MATERIAL AND METHODS (SUPPLEMENTAL DIGITAL CONTENT 1)

***Cell culture*.** Primary cultures of rat and human alveolar epithelial cells were used for the *in vitro* studies following approval of the University of California San Francisco and the University of Alabama at Birmingham Committees on Animal Research, rat alveolar epithelial type II (ATII) cells were isolated as previously described[1,2] with slight modifications. Briefly, the cells were isolated by elastase digestion followed by negative selection using four monoclonal antibodies against cell surface molecules expressed on rat macrophages (CD4/CD32/CD45/RMA) purchased from BD Biosciences-Pharmingen (San Diego, CA). These monoclonal antibodies were preincubated with Dynabeads M-450 (magnetic beads with sheep anti-mouse IgG; Dynal ASA, Oslo, Norway) in 0.1% bovine serum albumin/phosphate-buffered saline (PBS). After removing unbound monoclonal antibodies, rat ATII cells were mixed with the bead suspension and rocked gently for 30 min at 4°C. Unbound cells were isolated and plated on polycarbonate Snapwell membranes (Corning Costar Co., Cambridge, MA) with a 0.4-μm pore size. The cells were seeded at a concentration of 1.5×10^6 cells/cm^2 in Dulbecco's modified Eagle's medium/H21 medium containing 10% low endotoxin fetal bovine serum, 1% penicillin and streptomycin and kept at 37°C in a humidified 95% air, 5% CO_2 environment. Twenty-four hours later, nonadherent epithelial cells were removed by washing with PBS and fresh medium added to the lower compartments of the Transwells, thus maintaining the ATII cell monolayers with an air-liquid interface on their apical side. After 72–96 h, the cells that formed confluent monolayers reaching a transepithelial

electrical resistance greater than 1500 ohms·cm^2 were used for experimentation.

Following approval of the University of California, San Francisco Committee on Human Research, human alveolar epithelial type II cells were isolated using a modification of methods previously described[3] from human lungs that were not used by the California Transplant Donor Network.[4] Our studies indicate that these lungs were in good condition, both physiologically and pathologically. Cells were isolated after the lungs have been preserved for 4–8 h at 4°C. A lobe of the human lung was selected that had no evidence of injury on the preharvest chest radiograph, could be normally inflated, and had no area of consolidation or hemorrhage. The pulmonary artery for this segment was perfused with 37 °C PBS solution, and the distal airspaces of a segmental bronchus was lavaged 10 times with 37 °C Ca^{2+}- and Mg^{2+}-free PBS solution containing 0.5 mM EGTA and EDTA. 60–90 ml of pancreatic porcine elastase (8 units/ml) diluted in a Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution was instilled into the airspaces of 50 g of the chosen segment of lung tissue. The lung was incubated in a water bath for 30 min at 37°C and minced finely in the presence of fetal bovine serum and DNase I (500 μg/ml). The cell-rich fraction was filtered sequentially through one-layer gauze, two-layer gauze, 150- and 30-μm nylon meshes. The cell suspension was then layered onto a discontinuous Percoll density gradient of 1.04–1.09 g/ml solution and centrifuged at 400 × g for 20 min to remove red blood cells. The cells that accumulated at the interface of the solution and the Percoll were a mixture of type II pneumocytes and alveolar macrophages. These cells were recovered by centrifugation at 200 × g for 10 min at 4 °C. The pellet

was resuspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were incubated in Dulbecco's modified Eagle's medium containing magnetic beads coated with an anti-CD-14 antibody (Dynabeads M/450 CD14; Dynal) at 4 °C for 40 min under constant mixing to eliminate macrophages. The cell viability was assessed by trypan blue exclusion. The purity of isolated human alveolar type II cells was checked by Papanicolaou staining or by staining with anti-human type II cell antibody (obtained from Leland Dobbs, MD, PhD, University of California, San Francisco), and the purity has consistently been more than 90%. Human alveolar type II cells were seeded on collagen I-coated Transwells at a density of 1×10^6 cells/cm^2. The cells were grown in an air-liquid interface 72 h after seeding. Five days after the cells are seeded, the monolayer developed a transepithelial electrical resistance greater than 1500 ohms·cm^2, as reported for rat ATII cell monolayers.

Short-circuit current studies of rat and human ATII cell monolayers. Short-circuit current studies were performed as described previously.[5] Freshly isolated rat or human ATII cells (0.75×10^6) were seeded on polycarbonate Snapwell membranes (pore size, 0.4 μm; surface area, 1.13 cm^2). The culture medium was changed daily. Hydrocortisone (10^{-7} M) and insulin-transferrin-Sel-G were added to the culture medium for human cells. The cells were grown in an air-liquid interface 48–72 h after seeding. At 120–144 h, the Snapwell inserts were mounted in an Ussing chamber system (Physiologic Instruments Inc., San Diego, CA). The bathing solution in the apical chamber contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-HEPES,

and 10 mM glucose, pH 7.3. The bathing solution in the basolateral chamber was modified by substituting all of the NaCl with sodium gluconate and increasing the $CaCl_2$ concentration to 2 mM to compensate for calcium buffering caused by gluconate. The basolateral membrane of the cells was permeabilized with 40 μM nystatin. Complete permeabilization was verified by adding ouabain (1 mM), a specific Na^+/K^+ ATPase blocker, which did not reduce the I_{sc} level in the permeabilized monolayer compared with intact monolayers. The measurements were performed at 37 °C. The hemichambers were connected to a VCC MC6 voltage clamp (Physiologic Instruments Inc.) via Ag/AgCl electrodes and 1 M KCl agar bridges. Short circuit current (I_{sc}) data were acquired by PowerLab (ADInstruments Inc., Colorado Springs, CO) and recorded in Chart software (ADInstruments Inc.).

Isolation of plasma membrane-enriched fraction. Isolation of plasma membrane-enriched fraction was performed as described previously.[5] The cells were scraped into with a hypotonic buffer (10 mM HEPES, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, protease inhibitors) and allowed to swell for 30 min at 4°C with rotation. The cells were broken open with a Dounce apparatus using a loose fitting pestle and 20 strokes. The nuclei were pelleted by centrifugation at 1000 × g for 10 min at 4 °C. The supernatant was centrifuge at 15,000 × g for 15 min at 4°C to pellet the enriched membrane fraction. This fraction was resuspended in Laemmeli sample buffer and further analyzed by SDS-PAGE and Western blot.

Saturation binding experiments. Saturation binding experiments were

performed as described previously.[5] The plasma membrane-enriched fractions (1 µg of membrane protein) from primary rat alveolar type II cells were incubated with different concentrations (0–160 pM) of the β-AR antagonist [^{125}I]ICYP for 90 min at 37 °C in the following binding buffer: 50 mM Tris·HCl, 100 mM KCl, and 5 mM $MgCl_2$, pH 7.4. Nonspecific binding was determined by displacement of ICYP binding with the specific β_2-AR antagonist ICI-118,551 (100 µM). The reactions were stopped by dilution with 4 ml of ice-cold washing buffer (50 mM Tris·HCl, 154 mM NaCl, pH 7.4), followed by rapid filtration through presoaked Whatman GF/C glass fiber filters in a Millipore cell harvester (Millipore, Billerica, MA). The filters were washed three times with 4 ml of washing buffer. The radioactivity on the filters was counted with a γ-counter (PerkinElmer Life Sciences, Santa Clara, CA). We found that specific [^{125}I] ICYP binding was saturable and of high affinity.

Western blot analyses. Western blot analyses from cells homogenates were performed as described previously.[5] After equal amounts of protein were loaded in each lane and separated by 10% SDS-PAGE, the proteins were transferred to Invitrogen iBlot™ polyvinylidene difluoride membranes (Invitrogen, Grand Island, NY). The membranes were blocked for 1 h with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), which was also used as primary and secondary antibodies incubation buffer. The primary antibodies were used at dilutions of 1:500 and 1:1000, incubated overnight at 4°C. Near-infrared detection was used with the IRDye®-conjugated secondary antibodies (LI-COR Biosciences), which were either goat anti-mouse IRDye® 800CW or goat anti-rabbit IRDye® 680, used at 1:10,000 dilution and imaged at 84-µm resolution with the Odyssey

infrared imaging system (LI-COR Biosciences). Quantification was performed with the LI-COR Biosciences analysis software.

CINC-1 ELISA. CNC-1 levels in cell culture supernatant from ATII cell monolayers were measured by an ELISA purchased from R&D Systems (Minneapolis, MN) following the manufacturer's instructions.

Cell viability. The cell viability after exposure to different experimental conditions was measured by the Alamar Blue assay.[6] The cell media were replaced by medium containing 10% Alamar Blue and placed at 37 °C in the cell incubator for 2–3 h. The medium was then collected and read on a plate reader at 570 nm.

Rat studies. The protocol for measurement of AFC in rats was approved by the University of California, San Francisco, Committee on Animal Research, and was performed as previously described.[5,7] Briefly, male Sprague Dawley rats weighing 300–350 g were anesthetized with pentobarbital (60 mg/kg intraperitoneally), and anesthesia was maintained with 30 mg/kg of sodium pentobarbital every hour. An endotracheal tube (14G) was inserted through a tracheotomy. Catheters (PE50) 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) with an inspired oxygen fraction of 1.0, peak airway pressures of 8–12 cm H_2O, supplemented with positive end-expiratory pressure of 3 cm H_2O. The respiratory rate was adjusted to maintain the $PaCO_2$ between 35 and 40 mm Hg during the baseline

period. After the surgery, heart rate and systemic blood pressure were allowed to stabilize for 60 min. TGF-β1 was administered intraperitoneally (1 mg/kg). Six hours after the administration of TGF-β1, rats were exsanguinated and AFC was determined in the absence of ventilation or blood flow by measuring the increase in protein tracer concentration (^{125}I-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 ^{125}I-albumin, aspirated and reinstilled the solution three times and applied continuous positive airway pressure (continuous positive airway pressure: 8 cm H_2O, 100% F_IO_2) to prevent alveolar collapse and kept the animals at 37°C body core temperature. The instillate, an initial sample (after aspiration and reinstillation), and a sample after 30 min were obtained and analyzed. In some experiments, rats were pretreated with a CINC-1 blocking antibody or its isotype control antibody that were administered intraperitoneally 30 min before administration of TGF-β1. Sample sizes were chosen based upon previous experience and randomization and blinding methods were not feasible for these experiments.

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