Redox Regulation of Epithelial Sodium Channels Examined in Alveolar Type 1 and 2 Cells Patch-clamped in Lung Slice Tissue*

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The alveolar surface of the lung is lined by alveolar type 1 (AT1) and type 2 (AT2) cells. Using single channel patch clamp analysis in lung slice preparations, we are able to uniquely study AT1 and AT2 cells separately from intact lung. We report for the first time the Na+ transport properties of type 2 cells accessed in live lung tissue (as we have done in type 1 cells). Type 2 cells in lung tissue slices express both highly selective cation and non-selective cation channels with average conductances of 8.8 ± 3.2 and 22.5 ± 6.3 picosiemens, respectively. Anion channels with 10-picosiemens conductance are also present in the apical membrane of type 2 cells. Our lung slice studies importantly verify the use of cultured cell model systems commonly used in lung epithelial sodium channel (ENaC) studies. Furthermore, we identify novel functional differences between the cells that make up the alveolar epithelium. One important difference is that exposure to the nitric oxide (NO) donor, PAPA-NONOate (1.5 μM), significantly decreases average ENaC NPo in type 2 cells (from 1.38 ± 0.26 to 0.82 ± 0.16; p < 0.05 and n = 18) but failed to alter ENaC activity in alveolar type 1 cells. Elevating endogenous superoxide (O2•-) levels with Ethiolat, a superoxide dismutase inhibitor, prevented NO inhibition of ENaC activity in type 2 cells, supporting the novel hypothesis that O2•- and NO signaling plays an important role in maintaining lung fluid balance.

The alveolar epithelium is responsible for maintaining effective gas exchange and is composed of two morphologically distinct types of cells referred to as type 1 (AT1) and type 2 (AT2) cells. AT1 cells are believed to be terminally differentiated squamous cells and cover >95% of the alveolar surface with extensive flattened processes. In the past type 1 cells have been thought to be predominantly responsible for gas exchange and possibly water permeability. Our research efforts, however, reveal that type 1 cells play a more complex role in the alveoli. Type 2 cells are easily discernable from the long, flask-shaped type 1 cells in the alveolar epithelium because they tend to be located at the corners of the alveoli (covering only 2–5% of the alveolar surface) and are cuboidally shaped. In addition to contributing to net fluid transport, type 2 cells are responsible for surfactant synthesis (14) and are considered to be progenitors of AT1 cells (3, 6, 7, 11). To date the biophysical properties of type 2 cells have only been studied in cultured cell models. Furthermore, the mechanisms by which Na+ transport is regulated when both cell types are present in the same preparation is unclear.

Together the two cell types (AT1 and AT2 cells) provide an effective barrier against leakage of water and solutes into the airspace by maintaining a thin fluid layer on the luminal surface of the alveoli, promoting proper gas exchange. The thickness of the fluid layer must be maintained within very narrow limits to allow for proper oxygenation of the blood. We and others have shown that epithelial sodium channels play a critical role in maintaining lung fluid clearance under both normal conditions and in the event of lung injury. For instance, α-ENaC subunit knock-out mice die within 40 h of birth due to an inability to clear lung fluid (21), highlighting the importance of normal transepithelial Na+ transport in the neonatal lung. Moreover, we have shown that therapeutic agents, such as dopamine (18, 19), nitric oxide (22), and β-adrenergic agonists (5) (all commonly used to treat respiratory illnesses) have significant effects on lung ENaC function. Hence, a better understanding of lung ENaC regulation will lead to a greater understanding of how the alveoli maintain a clear breathing space.

In addition to actively transporting salt, AT1 and AT2 cells must be able to sense and immediately respond to changes in oxygen tension as well as harmful pollutants such as reactive oxygen and nitrogen species in inspired air. Therefore, it is particularly important to understand redox signaling in the alveoli, especially as it relates to normal ENaC function. Several studies have indeed suggested that ENaC activity may be regulated by changes in oxygen tension or by the associated increase in the production of reactive oxygen species (such as the superoxide anion, O2•-) after an increase in oxidative metabolism and mitochondrial activity. For example, at birth an increase in O2 tension contributes to increased Na+ reabsorption, which helps clear the newborn lung of excess fluid (for review, see Ref. 12). O’Brodovich and co-workers (29) and Matalon co-workers (31)
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have shown experimentally that maintaining rat fetal distal lung cells in high (20%) O₂ concentrations increased ENaC mRNA (29) and protein (31) expression. In fetal lung cells, a switch from low PO₂ to high PO₂ environments increases amiloride-sensitive short-circuit current and also increases total ENaC protein expression (1, 29, 31). Conversely, in adult rat alveolar type 2 cells, hypoxic culture conditions decreases amiloride-sensitive ²²Na⁺ influx as well as α,β- and γ-ENaC mRNA expression and α-ENaC protein levels (28). Thus, oxygen signaling is important in ENaC regulation; however, it remains unclear whether the regulation results from direct changes in PO₂ or the associated increases in O₂ production after elevated oxygen tension.

In our present study we successfully made single channel recordings from alveolar type 2 cells from live lung tissue (as we have recently done in type 1 cells) using cell-attached patch clamp to further investigate redox signaling in the alveoli. In addition to allowing us to make comparisons between ENaC regulatory processes in AT1 and AT2 cells, this novel approach also allows us to validate the standard use of cultured cells as models for type 1 and 2 cell transport properties. By examining the individual transport properties of each cell type as would naturally occur in tissue slices, we gain a better understanding of how type 1 and 2 cells work together in the alveoli to regulate net ion transport and balance lung fluid levels. Our results indicate that type 1 and 2 cells share similar biophysical properties yet display distinctly different cellular responses to reactive oxygen and nitrogen species.

METHODS AND MATERIALS

Lung Tissue Preparation—We have recently recorded single channel activity from live rat lung tissue (19). Using similar methodologies, we have characterized the single channel properties and redox response of alveolar type 1 and 2 cells in slices of rat lung tissue. Briefly, we housed male Sprague-Dawley rats with access to standard rat diet and water ad libitum. Between weeks 8 and 12, animals were anesthetized and killed for experimentation in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. All animal protocols conform to National Institutes of Health animal care and use guidelines and were approved by Emory University IACUC. After lung perfusion via the pulmonary artery with 75 ml of PBS, 2% low melting point agarose in PBS (kept warm in a 35 °C water bath) was intratracheally instilled into the lungs to expand airspaces and to provide support for the tissue during the slicing process. Excised lungs were removed en bloc and iced to solidify the agarose before mounting onto a vibratory microtome (model VT1000S, Leica Microsystems). The Vibratome blade was set to high frequency, slow forward advancement, and 250–300 μm for lung slice preparations. Immediately after the lung slice procedure, tissue for patch clamp recordings was placed in 50:50 ice-cold Dulbecco’s modified Eagle’s medium/F-12 (containing 10% fetal bovine serum, 2 mM l-glutamine, 1 mM dexamethasone, 84 μM gentamicin, and 20 units/ml penicillin-streptomycin). Lung slices were treated and patched within 6 h of the initial tissue preparation.

Single-channel Patch Clamp Analysis of Na⁺ Channels in Alveolar Cells—Lung slices were thoroughly washed in patch clamp solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4. Alveolar epithelial cells on the top surface of lung tissue slices were brought into focus using a Nikon inverted microscope with Hoffman modulation contrast under a 40× objective. Gigohm seals were formed on type 1 or 2 cells using a fire-polished glass microelectrode back-filled with patch solution, and lung slices were kept immersed in the same patch solution. An Axopatch 1-D (Molecular Devices) amplifier interfaced through an analog-to-digital board to a personal computer collected single-channel data. Channel currents were recorded at 5 kHz and filtered at 1 kHz with a low-pass Bessel filter.

Fluorescent Microscopy—Alveolar type 2 cells were positively identified by labeling lung slice preparations either with surfactant protein A-specific antibody (purchased from Chemicon, Billerica, MA), rat type II antibody developed by Dr. Leland Dobbs (10), or LysoTracker Red (available commercially from Invitrogen), which recognizes the lamellar bodies in type 2 cells that are not present in alveolar type 1 cells (as described in Refs. (13, 17, 32)). When used to label AT2 cells for single-channel recording purposes, LysoTracker Red was diluted 1:1000 directly into the patch solution in which the lung tissue was immersed. For immunolocalization of type 2 cells, lung slices were fixed with 1 μg/ml Ethiolat, a cell-permeable superoxide dismutase inhibitor purchased from Sigma Aldrich.

Measurement of O₂ Release—Dihydroethidium (DHE; purchased from Invitrogen-Molecular Probes) is a fluorescent probe that intercalates into DNA and has an excitation wave-
length of 520 nm and an emission of 610 nm. We have recently established that 2-hydroxyethidium production from DHE can be used as a quantitative measure of O$_2^-$ production in epithelial monolayers (33). Additionally, other groups have shown that DHE fluorescence can be used to measure changes in intracellular O$_2^-$ levels in cultured cells and frozen tissue sections (8, 27). In this study alveolar type 2 cells were isolated using methodologies described in Dobbs et al. (9) and Jain et al. (24) and maintained in culture for up to 7 days in Dulbecco’s modified Eagle’s medium/F-12-50/50 media with 10% fetal bovine serum, 2 mM L-glutamine, 20 units/ml penicillin-streptomycin, 84 µM gentamycin, 1 µM dexamethasone, and 10 ng/ml keratinocyte growth factor/fibroblast growth factor-7 growth factors for O$_2^-$ measurements. Before O$_2^-$ levels were measured, cells were rinsed with PBS and then incubated with 2 µM DHE in PBS solution for 30 min in a light-protected humidified 5% CO$_2$ chamber maintained at 37 °C. After DHE labeling, cells were fixed in 4% paraformaldehyde and sealed between a glass slide and coverslip with Vectashield (Vector Laboratories) mounting medium. DHE fluorescence was detected and quantified with a Zeiss LSM 510 NLO META laser scanning confocal microscope and compatible LSM 5 Image Browser software.

**Western Blot Analysis**—Standard Western blot analysis was used to demonstrate that alveolar type 2 cells cultured on plastic begin to express proteins that are specific for type 1 cells as early as 72 h in culture. Briefly, protein lysate was electrophoresed on a 7.5% acrylamide gel under denaturing conditions and then transferred to Protran nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in TBST buffer (10 mM Tris, pH 7.5, 70 mM NaCl, and 0.1% Tween) with 5% dry milk and then incubated with rat type 1-40 specific antibody (obtained from Dr. Leland Dobbs (11) and used at 1:100-fold dilution) for 1 h at room temperature. IgG-alkaline phosphatase-labeled secondary antibody (KPL, Gaithersburg, MD) was added at a concentration of 1 µg/10 ml of TBST and incubated for another 1 h at room temperature. After being washed thoroughly, alkaline phosphatase (AP) signal was detected using CDP-Star chemiluminescent substrate for AP (Tropix, Bedford, MA). Blots were analyzed on a Kodak Image Station 2000MM (Carestream Molecular Imaging, New Haven, CT) and compatible Kodak MI software.

**Methods for Statistical Analysis**—ENaC $P_o$ values were examined before and after the redox state of the cell had been pharmacologically altered in either alveolar type 1 or type 2 cells. Therefore, the same patch clamp recording before drug treatment could be used as its own control, and statistical significance could be determined by paired $t$ test analysis, with $p$ values <0.05 considered significant. Statistical significance for O$_2^-$ release was determined using one-way analysis of variance and the post hoc Holm-Sidak test, with $p$ ≤ 0.05 assumed to be statistically significant.

**RESULTS**

**Immunolocalization of Alveolar Cells for Patch Clamp Analysis**—We have recently reported epithelial Na$^+$ transport properties of alveolar type 1 cells in lung tissue slices, which represent a novel and physiologically relevant model for studying the ion transport mechanism responsible for lung fluid balance. Here, we report for the first time ion transport properties of type 2 cells in the same model. We positively identified type 2 cells in lung slices by labeling type 2 cells with either polyclonal antibodies (Fig. 1, A and C) or vital cell-permeable dyes. Our
immunohistochemical data identified the cuboidal cells, located at the junction of type 1 cells, for patch clamp analysis. Fig. 1A shows that the cells identified are indeed secreting surfactant protein A, an essential protein for lung compliance produced only by type 2 cells. LysoTracker Red (Fig. 1, B, 40× magnification, and D, 100× magnification) is a vital dye that can permeate the cell membrane and binds to spherical organelles with low internal pH or surfactant-producing lamellar bodies in type 2 cells (not found in type 1 cells). In Fig. 1C, we additionally verified that type 2 cells were indeed immunoreactive with specific rat type II-70 (RTII-70) antibody, which has been extensively characterized by others as a type 2-specific marker. Alongside each panel of labeled alveolar type 2 cells (using either LysoTracker Red, surfactant protein A, or rat type II-70 antibodies in 1A-C), we also counter-labeled alveolar type 1 cells with ECL to contrast the distinct morphology and localization of type 1 and type 2 cells.

Single Channel Activity in Alveolar Type 2 Cells in Lung Slices—We have measured for the first time single channel activity from type 2 cells in situ and found that type 2 cells indeed have functional epithelial sodium channels with highly selective and non-selective transport properties when accessed from live lung tissue. A representative single channel trace is shown in Fig. 2A at −60 through +60 mV (Vp) holding potentials. Figs. 2, B and C, shows current-voltage relationships that are typical of highly selective channels (HSC) and non-selective channels (NSC), respectively. For clarity, we enlarged a portion of the recording taken at −80 mV (Vp) in Fig. 2D highlighting both the smaller conducting HSC as well as the larger NSC, found within the same patch of cell surface membrane. Additionally, in Fig. 2E we generated an all-points histogram (and fitted the data to a Gaussian function) from the representative trace in Fig. 2D to show that, indeed,
HSCs and NSCs are present in alveolar type 2 cells patch-clamped from our lung slice model, and on occasion are both opened simultaneously.

We also observed channels that have the electrophysiological properties commonly associated with the cystic fibrosis transmembrane conductance regulator (CFTR) channels in our cell-attached recordings. Fig. 2F shows a representative patch clamp recording of anion channels in type 2 cells (using the same conditions used to examine Na⁺ current). In this trace the downward deflections likely represent Cl⁻ movement across the cellular membrane. The gating of this channel, with quick bursts of activity interrupted by brief flickering closures, is not different from what we currently describe in alveolar type 1 and type 2 cells patch-clamped from lung tissue.

Reactive Nitrogen Signaling in the Alveolar Epithelium—Our previous research has identified nitric oxide as a negative regulator of Na⁺ channel function in cultured cells (20, 22, 33). However, nitric oxide signaling in type 1 cells has not been examined at a single channel level. It is important to test the effect of nitric oxide on sodium transport properties of all cells that make up the alveolar epithelium in live tissue slices, as inhibition of ENaC may lead to undesirable effects related to lung edema clearance. This is also particularly important since inhaled NO is currently being used to treat pulmonary hypertension in newborns and older patients.
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Fig. 4, A and B, shows that PAPA-NONOate significantly decreased sodium transport in AT2 cells patched from lung slices but failed to alter ENaC NPo in AT1 cells. In 18 independent patch clamp studies of type 2 cells accessed in situ, 1.5 μM PAPA-NONOate significantly decreased ENaC NPo from control values of 1.38 ± 0.26 to 0.82 ± 0.16 (p < 0.05). Higher concentrations of the NO donor (500 μM) further decreased ENaC NPo values to 0.37 ± 0.22 in type 2 cells. In type 1 cells, however, ENaC NPo values did not differ from control values of 0.63 ± 0.12 when either 1.5 or 500 μM nitric oxide donor was applied to the bath media. We show segments of the single channel recordings from more than 20 min of recording after nitric oxide treatment in AT2 and AT1 cells in Figs. 5 and 6, respectively. Fig. 5 shows that before 1.5 μM nitric oxide application to the patch clamp bath, the probability of seeing HSC and NSC channel with 4.3- and 16-pS conductance can be seen under control conditions. B, after ~5 min of low μM nitric oxide treatment, HSC and NSC ENaC activity was significantly decreased in type 2 cells. 

Cells with Type 1 Phenotypes Have Elevated O₂ Levels Compared with Alveolar Type 2 Cells—Given that nitric oxide can easily diffuse across cellular membranes and that type 2 cells are found dispersed throughout the areas between type 1 cells, the reason why each cell type that makes up the alveolar epithelium responds differently to PAPA-NONOate is unclear. Because of the natural interaction between O₂ and NO and based largely on our recent finding that it is possible to...
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FIGURE 7. Oxidative state of alveolar epithelial cells. A, alveolar type 2 cells maintained in culture beyond 72 h differentiate into cells with AT1 properties and provide a useful model for studying the oxidative state of alveolar epithelial cells. Cell lysate prepared from type 2 cells maintained in culture conditions (described under “Materials and Methods”) become more immunoreactive with an RT-140 antibody specific for alveolar type 1 cells in Western blot analysis. B, after days 5 and 10 in culture, morphological changes and reactivity with ECL resemble that of type 1 cells found in lung tissue. C, DHE labeling of cells show that differentiated type 1 cells on days 3 and 5 in culture have significantly higher levels of superoxide anion compared with freshly isolated type 2 cells on day 1 (the asterisk indicates statistical significance determined between days 1 and 3 and day 1 versus day 5 values; p = 0.05 and n = 180 performed in 3 independent experiments shown).

prevent NO inhibition of ENaC by increasing local concentrations of \( \mathrm{O}_2^- \) (33), we tested whether cells of the alveolar epithelium could be in different oxidative states. Such a difference would suggest that type 1 and 2 cells generate \( \mathrm{O}_2^- \) at different rates and, therefore, have different biological responses to NO.

To study the oxidative state of alveolar cells, we used a well established cell culture model for alveolar type 1 cells. We and others have shown that when type 2 cells remain in culture on permeable supports, they trans-differentiate into type 1 cells (Fig. 7, A and B). This is an innate property of the alveoli; type 2 cells naturally transform into type 1 cells in vivo to repopulate the alveolar epithelium. In Fig. 7A we collected the protein lysate of cells maintained up to 7 days in culture for Western blot analysis. Using an RT-140 antibody specific for type 1 cells (provided by Dr. Leland Dobbs), we show that after 72 h in culture the cell lysates collected become increasingly immunoreactive with RT-140 antibody. Several groups have verified that type 2 cells can transdifferentiate into alveolar type 1 cells when maintained in culture (2, 11, 25). Although the data are not shown, we also confirmed that when type 2 cells remain in culture past day 3, expression of surfactant proteins also significantly decrease. Fig. 7B shows the morphological changes associated with type 2 cells on days 1, 5, and 10 in culture and that cells become more positive for ECL labeling. In Fig. 1 we established that ECL binds to type 1 cells with high specificity over type 2 cells in lung slices. In Fig. 7B we applied the same labeling protocol to show that as type 2 cells remain in culture, they begin to express surface proteins specific to type 1 pneumocytes. Last, in Fig. 7C we show in 3 independent experiments that AT1-like cells have elevated levels of \( \mathrm{O}_2^- \) compared with freshly isolated type 2 cells (kept in culture less than 24 h (day 1)). Isolated AT2 cells have average DHE (an effective indicator of intracellular \( \mathrm{O}_2^- \)) intensities of 250 ± 33 relative light units, calculated from 3 independent assays. However, by days 3 and 5 in culture the cells have differentiated to an AT1 phenotype and have significantly increased DHE values 169 ± 61 and 151 ± 27% above the level in AT2 cells at D1, respectively (Fig. 7C, p < 0.05). After remaining in culture for 1 week, alveolar cells begin to show a decrease in both RTI-40 protein expression (Fig. 7A) as well as \( \mathrm{O}_2^- \) production (Fig. 7C, last set of columns). Presumably, the lengthy culture of alveolar cells (past 5 days) on permeable supports leads to cell death/damage and a decrease in metabolic activity. It is, therefore, reasonable to find that \( \mathrm{O}_2^- \) levels on day 7 do not differ significantly from cells on day 1.

**Elevating Endogenous \( \mathrm{O}_2^- \) Levels Prevents Nitric Oxide Inhibition of ENaC Activity in Alveolar Type 2 Cells**—Our observation that transdifferentiated type 1 cells have elevated levels of reactive oxygen species led to the interesting hypothesis that the difference in response to nitric oxide in type 1 and 2 cells may be due in part to differences in the oxidative state of each cell type found in the alveoli. We have recently shown that oxygen radicals such as superoxide anions (\( \mathrm{O}_2^- \)) play a very important role in regulating ENaC activity, presumably by binding to nitric oxide and blocking the inhibitory effect of nitric oxide in epithelial cells (33). Indeed, our current results suggest that nitric oxide is capable of exerting an inhibitory effect only on type 2 cells (and not type 1 cells) because type 2 cells may have lower concentrations of reactive oxygen species.

If elevated \( \mathrm{O}_2^- \) levels in type 1 cells indeed limit NO inhibition of Na\(^+\) transport, then we would expect increasing \( \mathrm{O}_2^- \) levels in type 2 cells to similarly prevent nitric oxide inhibition of ENaC activity in these cells. In Fig. 8 lung slices were pretreated with a superoxide dismutase inhibitor (Ethiolat) before exposure to nitric oxide. In cell-attached patches on type 2 cells from lung slices pretreated with Ethiolat (in which endogenous levels of \( \mathrm{O}_2^- \) are elevated), there was no significant decrease in ENaC activity after 1.5 and 500 \( \mu \)M PAPANOate treatment. This trend was observed in nine independent observations (Fig. 8B).
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**A.**

FIGURE 8. Elevating O2 in AT2 cells with superoxide dismutase inhibitor prevents nitric oxide inhibition of ENaC in lung slice recordings. A, representative trace is of NSC with 24-pS conductance. In general, lung slices were pretreated with 5 mM Ethiolat, a superoxide dismutase inhibitor, for −5 min before obtaining a high gigohm resistance seals on type 2 cells in slices. In the same cell-attached patch of Ethiolat pretreated cells, 1.5 μM nitric oxide failed to significantly inhibit ENaC current (breaks in continual single channel recording are indicated with # symbols). B, average ENaC NPo from 9 independent observations show no significant change after 1.5 μM PAPA NONOate treatment. Quantification of ENaC NPo values reflect both HSCs and NSCs.

**DISCUSSION**

Examination of Type 2 Cells in Lung Slices Advances Our Understanding of Fluid Balance in Alveoli—Lung slice preparations represent a physiological model for alveolar fluid clearance which cultured cells are unable to duplicate. The benefits of studying ENaC in lung tissue preparations include circumventing technical limitations associated with harsh enzymatic digestion involved in primary cell isolation techniques (9) and the need to determine appropriate cell culture conditions for optimal surface expression of ENaC (24). Additionally, our data obtained from *in situ* patch clamp analysis of type 1 and 2 cells can be used to substantiate previous (and future) conclusions gleaned from tissue culture models. Indeed, many of the ion transport properties of the alveolar epithelium have been studied in cell culture systems. We have recently reported that type 1 cells have both highly selective cation and non-selective cation channels when studied *in situ*. We show here that type 2 cells also have HSC and NSC channels (similar to those reported in type 1 cells (19) and to primary type 2 cells studied in culture (15, 24)) with average conductances of 8.8 ± 3.2 and 22.5 ± 6.3 pS, respectively. Additionally, anion channels with properties like CFTR were observed, which presumably contribute to maintaining the electroneutrality of Na+. Our work with cell-attached patches of lung slices show that both type 1 and 2 cells contribute significantly to lung fluid clearance, each having the ability to absorb Na+ ion through HSC and NSC channels *in situ*.

**Important Differences in Redox Signaling between Type 1 and 2 Cells**—Because we can make single channel measurements from both cell types that comprise native alveoli, we can determine differences in signaling mechanisms between alveolar type 1 and type 2 cells. One important difference identified in our current study between type 1 and 2 cells is that nitric oxide only inhibits sodium transport in type 2 cells and not in alveolar type 1 cells. In Fig. 4 we show that type 2 cells respond to 1.5 μM nitric oxide with a significant decrease in ENaC NPo. Some cells in Fig. 4 show a modest decrease in ENaC NPo after nitric oxide treatment. Presumably, these type 2 cells with modest decreases in ENaC response may have slightly higher endogenous levels of superoxide compared with type 2 cells with bigger decreases in ENaC NPo values. However, it is interesting to compare the lack of effect of nitric oxide treatment in type 1 cells to NO inhibition in both type 2 cells in live lung slices and in cultured type 2 cells (22). To ensure that PAPA-NONOate effectively releases NO in both sets of experiments involving type 1 and 2 cells, we used the same lot of PAPA-NONOate for type 1 and 2 cell studies performed concurrently. In this way we can be confident that differences in NO responses could not be attributed to drug (in)efficacy.

Another potentially important difference identified between AT1 and AT2 cells may explain the different response to NO in pneumocytes. Our data suggest that type 1 and 2 cells may be in different oxidative states. Using DHE labeling we established that as alveolar type 2 cells transdifferentiate into type 1 cells, there is an associated increase in O2− release in type 1 cells. We and others routinely use DHE labeling as a measure of intracellular O2−. However, because the emission wavelength of DHE (610 nm) overlaps with that of the vital dye Lysotracker Red, used to positively identify type 2 cells, we were technically unable to determine the oxidative state of alveolar epithelial cells *in situ*. Alternatively, we measured the release of O2− as cells differentiated from type 2 progenitor cells to cells with an alveolar type 1 phenotype and found compelling evidence indicating that the redox state of type 1 and 2 cells is different. It is important to understand these differences between type 1 and type 2 cells, particularly since administration of high ambient oxygen (which necessarily elevates cellular O2− release) often accompanies inhaled nitric oxide therapy for lung illnesses. The therapeutic benefit of administering nitric oxide (particularly into alveoli with elevated O2−) is now well established in newborns with pulmonary hypertension but remains unclear in older patients with acute lung injury. Typically, NO delivery to ventilated patients is administered in a way that keeps NO and oxygen contact times at a minimum (16).

**Possible Clinical Implications**—Nitric oxide continues to be used in clinical practice to manage acute respiratory distress
syndrome and chronic obstructive pulmonary disease despite the lack of clear evidence of efficacy. Our data indicate that Na⁺ transport properties of alveolar type 1 cells cannot be altered by even very high concentrations of NO, whereas type 2 cells respond to nitric oxide with an immediate decrease in ENaC activity. Our ability to make single channel measurements in lung tissue slices makes it clear now that both type 1 and 2 cells play an important role in maintaining lung fluid balance and provides a plausible explanation for the general lack of consensus on whether nitric oxide works with any efficacy in clearing alveolar flooding or worsens it. Our data suggest that differences in the redox state of lung cells determine responsiveness to nitric oxide. Both our current and previous findings (33) indicate that O₂⁻ limits the inhibitory effect of nitric oxide signaling on ENaC. Given the natural interaction between O₂⁻ and NO molecules, this is a natural and plausible mechanism that could aid in regulating lung fluid balance and protecting the alveoli from reactive nitrogen species that are prevalent in lung injury, polluted atmospheres, and especially during NO therapy. O₂⁻ molecules bind quickly and irreversibly to nitric oxide with extremely high affinity (k = 6.7 × 10⁷ M⁻¹ s⁻¹). Our data indicate that alveolar cells may generate different quantities of O₂⁻ and that superoxide anion can diminish NO biological effects. Although O₂⁻ release in type 1 cells protects the major portion of the lung epithelia from harmful effects of nitric oxide, the strong interaction between O₂⁻ and NO may also limit NO known beneficial vasodilative effects. Seemingly, the rapid interaction between O₂⁻ and nitric oxide may be one mechanism in which epithelial cells maintain tonic fluid balance in the alveoli and protect lung epithelial cells from excessively high levels of reactive nitrogen species.

In summary, the results of our current study contribute to a better understanding of the functions of alveolar type 1 and 2 cells together and separately. We describe here for the first time the biophysical properties of alveolar type 2 cells in situ from live tissue. These measurements validate the use of cultured type 2 pneumocytes in ion transport studies by verifying that intact type 2 cells express both HSC and NSC channels involved in transepithelial sodium transport and fluid balance like cultured type 2 cells. Because we are able to obtain single channel recordings from both cells that make up the alveoli in an environment very close to their native environment, we can determine differences in type 1 and 2 cell function. It is generally accepted that type 2 cells are responsible for surfactant production and serve as precursor cells for renewal of the epithelium. Our patch clamp analysis of type 2 cells in lung slices provides additional conclusive evidence that type 2 cells are important contributors to net ion transport (with cation and anion channels at the apical membrane) despite the fact that these cells represent less than 5% of the total surface area of the alveolar epithelium. The functional role of type 1 cells has been less well characterized. Our current study as well as a recent publication by Chen et al. (4) describes a new protective role for type 1 cells. Our results support the idea that type 1 cells, which make up the majority of the alveolar lung surface, generate elevated levels of O₂⁻, which by and large protect the alveolar epithelium from excessive nitric oxide and maintains normal lung fluid balance.