α_1 -Antitrypsin Inhibits Epithelial Na^+ Transport *In Vitro* and *In Vivo*

Ahmed Lazrak¹, Izabella Nita^{1,2*}, Devipriya Subramaniyam^{1,2*}, Shipeng Wei¹, Weifeng Song¹, Hong-Long Ji¹, Sabina Janciauskiene^{2‡}, and Sadis Matalon^{1,3,4‡}

¹Departments of Anesthesiology and ³Physiology and Biophysics, and ⁴Center of Pulmonary Injury and Repair, University of Alabama at Birmingham, Birmingham, Alabama; and ²Lund University, Department of Clinical Sciences, Wallenberg Laboratory, Malmö University Hospital, Malmö, Sweden

A variety of studies have shown that Na^+ reabsorption across epithelial cells depends on the protease–antiprotease balance. Herein, we investigate the mechanisms by which α_1 -antitrypsin (A1AT), a major anti-serine protease in human plasma and lung epithelial fluid and lacking a Kunitz domain, regulates amiloride-sensitive epithelial Na^+ channel (ENaC) function *in vitro* and *in vivo*. A1AT (0.05 mg/ml = 1 μM) decreased ENaC currents across *Xenopus laevis* oocytes injected with human α, β, γ -ENaC (hENaC) cRNAs, and human lung Clara-like (H441) cells expressing native ENaC, in a partially irreversible fashion. A1AT also decreased ENaC single-channel activity when added in the pipette but not in the bath solutions of ENaC-expressing oocytes patched in the cell-attached mode. Incubation of A1AT with peroxynitrite (ONOO^-), an oxidizing and nitrating agent, abolished its antiprotease activity and significantly decreased its ability to inhibit ENaC. Intratracheal instillation of normal but not ONOO^- -treated A1AT (1 μM) in C57BL/6 mice also decreased Na^+ -dependent alveolar fluid clearance to the same level as amiloride. Incubation of either H441 cells or ENaC-expressing oocytes with normal but not ONOO^- -treated A1AT decreased their ability to cleave a substrate of serine proteases. A1AT had no effect on amiloride-sensitive currents of oocytes injected with hENaC bearing Liddle mutations, presumably because these channels remain at the surface longer than the wild-type channels. These data indicate that A1AT may be an important modulator of ENaC activity and of Na^+ -dependent fluid clearance across the distal lung epithelium *in vivo* by decreasing endogenous protease activity needed to activate silent ENaC.

Keywords: alveolar fluid clearance; serine proteases; H441 cells; *Xenopus* oocytes; ENaC

The amiloride-sensitive epithelial Na^+ channels (ENaCs) play an important role in Na^+ and fluid homeostasis across a number of epithelial cells, including those in airway and alveolar spaces (1, 2). ENaC mRNA, protein levels, and ENaC activity are regulated by a variety of hormones (such as aldosterone and dexamethasone), second messengers cAMP/protein kinase A, and reactive intermediates (2–4). In addition, endogenous channel-activating proteases (CAPs), as well as proteases released by inflammatory cells (trypsin, elastase), activate ENaC either by

CLINICAL RELEVANCE

Our data show that α_1 -antitrypsin (A1AT) inhibits active Na^+ reabsorption and lung fluid clearance across distal lung epithelial cells and highlight a potential mechanism by which A1AT administration may prove beneficial in clinical situations in which increased ENaC activity may contribute to lung pathology (such as cystic fibrosis).

cleaving critical amino acids in α - and γ -ENaC subunits, or by activating signaling pathways (1, 2, 5–7).

There has been considerable interest in identifying the mechanisms by which endogenous and exogenous proteases activate ENaC. Aprotinin, a potent and reversible Kunitz-type inhibitor of several serine proteases, including trypsin, plasmin, and kallikreins (8), has been reported to inhibit sodium transport among a variety of epithelial cells, including A6 (9), human bronchial epithelial (HBE) cells (10, 11), and rat and mouse lung alveolar epithelial cells (1). Other Kunitz-type serine protease inhibitors, such as hepatocyte growth factor activator inhibitor (HAI)-1 and HAI-2 (placental bikunin), have also been demonstrated to inhibit prostasin and ENaC activity (12). Thus, it is generally accepted that Kunitz-type protease inhibitors block epithelial serine proteases needed for ENaC activation, and these inhibitors have been suggested as a novel treatment for the Na^+ hyperabsorption in cystic fibrosis (CF) airways (10).

α_1 -Antitrypsin (A1AT) is an acute-phase glycoprotein and a member of the serine protease inhibitor (SERPIN) superfamily (13). Until recently, it was thought that its primary function was to neutralize elastase *in vivo*, protease-3, and other serine proteases released from activated human neutrophils. In fact, the rate of formation of the A1AT/neutrophil elastase inhibitory complex is one of the fastest known for SERPINs ($6.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$). However, recent findings indicate that A1AT also directly inhibits the activity of caspase-3, a cysteine protease that plays an essential role in apoptosis (14, 15), and the catalytic domain of recombinant human matriptase-1 (16), a multidomain type-II transmembrane serine protease, which is involved in ENaC proteolysis and activation (17). These findings lead to the hypothesis that both Kunitz-type and non-Kunitz-type serine protease inhibitors may inactivate epithelial serine proteases, and thus prevent proteolytic activation of ENaC *in vivo*.

Herein we performed a series of biophysical, biochemical, and physiological studies in *Xenopus* oocytes, injected with human α, β, γ -ENaC (hENaC) cRNAs, human lung Clara-like cells (H441), which express ENaC type-single channel activity (18), as well as in anesthetized mice, which clear alveolar fluid across their distal epithelium secondary to active Na^+ transport (19, 20), to identify the mechanisms by which A1AT inhibits amiloride-sensitive Na^+ transport and alveolar fluid clearance (AFC) both

(Received in original form September 10, 2008 and in final form December 17, 2008)

* These authors contributed equally as second authors.

‡ These authors contributed equally as senior authors.

This work was supported by NIH grants HL031197, HL051173, and SU01ES015676 (S.M.), and grants from the Swedish Medical Research Council, Crafoords Foundation, MAS Foundation, and Grifol's Institute (S.J.).

Correspondence and requests for reprints should be addressed to Sadis Matalon, Ph.D., Department of Anesthesiology, University of Alabama at Birmingham, 224 BMR II, 901 South 19th Street, Birmingham, AL 35205-3703. E-Mail: sadis@uab.edu

Am J Respir Cell Mol Biol Vol 41, pp 261–270, 2009

Originally Published in Press as DOI: 10.1165/rcmb.2008-03840C on January 8, 2009

Internet address: www.atsjournals.org

in vitro and *in vivo*. Our results indicate that very low concentrations (0.05 mg/ml or 1 μ M) of A1AT decreased amiloride-sensitive currents (I_{amil}) in oocytes and H441 cells in a partially irreversible fashion, and reduced Na^+ -dependent AFC *in vivo*. Additional studies showed that these effects were the result of A1AT inhibition of membrane-bound proteases present on the surface of oocytes and H441 cells. These new findings indicate that the inhibitory effect of A1AT might be an important regulator of ENaC activity and Na^+ -dependent AFC *in vivo*.

MATERIALS AND METHODS

A1AT Preparations

Purified human A1AT was purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich (St. Louis, MO). Prolastin was provided by Talecris Biotherapeutics, Inc. (Research Triangle Park, NC). The purity of A1AT preparations was greater than 97%, and their inhibitory activity was higher than 75%. Native A1AT was diluted in PBS (pH 7.4). To ensure the removal of endotoxin, A1AT was eluted through Detoxi-Gel AffinityPak columns according to manufacturer instructions (Pierce, Rockford, IL). Purified batches of A1AT were then tested for endotoxin with the limulus amebocyte lysate endochrome kit (Endosafe; Charles River, Charleston, SC). Endotoxin levels were less than 0.1 enzyme units/mg protein in all preparations used.

Treatment of A1AT with Peroxynitrite

A1AT (1.25 mg/ml) was suspended in PBS containing 10 mM HEPES (pH 7.4) and incubated with peroxynitrite (ONOO^- ; 1 mM). Because ONOO^- has a half-life of less than 1 second at pH 7.4 (21), it was added while still in NaOH as the solution was vigorously mixed. The pH was carefully monitored to ensure that reactions were performed at neutral pH. A1AT was then separated by SDS-PAGE (8 or 12% separating gel) in the presence of 2-mercaptoethanol, and visualized by staining the gels with GelCode (Thermo Fisher Scientific, Rockford, IL).

Construction of ENaC cRNAs with the Liddle Mutations

α , β , γ -hENaC cDNAs with the Liddle mutations (hENaC- α 595x, hENaC- β 566x, hENaC- β S520K, hENaC- γ 575x in a pMT3 vector) were a kind gift from Dr. Peter Snyder (University of Iowa). These plasmids were designed for nuclear injection. ENaC subunits were subcloned into pCDNA3.1 vector for *in vitro* transcription. hENaC- α 595x was excised by NotI and XhoI and cloned into pCDNA3.1 at NotI and XhoI. hENaC- β 566x and β S520K were excised by NotI and Acc65I and cloned into pCDNA3 at NotI and XhoI, with Acc65I and XhoI blunt ended. hENaC- γ 575x was excised by NotI and EcoRI and cloned into pCDNA3.1 at NotI and XhoI, with EcoRI and XhoI blunt ended. All the constructs were verified by DNA sequencing. ENaC subunit plasmids were linearized and cRNAs were prepared with a cRNA synthesis kit (Message Machine T7; Ambion, Austin, TX) according to the manufacturer's protocol. cRNAs were dissolved in RNase-free water, and the concentrations were determined spectrophotometrically.

Measurement of Current-Voltage Relationships in *Xenopus* Oocytes

Detailed description of these techniques has been previously reported (22–24). In brief, defolliculated oocytes, isolated from *Xenopus laevis* frogs, were injected with cRNAs encoding for wild-type α , β , γ -hENaC (8.4 ng each), dissolved in 50 nl of RNase-free water per oocyte, and incubated in half-strength L-15 medium for 24 to 48 hours. Whole-cell cation currents were measured by the two-electrode voltage clamp technique before and after perfusion with amiloride (10 μ M), which inhibited more than 90% of the inward Na^+ currents. The oocytes were held in a small groove in an experimental chamber of 1-ml volume at room temperature (21°C). The chamber was filled with ND96 solution containing: 96 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , and 10 mM HEPES at pH 7.6 (osmolarity, 200–220 mosM). Oocytes were impaled with two 3 M KCl-filled electrodes, with resistances of 0.5–1.5 M Ω . A TEV 200 voltage clamp amplifier (Dagan Corp., Minneapolis, MN) was used to voltage clamp oocytes. Two reference electrodes

were connected to the bath. The membrane potential (V_m) was held at -40 mV. Current-voltage (I - V) relationships were obtained by stepping the V_m from -120 mV to $+80$ mV in 20-mV increments for 600 milliseconds. Sampling protocols were generated by pCLAMP 8.0 (Axon Instruments, Molecular Devices, Union City, CA). Currents were sampled at the rate of 1 kHz, filtered at 500 Hz, and simultaneously stored electronically and displayed in real time on a chart recorder.

Effects of A1AT on Amiloride-Sensitive Na^+ Currents

Two different sets of experiments were performed: in the first set, we measured I - V relationships before and after addition of amiloride (10 μ M) into the bath solution containing ND96. Na^+ currents were continuously recorded while stepping the V_m from -40 to -140 mV (to record inward Na^+ currents) and from -40 to $+60$ mV (to record outward currents). Oocytes were then perfused with ND96 until stable currents were obtained at which point native or ONOO^- -treated A1AT (0.05 mg/ml; 1 μ M) were added into the bath solution and currents were continuously recorded until new steady-state values were reached (usually within 45 min). At that time, amiloride (10 μ M) or trypsin (1 μ M) was added to the bath and currents were recorded until new steady-states were reached. In a second set of experiments, hENaC-expressing oocytes were incubated for 2 hours in half-strength L-15 medium with and without A1AT (0.05 mg/ml; 1 μ M). At the end of this period, they were placed in the recording chamber under the microscope (filled with ND96) and I - V relationships were recorded as described above.

In some cases, oocytes microinjected with hENaC were incubated with MG-132 (a proteasome inhibitor; Calbiochem) for either 24 hours (100 nM) or 2 hours (4 μ M). After completion of I - V curves, they were perfused with A1AT (1 μ M) and inward currents were recorded as described above.

Assessment of Total and Surface ENaC Levels

Xenopus oocytes injected with either H_2O , or hENaC cRNAs were incubated with either vehicle or A1AT (1 μ M) for 45 minutes and then homogenized in 1 ml of 100 mM PBS (pH 7.4) containing one tablet of Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche Applied Sciences, Indianapolis, IN) and 10 mM N-ethylmaleimide (NEM). Lysates were centrifuged twice at $300 \times g$ for 10 minutes, and the supernatants collected and centrifuged at $16,000 \times g$ for 30 minutes. The upper layer, consisting of lipid and yolk, was removed, and the remaining pellet and supernatants were suspended in 1% triton X100 and solubilized for 1 hour at 4°C. After centrifugation at $16,000 \times g$ for 30 minutes, 300 μ l of the extract was suspended in 1 ml of the same buffer, and γ -ENaC was immunoprecipitated with 5 μ l of a polyclonal anti- γ -ENaC rabbit antibody (Abcam, Cambridge, MA) overnight at 4°C followed by 50 μ l of 50% protein G beads for 3 hours at 4°C. The beads were washed extensively with the same buffer, followed by a final wash in 50 mM Tris (pH 7.5). Immune complexes were dissociated by heating in 60 μ l of sample buffer at 95°C for 5 minutes, and probed with a rabbit polyclonal anti- γ -ENaC antibody (1:1,000 dilution; Abcam) followed by a secondary antibody (horseradish peroxidase protein A; 1:10,000 dilution; Pierce) and detected by chemiluminescence.

Surface expression of γ -hENaC was examined by a cell surface biotinylation assay, as previously described by us (25). cRNAs were coinjected into *Xenopus* oocytes. After 48 hours, oocytes were mechanically stripped of their vitelline membranes in hypertonic medium, and surface proteins were labeled with membrane-impermeable sulfo-NHS-Biotin (Pierce). Oocytes (10 per group) were subsequently lysed and centrifuged at $13,000 \times g$ for 15 minutes at 4°C. Biotinylated proteins were precipitated with streptavidin-agarose (Pierce), and subjected to SDS-PAGE, followed by Western blotting with anti- γ -ENaC antibodies, as described above.

Single-Channel Recordings in *Xenopus* Oocytes

The vitelline membranes of oocytes expressing α , β , γ -hENaC were surgically removed after shrinking the oocytes by immersing them in a hypertonic sodium aspartate solution (400 mM). Immediately after removal of this membrane, oocytes were transferred to the recording chamber containing 0.5 ml ND96. Single channels were recorded in cell-attached mode using an Axopatch 200B amplifier (Axon Instru-

ments). Signals were digitized at 5 KHz (DigiData 1,322 A, 16 bit; Molecular Devices, Sunnyvale, CA), filtered at 1 KHz, and acquired using an IBM compatible computer using the Axon pCLAMP 9.2 software (Axon Instruments).

Pipettes were made from a thick-walled capillary glass (LG 16; Dagan) using a two-stage puller (PC-10; Narishige, Tokyo, Japan). Pipette resistance was $9.7 (\pm 0.9) \text{ M}\Omega$ when filled with ND 96. The ground electrode (Ag-AgCl) was connected to the bath using a 3% agar bridge (1 M KCl). Single channels were recorded at a V_m of -100 mV . The mean value of the V_m , measured in a different set of oocytes using the double voltage clamp technique (18), was around 5.0 mV .

Data Analysis

Open probability (P_o) was measured as the total open time of a channel normalized to the total time of recording at -100 mV . For recordings showing more than one active channel (as was the case in most of our recordings), the average channels activity was reported as $N P_o$ (number of active channels multiplied by their P_o), which is determined by integrating the area under the Gaussian curves to the all-points current-amplitude, and normalized to the peak area of the closed level or baseline current level. This routine is included in Clampfit software (Axon Instruments).

Whole-Cell Recording of Na^+ Currents in H441 Cells

Clara-like H441 human lung cells were cultured on coverslips, as previously described (18). We added dexamethasone (100 nM) into the medium to enhance expression of ENaC and amiloride-sensitive Na^+ currents. Whole-cell currents were elicited by applying a step-pulse protocol from -140 to $+60 \text{ mV}$ in 10-mV increments for 500 milliseconds from a holding potential of -40 mV . I-V relationships were constructed by averaging the current values between 400 and 500 milliseconds from the start of recording with the Clampfit program and plotted using Origin Software (Northampton, MA). Only cells with stable baseline currents were included in the analysis. After obtaining stable whole-cell currents, cells were perfused with an external solution containing amiloride ($2 \text{ }\mu\text{M}$) or an equivalent amount of H_2O as vehicle, and amiloride-sensitive I-V relationships were calculated as described above.

Determination of Protease Enzymatic Activity

Serine protease activity on the oocyte membrane and H441 cells were monitored by measuring the release of a fluorescent 7-amido-4-methyl coumarin (AMC) from a peptide substrate, t-Butyl oxy-carbonyl (Boc)-Gln-Ala-Arg-AMCHCl (R&D Systems, Minneapolis, MN) using a fluorescence plate reader (Fluorster Optima; BMG; Labtech GmbH, Durham, NC). Briefly, 10 oocytes were added to a cuvette containing $200 \text{ }\mu\text{M}$ of ND96 medium, and the reaction was started by adding Boc-Gln-Ala-Arg-AMCHCl ($50 \text{ }\mu\text{M}$ final concentration). The reaction was monitored for up to 120 minutes by recording fluorescence at 460 nm after excitation at 380 nm . In parallel experiments, oocytes were pretreated for 45 minutes with A1AT ($1 \text{ }\mu\text{M}$; either native or ONOO $^-$ treated) before recording fluorescence. The same protocol was followed with H441 cells, except that the cell number was adjusted to $10^5/\text{ml}$ in Ringer's solution. Finally, in some cases, measurements were obtained after disrupting the cellular membranes of either oocytes or H441 cells treated with $1 \text{ }\mu\text{M}$ A1AT for 45 minutes.

Na^+ -Dependent AFC Measurements in Anesthetized Mice

AFC was measured as previously described (19, 20, 26). Briefly, C57BL/6 mice with body weights between 20 and 25 g were anesthetized with diazepam ($1.0 \text{ mg}/100 \text{ g}$, intraperitoneally; Abbott Laboratories, Abbott Park, IL) and ketamine ($20 \text{ mg}/100 \text{ g}$, intraperitoneally), and placed on a heating pad (Braintree Scientific, Cambridge, MA) to maintain body temperature at 37°C . The trachea was exposed and cannulated with a trimmed 18 -gauge intravenous catheter, which was then connected to a mouse respirator (model 687; Harvard A1 Apparatus, Holliston, MA). Mice were paralyzed with pancuronium bromide (0.04 mg , intraperitoneal; Gensia Pharmaceuticals, Irvine, CA) and ventilated with 100% O_2 with a $200\text{-}\mu\text{l}$ tidal volume ($8\text{--}10 \text{ ml}/\text{kg}$ body wt) at 160 breaths/min. Once stable anesthesia was achieved, mice were positioned in the left decubitus position, and $300 \text{ }\mu\text{l}$ of isosmolar NaCl containing 5% fatty acid-free BSA was instilled via the

tracheal cannula, followed by $100 \text{ }\mu\text{l}$ of room air to clear dead space. In experimental groups, normal or ONOO $^-$ -treated A1AT ($1 \text{ }\mu\text{M}$ final concentration) was added into the instillate. Postinstillation, mice were ventilated for a 30 -minute period, then the alveolar fluid was aspirated. AFC was calculated from the ratio of the protein concentration of the instillate before instillation and of the alveolar sample at 30 minutes.

Statistical Analysis

All values are expressed as means ($\pm 1 \text{ SEM}$), along with the number of measurements. I_{amil} values were calculated from the difference between steady-state values before and after addition of amiloride ($10 \text{ }\mu\text{M}$) into the bath. Data were analyzed by one-way ANOVA with Bonferroni's test or Student's paired t test, as appropriate. P values of less than 0.05 were considered significant.

RESULTS

A1AT Inhibits I_{amil} Values in *Xenopus* Oocytes Expressing ENaC

In the first set of experiments, we assessed the extent and time course of inhibition of I_{amil} values by A1AT. Uninjected oocytes showed near-zero levels of inward currents. Thus, inward currents of ENaC-injected oocytes were attributed to the movement of Na^+ ions, and will be referred to as " I_{Na} ." At a V_m of -100 mV , I_{Na} currents 48 hours after injection were $-7,368 (\pm 705; n = 171)$ ($X \pm 1 \text{ SEM}$; n = number of oocytes). Addition of amiloride ($10 \text{ }\mu\text{M}$) into the bath inhibited more than 95% of I_{Na} . Addition of A1AT (obtained either from Calbiochem or Sigma) in the bath at a final concentration of 0.05 mg/ml ($1 \text{ }\mu\text{M}$) inhibited I_{Na} by more than 90% within 45 minutes (Figures 1A and 1B). The mean rate constant (τ) of decay, calculated by fitting the I_{Na} with a monoexponential decay equation, $y = A \times \exp(-I_{\text{Na}}/t) + B$, was $13 (\pm 0.6) \text{ minutes}$ ($X \pm 1 \text{ SEM}$; $n = 13$). Adebamiro and colleagues (27) reported that aprotinin inhibited I_{Na} across A6 cells with a τ of $18 (\pm 1.2) \text{ minutes}$. The I_{Na} inhibition by A1AT is most likely due to a decrease in the number of active ENaC channels at the plasma membrane.

I_{amil} values increased gradually after replacing the oocyte bath solution with ND96 medium without A1AT, reaching values of approximately 40% of controls by 6 hours (Figure 1C). Addition of trypsin ($1 \text{ }\mu\text{M}$) into the bath after 45 minutes of incubation with A1AT restored I_{Na} to its control value within minutes (Figure 1B). Because trypsin is known to activate silent ENaC channels present in the cell membrane (28), our data suggest that A1AT decreases I_{Na} by inhibiting proteases that are needed for ENaC activation at the cell membrane. Both Prolastin ($1 \text{ }\mu\text{M}$) and aprotinin ($5 \text{ }\mu\text{M}$) inhibited I_{Na} to the same extent as A1AT (data not shown).

ONOO $^-$ -Treated A1AT Does Not Inactivate ENaC Activity

Exposure of A1AT to ONOO $^-$ oxidizes and nitrates a number of amino acids, including a key methionine (Met 358), located in the A1AT active site. These modifications were associated with loss of its ability to inactivate elastase (29, 30). SDS-PAGE analysis of normal A1AT revealed a single band at 52 kD , indicating that our preparations were free of contaminants from other antiproteases (Figure 2). On the other hand, higher molecular weight bands (in addition to the 52-kD band) were seen in SDS-PAGE blots of ONOO $^-$ -treated A1AT, consistent with self-aggregation (Figure 2). Western blotting studies of ONOO $^-$ (1 mM)-treated A1AT with a monoclonal anti-nitrotyrosine antibody and an alkaline phosphatase-conjugated monoclonal anti-dinitrophenyl group antibody (IgE) confirmed the presence of extensive nitration and oxidation (data not shown), in addition to the aggregation. In contrast to native A1AT, perfusion of ENaC-expressing oocytes with ONOO $^-$ -treated A1AT decreased I_{Na}

Figure 1. Time course of inward Na⁺ current (I_{Na}) and amiloride-sensitive current (I_{amil}) inhibition across epithelial Na⁺ channel (ENaC)-expressing oocytes by native or peroxynitrite (ONOO⁻)-treated α_1 -antitrypsin (A1AT). *Xenopus* oocytes were injected with human α , β and γ -hENaC cRNAs (8.4 ng each). (A) I_{Na} values were recorded 48 hours later while the membrane potential (V_m) was altered from -40 to -140 mV for 600 milliseconds, before and after addition of either normal (closed circles) or ONOO⁻-treated (open circles) A1AT (0.05 mg/ml = 1 μ M) into the perfusing solution (ND96). Amiloride (Amil.; 10 μ M) was added into the bath as indicated. Results are displayed of a typical experiment, which was repeated 15 times using three different batches of oocytes with identical results (see C for mean values). (B) The same as in (A), except that oocytes were perfused with an ND96 containing trypsin (1 μ M) and no amiloride at the indicated times. Results are shown of a typical experiment that was repeated at least 10 times. (C) I_{amil} -voltage relationships for oocytes incubated with either vehicle or A1AT (1 μ M) for 45 minutes and then placed in ND96 for the indicated times. I_{amil} values were calculated by subtracting currents remaining after addition of amiloride (10 μ M) from I_{Na} . Values are means ($\pm$ 1 SEM); n = 15 oocytes for each condition. Closed squares, vehicle; immediately after perfusion; open circles, 2 hours after A1AT perfusion; closed triangles, 4 hours after A1AT perfusion; open inverted triangles, 6 hours after A1AT perfusion. All values of A1AT-perfused oocytes are significantly different from control (ANOVA followed by the Bonferroni t test; P < 0.01).

Figure 2. SDS-PAGE of normal and ONOO⁻-treated A1AT. A1AT (2 μ g) was incubated with vehicle (lanes 1 and 2) or ONOO⁻ (1 mM; lanes 3 and 4) for 15 minutes. Proteins were then separated by SDS-PAGE and stained with GelCode. Molecular weights (kDa) of standards are shown. A typical blot is shown that was repeated at least three different times.

by less than 15% within 45 minutes (Figures 1B). Similarly, incubation of ENaC-expressing oocytes with ONOO⁻-treated A1AT for 2 hours decreased I_{Na} by less than 15% (Figure 3). These data indicate that intact antiprotease activity is necessary for the A1AT inhibition of ENaC.

Effects of A1AT on ENaC Single-Channel Activity

Recordings of single-channel activity in cell-attached patches of oocytes injected with α , β , and γ hENaC showed the presence of 4 pS channels with long open and closing times, characteristic of ENaC (Figure 4A). No change in channel

Figure 3. I_{Na} at -140 mV after exposure of ENaC-expressing oocytes to native and ONOO⁻-treated A1AT (1 μ M) for 2 hours. Box-whisker graph showing individual data points, boxes (25th to 75th percentiles of the data), whisker bars (5th to 95th percentiles), and means ($\pm$ 1 SEM) of I_{Na} just before and after perfusion with amiloride (Amil.; 10 μ M) for the indicated conditions. P < 0.01 compared with its corresponding control using the t test.

Figure 4. Single-channel currents recorded from cell-attached patches of ENaC-expressing oocytes. Oocytes were patched in the cell-attached mode, as described in the text, and currents were measured at a pipette holding potential of -100 mV ($V_{\text{holding}} = V_{\text{apical}} - P_{\text{pipette}}$); amplitude distribution histograms were generated as described in MATERIALS AND METHODS. Each trace represents 1-minute recordings for the indicated time after formation of the Giga seal. The close states are indicated by the *small bar* to the left of each record. (A and B) Control; (C and D) addition of A1AT ($1 \mu\text{M}$) in the bath at time zero; (E and F) A1AT ($1 \mu\text{M}$) was included in the pipette solution at the beginning of the patch. Results are shown from typical experiments reproduced at least 13 times with different oocytes from different batches.

activity (as assessed by NP_o , the product of number of channels multiplied by their open probability) of cell-attached patches was seen for up to 15–20 minutes after A1AT addition into the bath. However, after 30 minutes, NP_o increased from $0.15 (\pm 0.014; n = 6)$ (control) to $3.02 (\pm 0.52; n = 6)$ (A1AT; $X \pm 1$ SEM; $n =$ number of oocytes; $P = 0.0003$) (Figure 4B). The increase in NP_o was most likely the result of decreased intracellular Na^+ (due to the A1AT inhibition of ENaC), which, in turn, stimulated silent channels across the cell-attached patch, which was not exposed to A1AT (31). In contrast, addition of A1AT into the pipette decreased NP_o from the control value of $0.15 (\pm 0.014)$ to $0.077 (\pm 0.019)$ ($X \pm \text{SEM}; n = 6; P < 0.01$) (Figure 4B). These data indicate that the inhibitory effects of A1AT on ENaC require that A1AT must be present in its immediate vicinity.

A1AT Inhibits Whole-Cell Amiloride-Sensitive Na^+ Currents of Human Airway (H441) Cells

Results shown so far indicate that A1AT inhibits amiloride-sensitive currents across oocytes expressing human ENaC. To

assess the relevance of these findings in human lung epithelial cells, we patched H441 cells at the whole-cell mode and measured sodium currents (I_{Na}) before and after perfusion with $1 \mu\text{M}$ A1AT for 45 minutes. As shown in Figure 5, A1AT decreased H441 I_{Na} to the same extent as in *Xenopus* oocytes (Figure 1). Furthermore, trypsin restored a large fraction of the I_{Na} (Figure 5B). These results indicate that A1AT inhibits the activity of both heterologously expressed and native ENaC channels.

A1AT Inhibits the Activity of Membrane Proteases in *Xenopus* Oocytes and H441 Cells

In the next series of experiments, we tested the possibility that the A1AT inhibition of ENaC was due to inactivation of endogenous proteases in *Xenopus* oocytes needed to cleave, and thus activate, ENaC. We preincubated ENaC-expressing oocytes and H441 cells with A1AT for 45 minutes, then added the fluorogenic peptide, Boc-Gln-Ala-Arg-AMC-HCl, which is cleaved by a number of serine and cysteine proteases, and

Figure 5. A1AT decreases inward I_{Na} of human cells. H441 cells were patched in the whole-cell mode and held at -40 mV. A1AT (0.05 mg/ml; 1 μ M) or vehicle was then added in the recording chamber and I_{Na} values were then recorded while pulsing the V_m from -40 to -140 mV every 5 seconds for a 50-minute period. Amiloride (2 μ M) was added when steady-state I_{Na} values were obtained after addition of A1AT. (A) Typical time courses of I_{Na} while pulsing the V_m from -40 to -140 mV. (B) The same protocol as in (A) showing that addition of 1 μ M trypsin increased I_{Na} . (C) Current-voltage curves. Values are means (± 1 SEM; $n = 6$). Solid circles, vehicle; open inverted triangles, A1AT; solid triangles, amiloride (2 μ M) after vehicle. I_{Na} values of A1AT-treated oocytes were significantly different from the corresponding controls ($P < 0.01$).

measured fluorescence levels during the next 2 hours. As seen in Figure 6, there was a time-dependent increase of fluorescence levels when either oocytes or H441 cells were incubated with Boc-Gln-Ala-Arg-AMC-HCl, indicating the presence of active membrane-bound serine and cysteine proteases. Significantly lower fluorescence levels were seen when oocytes or H441 cells were preincubated with 1 μ M A1AT. Interestingly, this decrease in fluorescence was reversed when oocytes and H441 cell membranes were ruptured by vigorous mixing while continuously measuring the fluorescence. This indicates the presence of intracellular proteases that were not inactivated by A1AT. Finally, normal levels of fluorescence were observed when either oocytes or H441 cells were preincubated with ONOO $^-$ -treated A1AT. These data indicate that normal but not ONOO $^-$ -treated A1AT inactivated membrane-bound serine proteases.

A1AT Does Not Inhibit Amiloride Sensitive Currents in *Xenopus* Oocytes Expressing ENaC with Liddle Mutations

To further test the hypothesis that A1AT decreases ENaC activity by inactivating surface proteases necessary for its cleavage and activation, we tested the effects of A1AT on amiloride-sensitive currents of *Xenopus* oocytes injected with Liddle cRNAs. Liddle mutants have reduced binding for Nedd4-2 (32), a critical step leading to ubiquitination, internalization, and destruction by the proteasome and lysosome

systems (32). Oocytes microinjected with hENaC cRNAs with the Liddle mutations, expressed higher levels of I_{amil} values 48 hours after injection as compared with those injected with wild-type hENaC (Figure 7A; -9991.908 ± 823.667 nA versus -6852.675 ± 727.541 nA for Liddle versus wild-type hENaC; $n = 8$; $P = 0.01$). A1AT had no effect on I_{Na} of these oocytes (Figure 6A), in sharp contrast to what was observed in oocytes injected with wild-type hENaC cRNAs (Figure 1). Based on these findings, we considered the possibility that A1AT decreased wild-type ENaC by promoting ubiquitination. We were unable to detect increased levels of ubiquitinated ENaC after treatment of *Xenopus* oocytes with A1AT (data not shown). Furthermore, incubation of oocytes expressing wild-type ENaC with a well known proteasome inhibitor (MG-132; 100 nM for 24 hours or 4 μ M for 2 hours) delayed but did not alter the extent of A1AT inhibition of I_{Na} (Figure 7B). These findings argue against the possibility that A1AT enhanced ENaC ubiquitination. Instead, they suggest that Liddle ENaCs, because of their long residence time at the surface, were already cleaved before the incubation with A1AT, and did not require additional processing.

A1AT Decreases Surface ENaC Levels

Incubation of ENaC-expressing oocytes with A1AT did not alter total levels of γ -ENaC; however, 20 minutes after incubation, at a time when I_{Na} was decreased by at least 50%,

Figure 6. Measurements of oocyte and H441 cell protease activity after addition of A1AT. A total of 20 oocytes (A) or 10^5 H441 cells (B) were incubated with vehicle (solid squares), native (solid circles), or ONOO $^-$ -treated (1 mM; open squares) A1AT (1 μ M) for 45 minutes and then added to a cuvette containing Boc-Gln-Ala-Arg-AMC (50 μ M). Vertical arrow in A indicates the time that oocytes were ruptured by vigorous pipetting. Fluorescence was measured continuously for the next 60 minutes at 460 nm after excitation at 380 nm. Results are shown of typical experiments that were repeated three times.

Figure 7. (A) A1AT does not decrease currents in oocytes injected with Liddle ENaC. Continuous recording of inward I_{Na} by pulsing the V_m from -40 to -140 mV. In contrast to results obtained with wild-type ENaC, incubation of Liddle ENaC-injected oocytes with A1AT (0.05 mg/ml; 1 μ M) had no effect on I_{Na} . On the other hand, amiloride (10 μ M) decreased I_{Na} to zero. Results are shown of a typical experiment that was repeated nine times with identical results. (B) Inhibition of the proteasome system does not prevent the A1AT inhibition of ENaC. Mean values are displayed of I_{Na} of oocytes injected with wild-type ENaC and incubated with either MG-132 (red line, 0.1 μ M for 24 hours; orange line, 4 μ M for 2 hours) or vehicle (black line) before being perfused with A1AT (1 μ M). For purposes of clarity, SEs were not included.

surface levels of cleaved, mature γ -ENaC (75-kD band), assessed by Western blotting of biotinylated (surface) proteins, were decreased considerably (Figures 8A–8C). Both *in vitro* and *in vivo* studies have shown that γ -ENaC at the plasma membrane is expressed in two distinct pools: the mature, active

channels (~ 75 -kD band; Figure 9B) that had undergone proteolytic processing, and immature, noncleaved channels (~ 93 -kD band, Figure 9B), thought to be a reserve pool that can be activated by extracellular serine proteases (6, 33–35). Our results show that A1AT treatment decreased significantly the proteolytic cleavage and activation of surface γ -ENaC.

Native but Not ONOO⁻-Treated A1AT Inhibits AFC *In Vivo*

We and others have shown that anesthetized, ventilated mice clear a large fraction of intratracheally instilled fluid secondary to the active reabsorption of Na^+ ions (20, 36–38). The first step in the vectorial transport of Na^+ ions is their entry into the cytoplasm of alveolar epithelial cells through amiloride-sensitive channels down their electrochemical gradients (39, 40). As shown in Figure 9, AFC in normal C57BL/6 mice was approximately 30% of instillate per 30 minutes, and amiloride inhibited 50% of this value, in agreement with our previous findings in both C57BL/6 and BALB/c mice (20, 37). Addition of A1AT into the instilled fluid completely inhibited AFC to the same extent as amiloride. On the other hand, A1AT exposed to 1 mM ONOO⁻ had no effect on AFC (Figure 9).

DISCUSSION

We report, for the first time, that native but not ONOO⁻-treated A1AT (which lacks antiproteolytic activity) down-regulates ENaC activity in both *Xenopus* oocytes expressing α , β , γ hENaC and in a human airway cell line (H441) expressing both Na^+ -selective channels with conductance of roughly 4 pS (characteristic of ENaC) and nonselective 20 pS cation channels (18). In addition, we show that intratracheal instillation of native but not ONOO⁻-treated A1AT in anesthetized mice decrease amiloride-sensitive Na^+ -dependent AFC across their distal lung epithelium. Only 40% of the I_{amil} values being restored 6 hours after A1AT washout from the bathing medium indicates that a fraction of the inhibition was irreversible at least within this time frame. A1AT plasma levels in normal individuals are approximately 1.3 mg/ml, and only 5–10% of plasma A1AT levels are found in the interstitial fluid and in the alveolar space (41, 42). Thus, the concentration of A1AT used both in our *in vivo* and *in vitro* studies (0.05 mg/ml = 1 μ M) is close to its value in the mammalian epithelial lining fluid.

Figure 8. Incubation of ENaC-expressing oocytes with A1AT decreases surface but not total γ -ENaC levels. (A) Total γ -ENaC. (B) Surface γ -ENaC. Lane 1, water injected oocytes; lane 2, α,β,γ -hENaC-injected oocytes; lanes 3 and 4, α,β,γ -hENaC-injected oocytes exposed to 0.05 mg/ml A1AT for 20 and 40 minutes, respectively, before lysis. Results are shown of a typical experiment that was repeated three different times using three different batches of oocytes. For each lane, proteins from 5 (total) or 25 (surface) different oocytes were pooled. (C) Density values of cleaved (lightly shaded bars) and not-cleaved (darkly shaded bars) γ -ENaC bands shown in (B) using AlphaEaseFC 4.0 software (Alpha Innotech Corp., San Leandro, CA). Values shown are $X \pm SEM$; $n = 3$ (three distinct sets of oocytes were used).

Figure 9. A1AT decreases alveolar fluid clearance (AFC) *in vivo*. AFC was measured across the distal lung spaces of anesthetized and ventilated C57BL/6 mice 30 minutes after instillation of a solution containing 5% albumin and vehicle, A1AT (1 μ M), ONOO⁻-treated A1AT (1 μ M), or 1.5 mM amiloride. Values are means ($\pm$ SEM); numbers in parentheses are numbers of mice. * P < 0.05 and ** P < 0.01 compared with control.

The number of active ENaC channels present at the cell membrane is regulated by two major mechanisms: (1) by proteolysis, which converts inactive channels to active channels; and (2) by removal of ENaC from the surface. The former requires the presence of membrane-bound serine proteases; the latter involves ubiquitination, internalization, and degradation of surface ENaC (2). We found that A1AT does not inhibit amiloride-sensitive currents in *Xenopus* oocytes expressing ENaC bearing Liddle mutations. We were unable to detect increased levels of ubiquitinated ENaC after treatment of *Xenopus* oocytes with A1AT, and, furthermore, incubation of oocytes expressing wild-type ENaC with proteasome inhibitor (MG-132) delayed, but did not alter, the extent of A1AT inhibition of I_{Na} . Taken together, our results allow us to conclude that A1AT regulates ENaC activity via the inhibition of the CAPs.

CAPs are membrane-anchored proteins with extracellular serine protease domains (9). Matriptase (CAP3) is an autoactivating protease expressed in most epithelia, and plays a major role in the activation of prostasin (CAP1), the protease that is considered responsible for γ -ENaC cleavage and maturation at the cell surface, in addition to the role played by furin (1, 5, 9). CAP1, CAP2, and CAP3 mRNAs, as well as CAP1 protein, have been shown to be present in mouse alveolar epithelial cells (1). Herein, we demonstrate the presence of such proteases on cell surface of oocytes and H441 cells using a specific substrate that releases a fluorescent probe (AMC) indicative of its cleavage by a serine protease at an arginine residue, similar to the site of ENaC subunits cleavage. Our data did not allow us to definitively identify which proteases were inhibited by A1AT. To the best of our knowledge, A1AT inhibits neither prostasin nor furin. Because prostasin is synthesized as an inactive zymogen, requiring activation by an upstream protease (matriptase) (43), we propose that one mechanism by which A1AT inhibits ENaC activity is by inactivating matriptase. In support of this hypothesis, recently, Tseng and colleagues (44) isolated A1AT/matriptase complexes from human milk that are resistant to dissociation in boiling SDS, suggesting that A1AT

may bind to matriptase in an irreversible fashion. We also reported that native but not the inactivated A1AT inhibits the catalytic domain of matriptase with a second-order rate constant of $0.31 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ *in vitro* in an irreversible fashion (16). Other serine protease inhibitors, like aprotinin and nexin-1 (PN-1) have also been shown to inhibit the activity of matriptase, thereby decreasing ENaC currents.

It is important to point out that, previously, A1AT (a member of the SERPIN family, which lacks the Kunitz-type domain) was found to be ineffective in inhibiting Na^+ short-circuit currents across HBE cells mounted in Ussing chambers (10). A1AT (which does not inactivate furin) had also no effect on Na^+ equivalent short-circuit current of mouse cortical collecting duct cells (34). On the other hand, a variant of A1AT (α 1-PDX), which inhibits furin, significantly decreased the equivalent short-circuit current of cortical collecting duct (CCD) cells (34). In contrast, our data show that A1AT down-regulates both I_{amil} values *in vitro* and Na^+ -dependent AFC across the distal lung epithelia of anesthetized mice *in vivo*. Recently, Myerburg and colleagues (45) reported that protease inhibitor, PN-1, another member of the SERPIN family, inhibited Na^+ currents across human airway epithelial cells isolated from CF lungs (45). Protease inhibitor PN-1, like A1AT, inhibits matriptase (45). Thus protease inhibitors with or without a Kunitz domain may play an important role in the protease-antiprotease balance responsible for ENaC processing and activation. Currently, the reasons for the different effects of A1AT on ENaC activity among these studies and ours are not clear. It is possible that ENaC cleavage in HBE and CCD cells was independent of matriptase or another protease inhibited by A1AT.

Aprotinin inhibition of airway cell I_{Na} was reversed either by removal of aprotinin or by addition of excess exogenous proteases, such as trypsin and elastase (10). Trypsin and elastase are serine proteases, known to activate ENaC channels in oocytes, human bronchial cell lines, and rat alveolar epithelial cells (9, 46), either by activating near-silent channels that are already present in the membrane (28), or by activating signal transduction pathways (7, 47). In our experimental model, the effect of A1AT on ENaC in oocytes was not immediately reversible; only 40% of the initial current was restored 6 hours after A1AT washout from the bathing medium, indicating that a fraction of the inhibition was irreversible at least within this time frame. One possible explanation can be that A1AT builds a stable complex with membrane-bound proteases (such as matriptase), and, therefore, part of A1AT remains on the membrane after A1AT washout from the medium. We also found that the effect of A1AT can be overturned by addition 1 μ M trypsin to the bathing solution. Considering the rate at which I_{Na} is inhibited in the presence of A1AT (τ = 13 minutes), and the restitution of the current by trypsin, it is consistent with the notion that A1AT acts by inhibiting serine proteases needed for the activation of silent ENaC channels at the cell surface.

It is interesting to note that, although aprotinin has been shown to decrease amiloride-sensitive short-circuit currents across confluent monolayers of HBE cells (10), mouse alveolar epithelial cells (1), and A6 cells (27), it had no effect on baseline rat and mouse Na^+ -dependent AFC (although it prevented the terbutaline increase of AFC [1]). In contrast, A1AT decreased baseline AFC in both rats (47) and C57BL/6 mice (present data). One difference between the studies of Planes and colleagues (1) and ours is the baseline values of AFC in C57BL/6 mice. Planes and colleagues reported values of 17% of instilled volume per 30 minutes, whereas our AFC values (consistent with our previous work [20, 37, 48]) are closer to 30% of instilled volume per 30 minutes. In our experiments, A1AT decreased AFC to 12%, which is similar to the baseline value of Planes and colleagues (1).

The structural properties that allow A1AT to inhibit proteases are susceptible to post-translational modifications by oxidation, nitration, and polymerization (49, 50). Neutrophils and macrophages, which are capable of secreting high levels of oxidants at sites of inflammation, have been shown to oxidize two of the nine methionines in A1AT (358 and 351), leading to loss of anti-elastase activity (51). Similarly, exposure to ONOO⁻ was shown to result in loss of A1AT activity (29). Our data clearly show that ONOO⁻-treated A1AT does not decrease ENaC function, and suggest that its effects on ENaC are mediated via inactivation of a resident protease present on the cell membrane of oocytes, H441 cells, and on the apical membrane of lung epithelial cells *in vivo*. Increased levels of reactive oxygen nitrogen intermediates are present in the alveolar spaces of patients with a number of inflammatory diseases (52, 53), which may result in post-translational modifications (such as oxidation and nitration) of important lung proteins (52). Decreased levels of A1AT have also been observed in the lungs of animals exposed to cigarette smoke (54). Decreased levels of native A1AT may be beneficial in some diseases, such as acute lung injury, in which excess amounts of fluid may be present in the alveolar space, which can be cleared via osmotic forces generated by active, vectorial reabsorption of Na⁺ ions. On the other hand, decreased levels of A1AT in CF may contribute to the pathogenesis of the disease, providing the basis for A1AT administration.

In addition, at the cellular level, A1AT, independently of its protease inhibitory activity, was found to inhibit endotoxin (LPS)-stimulated TNF- α expression and release, and enhance IL-10 expression in human monocytes and endothelial cells by signaling through the antiinflammatory cAMP pathway, which is shared by a number of antiinflammatory drugs currently under development (55). Presently, A1AT is administered to patients with CF to restore protease-antiprotease balance and to reduce lung inflammation (56).

Taken together, the activation of channels by proteases at the apical membrane may be blocked by specific inhibitors, producing a time-dependent reduction in the number of active channels that results from internalization and degradation of already activated channels (27). Our data confirm previous findings on the importance of protease-antiprotease balance *in vitro*, and offers new insights as to the regulation of ENaC by A1AT, the most abundant circulating antiprotease *in vivo*. They also highlight a potential mechanism by which A1AT administration may prove beneficial in clinical situations in which increased ENaC activity may contribute to lung pathology.

Conflict of Interest Statement: S.M. and S.J. received a grant from Talecris Biotherapeutics, Inc., which manufactures Prolastin. S.M. was paid \$2,000 to attend the Sepracor Scientific Research Forum at the Windsor Court Hotel (New Orleans, LA), September 18–20, 2008 (travel costs were also paid for by Sepracor), received a grant from Sepracor, Inc. in the amount of \$120,127 (direct costs) for March 1, 2007 to February 28, 2008 to study “*In Vivo* Studies on Efficacy of Aerosolized Alpha1-Antitrypsin,” has a U.S. Provisional Patent Application (no. 61/052,414, “DPPC Formulates and Methods for Using” [filed May 12, 2008; inventors, E.I. Franses, S.H. Kim, Y. Park, and S.M.), and U.S. Provisional Patent Application (no. 60/573,558: “Methods for Using Pyrimidine Synthesis Inhibitors to Increase Airway Epithelial Cell Fluid Uptake” [filed May 21, 2004; inventors: I.C. Davis, W. Sullender, and S.M.), converted to International Patent Cooperation Treaty application (no. PCT/UC2005/017939); in May 2005, D.S. and S.J. received a grant from Talecris for 2006–2007 for \$150,000. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors are grateful to Dr. Peter Snyder, University of Iowa School of Medicine, for the generous gift of Liddle epithelial Na⁺ channel cRNAs, and to Ms. Teri Potter for editorial assistance in the preparation of this manuscript.

References

- Planes C, Leyvraz C, Uchida T, Angelova MA, Vuagniaux G, Hummler E, Matthay M, Clerici C, Rossier B. *In vitro* and *in vivo* regulation of transepithelial lung alveolar sodium transport by serine proteases. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L1099–L1109.
- Snyder PM. Minireview: regulation of epithelial Na⁺ channel trafficking. *Endocrinology* 2005;146:5079–5085.
- Rossier BC, Canessa CM, Schild L, Horisberger JD. Epithelial sodium channels. *Curr Opin Nephrol Hypertens* 1994;3:487–496.
- Matalon S, O’Brodoovich H. Sodium channels in alveolar epithelial cells: molecular characterization, biophysical properties, and physiological significance. *Annu Rev Physiol* 1999;61:627–661.
- Rossier BC. The epithelial sodium channel: activation by membrane-bound serine proteases. *Proc Am Thorac Soc* 2004;1:4–9.
- Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Poland PA, Harkleroad KL, Carattino MD, Kleyman TR. Maturation of the epithelial Na⁺ channel involves proteolytic processing of the alpha- and gamma-subunits. *J Biol Chem* 2003;278:37073–37082.
- Bengrine A, Li J, Hamm LL, Awayda MS. Indirect activation of the epithelial Na⁺ channel by trypsin. *J Biol Chem* 2007;282:26884–26896.
- Vogel R, Werle E, Zickgraf-Rudel G. Current aspects of kinin research. I. Potentiation and blocking of biological kinin activity. *Z Klin Chem Klin Biochem* 1970;8:177–185.
- Vallet V, Chraïbi A, Gaeggeler HP, Horisberger JD, Rossier BC. An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature* 1997;389:607–610.
- Bridges RJ, Newton BB, Pilewski JM, Devor DC, Poll CT, Hall RL. Na⁺ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L16–L23.
- Donaldson SH, Hirsh A, Li DC, Holloway G, Chao J, Boucher RC, Gabriel SE. Regulation of the epithelial sodium channel by serine proteases in human airways. *J Biol Chem* 2002;277:8338–8345.
- Fan B, Wu TD, Li W, Kirchhofer D. Identification of hepatocyte growth factor activator inhibitor-1B as a potential physiological inhibitor of prostasin. *J Biol Chem* 2005;280:34513–34520.
- Carrell RW, Lomas DA. Alpha1-antitrypsin deficiency—a model for conformational diseases. *N Engl J Med* 2002;346:45–53.
- Petrache I, Fijalkowska I, Medler TR, Skirball J, Cruz P, Zhen L, Petrache HI, Flotte TR, Tudor RM. Alpha-1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am J Pathol* 2006;169:1155–1166.
- Petrache I, Fijalkowska I, Zhen L, Medler TR, Brown E, Cruz P, Choe KH, Taraseviciene-Stewart L, Scerbavicius R, Shapiro L, et al. A novel antiapoptotic role for α_1 -antitrypsin in the prevention of pulmonary emphysema. *Am J Respir Crit Care Med* 2006;173:1222–1228.
- Janciauskiene S, Nita I, Subramaniam D, Li Q, Lancaster J Jr, Matalon S. α_1 -Antitrypsin inhibits the activity of the matriptase catalytic domain *in vitro*. *Am J Respir Cell Mol Biol* 2008;39:631–637.
- Hughey RP, Carattino MD, Kleyman TR. Role of proteolysis in the activation of epithelial sodium channels. *Curr Opin Nephrol Hypertens* 2007;16:444–450.
- Lazrak A, Matalon S. cAMP-induced changes of apical membrane potentials of confluent H441 monolayers. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L443–L450.
- Hardiman KM, Lindsey JR, Matalon S. Lack of amiloride-sensitive transport across alveolar and respiratory epithelium of iNOS(–/–) mice *in vivo*. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L722–L731.
- Hickman-Davis JM, Nicholas-Bevensee C, Davis IC, Ma HP, Davis GC, Bosworth CA, Matalon S. Reactive species mediate inhibition of alveolar type II sodium transport during mycoplasma infection. *Am J Respir Crit Care Med* 2006;173:334–344.
- Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990;87:1620–1624.
- Ji HL, Su XF, Kedar S, Li J, Barbry P, Smith PR, Matalon S, Benos DJ. Delta-subunit confers novel biophysical features to alpha beta gamma-human epithelial sodium channel (ENaC) via a physical interaction. *J Biol Chem* 2006;281:8233–8241.
- Chen L, Fuller CM, Kleyman TR, Matalon S. Mutations in the extracellular loop of alpha-rENaC alter sensitivity to amiloride and reactive species. *Am J Physiol Renal Physiol* 2004;286:F1202–F1208.
- Duvall MD, Zhu S, Fuller CM, Matalon S. Peroxynitrite inhibits amiloride-sensitive Na⁺ currents in *Xenopus* oocytes expressing $\alpha\beta\gamma$ ENaC. *Am J Physiol* 1998;274:C1417–C1423.
- Langlois AL, Berdiev B, Ji HL, Keyser K, Stanton BA, Benos DJ. Charged residues in the M2 region of alpha-hENaC play a role in channel conductance. *Am J Physiol Cell Physiol* 2000;278:C277–C291.

26. Davis IC, Lazarowski ER, Chen FP, Hickman-Davis JM, Sullender WM, Matalon S. Post-infection A77-1726 blocks pathophysiologic sequelae of respiratory syncytial virus infection. *Am J Respir Cell Mol Biol* 2007;37:379–386.
27. Adebamiro A, Cheng Y, Johnson JP, Bridges RJ. Endogenous protease activation of ENaC: effect of serine protease inhibition on ENaC single channel properties. *J Gen Physiol* 2005;126:339–352.
28. Chraïbi A, Vallet V, Firsov D, Hess SK, Horisberger JD. Protease modulation of the activity of the epithelial sodium channel expressed in *Xenopus* oocytes. *J Gen Physiol* 1998;111:127–138.
29. Moreno JJ, Pryor WA. Inactivation of alpha 1-proteinase inhibitor by peroxyntirite. *Chem Res Toxicol* 1992;5:425–431.
30. Pryor WA, Stone K. Oxidants in cigarette smoke: radicals, hydrogen peroxide, peroxyntirite, and peroxyntirite. *Ann NY Acad Sci* 1993; 686:12–27. (discussion 27–28).
31. Frindt G, Masilamani S, Knepper MA, Palmer LG. Activation of epithelial Na channels during short-term Na deprivation. *Am J Physiol Renal Physiol* 2001;280:F112–F118.
32. Knight KK, Olson DR, Zhou R, Snyder PM. Liddle's syndrome mutations increase Na⁺ transport through dual effects on epithelial Na⁺ channel surface expression and proteolytic cleavage. *Proc Natl Acad Sci USA* 2006;103:2805–2808.
33. Terasaki M, Runft LL, Hand AR. Changes in organization of the endoplasmic reticulum during *Xenopus* oocyte maturation and activation. *Mol Biol Cell* 2001;12:1103–1116.
34. Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP, Stockand JD, Kleyman TR. Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem* 2004;279:18111–18114.
35. Bruns JB, Carattino MD, Sheng S, Maarouf AB, Weisz OA, Pilewski JM, Hughey RP, Kleyman TR. Epithelial Na⁺ channels are fully activated by furin- and prostaticin-dependent release of an inhibitory peptide from the gamma-subunit. *J Biol Chem* 2007;282:6153–6160.
36. Matalon S, Hardiman KM, Jain L, Eaton DC, Kotlikoff M, Eu JP, Sun J, Meissner G, Stamler JS. Regulation of ion channel structure and function by reactive oxygen-nitrogen species. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L1184–L1189.
37. Davis IC, Lazarowski ER, Hickman-Davis JM, Fortenberry JA, Chen FP, Zhao X, Sorscher E, Graves LM, Sullender WM, Matalon S. Leflunomide prevents alveolar fluid clearance inhibition by respiratory syncytial virus. *Am J Respir Crit Care Med* 2006;173:673–682.
38. Matthay MA, Folkesson HG, Clerici C. Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiol Rev* 2002;82: 569–600.
39. Johnson MD, Bao HF, Helms MN, Chen XJ, Tigue Z, Jain L, Dobbs LG, Eaton DC. Functional ion channels in pulmonary alveolar type I cells support a role for type I cells in lung ion transport. *Proc Natl Acad Sci USA* 2006;103:4964–4969.
40. Yue G, Russell WJ, Benos DJ, Jackson RM, Olman MA, Matalon S. Increased expression and activity of sodium channels in alveolar type II cells of hyperoxic rats. *Proc Natl Acad Sci USA* 1995;92:8418–8422.
41. Gadek JE, Klein HG, Holland PV, Crystal RG. Replacement therapy of alpha 1-antitrypsin deficiency: reversal of protease-antiprotease imbalance within the alveolar structures of PiZ subjects. *J Clin Invest* 1981;68:1158–1165.
42. Rosenfeld MA, Ronald G, Crystal RG. Gene therapy for pulmonary diseases. *Pathol Biol (Paris)* 1993;41:677–680.
43. Andreassen D, Vuagniaux G, Fowler-Jaeger N, Hummler E, Rossier BC. Activation of epithelial sodium channels by mouse channel activating proteases (mCAP) expressed in *Xenopus* oocytes requires catalytic activity of mCAP3 and mCAP2 but not mCAP1. *J Am Soc Nephrol* 2006;17:968–976.
44. Tseng IC, Chou FP, Su SF, Oberst MD, Madayiputhiya N, Lee MS, Wang JK, Sloane DE, Johnson M, Lin CY. Purification from human milk of matriptase complexes with secreted serpins: mechanism for inhibition of matriptase other than HAI-1. *Am J Physiol Cell Physiol* 2008;295:C423–C431.
45. Myerburg MM, McKenna EE, Luke CJ, Frizzell RA, Kleyman TR, Pilewski JM. Prostaticin expression is regulated by airway surface liquid volume and is increased in cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L932–L941.
46. Caldwell RA, Boucher RC, Stutts MJ. Serine protease activation of near-silent epithelial Na⁺ channels. *Am J Physiol Cell Physiol* 2004; 286:C190–C194.
47. Swystun V, Chen L, Factor P, Siroky B, Bell PD, Matalon S. Apical trypsin increases ion transport and resistance by a phospholipase C-dependent rise of Ca²⁺. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L820–L830.
48. Davis IC, Xu A, Gao Z, Hickman-Davis JM, Factor P, Sullender WM, Matalon S. Respiratory syncytial virus induces insensitivity to beta-adrenergic agonists in mouse lung epithelium *in vivo*. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L281–L289.
49. Nita I, Hollander C, Westin U, Janciauskiene SM. Prolastin, a pharmaceutical preparation of purified human alpha1-antitrypsin, blocks endotoxin-mediated cytokine release. *Respir Res* 2005;6:12.
50. Hollander C, Westin U, Wallmark A, Piitulainen E, Sveger T, Janciauskiene SM. Plasma levels of alpha1-antichymotrypsin and secretory leukocyte proteinase inhibitor in healthy and chronic obstructive pulmonary disease (COPD) subjects with and without severe alpha1-antitrypsin deficiency. *BMC Pulm Med* 2007;7:1.
51. Taggart C, Cervantes-Laurean D, Kim G, McElvaney NG, Wehr N, Moss J, Levine RL. Oxidation of either methionine 351 or methionine 358 in alpha 1-antitrypsin causes loss of anti-neutrophil elastase activity. *J Biol Chem* 2000;275:27258–27265.
52. Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S. Increased levels of nitrate and surfactant protein A nitration in the pulmonary edema fluid of patients with acute lung injury. *Am J Respir Crit Care Med* 2001;163:166–172.
53. Sittipunt C, Steinberg KP, Ruzinski JT, Myles C, Zhu S, Goodman RB, Hudson LD, Matalon S, Martin TR. Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001;163:503–510.
54. Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, Belaouaj A. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am J Pathol* 2003;163:2329–2335.
55. Nita IM, Serapinas D, Janciauskiene SM. Alpha1-antitrypsin regulates CD14 expression and soluble CD14 levels in human monocytes *in vitro*. *Int J Biochem Cell Biol* 2007;39:1165–1176.
56. Griese M, Latzin P, Kappler M, Weckerle K, Heinzlmaier T, Bernhardt T, Hartl D. Alpha1-antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients. *Eur Respir J* 2007;29:240–250.