mediates injury from smoke inhalation to both the lung endothelial and the alveolar epithelial barriers in rabbits. *Am. J. Respir. Crit. Care Med.* **160**, 1443–1449
51. Modelska, K., Pittet, J. F., Folkesson, H. G., Courtney Broaddus, V., and Matthay, M. A. (1999) Acid-induced lung injury. Protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. *Am. J. Respir. Crit. Care Med.* **160**, 1450–1456
52. De Perrot, M., Sekine, Y., Fischer, S., Waddell, T. K., McRae, K., Liu, M., Wigle, D. A., and Keshavjee, S. (2002) Interleukin-8 release during early reperfusion predicts graft function in human lung transplantation. *Am. J. Respir. Crit. Care Med.* **165**, 211–215
53. Perrino, C., Naga Prasad, S. V., Schroder, J. N., Hata, J. A., Milano, C., and Rockman, H. A. (2005) Restoration of beta-adrenergic receptor signaling and contractile function in heart failure by disruption of the β ARK1/phosphoinositide 3-kinase complex. *Circulation* **111**, 2579–2587
54. Gregg, C. J., Steppan, J., Gonzalez, D. R., Champion, H. C., Phan, A. C., Nyhan, D., Shoukas, A. A., Hare, J. M., Barouch, L. A., and Berkowitz, D. E. (2010) β 2-adrenergic receptor-coupled phosphoinositide 3-kinase constrains cAMP-dependent increases in cardiac inotropy through phosphodiesterase 4 activation. *Anesth. Analg.* **111**, 870–877
55. Marone, R., Cmiljanovic, V., Giese, B., and Wymann, M. P. (2008) Targeting phosphoinositide 3-kinase-Moving towards therapy. *Biochim. Biophys. Acta* **1784**, 159–185
56. Tsai, W. C., Strieter, R. M., Wilkowski, J. M., Bucknell, K. A., Burdick, M. D., Lira, S. A., and Standiford, T. J. (1998) Lung-specific transgenic expression of KC enhances resistance to *Klebsiella pneumoniae* in mice. *J. Immunol.* **161**, 2435–2440
57. Batra, S., Cai, S., Balamayooran, G., and Jeyaseelan, S. (2012) Intrapulmonary administration of leukotriene B(4) augments neutrophil accumulation and responses in the lung to *Klebsiella* infection in CXCL1 knockout mice. *J. Immunol.* **188**, 3458–3468

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