Acute Effects of Cigarette Smoke Extract on Alveolar Epithelial Sodium Channel Activity and Lung Fluid Clearance

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Cigarette smoke contains high levels of reactive species. Moreover, cigarette smoke can induce cellular production of oxidants. The purpose of this study was to determine the effect of cigarette smoke extract (CSE)-derived oxidants on epithelial sodium channel (ENaC) activity in alveolar type 1 (T1) and type 2 (T2) cells and to measure corresponding rates of fluid clearance in mice receiving a tracheal instillation of CSE. Single-channel patch clamp analysis of T1 and T2 cells demonstrate that CSE exposure increases ENaC activity (N_{po}), measured as the product of the number of channels (N) and a channels open probability (P_o), from 0.17 ± 0.07 to 0.34 ± 0.10 ($n = 9$; $P = 0.04$) in T1 cells. In T2 cells, CSE increased N_{po} from 0.08 ± 0.03 to 0.35 ± 0.10 ($n = 9$; $P = 0.02$). In both cell types, addition of tetramethylpiperidine and glutathione attenuated CSE-induced increases in ENaC N_{po}. Biotinylation and cycloheximide chase assays indicate that CSE-derived ROS increases channel activity, in part, by maintaining cell surface expression of the α -ENaC subunit. *In vivo* studies show that tracheal instillation of CSE promoted alveolar fluid clearance after 105 minutes compared with vehicle control ($n = 10$ /group; $P < 0.05$).

Keywords: lung injury; COPD; emphysema; *in vivo* imaging of lung fluid volume

Ion channels, such as the amiloride-sensitive epithelial sodium channel (ENaC), play an important role in health and disease (1, 2). ENaC is composed of α , β , and γ subunits arranged in a fixed stoichiometry. ENaC participates in maintaining appropriate salt and water balance by reabsorbing Na⁺ at the apical membrane, thereby creating an osmotic gradient that facilitates the reabsorption of fluid (3). ENaC channels can be further classified as highly specific cation (HSC) and nonspecific cation (NSC) channels based on specific measurements of conductance and amiloride sensitivity. HSC channels are highly selective for Na⁺ (Na⁺:K⁺ of $> 40:1$), whereas NSC channels are less selective (Na⁺:K⁺ of 1:1) (1). In the lung, HSC and NSC channel activity plays an important role in maintaining airway surface liquid volume within normal limits (1), but the mechanisms of activation remain unclear. In the current study, we examined the role of cigarette smoke-derived oxidants on lung ENaC activity. Cigarette smoking and cigarette smoke are the single greatest risk factors for the development of chronic obstructive pulmonary disease (COPD). The two main forms of COPD include chronic bronchitis and emphysema (4), with each affecting

CLINICAL RELEVANCE

The work informs our understanding of the acute effects of cigarette smoke on epithelial sodium channel activity and subsequently lung fluid balance. Inappropriate sodium reabsorption may play an important role in promoting disease development.

distinct regions of the lung. Chronic bronchitis predominantly affects the airways, whereas emphysema primarily affects the distal portion of the lungs responsible for effective gas exchange (the alveolus). Hogg and colleagues (5, 6) reported that obstruction of the small airways in COPD is associated with a thickening of the airway wall and is accompanied by airway remodeling and mucociliary dysfunction. The pathogenesis of chronic bronchitis is complex and remains unclear; however, it is clear that COPD can be attributed to cigarette smoking. Specifically, studies using human bronchial airway epithelial cells show that cigarette smoke inhibits cAMP-mediated chloride secretion, leading to dehydration of the airway surface liquid (7–9). A reduction in the height of the airway surface liquid may lead to a cystic fibrosis–like phenotype with impaired mucus clearance that increases susceptibility to chronic lung infections.

Less attention has been given to the effect of cigarette smoke on ENaC activity in the distal portion of the lung. Alveolar type 1 (T1) and type 2 (T2) cells comprise the alveolar epithelium where gas exchange occurs. We have recently shown that both cell types express functional HSC and NSC channels and that both cell types play an important role in lung fluid homeostasis (10–12). Although one of the major problems associated with emphysema can be attributed to loss of T1 and T2 cells, we hypothesize that, similar to airway cells, cigarette smoke extract (CSE) can alter ion transport processes in the distal lung and lead to COPD.

Amiloride-sensitive channels in airway and alveolar cells play an important role in maintaining lung fluid volumes within narrow limits. Fluid naturally accumulates in the airspace due to the hydrostatic pressure favoring water flow out of the pulmonary capillaries across the lung epithelium. As such, vectorial transport of salt and water out of the airspace by normal ENaC activity is critical in maintaining the appropriate amount of fluid on the airway surface. Hyperactive sodium reabsorption can lead to airway drying, infection, inflammation, and cell death (2, 13). Therefore, it is important to study the signal transduction pathways that regulate lung ENaC.

Because cigarette smoke contains many ROS and induces oxidative stress in the lung (14), we examined the role of cigarette smoke–derived ROS on lung ENaC. Previously, we have shown that ENaC is regulated by free radicals, such as superoxide (15) and H₂O₂ (2, 8, 16, 17–19). Therefore, we tested the hypothesis that cigarette smoke extract (CSE) exposure would increase ENaC activity in lung T1 and T2 cells via oxidant signaling.

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We performed single-channel patch clamp measurements from alveolar T1 and T2 cells accessed from lung slices. We also examined changes in X-ray density of mouse chest radiographs to determine the effect of cigarette smoke on lung fluid balance after tracheal instillation of CSE or vehicle control. Together, these studies allowed us to determine the effects of CSE exposure on HSC and NSC channel activity in the alveolar epithelium and verify that changes in single-channel activity translate into whole lung responses.

MATERIALS AND METHODS

Animals

Twelve-week-old female C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) for lung fluid clearance studies. Male Sprague Dawley rats (8–10 wk old) were purchased from Charles River (Wilmington, MA) for lung tissue slices and cell isolation. Animals had *ad libitum* access to water and standard chow. All procedures conformed to National Institutes of Health and institutional animal care and use guidelines.

Reagents

Unless stated otherwise, all reagents were purchased from Sigma Aldrich (St. Louis, MO).

Lung Slices and Primary Cell Isolation

All procedures were performed as previously described (10).

CSE Preparation

CSE (100%) was prepared using 1R5F research-grade cigarettes (University of Kentucky, Lexington, KY) in saline solution containing 96 mM NaCl, 3.4 mM KCl, 0.8 mM CaCl₂, 0.8 mM MgCl₂, and 10 mM HEPES (pH 7.4). The extract from one cigarette was collected into 10 ml of the saline solution using a vacuum syringe and smoking apparatus. Preparations of 100% CSE within absorbance values of 0.20 ± 0.2 (320 nm) were used, and 100% CSE was diluted in appropriate buffers.

Lung Fluid Clearance

Lung fluid clearance was performed as previously described (15, 17).

Electrophysiology

Single-channel patch clamp analysis was performed as previously described (15). CSE, TEMPO, and glutathione treatments are as indicated in RESULTS.

Western Blot

T2 cells were resuspended in DMEM-F12 medium (with 10% FBS, 2 mM L-glutamine, 20 U/ml of penicillin-streptomycin, 84 μ g gentamycin, 1 μ M

dexamethasone) and treated with 1% CSE diluted in DMEM-F12. After 1 hour of CSE treatment, Western blots were performed as described without deviation.

Biotinylation of Apical Membrane Proteins

Cells were treated with 1% CSE diluted in DMEM-F12 medium for 1 hour at room temperature. T2 cells were washed with PBS, and apical membrane proteins were biotinylated with 0.5 mg/ml of S-S biotin (Pierce, Rockford, IL) in borate buffer containing 85 mM NaCl, 4 mM KCl, and 15 mM Na₂B₄O₇ (pH 8.0) for 30 minutes at room temperature. Cells were washed three times in PBS, and biotinylation was quenched with DMEM supplemented with 10% horse serum and 125 mM lysine. Protein concentrations were determined using Bradford assay. Neutravidin beads (Pierce) were combined with 300 μ g biotinylated protein and incubated overnight at 4°C. Streptavidin-bound protein was collected in 1X-SDS sample buffer, heated to 95°C for 3 minutes, and separated by SDS-PAGE.

Cycloheximide Chase

Primary rat alveolar T2 cells were treated in 0.01% solution of cycloheximide alone or with 1% CSE $\pm$ 25 nM TEMPO. Protein was harvested at 0, 30, 60, and 120 minutes and immunoblotted for α -ENaC to determine the effect of CSE exposure on sodium channel degradation. Protein expression was normalized to β -actin; data are representative of three observations.

ROS Assays

Superoxide levels were quantified using dihydroethidium and expressed as percent of control, and H₂O₂ levels were determined from the supernatant of lung slices using Amplex Red (Invitrogen, Carlsbad, CA). Assays have been described previously (15, 17).

Statistics

Statistical analysis was determined by paired *t* test using Sigma Plot 10.1, and *P* values ≤ 0.05 were considered significant. Data are presented as means $\pm$ SE. Power analysis was performed using SAS 9.3.

RESULTS

CSE-Derived Oxidants

We established that CSE is a source of oxidants and that CSE can induce ROS production in alveolar cells. The level of ROS in alveolar T2 cells acutely exposed to CSE (< 15 min exposure) showed significantly greater levels of ROS compared with untreated control cells ($n = 3$; $P = 0.009$) (Figure 1A). Using a dose-response curve (data not shown), we determined the level of H₂O₂ after acute treatment of CSE to be approximately 1 to 2 μ M. However, after 4 hours of exposure to CSE, the level of H₂O₂ production increased to nearly 25 μ M ($n = 3$; $P = 0.0001$) in rat lung cells (Figure 1B). This robust increase in

Figure 1. Cigarette smoke extract (CSE)-derived reactive oxygen species (ROS). (A) Measurements of superoxide production using dihydroethidium show a significant increase in superoxide production by type 2 cells after <15 minutes of CSE exposure. ($n = 3$; $P = 0.009$). (B) Four hours of CSE exposure increased H₂O₂ production from lung tissue slices cells from approximately 2 to approximately 25 μ M ($n = 3$; $P = 0.0001$) as measured by Amplex Red.

measured ROS can only be attributed to cellular responses in the generation of ROS. The studies reported herein do not distinguish between CSE and cellular sources of ROS but rather examine the overall effect of CSE-derived oxidants on lung ENaC.

CSE Increases Epithelial Sodium Channel Activity via Oxidant Signaling in Alveolar Epithelial Cells

Figure 2A shows a representative trace of a cell-attached patch clamp recording (~ 20 min in duration) obtained from an alveolar T1 cell before and after CSE and TEMPO were applied to the extracellular bath as indicated. Alveolar T1 cells were accessed from rat lung, where a hemisection of the alveolar

lumen is exposed in a 250- μ m preparation of live lung tissue slices. Enlarged portions of the continuous trace are shown in Figures 2B, 2C, and 2D with point amplitude histograms of shorter segments, which together reveal channel properties. Figure 2B shows that before CSE treatment, HSC and NSC channels could be detected in the same cell-attached patch at similar frequencies. The HSC had a 0.15 pA opening at 10 mV ($-V_p$) potential, and the larger conducting NSC had approximately 0.4 pA openings. Chord conductance (γ) was calculated from -20 , -10 , and $+10$ mV ($-V_p$) holding potentials and indicates that HSC $\gamma = 4.2$ pS and NSC $\gamma = 12.1$ pS (data not shown). Figure 2C shows that CSE markedly increased net sodium reabsorption by activating the HSC and NSC channels, as indicated by the increase in downward deflections (which represents inward Na^+

Figure 2. CSE-induced oxidants regulate epithelial sodium channel (ENaC) activity in alveolar type 1 cells. (A) Continuous cell-attached patch recording of a primary alveolar type 1 cell accessed from a lung slice preparation. Arrow denotes closed state, and downward deflections represent inward Na^+ channel openings (-10 mV [$-V_p$] holding potential). Enlarged portions of the representative recording represent control (B), CSE-treated (C), and TEMPO-treated (D) conditions; associated point amplitude histograms show the frequency of channel openings. The highly selective cation (HSC) and nonselective cation (NSC) channels were observed with calculated conductances of 4.2 pS for HSC channels and 12.1 pS for NSC channels (data not shown). (E) Results from nine independent observations shown on dot-plot with y axis = ENaC activity (N = number of channels; P_o = open probability). CSE exposure increased NPo values from 0.19 ± 0.07 to 0.34 ± 0.10 ($P = 0.04$). Subsequent addition of TEMPO decreased NPo to 0.18 ± 0.10 ($P = 0.02$). There was not a significant difference between control conditions and CSE + TEMPO conditions ($P = 0.26$).

Figure 3. CSE-induced oxidants regulate ENaC activity in alveolar type 2 (T2) cells. (A) Continuous cell-attached patch clamp recording of a primary alveolar T2 cell. Arrow denotes closed state, and downward deflections represent Na^+ channel openings (-20 mV) ($-V_p$). Enlarged portions of representative recording represent control (B), CSE-treated (C), and TEMPO-treated (D) conditions; associated point amplitude histograms show frequency of channel openings. Calculated conductances were 4.2 pS for HSC and 12 pS for NSC (data not shown). (E) Results from nine independent observations shown on dot-plot with y axis = ENaC activity (N = number of channels; P_o = open probability). CSE exposure increased NPo values from 0.09 ± 0.03 to 0.35 ± 0.10 ($P = 0.02$); addition of TEMPO decreased NPo to 0.06 ± 0.02 ($P = 0.05$). There was no significant difference between control conditions and CSE + TEMPO ($P = 0.41$). (F) Continual cell-attached patch clamp analysis where TEMPO was added before CSE application, with the “##” symbols indicating breaks in continuous patch clamp recording. Pretreating cells with TEMPO attenuates CSE-induced increases in ENaC activity in T2 cells.

current) away from the closed state of the channel. Point amplitude histograms show multiple channel openings occurring with greater frequency after CSE exposure. Figure 2D shows an enlarged portion of the same cell-attached patch after TEMPO (a superoxide dismutase mimetic) treatment; the point amplitude histogram shows significant reduction in NSC and HSC ENaC activity. Figure 2E summarizes the effect of CSE and CSE+TEMPO on ENaC activity from six independent observations of T1 cells accessed from lung slice preparations. CSE increased ENaC activity, measured as the product of the number of channels and a channel's open probability (NPo), from 0.17 ± 0.07 to 0.34 ± 0.10 pA ($P = 0.04$ using paired t test analysis). Subsequent treatment with TEMPO decreased ENaC activity to 0.17 ± 0.10 pA ($P = 0.02$; paired t test analysis). *Post hoc* power analysis indicated that the sample size was adequate.

Similarly, Figure 3A shows a continual cell-attached patch clamp recording performed in an alveolar T2 cell before and after CSE and TEMPO were applied to the extracellular bath as indicated. Enlarged portions of the trace, shown alongside the point amplitude histograms of the respective segments (Figures 3B–3D), demonstrate that CSE increases channel activity (as indicated by downward deflections away from the closed state and in the point amplitude histograms) in an ROS-dependent manner because TEMPO sequesters ROS and decreases channel activity. CSE increased ENaC activity (NPo) from 0.08 ± 0.03 to 0.35 ± 0.10 pA ($P < 0.05$ (Figure 3E). Subsequent treatment with TEMPO decreased ENaC activity to 0.06 ± 0.04 pA ($P < 0.05$). *Post hoc* power analysis indicated that the sample size was adequate.

Figure 3F further indicates that CSE-induced oxidants regulate ENaC. We pretreated alveolar T2 cells with TEMPO before adding CSE (the inverse of Figures 3A–3D). A representative trace using this experimental approach (Figure 3F) shows the closing and opening of a 0.25 pA channel at a slightly depolarizing potential of -10 mV ($-V_p$). In the same cell-attached patch recording, application of TEMPO to the cell bath decreased ENaC activity, as we have reported previously (10, 15), and subsequent treatment with CSE abrogated channel activity by 6 minutes. The presence of TEMPO, compared with the same time course of drug action observed in Figures 2 and 3 (performed in T1 and T2 cells, respectively), also decreased channel activity after more than 6 minutes of CSE exposure in T2 cells.

To verify that CSE exposure increased ENaC, we performed amiloride sensitivity studies using alveolar T2 cells. Amiloride (10 μM) was back-filled into the pipette and allowed to diffuse down the tip to occlude the channel opening (Figure 4A). Because amiloride blocks the outer pore of sodium channels with specificity, we are able to show that CSE alters ENaC activity. Before amiloride's diffusion to the tip of the electrode (< 10 min), downward deflections from the closed state (Figure 4A, arrows) in the cell-attached patch is indicative of inward sodium current. ENaC activity was abrogated with amiloride, thereby inhibiting CSE-induced ENaC activity (Figure 4A). Channel activity did not resume when hyperpolarizing potentials were

applied (Figures 4B and 4C), indicating that CSE does not stimulate voltage-gated channels. Last, we quantified mean dwell time before and after complete amiloride diffusion to the electrode tip (near 10 min and in the presence of CSE). A significant reduction in channel dwell time (from $3,323 \pm 490$ ms to $1,550 \pm 517$ ms; $P = 0.03$) suggests that CSE activates amiloride-sensitive epithelial sodium channels in the lung. *Post hoc* power analysis indicated that the sample size was adequate.

We performed additional studies using glutathione (GSH) as an alternative antioxidant for TEMPO. Glutathione is a natural antioxidant, produced by cells in an ATP-dependent reaction (involving γ -glutamylcysteine and glycine), and has been shown to protect against oxidative injury (20). To provide further support that CSE-induced oxidants regulate ENaC, we pretreated alveolar T2 cells with 400 μM GSH and assessed for changes in ENaC activity after a CSE challenge (Figure 5D). Pretreatment with 400 μM GSH abrogated ENaC's response to CSE ($n = 9$) (Figure 5E). These data suggest that CSE-derived oxidants regulate ENaC.

CSE-Derived ROS Increases Surface Expression of α -ENaC and Prevents Subunit Degradation

CSE treatment increases cell surface expression of α -ENaC, which represents one possible mechanism for the observed increase in ENaC NPo after CSE exposure (Figure 6A). Densitometric evaluation of biotinylated α -ENaC shows that CSE exposure significantly increased cell surface expression of α -ENaC. Sequestering ROS with TEMPO in the presence of CSE significantly reduced surface expression α -ENaC. This implies that ROS plays an important role in cell surface expression of α -ENaC. The absence of β -tubulin detection indicates that only cell surface protein was evaluated in the biotinylation assays. Figure 6A also shows that CSE significantly increases α -ENaC subunit expression using whole T2 cell lysate (normalized to β -tubulin expression and expressed relative to control). Similar to the biotinylation assays, TEMPO attenuated the CSE-induced increase in α -ENaC protein obtained from T2 cell homogenate.

Cycloheximide chase assays indicate that CSE prevents sodium channel subunit degradation (Figure 6B). The mature ENaC (~ 65 kD protein) at the cell surface is an unstable protein with a reported half-life of approximately 40 to 120 minutes (21). In the presence of a protein synthesis inhibitor (cycloheximide), however, CSE-derived ROS attenuated α -ENaC degradation (Figure 6B).

CSE Promotes Alveolar Fluid Clearance and Increases Surface Expression of α -ENaC

We assessed the alveolar fluid clearance in mice receiving a tracheal instillation of CSE compared with vehicle control (Figure 7). CSE instillation (Figure 7, *closed squares*) promoted alveolar fluid clearance in mice, which differed significantly compared with vehicle control-treated mice (Figure 7, *open circles*). The observed effect of CSE on alveolar fluid balance was observed

Figure 4. Amiloride blocks CSE-induced increases in ENaC activity. (A) Representative single-channel patch clamp recording of a primary alveolar T2 cells in the cell-attached configuration with 10 μ M of amiloride back-filled in the microelectrode. Arrow denotes the closed state, and downward deflections represent channel openings (0 mV [-Vp]). Enlarged portions show channel recording after 3 and 10 minutes of exposure to CSE, respectively. (B and C) Cell-attached patch clamp recording at hyperpolarizing potentials (-20, -40 mV [-Vp], respectively) show lack

of channel activity. (D) Dot plot graph showing a reduction in mean dwell time before and after diffusion of amiloride to the tip of the electrode with CSE present ($n = 3$ independent observations), $P = 0.03$.

beginning at 105 minutes and was sustained throughout the 240-minute acquisition period.

DISCUSSION

In the current study, we show that CSE regulates lung ENaC via oxidant signaling, affecting alveolar fluid balance. In addition, CSE exposure increases HSC and NSC ENaC activity in alveolar T1 and T2 cells. Work from our group has previously shown that ROS regulate ENaC activity, and, because cigarette smoke contains many free radicals (22–24), we hypothesized that CSE-induced oxidants would increase ENaC activity in alveolar T1 and T2 cells. To demonstrate that ROS generated from exposure to CSE regulate ENaC, we performed patch clamp experiments in the setting of CSE with TEMPO, a superoxide dismutase

mimetic, or in the presence of glutathione, an antioxidant. By inhibiting ROS, ENaC activity was significantly reduced, indicating that cigarette smoke-derived ROS play a critical role in the regulation of lung ENaC. Furthermore, we show that CSE increases surface expression of α -ENaC while inhibiting protein degradation, suggesting that cigarette smoke may contribute to inappropriate Na^+ reabsorption, thereby affecting lung fluid balance.

The effect of cigarette smoke on ion transport in the airways has been reported using different methods for cigarette smoke exposure. In the current study we used aqueous CSE, a water-soluble extract from filtered cigarettes, for *in vitro* and *in vivo* use. Consequently, our CSE preparations did not contain the volatile fraction of cigarette smoke that is known to be cytotoxic to alveolar T2 cells and is not required for ion transport, as has

Figure 5. Glutathione (GSH) attenuates CSE-induced ENaC activity. (A) Representative trace obtained from an isolated rat alveolar T2 cell that has been pretreated with 400 μ M GSH before and after the addition of CSE. Lower panels are excerpts taken during control and CSE treatment, respectively. (B) Results from eight independent observations shown on dot-plot, with y axis = ENaC open probability (Po). In the presence of 400 μ M GSH, ENaC Po was unaffected (from 0.24 ± 0.05 to 0.26 ± 0.09 ; $P = 0.46$) by the addition of CSE.

Figure 6. CSE exposure increases surface ENaC expression. (A) The upper panel is a representative Western blot of biotinylated α -ENaC obtained from T2 cells. Cells were treated with vehicle control, 1% CSE, TEMPO, or CSE + TEMPO. Negative β -tubulin demonstrates that intracellular proteins were not labeled with biotin ($n = 4$). Lower panel: Alveolar T2 cell lysate immunoblotted for α -ENaC with quantification of blots ($n = 4$). (B) Cycloheximide (CHX) chase performed in the presence of 1% CSE or 1% CSE with 25 nM TEMPO. Representative immunoblots of α -ENaC and β -actin loading controls are provided. Averaged results from three independent observations normalized for β -actin are shown on y-axis, and the x-axis represents ENaC half-life (min). Closed circles: CSE + CHX; closed triangles: CSE + TEMPO + CHX; closed squares: CHX alone. * $P < 0.05$.

been previously described (25). The pH of our 1% CSE solution was 7.4 ± 0.01 . Moreover, our studies included single-channel evaluation of the effect of CSE on Na^+ transport in addition to *in vivo* evaluation of alveolar fluid clearance performed in freely breathing anesthetized mice. Prior studies describing cigarette smoke-(in)activation of channels used short-circuit current measurements, which may inadvertently include bioelectrical changes in cation and anion transporters and/or channels across a monolayer.

Most studies addressing responses to cigarette smoke focus on long-term exposure, and little attention has been given to its acute effects. As a result, our understanding of the immediate

effects of cigarette smoke is limited. Additionally, it is imperative to establish the effect of cigarette smoke on ENaC activity before long-term studies can be initiated. For these reasons, we evaluated the acute effects of CSE exposure.

Single-Channel Analysis of the Effect of CSE on Ion Transport

Clunes and colleagues (8) described no change in ENaC protein expression in human bronchial epithelial cells exposed to cigarette smoke for 10 minutes. Contrary to this finding, we show that CSE increases the surface expression of α -ENaC after 60

Figure 7. CSE exposure promotes alveolar fluid clearance *in vivo*. Line graph depicting changes in lung fluid clearance in mice receiving a tracheal challenge of saline or CSE (y-axis = lung fluid clearance $[I-I_0]$), where I represents fluid volume at a respective point in time and I_0 is fluid volume at the first X-ray exposure. More positive values represent greater fluid clearance ($n = 10$ per group).

minutes of exposure (Figure 6A) and that CSE inhibits protein degradation (Figure 6B). The time course of these changes is in line with our *in vivo* experiments (Figure 7) in which instillation of CSE promoted alveolar fluid clearance after approximately 105 minutes. Point amplitude histogram data from Figures 2C and 3C shows that the change in lung fluid volume is attributed to activation of HSC and NSC channels. Despite the differences in selectivity and conductances of HSC and NSC channels, prolonged unidirectional transport of Na^+ ions via both channel types would lead to severe dehydration of airway surface liquid volume. The molecular identity (e.g., subunit composition) of HSC and NSC channels has not been resolved. However, the biophysical properties of HSC channels are identical to channel activity measured in α , β , and γ subunits heterologously expressed. Additional expression studies and siRNA knockdown studies indicate that NSC channels may be composed of the α -ENaC subunit alone (reviewed Ref. 1). Our studies show that the 65-kD mature and the cleaved form of α -ENaC, which plays a role in HSC- and NSC-mediated changes in transport capacity, was indeed increased after acute CSE exposure (18). Based on this new observation of HSC and NSC activation by CSE exposure, we expect the acute effects of cigarette smoke-induced lung injury to be more severe than, for example, acute LPS-mediated injury because of differences in channel-mediated responses. We recently reported that acute 1 mg/ml LPS treatments resulted in a decrease in HSC activity concurrent with increases in NSC activity (17).

The precise mechanism responsible for CSE-induced ENaC activity requires further investigation. However, recent work from the Tarran laboratory has shown that cigarette smoke leads to cystic fibrosis transmembrane regulator (CFTR) internalization and a decrease in cAMP-mediated chloride secretion, which culminates in dehydration of the airway surface liquid (ASL) (7). The ASL is responsible for trapping inhaled particles and pathogens, and ASL depth is reduced in cystic fibrosis. Furthermore, cigarette smoke exposure dehydrates the ASL to comparable levels observed in cystic fibrosis, suggesting that inappropriate ion transport plays a critical role in obstructive lung disease. In the current study, instillation of CSE leads to increased fluid absorption, supporting our electrophysiological data. The functional outcome of CSE exposure, as reported with airway epithelia, is excessive reabsorption of water. Collectively, these data suggest that appropriate salt and water balance is vital to maintaining a healthy airway and alveolar epithelium. Conversely,

inappropriate ion channel activity would be expected to lead to lung disease, and studies show that mice overexpressing β -ENaC spontaneously developed emphysema and chronic bronchitis (26).

Cigarette Smoke Regulates CFTR and ENaC Function

It has been shown that CSE inhibits chloride secretion in human bronchial epithelial cells (9, 27, 28) and induces CFTR internalization to promote airway surface liquid dehydration (8). The aforementioned studies, together with our current observation that CSE activates ENaC activity in a ROS-dependent manner, argue for a causal connection for CFTR regulation of lung ENaC, an area of molecular research that has been widely investigated (see Ref. 29 for current perspective). Because a certain threshold in CFTR function is believed to be required for maintaining normal Na^+ reabsorption into the cell, cigarette smoke inhibition of CFTR expression at the gene, protein, and functional levels (7) may lead to changes in the resting membrane potential that favor hyperactive Na^+ uptake. Johnson and colleagues (12) have reported functional expression of CFTR and ENaC in alveolar epithelial T1 cells, which make up more than 95% of the alveolar surface area, and it is an intriguing possibility that CSE also indirectly regulates ENaC activity via impairment of CFTR function.

Oxidative Stress and COPD

Cigarette smoke causes COPD, but there is no consensus on how, and only α_1 -antitrypsin deficiency has been proven to result in emphysema (and ultimately COPD). The small airways, specifically the terminal bronchioles, appear to be the site of disease origin in COPD (6), and numerous studies describe an increase in immune cells in the small airways (30, 31). Studies show that all smokers develop inflammation in the small airways; however, only a small percentage of smokers develop COPD (32). ROS production increases during the inflammatory response and oxidative stress ensues. The effect of inflammation and oxidative stress on ENaC activity, putative or otherwise, is unclear and has limited our understanding of the role of ENaC in the pathogenesis of obstructive lung disease.

Inappropriate Na^+ reabsorption clearly results in cystic fibrosis and predominantly affects the airways. Mall and colleagues (26) showed that mice overexpressing β -ENaC spontaneously developed emphysema and chronic bronchitis. A perplexing issue with the pathogenesis of COPD is that tissue rarefaction in emphysema and tissue fibrosis in airways occur simultaneously and in close proximity to one another in the setting of ongoing inflammation. β -ENaC overexpressing mice develop emphysema with enlarged distal airspaces and increased lung compliance (26). There are several potential mechanisms through which altered Na^+ transport could contribute to emphysema formation. First, cigarette smoking increases mucus production and affects ASL hydration, which could lead to mucus plugging, causing mechanical overdistention of the distal airspaces. Second, a reduction in the volume of the epithelial lining fluid in the alveolus may limit the fluidity of antioxidants in the lining fluid, decreasing their distribution and increasing the potential for inhaled oxidants to cause necrosis and apoptosis. Under both conditions, a better understanding of sodium channel regulation could mitigate lung injury. Third, oxidative stress from chronic inflammation, which may occur after chronic channel hyperactivity, may result in a protease-antiprotease imbalance, leading to proteolytic degradation of alveolar structures. Because TEMPO and GSH attenuated ENaC activity, our findings indicate that antioxidant therapies could be further evaluated as possible therapeutics for COPD.

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