RNA interference for α-ENaC inhibits rat lung fluid absorption in vivo

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Lung fluid absorption (LFA) is secondary to vectorial Na transport across the alveolar epithelium, where Na enters alveolar epithelial cells through apically located Na channels and is subsequently extruded by basolaterally located Na-K-ATPases (for review see Refs. 27–29). β-Adrenoceptor agonists and other substances that increase intracellular cAMP levels stimulate LFA (2, 8, 33, 41). The mechanism by which intracellular cAMP stimulates LFA is not fully understood but is related to protein kinase A activation (32), recruitment of new Na-K-ATPases to basolateral cell membranes (41), recruitment of epithelial Na channels (ENaC) to apical cell membranes (45), and/or increased Na channel open probability (1, 27–29). Recent data suggest that chloride also may play a role in β-adrenoceptor agonist-mediated upregulation of ion and fluid transport across the alveolar epithelium (7, 16). In several animal species, the Na channel blocker amiloride inhibits a significant fraction of unstimulated and stimulated LFA (for review see Refs. 27–29). Amiloride is fairly nonspecific and inhibits a multitude of Na transporting proteins in the lung, among them ENaC (1, 8, 52) and possibly also nonspecific cation channels (24). Some recent studies demonstrated that the amiloride-insensitive fraction of 8-bromoguanosine 3',5'-cyclic monophosphate-stimulated short-circuit current and 22Na uptake in rat tracheal epithelia could be inhibited by dichlorobenzamil or L-cis-diltiazem, both inhibitors of cyclic nucleotide-gated (CNG) cation channels (44). In subsequent studies, dichlorobenzamil or L-cis-diltiazem inhibited a significant fraction of LFA in sheep (17) and rats (34), which suggested that CNG channels may play a role in LFA.

RNA interference (RNAi) is a posttranscriptional process triggered by the introduction of gene-specific double-stranded RNA (dsRNA). This then leads to specific gene silencing in a sequence-specific manner (30). This dsRNA-mediated inhibition of gene expression has rapidly become a major tool for in vitro analysis of gene function. Specific gene silencing has been achieved in a variety of cell systems using chemically synthesized or in vitro-transcribed small interfering RNA (siRNA) (6) as well as PCR or DNA vector-based short hairpin RNA (3, 12, 46, 50). However, in vivo gene silencing has not commonly been carried out but is expected to gain in importance for target validation. In a recent study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA was utilized as a proof of principle for noninvasive siRNA delivery to mice lungs (25). Also, siRNA administration via intranasal instillation has recently been tested for the capacity to inhibit respiratory syncytial virus infection (53). In mice, siRNA-induced knockdown of caveolin-1 was recently found to increase lung vascular permeability (31).

In the current study, we employed the RNAi technique to further the understanding of the importance of α-ENaC for LFA in the adult rat. Our specific aims for this study were 1) to generate a plasmid that could be used to transfect the rat lung in vivo, 2) to investigate the effect of this specific α-ENaC siRNA on baseline, unstimulated LFA, 3) to investigate the effect of this specific α-ENaC siRNA on terbutaline-stimulated LFA, 4) to investigate whether the amiloride sensitivity of the baseline and stimulated LFA correlated with the specific α-ENaC siRNA pretreatment, 5) to determine IC_{50} values for pSi-4 inhibition of α-ENaC mRNA and protein expression, and 6) to study the localization of the siRNA effect by isolating alveolar epithelial type II cells 24 h after the α-ENaC siRNA-generating plasmid DNA instillation. The gene and tissue specificity of α-ENaC siRNA silencing was also investigated.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (wt 300–350 g, n = 86; Harlan, Indianapolis, IN) were used in the study. The rats were kept

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at a 12:12-h day-night rhythm and had free access to standard rat chow (Purina; Copley Feed, Copley, OH) and tap water. All studies were reviewed and approved by the Institutional Animal Care and Use Committee at the Northeastern Ohio Universities College of Medicine.

**Plasmid construction.** The rat α-ENaC mRNA (GenBank accession no. NM_031548) secondary structure was predicted based on the principle of minimizing free energy, using the RNA structure version 3.71 software. Four 19-nucleotide double-stranded siRNAs were designed to target rat α-ENaC mRNA. These four sequences correspond to rat α-ENaC cDNA nucleotide positions 1667-1685, 467-485, 429-447, and 1617-1635, respectively. Each target was specific for rat α-ENaC and did not match other sequences in the GenBank. For the construction of each siRNA-expressing vector, two complementary oligonucleotides (forward and reverse), containing a sense strand, followed by a short spacer (5’-TTCAGAGA-3’), an antisense strand, and an RNA polymerase III termination signal (5’-TTTTTTGGAAA-3’), were synthesized, annealed, and ligated into psSilencer 3.0-H1 (Ambion, Austin, TX). The four sets of synthesized oligonucleotides with BamHI and HindIII overhangs were as follows: psi-1, forward 5’-GATCCGTCAAGGTTGTTAGTTTTTTGGAAA-3’; pSi-2, forward 5’-GATCCGTCACTGTGCACTGCTTAATTTAAGGTTGTTAGTTTTTTGGAAA-3’; reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’, reverse 5’-AGCTTTCTCCAAAATCATTGCTTAGCCATTTAATTGGAATTTTGTTTGTTTGGAAA-3’. As a negative control, we used a nonsilencing sequence, 5’/H11032TGAATTTGTTGTTAATAGTGTAACG-3’.

**Surgical procedure and ventilation.** The rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt; Nembutal; Abbott, Chicago, IL). A 2.0-mm [inner diameter (ID)]; endotracheal tube (PE-240, Clay Adams; Becton Dickinson, Sparks, MD) was inserted through a tracheotomy, and a 0.58-mm (ID) catheter (PE-50, Clay Adams; Becton Dickinson) was inserted into the left carotid artery. Pancuronium bromide (0.3 mg/kg body wt × h; Sigma Chemical) was administered through the artery catheter for neuromuscular blockade. Pupil dilation, blood pressure, and heart rate were used as indicators of anesthesia. The anesthesia was complemented when necessary, e.g., if blood pressure began to increase. The rats were maintained in the left lateral decubitus position during the experiment and were ventilated with a constant-volume piston pump (Harvard Apparatus, Nantucket, MA) with an inspired oxygen fraction of 1.0, a respiratory rate of 45–50 breaths/min, and 2.8 ± 0.2 ml tidal volumes. Positive end-expiratory pressure was kept at 2–3 cmH2O. The measured peak airway pressure was 12 ± 2 cmH2O for both the psi-0- and the psi-4-pretreated rats. Mean arterial blood pressure remained stable at 160 ± 20 Torr for psi-0-pretreated rats and at 148 ± 10 Torr for psi-4-pretreated rats.

Peak airway pressure, arterial blood pressure, and heart rate were measured with calibrated pressure transducers (ADInstruments, Colorado Springs, CO) connected to analog-to-digital converters and amplifiers (MacLab QUAD Bridge and MacLab/8, respectively, ADInstruments) and continuously recorded, with Chart version 4.2.4 software (ADInstruments) on an IBM PC-compatible computer.

**General protocol.** A blood sample was collected 5 min before the fluid instillation in an air-tight 1-ml syringe for arterial blood gas measurements and pH measurement and analyzed using a Radiometer system. Average blood gas determinations were as follows: pSi-0, PO2: 520±40 Torr, PCO2: 42±4 Torr, and pH 7.43±0.04, and for pSi-4, PO2: 504±87 Torr, PCO2: 36±4 Torr, and pH 7.47±0.04. After a 30-min baseline period of stable heart rate and blood pressure, the instillation tube (PE-50) was passed through the tracheal tube into the left lung without interrupting ventilation. The instillate (3–4 ml/kg body wt) was instilled over 20 min by infusing 0.05 ml/min with a 1-ml syringe. The study was carried out over 1 h and started at the beginning of fluid instillation. After instillation, the tubing was withdrawn. Blood gases and pH were measured during the baseline and at the end of the study. At the end of the experiment, a blood sample (2 ml) was withdrawn from the carotid artery. The abdomen was opened, and the rats were exsanguinated by transecting the renal artery. The lungs were removed from the chest through a midline sternotomy. A PE-50 catheter was passed into the instilled lung, and a sample of the remaining lung fluid was collected. A previous study demonstrated that the protein concentration in fluid aspirated with a catheter wedged into the distal airs spaces is a good reflection of the lung fluid protein concentration (28, 29). Protein concentrations in the instillate and in final lung fluid samples were measured spectrophotometrically (Multiscan; Labsystems, Helsinki, Finland) with the Lowry method (22) adapted for microtiter plates. The lungs were then snap-frozen in liquid nitrogen and stored at −80°C until further analysis.
Specific protocol. All rats were surgically prepared as described above. The rats were randomly divided into the groups below. All rats were studied for 1 h as described in General protocol.

Pilot studies. These rats (n = 2 in each group) were pretreated with one of the five generated siRNA plasmid DNAs (pSi-0 to pSi-4) 24 h before the LFA experiments were carried out. The rats were then instilled with the 5% albumin instillate containing 10^{-4} M terbutaline. We tested in the preliminary pilot experiments 30 and 40 amplification cycles of pSi-0 to pSi-4. At 30 amplification cycles, we observed smaller decreases in αENaC mRNA expression for pSi-1 to pSi-3 than for pSi-4 (data not shown), thus supporting our initial selection of this siRNA-generating plasmid DNA for the rest of the studies. However, it also indicated that pSi-1 to pSi-3 may have had some silencing effects. We elected to use 40 cycles after the careful initial analysis of outcome vs. number of cycles and because we found that this optimal amplification generated repeatable results. The effect at the mRNA level for pSi-1 to pSi-3 may not have been large enough to generate a difference at the protein expression level that could be observed by Western blot analysis. After all analyses (LFA measurement, RT-PCR, and ENaC protein expression) were carried out on these rats (Fig. 1), it was determined that pSi-4 had the best silencing efficiency and was used for the remaining studies.

Control studies. These rats were pretreated with an irrelevant plasmid DNA (pSi-0; n = 6) or the α-ENaC siRNA-generating plasmid DNA (pSi-4; n = 6) 24 h earlier, respectively. The rats were then instilled with the 5% albumin instillate.

Terbutaline studies. These rats were pretreated with an irrelevant plasmid DNA (pSi-0; n = 6) or the α-ENaC siRNA-generating plasmid DNA (pSi-4; n = 6) 24 h earlier, respectively. The rats were then instilled with the 5% albumin instillate containing 10^{-4} M terbutaline.

Amiloride studies. These rats were pretreated with an irrelevant plasmid DNA (pSi-0; n = 4 baseline and n = 4 terbutaline-instilled) or the α-ENaC siRNA-generating plasmid DNA (pSi-4; n = 4 baseline and n = 4 terbutaline-instilled) 24 h earlier, respectively. The rats were then instilled with the 5% albumin instillate containing 10^{-4} M of the sodium channel inhibitor amiloride or 10^{-4} M amiloride plus 10^{-4} M terbutaline. We used 10^{-4} M amiloride because ~50% of amiloride is protein bound and another significant fraction escapes from the air spaces, resulting in lower functional concentrations (36, 52), and the same or higher concentration of amiloride has been used in multiple earlier studies (8, 33, 37).

Dose-response studies. These rats were pretreated with increasing doses of the α-ENaC siRNA-generating plasmid DNA (pSi-4) 24 h
before harvesting the lungs for α-ENaC mRNA and protein expression. We used pSi-4 at the following concentrations: 0 μg/kg body wt \( (n = 4) \), 2.5 μg/kg body wt \( (n = 4) \), 25 μg/kg body wt \( (n = 4) \), 50 μg/kg body wt \( (n = 4) \), 100 μg/kg body wt \( (n = 4) \), and 200 μg/kg body wt \( (n = 4) \) for these experiments.

**LFA measurements.** LFA was calculated from the increase in lung fluid albumin concentration over the 1-h study, as has been done in several studies before (for review see Refs. 28 and 29). Data are presented as LFA calculated by the following equation:

\[
LFA = \frac{(V_t - V_p)}{V_t} \times 100
\]

where \( V_t \) is instilled fluid volume and \( V_p \) is final lung fluid volume calculated from the increase in protein concentration over the 1-h experiment:

\[
V_p = (V_t \times C_p)/C_p
\]

where \( V_t \) and \( V_p \) are as above and \( C_p \) and \( C_t \) are the protein concentrations of the final lung fluid and the instilled fluid, respectively.

**Endothelial and epithelial protein permeability.** In six rats (3 pSi-0 and 3 pSi-4), a solution containing 2.0 mg of FITC-conjugated dextran 70,000 (FD70; MP Biomedicals) was injected via the carotid artery catheter. FD70 was prepared as a 20-μm solution and run through a gel filtration column (D-Salt Excellulose Plastic Desalting Column; Pierce, Rockford, II) before the intraarterial injection to separate free unbound FITC from the injected FD70. The FD70 was given 15 min before lung fluid instillation. Blood samples were taken immediately before fluid instillation and at the end of the study for plasma FD70 fluorescence analysis. The lungs were then collected for determination of extravascular plasma equivalents as described earlier in various animal species (28, 33, 37, 47).

To estimate the clearance of the vascular tracer FD70 into the lung extravascular compartments (interstitium and air spaces), total extravascular FD70 accumulation in plasma, alveolar fluid, and in the lung homogenate was measured spectrophotofluorometrically (Fluoroscan FL; Labsystems). The passage of FD70 molecules across the endothelial-epithelial barriers was considered to be equal to that of albumin, since they have similar molecular weights (70 vs. 68 kDa). The theory and method behind this plasma equivalent determination have been described in multiple published reports and in various animal species (28, 33, 37, 47). Calculations of endothelial protein passage were done using the FD70 concentrations in the different compartments and applying them in the following equation:

\[
FD70_{(extravascular, lung)} = FD70_{(total, lung)} - FD70_{(vascular space, lung)}
\]

To calculate FD70\(_{(vascular space, lung)}\), the average FD70 measurements in the plasma samples were multiplied by the blood volume in the lungs. The blood volume \( (Q_B) \) in the lungs at the end of the experiment was calculated from the following relationship:

\[
Q_B = 1.039 \times (Q_H \times FWH \times Hb_S)/(FWA \times Hb_W)
\]

where \( Q_H \) is the lung homogenate weight, \( FW_H \) is the lung water fraction, \( Hb_S \) and \( FWS \) are the hemoglobin concentration and the water fraction, respectively, in supernatants obtained after lung homogenate centrifugation, and \( Hb_W \) is the hemoglobin concentration of the last blood sample. The lung water fraction was obtained by gravimetric measurements of the lung as has been done before (2, 28, 33). The density of blood was set to 1.039 g/ml.

To estimate endothelial-to-epithelial passage of FD70, the fluorescence was measured in instillates and in blood plasma samples before and after experiments as we have done before (33). The endothelial-to-epithelial FD70 passage was then calculated as a ratio between air space fluid FD70 and plasma FD70:

\[
FD70_{(passage)} = \frac{FD70_{(total, air space)}}{FD70_{(vascular space)}}
\]

where \( FD70_{(vascular space)} \) and \( FD70_{(air space fluid)} \) are the total amounts calculated by the equations, respectively.
5 min at +4°C. The supernatant (membrane and cytosol fraction) was collected and aliquoted in multiple tubes for each sample and snap-frozen in liquid nitrogen. One tube was designated to be used for determining total protein concentration of the sample to ensure equal loading of the electrophoresis gel. Aliquots were stored at −80°C until analysis.

Polyacrylamide gel electrophoresis and transfer to nitrocellulose membrane (Pierce) were carried out using standard protocols. After electrophoresis and transfer, the nitrocellulose membrane was placed in blocking buffer [SuperBlock Dry Blend blocking buffer in Tris-buffered saline (TBS); Pierce], and blocking was carried out for 1 h at room temperature.

**ENaC subunit detection.** The anti-ENaC antibodies used were purchased from Alpha Diagnostics International (San Antonio, TX) and were directed against 20 residues near the NH2 terminus. These antibodies specifically recognize membrane proteins of appropriate sizes (85–90 kDa for α-ENaC and 90–95 kDa for β-ENaC) in rats. After blocking, the membrane was incubated with the primary antibody [anti-α-ENaC (rabbit) and anti-β-ENaC (rabbit), respectively] on an orbital shaker overnight at +4°C. After incubation, the membrane was washed five times for 10 min with wash buffer (pH 7.5; TBS with 0.1% Tween 20). After the wash process, the membrane was incubated with the enzyme-conjugated secondary antibody (goat anti-rabbit IgG) for 1 h at room temperature. After incubation and wash, the substrate solution (SuperSignal West Femto) was added to the blot and incubated for 5 min. The luminescence signal was detected using a Kodak image analyzer and densitometrically analyzed using TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom).

**Na-K-ATPase subunit detection.** The anti-α1-Na-K-ATPase antibodies were obtained from Upstate Cell Signaling Solutions (Waltham, MA) and were directed against residues 338–518 of the α1-subunit of the Na-K-ATPase. These antibodies specifically recognize membrane proteins of appropriate sizes (~95 kDa for the α1-subunit) in rats. After blocking in SuperBlock, the membrane was incubated with the primary polyclonal antibodies [anti-α1-Na-K-ATPase (rabbit)] on an orbital shaker overnight at +4°C. After incubation, the membrane was washed 5 × 10 min in wash buffer. Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG) for 1 h at room temperature. After incubation and wash, the substrate solution (SuperSignal West Femto) was added to the blot and incubated for 5 min. The luminescence signal was detected using a Kodak image analyzer and densitometrically analyzed using TotalLab software.

**Statistics.** All data are presented as means ± SD. Data were analyzed with one-way ANOVA with Tukey’s test as post hoc or Student’s t-test as appropriate. Differences were considered significant when a P value of <0.05 was reached.

**RESULTS**

**Lung ENaC mRNA expression.** We investigated whether α-ENaC siRNA-encoding plasmid DNA pretreatment affected lung α-ENaC and β-ENaC mRNA levels. First, we determined that plasmid DNA pretreatment in itself did not affect lung ENaC mRNA and protein expression. As demonstrated in Fig. 2A, pretreatment with the irrelevant plasmid DNA (pSi-0) did not change either α-ENaC (A) or β-ENaC (B) mRNA expression. Second, we investigated the effects from pretreatment with the specific plasmid DNA (pSi-4) on α-ENaC and β-ENaC mRNA expression. As shown in Fig. 3, pSi-4 pretreatment significantly reduced α-ENaC mRNA levels compared with that after pSi-0 pretreatment (A). The band representing α-ENaC in the pSi-4 lane was virtually absent, whereas it was clearly visible in the pSi-0 lane. RT-PCR analysis of β-ENaC mRNA expression revealed that α-ENaC siRNA pretreatment had no effect on β-ENaC mRNA levels (B), and the gel displayed equal intensity bands for the α1-subunit in rats. After blocking in SuperBlock, the membrane was incubated with the primary polyclonal antibodies [anti-α1-Na-K-ATPase (rabbit)] on an orbital shaker overnight at +4°C. After incubation, the membrane was washed 5 × 10 min in wash buffer.

**Fig. 2.** A: representative lung α-ENaC RT-PCR gel with and without 24-h pretreatment with irrelevant siRNA (pSi-0)-generating plasmid DNA. pSi-0 pretreatment did not affect normal lung α-ENaC mRNA expression. B: representative lung β-ENaC RT-PCR gel with and without 24-h pretreatment with irrelevant siRNA (pSi-0)-generating plasmid DNA. pSi-0 pretreatment did not affect normal lung β-ENaC mRNA expression. C: representative lung α-ENaC Western blot with and without pretreatment (UT) with irrelevant siRNA (pSi-0)-generating plasmid DNA. pSi-0 pretreatment did not affect normal lung α-ENaC protein expression. D: representative lung β-ENaC Western blot with and without pretreatment with irrelevant siRNA (pSi-0)-generating plasmid DNA. pSi-0 pretreatment did not affect normal lung β-ENaC protein expression. OD, optical density.
RT-PCR β-ENaC product in both groups. GAPDH was always amplified as an internal standard to control for equal gel loading and did not vary in any condition.

Lung ENaC protein expression. We then investigated whether α-ENaC siRNA-encoding plasmid DNA pretreatment affected lung α-ENaC and β-ENaC protein expression. Gels were always loaded with equal protein amounts per well. Lung ENaC protein expression was examined under both stimulated (by terbutaline) and unstimulated (baseline) conditions. Pretreatment with the irrelevant plasmid DNA did not affect lung expression of α-ENaC (Fig. 2C) or β-ENaC (Fig. 2D) compared with untreated control rats. As shown in Fig. 3, pSi-4 pretreatment significantly reduced lung α-ENaC protein expression by nearly 80% during both baseline and terbutaline-stimulated conditions compared with that after pSi-0 pretreatment (Fig. 3C). The plasmid DNA pretreatment did not affect lung β-ENaC expression in either condition (Fig. 3D). Representative Western blots of both α-ENaC and β-ENaC are also shown in Fig. 3.

Dose-response curve and IC_{50} determination. We pretreated these rats with increasing doses of the α-ENaC plasmid DNA (0–200 μg/kg body wt pSi-4) 24 h before harvesting the lungs for α-ENaC mRNA and protein expression. The specific silencing of α-ENaC was more efficient for mRNA than α-ENaC protein expression (Fig. 4). The IC_{50} for α-ENaC mRNA knockdown by 50% was 32 μg/kg body wt pSi-4 (Fig. 4A). To knock down α-ENaC protein expression by 50%, an IC_{50} of 59 μg/kg body wt was required (Fig. 4B). Moreover, as can be seen from the dose-response curves (Fig. 4, A and B), our selected dose, 100 μg/kg body wt, offered a near-optimal pSi-4 concentration.

Lung α_{1}-Na-K-ATPase protein expression. We then investigated whether α-ENaC siRNA-encoding plasmid DNA pretreatment affected α_{1}-Na-K-ATPase protein expression in the lung. Gels were also here loaded with equal protein amounts per well. As shown in Fig. 5, plasmid DNA pretreatment, either with pSi-0 or pSi-4, did not affect the α_{1}-Na-K-ATPase protein expression in the lung (Fig. 5B). A representative Western blot of the α_{1}-Na-K-ATPase subunit is also shown in Fig. 5A.

LFA. Once we had determined the molecular basis for a functional study, we then investigated the consequences of α-ENaC siRNA-encoding plasmid DNA pretreatment on LFA. We used the measurement of LFA as a well-defined endpoint for the functional studies, since LFA has previously been linked in several studies to ENaC activity and function (for review see Refs. 27–29). The pretreatment with the plasmid DNA encoding the α-ENaC-specific siRNA (pSi-4) attenuated the terbutaline-induced stimulation of LFA seen in the pSi-0 rats (Fig. 6). However, pretreatment with the α-ENaC siRNA-generating plasmid DNA (pSi-4) of rats studied under unstimulated baseline conditions was less effective and blocked the baseline LFA by ~30% (Fig. 6).

Amiloride sensitivity of LFA. We then investigated the consequences of the α-ENaC siRNA-encoding plasmid DNA pretreatment with respect to amiloride sensitivity of the LFA. Amiloride was used because it has previously been linked in several studies to ENaC activity and function (8, 33, 36, 52). Pretreatment with the plasmid DNA encoding the α-ENaC-specific siRNA (pSi-4) attenuated the normal amiloride sensitivity of terbutaline-stimulated LFA seen in pSi-0-pretreated rats (Fig. 7). Pretreatment with the α-ENaC siRNA-generating plasmid DNA (pSi-4) of rats
studied under unstimulated baseline conditions also attenuated amiloride sensitivity of LFA (Fig. 7).

**Lung tissue specificity.** To examine the organ and tissue specificity of α-ENaC siRNA-encoding plasmid DNA pretreatment, we studied α-ENaC and β-ENaC mRNA and protein expression in the kidney. Representative RT-PCR gels and Western blot membranes are shown in Fig. 8, A–D. As seen in Fig. 8, α-ENaC and β-ENaC mRNA (A and C) and protein expression (B and D) remained the same in the kidney regardless of plasmid pretreatment.

**Localization of the RNAi effect.** To examine the alveolar distribution of the siRNA-generating plasmid for α-ENaC, we isolated alveolar epithelial type II cells from three pSi-0-instilled rats and three pSi-4-instilled rats. As seen in Fig. 9, α-ENaC mRNA, as determined by RT-PCR, was completely absent from the alveolar epithelial type II cells isolated from the pSi-4-instilled rats 24 h after plasmid DNA instillation, whereas it was clearly expressed in pSi-0-instilled rat alveolar epithelial type II cells.

**Assessment of lung endothelial and epithelial permeability.** Endothelial protein leak, when calculated as extravascular plasma equivalents, was low in the six examined rats, three pSi-0- and three pSi-4-pretreated rats, subjected to the plasmid DNA pretreatment and LFA study. An extravascular plasma equivalent of 0.06 ± 0.02 ml was detected in these rats after the 1-h LFA experiment. To estimate endothelial-to-epithelial protein leak, we analyzed this as a ratio between FD70 in the air space fluid samples and plasma as has been done earlier (33). The ratio was also uniformly low and at 0.07 ± 0.04 in the six rats subjected to the LFA study.

**DISCUSSION**

There were four important findings in these studies. First, pretreatment with the specific α-ENaC siRNA-generating plasmid DNA attenuated the terbutaline stimulation of the LFA. Second, α-ENaC siRNA pretreatment decreased baseline LFA by ~30%. Third, amiloride sensitivities during baseline con-
Effects on the expression of ENaC were highly specific as there were no pSi-4 pretreatment regardless of later terbutaline treatment or ENaC expression, both mRNA and protein, was attenuated by Western blot analysis of ENaC protein in the lung, where supported by the RT-PCR analysis of ENaC mRNA and exhibited by this amiloride concentration. This relationship is the majority of amiloride-sensitive ENaC channels would be in baseline LFA is estimated to be relatively high at 10–70% (8, 28, 29, 36, 37, 52) of both stimulated and baseline LFA. The apparent discrepancy suggests that there are two separate mechanisms, one that does not rely on ENaC entirely, although amiloride sensitive and responsible for baseline LFA, and one that relies fully on ENaC and is responsible for the terbutaline-stimulated LFA. Another possibility is that not all ENaC channels were inhibited by amiloride due to a loss of amiloride from the distal air spaces after the instillation, thus generating a potentially low amiloride concentration. We used amiloride at a concentration of 10^{-4} M, which in fact may be considered a fairly high amiloride concentration. Even after a significant leak and binding to protein, as shown before (52), the expected active amiloride concentration in the air spaces is still estimated to be relatively high at ~5 	imes 10^{-5} M. Thus we believe that the vast majority of amiloride-sensitive ENaC channels would be inhibited by this amiloride concentration. This relationship is supported by the RT-PCR analysis of ENaC mRNA and Western blot analysis of ENaC protein in the lung, where ENaC expression, both mRNA and protein, was attenuated by pSi-4 pretreatment regardless of later terbutaline treatment or not. Our pretreatment was highly specific as there were no effects on the expression of β-ENaC in the same lung tissue samples, either at the protein or mRNA levels. Thus β-adrenoceptor stimulation of LFA depends critically on ENaC expression in the lung, whereas baseline LFA seems less ENaC dependent.

Plasmid DNA delivery into the air spaces of the lungs represents a method for short-term drug delivery. Therefore, a broad array of plasmid DNA delivery techniques has been developed and utilized. Generally, the plasmid DNA of interest is mixed with some kind of carrier molecule that facilitates and/or mediates the plasmid DNA uptake into the target tissue. Over the past decade, numerous viral and nonviral approaches have been reported and developed for transferring genes to the lung, but most have significant limitations. Nonviral plasmid DNAs lack some of the risks inherent with viral vectors; the delivery is less toxic, less immunogenic, and easier to prepare, which attracts considerable interest as potential use in clinical treatments. Cationic liposome-mediated intravenous gene delivery has shown some promise in treating pulmonary diseases, including lung tumor metastases, pulmonary hypertension, and the acute respiratory distress syndrome (19, 35). Even in vivo electroporation has recently been utilized to transfect the lung (23). However, carriers are not always required; it may be possible to deliver naked DNA into cells via intramuscular injection (48) and systemic administration (20). These techniques have, however, serious limitations when studies are designed to investigate lung epithelial proteins and their importance. Our method utilized a modification of an original finding by Sawa and colleagues (43), where they demonstrated that intraluminal water instillation/aerosolization into the air spaces of the lungs increased the transfection efficiency of the instilled luciferase gene. We utilized this idea of low osmolar gene delivery for our studies but selected a medium-low osmolality solution of 113 ± 10 mosmol/kgH2O together with Lipofectamine 2000. This seemed to work very well in our hands and produced a clear result of specific silencing of the desired α-ENaC subunit.

Did the intratracheally instilled siRNA-generating plasmid DNAs reach the alveolar epithelium, which represents a principal barrier across which edema fluid is absorbed secondarily?...
to Na absorption via apical ENaC channels? To answer this question, we instilled the siRNA-generating plasmid DNA for α-ENaC intratracheally as we did for the functional studies. After 24 h, we isolated the alveolar epithelial type II cells, a cell type in the lung previously recognized to transport Na via ENaC channels (27–29), and measured α-ENaC expression by RT-PCR. We found that after α-ENaC siRNA pretreatment, mRNA expression of α-ENaC was completely absent in these isolated cells compared with α-ENaC mRNA expression in pSi-0-instilled rat cells. Thus we conclude that our delivery method of the siRNA-generating plasmid DNA successfully delivers plasmid DNA to the alveolar epithelium. Moreover, this intratracheal siRNA-generating plasmid DNA instillation functionally attenuates α-ENaC expression in the alveolar epithelium, one site previously documented as important for fluid absorption from the air spaces (27–29).

There are some methodological concerns of note. First, it could be possible that some endotoxin was present in the plasmid preparations, although every effort was made to reduce and exclude endotoxin from the plasmid preparations. We used an endotoxin-binding resin for the exclusion of it during plasmid DNA isolation. If present, endotoxin would have been present at similar levels in the pSi-0 preparation and the pSi-4 preparation and would likely have affected the outcome equally. Because the silencing of α-ENaC was specific, this was likely not a major issue. Second, an unspecified volume of fluid stemming from the plasmid DNA pretreatment 24 h earlier could be present in the lungs at the start of the LFA experiments. This possibility was significantly reduced by the use of the low osmolar plasmid DNA pretreatment solution, ensuring that this fluid rapidly got absorbed. Third, we used a plasmid containing the H1 promoter, thus allowing this plasmid to be expressed in a multitude of cell types, including the cell types believed to be the principal cells involved in trans-epithelial sodium and fluid absorption in the lung, the alveolar epithelial type I and type II cells, and the distal airway epithelial cells. In vivo, it is difficult to make sure that the administered siRNA is expressed in only one cell type. One possible way is to utilize a cell-specific promoter to assure the plasmid will only be expressed in certain cell types. There have been a few attempts to do so; a recent study utilized the surfactant protein C promoter to deliver siRNA to isolated lung epithelial cells in culture (13). However, few such studies are available to our knowledge in an in vivo situation where this has been tested as we have done in this study. In one recent study, it was demonstrated that in vivo gene silencing of pulmonary expression of macrophage inflammatory protein-2 protected the lung against neutrophil-mediated septic acute lung injury; however, these investigators administered siRNA directly into the lungs of mice (21). The drawback of the utilization of cell-specific promoters is the potential of negative results; it is difficult to interpret whether the negative result is due to a failed delivery, a no-expression, or that this particular...
cell did not participate in that specific process studied. The use of a more nonspecific promoter is more likely to overcome these problems but suffers from the limitation of being nonspecific. Functionally, this is likely overcome because the protein designed to silence may only be expressed by certain cells, and thus the silencing plasmid, although being expressed widely, is only expected to silence the gene where it is being expressed. Our study was designed deliberately to utilize a more nonspecific promoter and functionally inhibit the effect in the lung. Although we know that sodium transport occurs across a few cell types out of the 40 different cell types present in the lung, when the lung is fluid filled during recovery from pulmonary edema or immediately after birth, the majority of these sodium-transporting cell types are likely exposed to this edema fluid and participate in the absorption of the fluid. Moreover, as demonstrated in our studies using the freshly isolated alveolar epithelial type II cells, the intratracheal instillation of the siRNA-generating plasmid DNA attenuated α-ENaC mRNA expression in these cells, thus confirming that the intratracheally instilled siRNA-generating plasmid DNA reached the alveolar epithelium, a site where transepithelial Na and fluid absorption occur (26–29). However, the studies do not exclude exposure of the siRNA-generating plasmid DNA to bronchial-bronchiolar epithelial cells and the alveolar epithelial type I cells, which are other Na-absorbing cell types in the lungs (28).

Did nonspecific effects such as barrier protein leak in the lung affect the results? This is unlikely for several reasons. First, pretreatment with the noncoding plasmid DNA (pSi-0) did not affect the terbutaline stimulation and amiloride inhibition of the LFA compared with previously published studies (37) under these conditions. Because the LFA proceeded at rates similar to previously recognized data in ventilated rats (28, 29, 37) and was even able to be stimulated and inhibited suggested that any increased barrier permeability was likely of minor importance. Second, the low-extravascular plasma equivalents in the lung and the unchanged epithelial FD70 permeability during the LFA study support the assumption of an uninjured lung. Third, in vivo instillation of plasmid DNA may evoke a cytokine response that in turn could affect the lung’s ability to absorb fluid. Although cytokines have in the past been demonstrated to affect lung fluid balance (11, 40, 51), this was unlikely to be a major component of our study. Because the instillation of the nonspecific plasmid DNA (pSi-0) did not affect any of the studied parameters compared with untreated rats, release of a cytokine with the capacity to affect the lung was likely very limited. Fourth, the exhibition of a dose-response relationship of the plasmid DNA pretreatment solution also strengthened the notion that the lung remained intact after silencing α-ENaC by the siRNA-generating plasmid DNA pretreatment. Thus it is unlikely that nonspecific effects related to the model were of principal importance for the outcome of the study.

The efficiency of gene silencing by the α-ENaC-specific siRNA-generating plasmid DNA instillation was also investigated. We carried out a dose-response analysis of the α-ENaC silencing after intratracheal instillation of increasing pSi-4 doses (0–200 μg/kg body wt). The efficiency of α-ENaC silencing was greater for mRNA (IC50 = 32 μg/kg body wt) than for protein expression (IC50 = 59 μg/kg body wt). This was likely to be expected since mRNA knockdown precedes protein knockdown in time. More importantly, this analysis demonstrates that our selected pSi-4 dose (100 μg/kg body wt) was optimal to achieve a near-maximal or maximal specific α-ENaC silencing for our studies.

Compared with conventional gene knockout technology, gene silencing using specific siRNA has the following advantages: 1) siRNA may be a time-saving and cost-effective approach to generation of traditional knockout animals, 2) a genomic sequence is not required for siRNA, whereas it is essential for conventional gene knockout technology, and 3) for lethal genes, a conventional gene knockout animal cannot usually be obtained, whereas an siRNA with different potency might be used to produce different phenotypes of this kind of gene knockout animal as well as organ-specific knockout animals when the molecule of interest is vital to a multitude of organs. Knocking out α-ENaC in the mouse caused a neonatal mortality of 100% by 40 h postbirth (15), a result that initially was interpreted as ENaC was critical for the lung to be able to clear the fetal lung fluid at term. It is difficult to be absolutely sure of the phenotype when you completely knock out one gene, such as α-ENaC, from the whole animal as the animal is likely to have phenotypic variation related to knocking the gene, α-ENaC, out from distal organs relative to the organ of interest. This was likely of less importance in our experiments as the α-ENaC siRNA-generating plasmid had no effect on a remote organ with a well-documented ENaC expression, i.e., the kidney. Moreover, the nature of the specificity of this approach was also demonstrated in that the pretreatment with the α-ENaC siRNA-generating plasmid DNA had no effects on either β-ENaC expression or α1-Na-K-ATPase expression in the lung. siRNA technology with organ-specific silencing of the gene provides, in some circumstances, a superior tool to investigate the importance of one specific gene product in one defined organ. In this study, we demonstrated that silencing of α-ENaC in the lung was lung specific as well as subunit specific, with a functional response that strongly suggested that β-adrenoceptor-stimulated LFA in the adult rat was critically dependent on the presence of α-ENaC in the lung epithelium. On the other hand, baseline LFA did not seem to be solely dependent on α-ENaC.

In some models of lung injury, it has been shown that stimulatory effects from β-adrenoceptor agonists on alveolar fluid clearance may be attenuated. In bleomycin-induced lung injury, there was a decreased ENaC expression associated with an attenuated β-adrenoceptor response (9). In other models, such as after hypoxia (39), alveolar epithelial ENaC expression was also reduced when associated with an impaired β-adrenoceptor stimulation. Also, hemorrhagic shock attenuated β-adrenoceptor stimulation of LFA secondary to reactive nitrogen species (38). Together with our current data, these observations may strengthen the conclusion the β-adrenergic stimulation of LFA is heavily ENaC dependent.

In summary, these data demonstrate the siRNA expressed from intratracheally instilled nonviral vectors in vivo specifically reduce the expression of their target molecule, in our case, the α-ENaC subunit of the epithelial sodium channel. Along with the reduced expression, the functional role of ENaC in LFA was attenuated after stimulation with β-adrenoceptor agonists but less affected during normal nonstimulated
conditions. This strongly suggests that ENaC has a principal function in the lung as being responsible for β-adrenoceptor-stimulated LFA, whereas baseline LFA relies on both ENaC and some other mechanism. These findings may extend the application of siRNA to nonviral therapies and to basic research in pulmonary medicine and physiology, including defining specific gene function in the lung.

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