Interleukin-1β Decreases Expression of the Epithelial Sodium Channel α-Subunit in Alveolar Epithelial Cells via a p38 MAPK-dependent Signaling Pathway*§

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Acute lung injury (ALI) is a devastating syndrome characterized by diffuse alveolar damage, elevated airspace levels of pro-inflammatory cytokines, and flooding of the alveolar spaces with protein-rich edema fluid. Interleukin-1β (IL-1β) is one of the most biologically active cytokines in the distal airspaces of patients with ALI. IL-1β has been shown to increase lung epithelial and endothelial permeability. In this study, we hypothesized that IL-1β would decrease vectorial ion and water transport across the alveolar epithelium. Therefore, we measured the effects of IL-1β on transepithelial current, resistance, and sodium transport in primary cultures of alveolar epithelial type II (ATII) cells. IL-1β significantly reduced the amiloride-sensitive fraction of transport across the alveolar epithelium. Moreover, IL-1β decreased basal and dexamethasone-induced epithelial sodium channel (ENaC) mRNA and protein expression in alveolar epithelial type II (ATII) cells and was independent of the activation of αβ-integrin and transforming growth factor-β. These results indicate that IL-1β may contribute to alveolar edema in ALI by reducing distal lung epithelial sodium absorption. This reduction in ion and water transport across the lung epithelium is in large part due to a decrease in αENaC expression through p38 MAPK-dependent inhibition of αENaC promoter activity and to an alteration in ENaC trafficking to the apical membrane of ATII cells.

Acute lung injury (ALI) is a devastating clinical syndrome manifested by diffuse alveolar damage, capillary injury, and disruption of the alveolar epithelium. The acute phase of ALI is characterized by the influx of protein-rich edema fluid that impairs gas exchange, causing arterial hypoxemia and respiratory failure with an overall mortality rate of 30–40% (1). Along with an increase in lung endothelial and epithelial permeability to protein, this syndrome is associated with abnormal surfactant production and decreased vectorial fluid transport across the lung epithelial barrier (2, 3). A number of inflammatory mediators have been found to be elevated in the alveolar space during the early phase of ALI, including interleukin (IL)-1β, tumor necrosis factor-α, IL-6, and IL-8 (4). IL-1β is one of the most biologically active cytokines in pulmonary edema and bronchoalveolar lavage fluids of patients with ALI (4–6). Indeed, IL-1β increases microvascular lung epithelial permeability in vitro and in vivo models of ALI (7). IL-1β also enhances alveolar epithelial repair by increasing cell spreading (8) and fibroblast activation and proliferation (5). In contrast, the role of IL-1β in distal lung epithelial ion transport remains unclear. In other epithelia such as the colon, epithelium, IL-1β inhibits aldosterone-induced electrogenic sodium absorption and attenuates aldosterone-induced up-regulation of β- and γ-subunit mRNA expression (9). In the renal collecting duct, IL-1β inhibits sodium absorption and stimulates anion secretion (10). Recently, IL-1β has been shown to reduce amiloride-sensitive short circuit current (Isc) in normal human bronchial epithelial cells (11). However, the effect of IL-1β on vectorial sodium transport across the alveolar epithelium is unknown.

We report here that IL-1β inhibits alveolar epithelial sodium transport across the distal lung epithelium secondary to a reduction in epithelial sodium channel (ENaC) mRNA and protein expression in alveolar epithelial type II (ATII) cells. IL-1β affects αENaC transcription by inhibiting its promoter activity via a p38 MAPK-dependent mechanism, resulting in a decrease in the steady-state levels of both total and apical membrane αENaC proteins.

EXPERIMENTAL PROCEDURES

Reagents—All cell culture media were prepared by the University of California—San Francisco Cell Culture Facility using deionized water and analytical grade reagents. Amiloride, ouabain, decorin, and protease inhibitors were obtained from Sigma. Human recombinant IL-1β and IL-1β receptor antagonist (IL-1RA) were obtained from R&D Systems (Minneapolis, MN). NaCl was obtained from PerkinElmer Life Sciences.

The abbreviations used are: ALI, acute lung injury; IL, interleukin; ENaC, epithelial sodium channel; ATII, alveolar epithelial type II; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; GR, glucocorticoid receptor; IL-1RA, IL-1β receptor antagonist; TGF-β, transforming growth factor-β; MAPKAPK, MAPK-activated protein kinase; GRE, glucocorticoid response element; PBS, phosphate-buffered saline; TER, transepithelial electrical resistance; PD, potential difference; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TEC, transepithelial current.
Sciences. 35S and the chemiluminescence ECL Plus kit were obtained from Amersham Biosciences. Sulfuscuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate and streptavidin-garose beads were obtained from Pierce, MAPK inhibitors PD98059 (an inhibitor of the kinase upstream of ERK1/2), SB202190 (an inhibitor of p38 MAPK), and SP600125 (a reversible inhibitor of JNK) were obtained from Calbiochem. These MAPK inhibitors have been shown to block their respective MAPKs in lung epithelial cells (12–15). Mifepristone (RU486), a known glucocorticoid receptor (GR) antagonist, was purchased from BIOSEL Research Labs Inc. (Plymouth Meeting, PA). Soluble chimeric transforming growth factor-β (TGF-β) type II receptor was purchased from Biogen Idec (Cambridge, MA). αvβ6 integrin-blocking antibody 1D5 originated in the laboratory of D. Sheppard (16). Polyclonal and affinity-purified αENaC antibodies originated in the laboratory of B. C. Rossier (17). Antibodies and phospho-specific antibodies for p38 MAPK and MAPKAPK-2 were purchased from Cell Signalling Technology (Beverly, MA). Antibodies for Na−K+−ATPase α-, and β1-subunits were purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were purchased from ICN (Costa Mesa, CA). Catonic liposomes (FuGene 6) were obtained from Roche Applied Science. The following two plasmids were a kind gift from André Dagenais (Université de Montréal, Montreal, Canada): plasmid pGL-3 containing the luciferase gene subcloned downstream of the full-length murine mENaC promoter with a deletion in the glucocorticoid response element (GRE) (pG3L-basic/amENaC) and plasmid pGL-3 containing the luciferase gene subcloned downstream of the murine mENaC promoter with a deletion in the glucocorticoid response element (GRE) (pG3L-basic/amENaCAVXR-YbOl). The protein concentration of cell lysates was determined using a Bio-Rad protein assay kit or a BCA kit (Pierce).

Cell Culture—Primary cultures of rat and human alveolar epithelial cells were used for the in vitro studies. Rat ATII cells were isolated as described previously (18, 19) with slight modifications. Briefly, 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, and rat macrophage activator) purchased from Pharmingen. These monoclonal antibodies were prein- cubated with Dynabeads M-450 (magnetic beads with sheep anti-mouse IgG; Dynal Biotech ASA, Oslo, Norway) in 0.1% bovine serum albumin in 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 Transwell dishes (0.4-μm pore size; Corning). Cells were seeded at a density of 1.5 × 104 cells/cm2 in Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum and 1% penicillin/streptomycin and kept at 37 °C in a humidified 95% air and 5% CO2 environment. Twenty-four hours later, the cells were incubated with Dynabeads M-450 (magnetic beads with sheep anti-mouse IgG; Dynal Biotech ASA, Oslo, Norway) in 0.1% bovine serum albumin in 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 Transwell dishes (0.4-μm pore size; Corning). Cells were seeded at a density of 1.5 × 104 cells/cm2 in Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum and 1% penicillin/streptomycin and kept at 37 °C in a humidified 95% air and 5% CO2 environment. Twenty-four hours later, non-adherent epithelial cells were removed by washing with PBS, and fresh medium was added to the lower compartments of the Transwell dishes, thus maintaining the ATII cell monolayers with an airliquid interface on their apical side. After 72–96 h, cells that formed confluent monolayers reaching a transepithelial electrical resistance (TER) > 1500 ohms/cm2 were used for experimentation.

Human ATII cells were isolated using a modification of the method described previously (20). Briefly, alveolar type II cells were isolated from human lungs that were not used by the Northern California Transplant Donor Network. Our studies indicated that these lungs were in good condition, both physiologically and pathologically (21). Cells were isolated after the lungs had been preserved for 4–8 h at 4 °C. A lobe of human lung that had no evidence of injury on the pre-harvest chest radiograph, that could be normally inflated, and that had no area of consolidation or hemorrhage was selected. The pulmonary artery for this segment was perfused with 37 °C PBS solution, and the distal airspaces of a segmental bronchus were lavaged 10 times with 50 C Ca2+−Mg2+−free PBS solution containing 0.5 mM each EDTA and EGTA. Sixty to ninety milliliters of pancreatic porcine elastase (8 units/ml) diluted in Ca2+−Mg2+−free Hanks’ balanced saline solution were 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 minced lung was filtered sequentially through one layer of 150- and 30-μm nylon mesh. The cell suspension was then layered onto a discontinuous Percoll density gradient (1.04–1.09 g/ml) 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 anti-CD41 antibody (Dynabeads M-450 CD14, Dynal Biotech ASA) at 4 °C for 40 min under constant mixing to eliminate macrophages. Cell viability was assayed 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, University of California, San Francisco), and the purity was consistently >90% (data not shown). Human alveolar type II cells were seeded on collagen I-coated Transwell dishes at a density of 1 × 105 cells/cm2. Five days after the cells were seeded, the monolayer developed a TER >1500 ohms/cm2, as reported for rat ATII cell monolayers.

Cell Viability—Cell viability after exposure to different experimental conditions was measured by the Alamar Blue assay (22). Cell media were replaced with medium containing 10% Alamar Blue and placed at 37 °C by MI incubator for 2–3 h. The media were then collected and read on a plate reader at 530 nm.

Measurement of Monolayer Bioelectric Properties—The TER (kilo-ohms/cm2) and potential difference (PD; millivolts, apical side as reference) were measured using the Millicell-ERS Voltmohmeter (Millipore Corp., Bedford, MA). Transepithelial current (I[I])− micromamps/cm2) was calculated from the relationship I[I] = PD/R, (Ohm’s law). The effect of PD98059 (10−4 M) on both sides of the Transwell, the cells were washed twice with wash medium (150 mM NaCl and 2 mM HEPES, pH 7.4) at 37 °C and equilibrated with flux medium (140 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM MgCl2, 0.2 mM CaCl2, 10 mM glucose, and 20 mM HEPES, pH 7.4) for 10 min at 37 °C. After equilibration, only the apical medium was replaced with fresh flux medium containing “Na” at a concentration of 5 μM (123 h), and the monolayers were washed three times by overflow with cold wash medium to eliminate “Na” not taken up by the cells and to stop further uptake. The last wash solution was measured for “Na” and verified not to contain any radioactivity. The cells were then lysed with 0.1% NaOH, and the radioactivity in the lystate was measured in a β-counter. Protein determination was used for normalization of the results. Control experiment was done with 10% ethanol in 1% ethanol for 10 min. Thus, only one 6-min time point was taken. All fluxes were measured in triplicate.

Transepithelial Sodium Transport Measurements—The activity of the amiloride-sensitive sodium transport pathway across rat ATII cell monolayers was determined by unidirectional tracer uptake measurements using the technique described by Mairbaurl et al. (23). Briefly, after exposure to IL-1β or its vehicle on both sides of the Transwell, the cells were washed twice with wash medium (150 mM NaCl and 2 mM HEPES, pH 7.4) at 37 °C and equilibrated with flux medium (140 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM MgCl2, 0.2 mM CaCl2, 10 mM glucose, and 20 mM HEPES, pH 7.4) for 10 min at 37 °C. After equilibration, only the apical medium was replaced with fresh flux medium containing “Na” at a concentration of 5 μM (123 h), and the monolayers were washed three times by overflow with cold wash medium to eliminate “Na” not taken up by the cells and to stop further uptake. The last wash solution was measured for “Na” and verified not to contain any radioactivity. The cells were then lysed with 0.1% NaOH, and the radioactivity in the lystate was measured in a β-counter. Protein determination was used for normalization of the results. Control experiment was done with 10% ethanol in 1% ethanol for 10 min. Thus, only one 6-min time point was taken. All fluxes were measured in triplicate.

Western Blot Analysis—Western blot analysis was performed as described previously (24). After equal amounts of protein were loaded on each lane and separated by 10% SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore Corp.), and αENaC was detected using an affinity-purified antibody at 1:1000 dilution and horseradish peroxidase-conjugated goat anti-rabbit antibody at 1:10,000 dilution. Anti-p38 MAPK and anti-phospho-p38 MAPK antibodies were used at 1:500 dilution, and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were used at 1:5000 dilution. The protein bands were visualized by chemiluminescence. Quantification was done using ImageMaster digital image analysis system (Alpha Innotech, San Leandro, CA).

Membrane Protein Biotinylation—Biotinylation and recovery of apical membrane proteins were performed as described previously (25) using sulfuscuccinimidyl 2-biotinamido-ethyl-1,3-dithiopropionate. The protein concentration of the lysesates was determined using the BCA kit. Streptavidin-garose beads were added to equal amounts of total protein to recover the biotinylated proteins. The amount of added beads
was adjusted to ensure complete recovery of biotinylated proteins from lysates. Biotinylated proteins were eluted from the beads by heating to 90 ºC in SDS-PAGE sample buffer and analyzed by Western blotting. Actin, a cytoskeletal protein, was used as a control for the specificity of surface biotinylation. This protein was detected in cell lysates, but not in the pool of protein recovered after apical surface biotinylation. 

**Human GAPDH**

Forward
5'-'TGAAGATTTGAGCTGAGTCCAGC-3'
Reverse
5'-'AGGTTAATGACGACGGTGTGGA-3'
Probe
5'-'TTGGTTCTATTTGCAGTTGCC-3'

**Human αENaC**

Forward
5'-'CTGGCATGCTGTGCTGCC-3'
Reverse
5'-'CCGGAAGGAGCGAGGATTC-3'
Probe
5'-'TGCAAGACACGGAGACGGG-3'

**Rat GAPDH**

Forward
5'-'CTGCAACGTGCTGCTGGTCA-3'
Reverse
5'-'AGGCCCAGGTCCTCTTATG-3'
Probe
5'-'TCGGCCCGCTTCCTTCCACA-3'

**Rat αENaC**

Forward
5'-'TGAATTTCACCACCCCTCC-3'
Reverse
5'-'CCCCGAGGTGATTGAGTCC-3'
Probe
5'-'CCTCCTCACTGCTCCTCGCA-3'

**Rat βENaC**

Forward
5'-'CTGCGATGATCCGCTGCA-3'
Reverse
5'-'AACAGACGAGGCCACAAC-3'
Probe
5'-'CTGCCAAGTATGATGACATCAAGA-3'

**Rat αENaC GRE**

Forward
5'-'AAAAAGGACAGATGCTCTAGGAC-3'
Reverse
5'-'GTCCTAGGACCATGCTGCTTTT-3'

**Rat Na+K+-ATPase α1-subunit**

Forward
5'-'GGCTGTCATCTTCCTCATTGG-3'
Reverse
5'-'AAGACGAGGAGGGCGCA-3'
Probe
5'-'CTGCTAGGACATTCTGTTCTTTT-3'

**Rat Na+K+-ATPase β1-subunit**

Forward
5'-'GCCCAAGGACAGCATACTG-3'
Reverse
5'-'CCCGCTCTCTGCGTTCA-3'
Probe
5'-'TCCAGGATGTGGCAGATTGCCCA-3'

**Luciferase activity** was determined to verify that the transfection was comparable within and between experiments. 

**Electrophoretic Mobility Shift Assay**—Rat alveolar type II cells were plated in 24-mm filter insert culture dishes at a density of 5 × 10⁶ cells/well and cultured for 4 days in an air-liquid interface. Cells were subjected to dexamethasone with or without IL-1β and a p38 MAPK inhibitor for 30 min at 4 ºC. Nuclear protein isolation, cell pellets were resuspended in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 0.5 mM dithiothreitol, 0.4% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride). After incubation on ice for 10 min, crude nuclei were isolated by centrifugation at 10,000 × g for 10 min at 4 ºC. The supernatant was removed; 3 cell pellet volumes of high salt extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM dithiothreitol, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) were added; and the suspension was incubated on a rotary shaker for 30 min at 4 ºC. The sample was centrifuged at 16,000 × g for 30 min at 4 ºC; the supernatant (containing extracted nuclear proteins) was collected; the protein concentration was determined; and the lysate was stored at −70 ºC until further use. Electrophoretic mobility shift assay was performed using a GRE consensus oligonucleotide probe (designed based on GenBank™ accession number AF017853) that was end-labeled with 32P-ATP (see Table I). After labeling, forward and reverse oligonucleotides were annealed slowly from 95 ºC to room temperature. Nuclear protein (5 μg) was incubated with 100,000 cpm of 32P-labeled GRE consensus nucleotide for 20 min in binding buffer consisting of 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% glycerol, and 1 μg of poly(dI-dC). The specificity of the DNA/protein binding was determined by competition reactions in which a 100-fold molar excess of unlabeled GRE oligonucleotide was added. After incubation, the samples were analyzed by nondenaturing polyacrylamide gel electrophoresis, and the bands were visualized by autoradiography. 

**Primers and Probes**—Real-time reverse transcription (RT)-PCR primers and probes (Table I) were designed using Primer Express software (PE-Applied Biosystems, Warrington, United Kingdom). The TaqMan probes were labeled with a fluorophore reporter dye (6-carboxyfluorescein) at the 5’-end and a Black Hole Quencher dye (Biosearch Technologies, Inc.) at the 3’-end. 

**Quantitative Real-time RT-PCR**—Total RNA was extracted from rat or human ATII cells cultured for 4 days in an air-liquid interface using the RNAeasy mini kit (Qiagen Inc.). One microgram of total RNA was reverse-transcribed using the Superscript first-strand synthesis system (Invitrogen). RT-PCRs were performed, and the results were analyzed using the ABI PRISM 7700 sequence detection system (PE- Applied Biosystems). Briefly, RT-PCRs were carried out in a 25-μl reaction mixture containing 1 μM TaqMan Universal PCR Master Mix (PE-Bio systems), 10 pmol of primers, 5 pmol of TaqMan probe, and an equivalent of 100 ng of total RNA for 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. The number of cycles to threshold of fluorescence detection was normalized to the number of cycles to threshold of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample tested. Results are expressed as a percentage of cDNA abundance compared with the control. The percentages were used for all statistical comparisons.
**IL-1β** decreases the TEC across rat lung epithelial cell monolayers. A–C, IL-1β causes a dose-dependent decrease in the transepithelial PD and TEC across primary cultures of polarized rat ATII cell monolayers. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1β (10⁻³ to 10 ng/ml) or its vehicle for 24 h. The PD, TEC, and TER were measured with an epithelial ohmmveterimeter with Ag/AgCl electrodes. D–F, IL-1β induces a time-dependent decrease in the PD and current across the apical membrane of primary cultures of polarized rat ATII cell monolayers. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1β (10 ng/ml) or its vehicle for 1–24 h. The PD, TEC, and TER were measured as described above. For all experiments, control values for PD = 9.2 ± 1.3 mV, TEC = 1.63 ± 0.18 kohms/cm², and TEC = 5.8 ± 0.7 μA/cm². The results are the mean ± S.D. of at least four experiments done in triplicate.

### RESULTS

**IL-1β Decreases Sodium Transport across Rat Lung Epithelial Cell Monolayers**—We first determined whether IL-1β affects the PD, TER, or apical Na⁺ uptake across primary cultures of rat ATII cell monolayers. Exposure to IL-1β (0.01–10 ng/ml) significantly decreased the PD, an effect that was completely blocked by IL-1RA. IL-1β had no effect on the measured TER, resulting in a significant reduction in the calculated transepithelial current (TEC) (Fig. 1, A–C). The IL-1β-dependent reduction of the PD and TEC across ATII cell monolayers was observed as soon as 6 h after exposure to the cytokine (Fig. 1, D–F). IL-1β affected both the basal and dexamethasone-dependent TECs across ATII cell monolayers independently of whether IL-1β was tested on the apical or basal surface (Fig. 2, A and B). Furthermore, IL-1β affected mostly the amiloride-sensitive fraction of TEC (Fig. 2C). Measurements of the PD and TER in ATII monolayers before and after addition of amiloride (1.3 mM) in the PD with no change in the TER (data not shown), indicating that the resistance of ATII monolayers is determined mostly by the tight junctions. We then hypothesized that the IL-1β-mediated decrease in the calculated TEC is explained by a decrease in transepithelial ion transport. Therefore, we measured the effect of IL-1β on both ²²Na⁺ uptake across the apical membrane of rat ATII cells and ²²Na⁺ transepithelial transport across rat ATII monolayers. Exposure to IL-1β (10 ng/ml) for 6 h induced a significant decrease in the apical sodium uptake and the transepithelial ²²Na⁺ transport across ATII monolayers, which were not further affected by addition of amiloride at a saturating concentration (10⁻⁴ M) (Fig. 2, D and E). Taken together, the data indicate that IL-1β significantly decreases the amiloride-sensitive fraction of the transepithelial sodium transport across rat ATII monolayers.

**IL-1β Decreases αENaC Gene Expression in Rat and Human Lung Epithelial Cells**—The next series of experiments was designed to determine whether IL-1β alters αENaC gene expression. Exposure to IL-1β (0.1–10 ng/ml) for 6 h caused a dose-dependent decrease in basal αENaC mRNA expression that was blocked by pretreatment with IL-1RA (Fig. 3A). IL-1β also significantly decreased the dexamethasone-induced αENaC mRNA levels (Fig. 3B). In parallel, we examined the effect of IL-1β on the mRNA levels of the β- and γ-subunits of ENaC in both unstimulated and dexamethasone-stimulated cells. Quantitative real-time PCR analysis revealed that IL-1β also caused a decrease in both basal and dexamethasone-stimulated βENaC and γENaC mRNA levels (Fig. 3, C and D), with a greater effect on the α- and γ-subunits.

Because the MAPKs have been shown to be downstream mediators of IL-1β signaling (27, 28), ATII cells were preincubated with specific inhibitors of ERK1/2, p38, and JNK 1 h before addition of IL-1β. SB202190 (a p38 MAPK inhibitor), but not other MAPK inhibitors, blocked the IL-1β-dependent decrease in αENaC mRNA levels (Fig. 3E). To determine whether IL-1β directly affects mRNA transcription, we then examined the effect of actinomycin D and cycloheximide on the IL-1β-dependent decrease in αENaC mRNA abundance by quantitative real-time PCR. The results showed that IL-1β had a direct effect on αENaC gene expression because its effect was blocked by pretreatment with actinomycin D, but was unaffected by pretreatment with cycloheximide (Fig. 3F).

Finally, the studies on the effect of IL-1β on αENaC gene expression were extended to primary cultures of human ATII cells because of possible differences in αENaC gene regulation by IL-1β between rodents and humans. IL-1β caused a significant decrease in αENaC mRNA levels in human lung epithelial cells, which was blocked by preincubation with SB212090, a p38 MAPK inhibitor (Fig. 4). Taken together, these experiments indicate that IL-1β caused a decrease in basal and dexamethasone-dependent αENaC gene expression via a p38 MAPK-dependent mechanism in both rat and human ATII cells.
IL-1β decreases basal and dexamethasone-induced sodium transport across rat lung epithelial cell monolayers. A and B, IL-1β causes a non-polarized decrease in the basal and dexamethasone-dependent TECs across primary cultures of polarized rat ATII cell monolayers. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1β (10 ng/ml) for 6 h. Amiloride (10^{-4} M) was added for 15 min. The PD, TEC, and resistance were measured as described above. C and D, IL-1β decreases the amiloride-sensitive fraction of sodium transport across the apical membrane and sodium transport across monolayers of primary cultures of polarized rat ATII cells. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1β (10 ng/ml) or its vehicle for 6 h. Amiloride (10^{-4} M) was added to the flux medium. For all experiments, control values for PD = 8.6 ± 1.1 mV, TER = 1.6 ± 0.15 kilo-ohms/cm^{2}, TEC = 5.5 ± 0.6 μA/cm^{2}, and 22Na flux = 76 ± 8 nmol/mg of protein/6 min. The results are the mean ± S.D. of at least four experiments done in triplicate. *, p < 0.05 for monolayers exposed to IL-1β vehicle.

IL-1β Decreases Total and Membrane αENaC Protein Expression in Rat Lung Epithelial Cells—The next series of experiments were performed to determine the effect of IL-1β on αENaC protein levels in rat ATII cell monolayers. Exposure to IL-1β (10 ng/ml) caused a significant decrease in both basal and dexamethasone-induced αENaC protein expression (Fig. 5A). This effect was time-dependent and specific for IL-1β because it was reversed by IL-1RA (Fig. 5, A and B). Furthermore, IL-1β decreased αENaC protein expression via a p38 MAPK-dependent mechanism (Fig. 5C). IL-1β induced the phosphorylation of p38 MAPK and its downstream kinase MAPKAPK-2, an effect that was blocked by pretreatment with SB202190 in ATII cells (Fig. 5D).

The next series of experiments were designed to determine whether IL-1β would decrease the expression of αENaC protein on the apical membrane of rat ATII cell monolayers. IL-1β caused a time-dependent decrease in both basal and dexamethasone-induced αENaC expression on the apical membrane of rat ATII cells (Fig. 6, A and B). Taken together, these data demonstrate that IL-1β decreased total and apical membrane αENaC protein expression via a p38 MAPK-dependent mechanism.

IL-1β Inhibits αENaC Promoter Activity by Decreasing GR Nuclear Translocation via a p38 MAPK-dependent Mechanism—To further understand the mechanism of IL-1β-dependent down-regulation of αENaC gene expression in lung epithelial cells, we measured the activity of the αENaC promoter after exposure to IL-1β in rat ATII cells. Luciferase assays were performed by transiently transfecting rat ATII cells with plasmid pGL-3 containing the luciferase gene subcloned downstream of the murine αENaC promoter with or without a deletion in the GRE. IL-1β decreased the dexamethasone-dependent αENaC promoter activity by 90%. This effect was not observed in lung epithelial cells transiently transfected with a plasmid that did not respond to dexamethasone because of a deletion in the GRE (pGL3-basic/αENaCΔGRE-XhoI) (Fig. 7A). A comparable lack of response to dexamethasone was also observed upon pretreatment of the cell monolayers with mifepristone (RU486, a known GR antagonist, before exposing the cells to dexamethasone. Interestingly, IL-1β did not further inhibit the dexamethasone-dependent αENaC promoter activity in cells that had been pretreated with mifepristone (Fig. 7A). Pretreatment with the p38 MAPK inhibitor SB202190 (added 1 h before IL-1β) reversed the IL-1β-mediated inhibition of the dexamethasone-dependent αENaC promoter activity in lung epithelial cells (Fig. 7B). Furthermore, the results of electrophoretic mobility shift assays with GRE consensus oligonucleotides demonstrated that IL-1β decreased the nuclear translocation of the GR and its binding to the GRE. This effect was also blocked by SB202190 (Fig. 7, C and D).

Taken together, these experiments indicate that IL-1β decreased αENaC promoter activity via a p38 MAPK-dependent mechanism.

IL-1β Decreases αENaC Protein and Gene Expression Independently of αvβ6 Integrin Activation of TGF-β—We have previously reported that αvβ6 integrin activates TGF-β in
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FIG. 3. IL-1β decreases αENaC gene expression in rat lung epithelial cell monolayers via a p38 MAPK-dependent mechanism. A, IL-1β causes a dose-dependent decrease in αENaC mRNA levels in primary cultures of polarized rat ATII cell monolayers. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1β (10 ng/ml) or its vehicle for 6 h. αENaC mRNA levels were measured as described for A. In some experiments, dexamethasone (Dex; 100 nM) was added to the culture medium 1 h before the 6-h exposure to IL-1β (10 ng/ml) or its vehicle. In some experiments, PD98059 (an inhibitor of the kinase upstream of ERK1/2; 10 μM), SB202190 (an inhibitor of p38 MAPK; 10 μM), or SP600125 (an inhibitor of JNK; 10 μM) was added to the cell medium 30 min before the 6-h exposure to IL-1β (10 ng/ml) or its vehicle. For all experiments, the results are the mean ± S.D. of at least three experiments done in triplicate. *, p < 0.05 for monolayers exposed to IL-1β vehicle; **, p < 0.05 for monolayers exposed to dexamethasone and IL-1β vehicle.

FIG. 4. IL-1β decreases αENaC mRNA levels in primary cultures of polarized human ATII cell monolayers via a p38 MAPK-dependent mechanism. Human ATII cell monolayers cultured in an air-liquid interface for 5 days were exposed to IL-1β or its vehicle for 6 h. In some experiments, the p38 MAPK inhibitor SB202190 (10 μM) was added to the cell medium 1 h before the 6-h exposure to IL-1β (10 ng/ml) or its vehicle. The results are the mean ± S.D. of three experiments done in duplicate. *, p < 0.05 for monolayers exposed to IL-1β vehicle.

epithelial cells (29) and that active TGF-β1 decreases αENaC gene and protein expression in rat and human ATII cell monolayers (30). The next series of experiments were designed to elucidate the potential role of αvβ6 integrin or TGF-β in the IL-1β-dependent down-regulation of αENaC. Quantification of αENaC mRNA was performed in rat alveolar type II cells stimulated with IL-1β after preincubation with αvβ6 integrin-blocking antibody; a TGF-β type I, II, and III receptor-blocking agent (decorin); or a soluble chimeric TGF-β type II receptor. Quantitative real-time PCR revealed that αvβ6 integrin-blocking antibody, decorin, and the soluble chimeric TGF-β type II receptor did not prevent the decrease in αENaC mRNA abundance via a p38 MAPK-dependent mechanism. αENaC mRNA levels were measured as described for A. In some experiments, dexamethasone (Dex; 100 nM) was added to the culture environment at the same time as IL-1β or its vehicle. E, IL-1β decreases αENaC mRNA abundance via a p38 MAPK-dependent mechanism. αENaC mRNA levels were measured as described for A. In some experiments, PD98059 (an inhibitor of the kinase upstream of ERK1/2; 10 μM), SB202190 (an inhibitor of p38 MAPK; 10 μM), or SP600125 (an inhibitor of JNK; 10 μM) was added to the cell medium 1 h before the 6-h exposure to IL-1β (10 ng/ml) or its vehicle. F, reduction in αENaC mRNA expression by IL-1β occurs at the transcriptional level and does not depend on the de novo synthesis of proteins. αENaC mRNA levels were measured as described for A. In some experiments, either actinomycin D (Act D; 40 μM) or cycloheximide (CHX; 5 μM) was added to the cell medium 30 min before the 6-h exposure to IL-1β (10 ng/ml) or its vehicle. For all experiments, the results are the mean ± S.D. of at least three experiments done in triplicate. *, p < 0.05 for monolayers exposed to IL-1β vehicle; **, p < 0.05 for monolayers exposed to dexamethasone and IL-1β vehicle.

DISCUSSION

IL-1β has been shown to affect the function of the lung epithelial barrier by increasing protein paracellular permeability and causing surfactant abnormalities (33, 34), both charac-
teristic features of ALI. However, the effect of IL-1\(\beta\) on alveolar ion transport is still unknown. We therefore examined whether IL-1\(\beta\) would affect sodium transport across the distal lung epithelium, where most of the vectorial fluid clearance from the airspace occurs.

We found that IL-1\(\beta\) decreased the TEC across ATII cell

FIG. 5. **IL-1\(\beta\) decreases \(\alpha\)ENaC total protein expression in polarized rat ATII cell monolayers.** A, IL-1\(\beta\) decreases basal and dexamethasone-induced \(\alpha\)ENaC protein expression. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1\(\beta\) (10 ng/ml) or its vehicle for 6 h with or without dexamethasone (Dex; 100 nM) or IL-1RA (10 \(\mu\)g/ml). \(\alpha\)ENaC total protein expression was measured by Western blotting. One representative experiment is shown; three additional experiments gave comparable results. B, time-dependent effect of IL-1\(\beta\) on \(\alpha\)ENaC protein expression. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1\(\beta\) (10 ng/ml) or its vehicle for 2, 6, and 24 h. \(\alpha\)ENaC total protein expression was detected by Western blotting. One representative experiment is shown; three additional experiments gave comparable results. C, IL-1\(\beta\) decreases \(\alpha\)ENaC protein expression via a p38 MAPK-dependent mechanism. Rat ATII cell monolayers were cultured in an air-liquid interface for 4 days. In some experiments, the p38 MAPK inhibitor SB202190 (10 \(\mu\)M) was added to the cell medium 1 h before exposure to IL-1\(\beta\) (10 ng/ml) or its vehicle for 6 h. \(\alpha\)ENaC total protein expression was detected by Western blotting. One representative experiment is shown; three additional experiments gave comparable results. D, IL-1\(\beta\) induces p38 MAPK and MAPKAPK-2 phosphorylation in primary cultures of rat ATII cells. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1\(\beta\) (10 ng/ml) or its vehicle for 5, 10, 30, and 60 min. Total protein expression was determined by Western blotting. In some experiments, the p38 MAPK inhibitor SB202190 (10 \(\mu\)M) was added to the cell medium 1 h before exposure to IL-1\(\beta\) or its vehicle. One representative experiment is shown; three additional experiments gave comparable results. For all experiments, densitometry analysis results are the mean \(\pm\) S.D. of three experiments. *, \(p < 0.05\) for monolayers exposed to IL-1\(\beta\) vehicle.

FIG. 6. **IL-1\(\beta\) decreases apical membrane \(\alpha\)ENaC expression in polarized rat ATII cell monolayers.** A, IL-1\(\beta\) decreases basal \(\alpha\)ENaC expression on the apical cell membrane of polarized rat ATII cell monolayers. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to IL-1\(\beta\) (10 ng/ml) or its vehicle for 1, 4, and 6 h. \(\alpha\)ENaC protein expression on the apical cell membrane was measured by cell-surface biotinylation as described under "Experimental Procedures." One representative experiment is shown; three additional experiments gave comparable results. B, IL-1\(\beta\) decreases dexamethasone-induced \(\alpha\)ENaC expression on the apical cell membrane of polarized rat ATII cell monolayers. Rat ATII cell monolayers cultured in an air-liquid interface for 4 days were exposed to dexamethasone for 12 h (100 nM) and then to IL-1\(\beta\) (10 ng/ml) or its vehicle for 1, 4, and 6 h in the presence of dexamethasone (100 nM). \(\alpha\)ENaC protein expression on the apical cell membrane was measured by cell-surface biotinylation as described 'Experimental Procedures.' One representative experiment is shown; three additional experiments gave comparable results. For all experiments, densitometry analysis results are the mean \(\pm\) S.D. of three experiments. *, \(p < 0.05\) for monolayers exposed to IL-1\(\beta\) vehicle.
monolayers in a time- and dose-dependent manner, predominantly affecting the amiloride-sensitive alveolar transepithelial sodium transport. Our results are in accordance with those of previous studies showing that, in other epithelia such as the distal colon and the renal collecting duct, IL-1β inhibits apical sodium absorption (9, 10). In addition, IL-1β has also been shown to reduce amiloride-sensitive short circuit current in normal human bronchial epithelial cells (11). Interestingly, in contrast to the previously described effect of TGF-β on the TEC across alveolar type II cell monolayers, which occurs exclusively on the basolateral side (30), the effect of IL-1β occurred independently on the side of cytokine application. The lack of sidedness might be explained by a transient IL-1β-induced increase in paracellular permeability that could cause the cytokine to diffuse across the leaky cell monolayer.

The most common amiloride-sensitive sodium channel on the apical surface of lung epithelial cells is ENaC, formed by three homologous subunits (35). This ion channel has been shown to be critical in the absorption of salt and fluid by lung epithelial cells and has an important role in keeping the airspace dry to allow physiological gas exchange (36–38). We found that IL-1β caused a decrease in ENaC mRNA expression in both rat and human epithelial cells. Moreover, IL-1β caused a non-coordinated down-regulation of ENaC subunits, affecting the α- and γ-subunits more than the β-subunit. Other studies have also shown differential regulation of the three ENaC subunits. IL-4 has been shown to cause a greater decrease in γENaC mRNA levels in human bronchial epithelial cells (39), whereas tumor necrosis factor-α and hypoxia cause a greater decrease in the βENaC and γENaC mRNA levels in the distal colon and alveolar epithelium, respectively (9, 40).

Under physiological conditions, the αENaC gene is positively regulated by several factors, including, but not limited to, cortisol (41, 42), cAMP (43, 44), aldosterone (17, 45), and insulin.
IL-1β and Lung Epithelial Sodium Transport

A

IL-1β (10 ng/ml) αvβ6 antibody Decerin TGFβ-scRII

B

Dexamethasone-dependent decrease in αENaC mRNA and protein expression is independent of αvβ6 integrin and TGF-β activation. Primary cultures of polarized rat ATII cell monolayers maintained in an air-liquid interface for 4 days were exposed to IL-1β (10 ng/ml) or its vehicle for 6 h with or without αvβ6 integrin-blocking antibody (100 μg/ml), decorin (10 μg/ml), or the soluble chimeric TGF-β type II receptor (TGFβ-scRII; 20 μg/ml). αENaC mRNA levels were measured in ATII cells by real-time RT-PCR and normalized to GAPDH mRNA levels. The results are the mean ± S.D. of at least three experiments done in triplicate. *, p < 0.05 for monolayers exposed to IL-1β vehicle. C, IL-1β decreases αENaC protein levels independently of αvβ6 integrin or TGF-β activation. Primary cultures of polarized rat ATII cell monolayers maintained in an air-liquid interface for 4 days were exposed to IL-1β (10 ng/ml) or its vehicle for 6 h with or without αvβ6 integrin-blocking antibody (100 μg/ml) or an antibody against the three isoforms of TGF-β (10 μg/ml). αENaC total protein expression was detected by Western blotting. One representative experiment is shown; three additional experiments gave comparable results. Densitometry analysis results are the mean ± S.D. of three experiments. *, p < 0.05 for monolayers exposed to IL-1β vehicle.

(46, 47). Among them, cortisol plays a critical role in maintaining lung fluid balance at birth (48) and during adult life (41) via an increase in ENaC gene expression in lung epithelial cells. In particular, cortisol and other synthetic glucocorticoids such as dexamethasone increase mRNA expression of all three ENaC subunits in several epithelia, including the lung (49–51) and renal collecting duct (52). Furthermore, glucocorticoids increase αENaC mRNA expression in the lung (53). The effect of the glucocorticoids on αENaC gene expression is mediated by translocation of the glucocorticoid-GRE complex to the nucleus and its binding to the GRE on the αENaC promoter (51). We found that IL-1β decreased dexamethasone-dependent αENaC mRNA and total protein expression. Our results are consistent with previous in vitro studies showing that IL-1α and IL-1β inhibit GR nuclear translocation and dexamethasone-induced gene transcription (54, 55). Furthermore, after dexamethasone stimulation, IL-1β decreased αENaC total protein expression less than apical plasma membrane αENaC protein expression, suggesting that IL-1β might also modulate ENaC trafficking to the apical membrane of alveolar epithelial cells. In vivo, the effect of IL-1β on the vectorial transport of sodium across the distal lung epithelium is still unclear. We have previously reported that the release of IL-1β in the distal spaces of the lung after the onset of hemorrhage prevents the cAMP-mediated up-regulation of alveolar liquid clearance in rats (56). The effect of IL-1β is mediated by an NO-dependent mechanism (57). Taken together, these studies and our study show that IL-1β affects ion transport in the distal lung epithelium directly by inhibiting ENaC biosynthesis and indirectly by preventing the cAMP-mediated up-regulation of this transport.

By which mechanism does IL-1β affect sodium absorption in the distal lung epithelium? IL-1β binds to its membrane IL-1β type I receptor, triggering the activation of several signaling pathways such as the MAPKs. We found that inhibition of the p38 MAPK signaling pathway completely prevented the IL-1β-mediated reduction in both αENaC gene and protein expression in ATII cells. Indeed, IL-1β inhibited the dexamethasone-dependent αENaC promoter activity via a decrease in GR/GRE binding by a p38 MAPK-dependent mechanism. These results are in accordance with those of a previous study showing that IL-1α inhibits the nuclear translocation of the GR and its DNA binding in mouse fibroblast cells transfected with a GR-mediated reporter gene construct (54).

We have recently reported that active TGF-β1 is a critical mediator of ALI (24) and causes a decrease in αENaC protein and gene expression in rat and human ATII cell monolayers (30). Furthermore, we have previously found that αvβ6 integrin, expressed in conducting airways and alveoli (58–60), activates endogenous latent TGF-β1 in epithelial cells (29). In addition, several studies have shown that αvβ6 integrin expression is markedly up-regulated in epithelial cells of multiple epithelial organs, including the lung, in response to acute and chronic inflammation and in cancer (58, 60, 61). Therefore, we sought to determine whether the IL-1β-dependent down-regulation of αENaC would be mediated by αvβ6 integrin or TGF-β. In fact, the effect of IL-1β on αENaC protein and gene expression was αvβ6 integrin/TGF-β-independent. These results suggest that IL-1β and TGF-β may have independent roles in the
down-regulation of ENaC and the reduction of sodium transport in the distal lung epithelium via distinct molecular pathways that could be additive in inhibiting the removal of alveolar edema from airspaces in ALI patients. Furthermore, our results indicate that IL-1β does not affect Na⁺-K⁺-ATPase α₁- and β₁-subunit protein expression in ATII cells. However, these data do not exclude that IL-1β might modulate Na⁺-K⁺-ATPase pump activity, possibly by affecting the trafficking of the channel subunits to the basolateral membrane. A complete understanding of the possible effect of IL-1β on the expression and function of Na⁺-K⁺-ATPase will require further studies.

In summary, the results of this study indicate that IL-1β causes a reduction in sodium transport across ATII cells. This effect is attributable in large part to a decrease in apical membrane αENaC expression in lung epithelial cells resulting from p38 MAPK-mediated inhibition of the αENaC promoter activity and to an alteration in ENaC trafficking to the plasma membrane. Because a reduction in net alveolar fluid clearance has been associated with increased respiratory failure and higher mortality in patients with ALI (2, 3) and given that IL-1β is one of the most bioactive cytokines present in the distal airspaces of patients with ALI (4–6), IL-1β may be an important mediator of impaired fluid clearance in patients with ALI.

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