Regulation of ion transport by oxidants

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Downs CA, Helms MN. Regulation of ion transport by oxidants. Am J Physiol Lung Cell Mol Physiol 305: L595–L603, 2013. First published September 6, 2013; doi:10.1152/ajplung.00212.2013.—Ion channels perform a variety of cellular functions in lung epithelia. Oxidant- and antioxidant-mediated mechanisms (that is, redox regulation) of ion channels are areas of intense research. Significant progress has been made in our understanding of redox regulation of ion channels since the last Experimental Biology report in 2003. Advancements include: 1) identification of nonphagocytic NADPH oxidases as sources of regulated reactive species (RS) production in epithelia, 2) an understanding that excessive treatment with antioxidants can result in greater oxidative stress, and 3) characterization of novel RS signaling pathways that converge upon ion channel regulation. These advancements, as discussed at the 2013 Experimental Biology Meeting in Boston, MA, impact our understanding of oxidative stress in the lung, and, in particular, illustrate that the redox state has profound effects on ion channel and cellular function.

antioxidant stress; glutathione; lung ion channel; oxidative stress

THE ROLE OF REACTIVE SPECIES (RS) under normal and pathological pulmonary conditions is an intense and active area of investigation. This interest has spanned over a decade; in fact, it has been precisely 10 years since Drs. Eaton, Kotlikoff, Stamler, and Matalon discussed their findings concerning the investigation. This interest has spanned over a decade; in fact, it has been precisely 10 years since Drs. Eaton, Kotlikoff, Stamler, and Matalon discussed their findings concerning the effects of reactive intermediates on Ca\textsuperscript{2+}, K\textsuperscript{+}, Na\textsuperscript{+}, and Cl\textsuperscript{-} channels at the 2003 Experimental Biology meeting in San Diego (72). This important topic was recently revisited at the 2013 Experimental Biology Meeting held in Boston, MA. This year’s symposium on Oxidative Stress was chaired by Drs. M. N. Helms (Emory University) and S. Niederlechner (University of Colorado) with invited speakers: C. A. Downs (Emory University), R. R. Cox (University of South Florida), H. Ma (Emory University), C. E. Hill (Australian National University), L. Gliemann (University of Copenhagen), and M. Mendez (Henry Ford Hospital). Each speaker shared their most recent findings related to redox biology within the context of their respective fields. Based on the invited presentations, it is without doubt that advancements have been made within the last decade of research on oxidative stress and ion channel activity. These advancements include: 1) identification of nonphagocytic NADPH oxidases (NOX) as a source of regulated RS production in epithelia, 2) comprehension that excessive treatment with antioxidants can result in a greater degree of oxidative stress (a response now termed “antioxidant stress”), and 3) characterization of novel RS signaling pathways that converge upon ion channel regulation. The purpose here is to provide a contemporary overview of the state-of-the-sciene related to redox regulation of lung epithelial channels and transporters in health and disease.

Overview of ion channels and transporters in lung epithelia.

The membrane potential of airway and alveolar epithelial cells has been measured in pneumocytes and range between $-24$ and $-63$ mV (10, 12, 31, 32, 34, 65, 73). These values indicate that, under basal conditions, lung cells have depolarizing resting membrane potentials that favor passive Na\textsuperscript{+} reabsorption from apical to basolateral surfaces. In the lung, amiloride-sensitive epithelial Na\textsuperscript{+} channels (ENaC), composed of $\alpha$, $\beta$-, and $\gamma$-subunits, have been identified as the major contributor of net salt and water reabsorption. New studies, however, describe the presence of a $\delta$-ENaC variant in the human lung with unique biophysical properties (53, 102). The characterization of distinct $\delta$-ENaC splice variants may explain the diverse cation selectivity and amiloride sensitivity of native ENaC in the lung. Of particular interest, Matalon and colleagues (53) provide evidence that channels formed by the $\delta$-ENaC subunit are regulated by 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate-Na, indicating an important role for nitric oxide (NO) signaling in $\delta$-ENaC-containing channels. Classically, there are two families of ENaC characterized in the lung, the first of which are highly selective cation (HSC) channels that transport Na\textsuperscript{+} unidirectionally with low conductances of $\sim6$ pS. The second family of amiloride-sensitive Na\textsuperscript{+} channels in the lung is nonselective cation (NSC) channels that have Na\textsuperscript{+}–K\textsuperscript{+} permeability of $\sim1.4$ and larger conductances ranging between 19 and 30 pS. The identification of HSC and NSC amiloride-sensitive current in alveolar type 1 cells (46, 47) has changed the classical paradigm that alveolar type 2 cells purely drive Na\textsuperscript{+} absorption across the entire alveolar epithelium. Because type 1 cells form $>98\%$ of the lung surface area, and type 2 cells comprise $<2\%$ of the alveolar surface area (20), it is now clear that type 1 cells play a critical role in active transepithelial ion transport in addition to its classical role in passive gas exchange (29); this was not appreciated a decade...
ago. In addition to HSC and NSC activity in lung epithelia, amiloride-insensitive cyclic nucleotide-gated cation channels (CNG) have been characterized in primary alveolar type 1 cells (54). These channels have very low unitary conductances of ~2.9 pS and reversal potentials close to zero; as such, the CNG family of NSC channels is not likely to be key determinants of lung fluid transport under basal conditions. The apical surface of lung epithelia also transports K⁺ via several voltage-dependent K⁺ channels and Ca²⁺-dependent K⁺ channels located in the apical membrane (3, 66–68, 101). In the lung, these channels contribute to the high K⁺ content in adult airway surface liquid (ASL) (93) as well as influence the apical resting membrane potential. In the fetal lung, Cl⁻ secretion into the airway lumen via the cystic fibrosis transmembrane conductance regulator (CFTR) necessarily occurs in the development stages up to the period immediately preceding birth. In the adult lung, however, NOX-mediated Cl⁻ transport into the alveolar space has been implicated as a major contributor to cardiogenic lung injury (86). Because CFTR knockout mice do not display an abnormal lung phenotype (23, 40, 41, 70, 99), it is clear that additional Cl⁻ channels such as Ca²⁺-dependent Cl⁻ channels play key roles in maintaining ASL volumes in rodent lung. In all Na⁺-transporting epithelia, cells maintain a propensity for passive Na⁺ absorption down an electrochemical gradient by pumping three Na⁺ out for every two K⁺ pumped into the cell via basolaterally located Na⁺-/K⁺-AT-Pases (reviewed in Ref. 30). Basolaterally positioned Na⁺-/K⁺-/2Cl⁻ cotransporters maintain intracellular Cl⁻ concentrations. Together, these ion channels and transporters work to maintain alveolar fluid homeostasis. Airway epithelial cells, of course, also have electroneutral processes, such as Na⁺/H⁺ exchange and Cl⁻/HCO₃⁻ exchange that serve to primarily regulate intracellular pH (63, 75, 81).

Biological sources of RS production and channel regulation. RS, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical, hypochlorous acid, and NO, are by-products of oxidative metabolism and enzymatic reactions. Production of RS is increased during inflammation, sepsis, pulmonary edema, and in a variety of lung and systemic diseases (76, 84, 97). Excessive production of RS occurs in pathological states as evidence shows that excessively high levels of RS result in lipid peroxidation, DNA knicking, and irreversible oxidation of protein, which disrupts oxidant-mediated signaling (44). Despite their potential for serious cellular damage, several studies clearly articulate a role of RS in cell signaling pathways under important physiological conditions, such as ion channel regulation.

RS are important regulators of cell function and may be generated through a variety of metabolic pathways, including: 1) the electron transport system of mitochondria, 2) the xanthine/xanthine oxidase system, 3) the cyclooxygenase pathway of the arachidonic pathway, 4) the activated neutrophil system, 5) the amyloid β-protein system, and 6) nonphagocytic tissue NOX (59–62). The best-characterized intracellular source of RS generation is the electron transport system in mitochondria. Because mitochondrial O₂⁻ generation occurs as a by-product of respiration, very high O₂⁻ can quickly accumulate in the cell and lead to cell death. At the cell surface, however, RS (O₂⁻ and H₂O₂ in particular) are produced in much lower quantities by the family of NOX and serve as important signaling molecules (33). Based upon homology with gp91phox, the catalytic sub-unit of the phagocytic oxidase, the first new member of the NOX family was initially cloned in 1998 (45). There are now seven NOX isoforms identified (i.e., NOX 1–5, DUOX1, and DUOX 2), albeit the precise biological role of each NOX isoform remains unclear. It is clear, however, that NOX 1–3 require interaction between the cytosolic domain (gp47phox, gp47phox and gp67phox) and the catalytic domain (gp91phox and gp22phox) for O₂⁻ production. NOX 4 is constitutively active and produces H₂O₂ (89). NOX 5, DUOX1, and DUOX2 require Ca²⁺ for activation (see Fig. 1).

Several advancements have been made in delineating the role of NOX 2 and NOX 4 isoforms in ENaC regulation, and further work has explored novel redox-sensitive signal transduction pathways that regulate lung ENaC. In particular, it has been shown that NOX 2 and NOX 4 are expressed in alveolar epithelial type 1 and type 2 cells. Furthermore, gp91phox, the catalytic domain of Rac-1-dependent NOX enzymes, has been coimmunoprecipitated with the α-ENaC subunit (35, 90). The proximity of RS release with active Na⁺ channel subunit indicates that these unstable, reactive molecules can indeed regulate apically located channels embedded in the cell membrane before targeted dismutation of the RS. Further evidence indicating an important role of NOX-derived RS includes studies in which inhibition of the small G protein Rac-1, using NSC-23766 compound, inhibited both O₂⁻ production and ENaC activity in the alveolar epithelium. In the same study, tracheally instilling NSC-23766 alongside saline challenges of ~100 μl into the lung reduced the rate of lung fluid clearance in C57Bl6 mice. NOX 2 appears to be a significant contributor to O₂⁻-dependent regulation of ENaC, since NOX 2⁻/- mice also exhibited impaired lung fluid clearance (36).

In addition to working toward delineating the cellular source of RS that regulate lung ENaC, advancements have been made in delineating redox-sensitive pathways and mechanisms that regulate salt and water transport in the lung. With the use of a fluorescently conjugated maleimide compound, it has been recently shown that Cys thiols on α-ENaC can be directly modified by NOX-derived RS (27). RS can also alter ENaC activity via a signal transduction pathway involving the ubiquitin-like protein Nedd8 (25). Recently, it was shown that H₂O₂ inhibits Nedd8 conjugation to the E2 ligase Cullin-1, the effect of which is decreased ubiquitination and decreased proteolytic degradation of lung ENaC in the presence of RS. In the setting of cigarette smoke, which is known to contain many free radicals as well as induce RS production, it has been observed that exposure to an aqueous preparation of cigarette smoke upregulates alveolar ENaC that is coupled with attenuated ENaC degradation via an oxidant-mediated pathway (24). Importantly, in this study, it was also shown that cigarette smoke acts as an agonist of NOX enzyme activity, leading to upregulation of ENaC function. The important clinical implication is that smoking can exasperate dry lung disorders, such as emphysema, via inappropriate channel activation. Collectively, these studies advance understanding of the biological role of NOX enzyme activity in the lung and puts forward novel redox-sensitive pathways that regulate ENaC.

Despite these advancements, however, cross talk among RS, ENaC, and CFTR remains unclear and requires further study. Using precision-cut lung slices from CFTR knockout mice, Lazrak and colleagues showed that mice lacking normal Cl⁻ transporters have significantly elevated levels of proteolytically
processed ENaC in the lung (64). This finding indicates that effective antagonists of Cl\(^{-}\)/H\(_{11002}\) channel activity, perhaps reactive intermediates, can thereby serve as effective activators of ENaC (and hence salt and water transport out of the luminal airspace). The important implication here is that RS can alter ENaC activity by a variety of direct and indirect (via altered CFTR activity) mechanisms that require additional investigation.

The role of NOX-generated RS in the regulation of cellular functions extends beyond ion channels in lung epithelia as discussed during the symposium. More specifically, NOX 2- and NOX 4-induced RS production has been connected with a decrease in T-type and an increase in L-type Ca\(^{2+}\) channel activity in vascular cells, an effect that is reversible with the addition of superoxide dismutase mimetic, apocynin and diphenyliodonium (DPI). NOX 2 and NOX 4 are also expressed in juxtaglomerular cells and can alter Na\(^{+}\) sensing in the renal nephron. Furthermore, stimulation of juxtaglomerular cells with H\(_{2}O_{2}\) provides a dose-dependent response in renin release, an effect that was abrogated with the addition of catalase. Inhibition of NOX 2 and NOX 4 with Fulvene-5 has been shown to attenuate the regulatory effects of these NOX isoforms in the kidney (92). Collectively, data strongly support a role for biologically generated RS in the regulation of cellular functions and ion transport in several various organ systems.

Oxidative stress and ion transport. Oxidative stress occurs in many lung diseases and is characterized by an imbalance in oxidants and antioxidants that dysregulates oxidative signaling (85). Oxidative stress has been observed in edematous and nonedematous lung diseases, suggesting that oxidative stress, and ultimately oxidative regulation of ion channels, may play a critical role in many lung pathologies (13, 51). Because
ENaC, CFTR, and K⁺ channels play key roles in maintaining normal epithelial lining fluid (ELF) volume and composition, we focus the discussion on redox regulation of these channels and transporters in the lung. Indeed, multiple studies converge to illustrate the importance of oxidant regulation of ion channels and transporters in the pathogenesis of edematous and nonedematous lung diseases such as pulmonary edema, acute lung injury, chronic bronchitis, and cystic fibrosis.

Airway and alveolar epithelial cells are often exposed to increased concentrations of intracellular and extracellular RS because of exposure to environmental pollutants, cigarette smoke, noxious gases, or other NOX enzyme agonists. Furthermore, these irritants may induce an inflammatory response resulting in macrophage recruitment and even higher levels of RS. Accumulation of RS can indeed regulate ion channels, as discussed below.

**Oxidative regulation of ENaC.** ENaC is typically composed of three subunits (α, β, and γ) arranged in a fixed stoichiometry, and ENaC functions to reabsorb Na⁺ from the apical surface (49, 50). Reabsorbed Na⁺ is then extruded via the basolaterally located Na⁺/K⁺-ATPase pump; the net movement of Na⁺ produces an osmotic gradient that facilitates the diffusion of water from the alveolar space to the interstitial space where it is then reabsorbed by the vasculature (29). As a result, ENaC is considered the rate-limiting step in the resolution of pulmonary edema. ENaC is regulated by NOX production of O₂⁻⁻ to promote fluid clearance in mice receiving a tracheal instillation of saline, likely through NOX 2 (27, 35). H₂O₂ also upregulates ENaC activity (γ = 6 and 11 pS) in alveolar epithelial type 1 and type 2 cells and has significant effects on inhibition of ENaC ubiquitination by preventing Nedd8 conjugation to the E3-CRL (25). NO inhibits ENaC activity through increased inducible nitric oxide synthase production in Clara cells infected with respiratory syncytial virus (87). Others have reported that NO and related RS modulate ENaC activity via cGMP-dependent and -independent mechanisms. In particular, the δ-ENaC subunit, recently characterized in the human lung, is regulated by NO-mediated cGMP signaling (53, 102). Additionally, multiple studies converge to illustrate that the strong oxidant and nitrating agent peroxynitrite downregulates ENaC (42, 43).

**RS regulation of ENaC activity is complex and can vary depending on the type, duration, and amount of RS exposure as well as antioxidant availability. This point is highlighted in the report by Yue and colleagues showing that rat alveolar type 2 cells exposed to sublethal oxygen concentrations (85%) for 7 days upregulated ENaC, whereas 100% oxygen downregulated channel function (98). Septic rats treated with N-acetylcysteine showed significantly lower levels of oxidative stress and higher survival rates. This observation was coupled with significant increase in α-ENaC levels and enhanced lung fluid clearance (7), thus indicating that antioxidant availability can alter RS signal transduction pathways in the lung. The threshold level of RS and antioxidants, which determine whether a beneficial physiological response is elicited or whether an adverse pathological condition ensues, remains ill-defined.

A common injurious agent for the lung epithelium is cigarette smoke. It was recently shown that an aqueous form of cigarette smoke (cigarette smoke extract, CSE) increases ENaC activity (γ = 12 pS) in alveolar type 1 and type 2 cells through oxidant-mediated pathways (26). Furthermore, the effects of cigarette smoke on ENaC include prolonged retention of the active form of ENaC in the plasma membrane through inhibition of ubiquitination. Airway epithelia appear to respond in a similar fashion as evidenced by a reduction in the height of ASL from human bronchial airway epithelial cells exposed to CSE (15). The effects of CSE are, in part, dose dependent with moderate to high concentrations leading to disruption of the endothelial barrier, cellular necrosis, and a greater inflammatory response (79). Recent data demonstrate that resolvins-D1 has a potent anti-inflammatory and proresolving effect in cigarette smoke-induced lung inflammation (48).

**Oxidative regulation of CFTR.** CFTR is a 1,480-amino-acid protein and a member of the traffic ATPase family. CFTR functions as a cAMP-regulated Cl⁻ channel that controls other ion channel conductive pathways, including ATP transport and epithelial Na⁺ and Cl⁻ channels (16). Defective CFTR causes cystic fibrosis, a disease process characterized by impaired Na⁺ and Cl⁻ transport in the lungs, pancreas, liver, sweat glands, gastrointestinal tract, and male reproductive system (56). Studies show that Cl⁻ channels are sensitive to RS (4, 5). Cl⁻ channels and voltage-dependent anion-selective channels in the mitochondrial membrane of the surface of bovine trachea are regulated by oxidation-reduction mechanisms (28). NO modulates CFTR expression and function in CFTR-expressing airway epithelial cells. Using nasal potential difference in humans, Clunes and colleagues (15) reported that cigarette smoke exposure rapidly inhibits CFTR activity in vivo. Furthermore, human bronchial epithelial cells exposed to cigarette smoke demonstrate a reduction height of the ASL and internalization of CFTR into aggresomes through RS-mediated inhibition of autophagy (71). Velsor and colleagues assessed mitochondrial glutathione (GSH) levels and markers of DNA and protein oxidation in the lungs of CFTR⁻⁻ mice and observed elevated mitochondrial oxidative stress in CFTR⁻⁻ mice. Dysfunctional CFTR was found to increase ROS and lead to greater mitochondrial oxidative stress (94).

**Studies show that H₂O₂ increased CFTR-mediated Cl⁻ secretion in monolayers of the human submucosal gland serous cell line Calu-3 (19). In contrast, H₂O₂ was found to inhibit Ca²⁺-dependent chloride secretion by activating signaling pathways that inhibit membrane ion transport proteins in colonic epithelial cells (11). In differentiated human airway epithelial cells, H₂O₂ was found to activate CFTR through a CAMP-dependent, autocrine prostaglandin pathway (17). Collectively, evidence suggests that putative and potentially pathological levels of RS play a significant role in the regulation of cellular function and ion channels.

**Oxidative regulation of K⁺ channels.** K⁺ channels, including voltage-dependent and Ca²⁺-dependent K⁺ channels, contribute to the high K⁺ content in the ELF and are important for maintaining electroneutrality. K⁺ channels are ubiquitous and have been extensively studied in the vasculature where evidence shows that K⁺ channels are sensitive to changes in oxygen content (95). In the lung, K⁺ channels appear to play a role in modulating Na⁺ absorption and Cl⁻ secretion in...
bronchial epithelial cells (101) and ultimately effect fluid transport across alveolar monolayers (68).

Studies show that thiol oxidation of pulmonary vascular smooth muscle and endothelial cells affects pulmonary artery vascular tone by activating K\(^+\) channels and inhibiting store-operated Ca\(^{2+}\) channels (83). Numerous studies converge to illustrate the effects of H\(_2\)O\(_2\) on K\(^+\) channels (82, 100). However, little is known about the effects of oxidative stress on K\(^+\) channel function in the lung epithelia.

**Antioxidants and lung health.** The potential role of antioxidants in preventing and/or slowing the progression of lung disease is an area of active investigation. Although initial reports suggested that antioxidant supplements could promote better health, recent studies report limited success in treating pulmonary conditions with antioxidants such as vitamin E (21), vitamin C (22), α-tocopherol, and β-carotene (80). At the symposium, data presented by Dr. Gliemann showed that antioxidant supplementation blunts positive effects of exercise. Without completely understanding the role of antioxidants on ion transport and lung function, excess supplementation with known and putative antioxidants could even be harmful.

**Reducing agents augment ion transport.** Currently, the most commonly used synthetic reducing agent for proteins is dithiothreitol (DTT). DTT’s formula is C\(_4\)H\(_{10}\)O\(_2\)S\(_2\), and it is the common name for the small-molecule redox reagent first described by Dr. W. W. Cleland in 1964 (14). Under oxidative conditions, DTT forms a stable six-membered ring with an internal disulfide bond, making it a strong reducing agent. Because of its unique properties, DTT is often used in ion channel and transporter studies to prevent disulfide bond formation. Hence, ion transport activity under nonoxidizing conditions can be gleaned from electrophysiological recordings in which DTT, or other strong reducing compounds, have been experimentally applied.

The functional properties of several members of the ENaC degenerin family of ion channels have been shown to be modulated by reductants that act on sulfhydryl groups of cysteine residues (1, 55, 91) Acid-sensing ion channels (ASICs), while primarily expressed in mammalian sensory neurons, share high sequence homology with ENaC. The structure of ASIC has been resolved by X-ray crystallography at 1.9 Angstrom resolution (52), which will facilitate all subsequent structure/function studies under oxidizing and reducing conditions for the ENaC/degenerin family of ion channels. Using whole cell current clamp, Andrey et al. (1) have shown that 1–2 mm DTT reversibly potentiates proton-activated current. ENaC, however, does not respond to DTT treatment with any change in channel activity. This finding, however, is not indicative of the important role Cys thiol modification may play in Na\(^+\) channel regulation. In fact, early studies have shown that cells transfected with α- and γ-ENaC constructs with all Cys substituted lacked normal channel activity (91), indicating an important role of Cys thiols in Na\(^+\) channel subunits. The general view is that Cys disulfide bond formation is an important part of normal ENaC assembly and insertion into the membrane, and thus, subsequent application of reducing compounds such as DTT is not expected to alter channel activity under basal conditions. Interestingly, however, βS518C mutants, expressed alongside wild-type (WT) α- and γ-subunits in Xenopus laevis oocytes, responded to reducing agents MTSET and MTSEA not observed in heterologously expressed WT ENaC subunits. The important observation in this study is that the large sulfhydryl reagent MTSET accesses the mutated BS518C degenerin site only when the channel is in the open state, whereas the smaller reagent MTSEA can modify ENaC in the open and closed state without interfering with amiloride binding (55). While the precise mechanism of Na\(^+\) channel regulation by reductants remains unclear, these studies indicate that the cysteine-rich extracellular domain of ASIC and ENaC may serve as redox sensors, and respond with significant change in channel gating.

A role for thiol modification in modulating lung fluid secretion has been established by Cotten and Welsh (18), who reported that the cysteine-modifying agent N-ethylmaleimide activates CFTR channels in excised patches of HeLa cells expressing CFTR. Further evaluation of reducing agent on CFTR channel gating shows that increases in both opening and closing rates of the channel occur. Chemical modification of cysteine sulphydryl groups using DTT has also been reported to augment Ca\(^{2+}\)-activated K\(^+\) channels in smooth muscle cells (96) and lipid bilayer assays (88). Measurement of maxi-K\(^+\) channels using the inside-out configuration indicates that reducing agents alter K\(^+\) channel gating properties without altering unitary conductance. Additionally, in this study DTT reversed the inhibitory effect of H\(_2\)O\(_2\) on K\(_{\text{V}, \text{Ca}}\) channels, which indicates that oxidizing and reducing agents modify distinct amino acid residues in K\(^+\) channels (96). Generally speaking, reducing agents have permissive effects on ion gating properties of channels and transporters that are expressed in the lung epithelium; it is not clear whether these observations would be recapitulated in vivo given the lung’s exquisite ability to maintain redox balance.

**Cellular antioxidants: the role of GSH.** Several low-molecular-weight antioxidants can be found in the lung ELF. There are high levels of cysteine, ascorbic acid, α-tocopherol, urate, thiocyanate, and GSH that can act as antioxidants in the lung (44). In terms of progressive lung disorders, GSH is perhaps the most widely investigated low-molecular-weight antioxidant. GSH levels are also ~100-fold higher in lung ELF than in the plasma (8, 9, 44). In disorders associated with abnormal ELF fluid volumes, such as acute respiratory distress syndrome (74), cystic fibrosis (37), and late-stage chronic obstructive pulmonary disease (COPD) (38), GSH is oxidized by RS and is converted to its disulfide form, GSSG. Lung cells have the ability to synthesize GSH de novo. However, there is also evidence that systemic GSH can be taken up by alveolar type 2 cells and transported to the ELF (2). The important implication here is that depletion of hepatic supplies of GSH can contribute to the development or progression of lung disease if a systemic supply of GSH is indeed required for normal lung function.

The majority of GSH in the lung is in the reduced state. Similar to reducing agents, GSH and cellular antioxidants can modify protein function. For example, oxidized protein cysteine can react with GSH, forming a protein-GSH conjugate (S-SG) and yielding water as a by-product (77). Hence, GSH conjugations to sulfenic acids (Cys-SOH) have important roles as catalytic center and oxidative stress sensors. Functioning as an antioxidant under oxidative stress, GSH itself is oxidized by RS, forming GSSG. In turn, GSSG posttranslationally modifies protein cysteine thiols (R-SH) in a process termed S-glutathionylation (S-GSSG). There is ample evidence from the literature...
indicating that oxidized and reduced GSH can serve as important cell signaling factors (39, 69, 103). However, the role of $\gamma$-glutathionylonation in ion transport remains unclear, despite widespread clinical treatment of respiratory-related conditions (in oxidatively stressed lung) with inhaled GSH (78).

There is indeed an intimate relationship between GSH and apical transport, given that CFTR protein serves as an apical transporter of GSH. Additionally, GSSG has indeed been shown to modulate cation channel activation in calf vascular endothelial cells (57, 58) and seemingly alters ENaC activity in alveolar type 1 and 2 cells. Different from the GSSG-induced increase in whole cell current in endothelial cells, our unpublished observations indicate that $\gamma$-glutathionylonation inhibits ENaC function and that the reduced form of GSH increases ENaC activity. The associated increase in GSH content in smoker ELF (104), coupled with the fact that the main cause of COPD (a disorder in which ELF volumes are depleted) is cigarette smoking, provides additional indirect evidence that GSH has a permissive effect on net salt and water transport across the alveolar epithelium that could lead to dry lung disorders such as COPD. Clearly more work is needed to understand the precise signaling mechanisms; however, this is one of several important examples of antioxidant stress discussed at the symposium.

DISCUSSION

Possibly, the most valuable aspect of the symposium was the discussion that ensued. In particular, the challenges associated with the methodological approaches used in the investigation of oxidative stress resonated with all participants. For example, the common application of exogenous H$_2$O$_2$ to isolated cell preparations or tissue slices and its efficacy in modeling oxidative stress were discussed. Many investigators reported using extracellular concentrations of H$_2$O$_2$ in the micromolar to millimolar range without compromising cell viability. Additionally, Dr. Sadis Matalon (University of Alabama, Birmingham, AL), a leader in the field of redox signaling, proposed that the effect of antioxidants should be considered alongside utilizing oxidizing stimuli, when possible, to effectively model the in vivo environment. For example, isolated lung culture experiments in which oxidants are applied exogenously or generated should be performed and/or validated in the presence of ELF, which is rich in antioxidants, to more accurately reflect redox signaling in the lung. Along the same vein, Dr. Sergei Dikalov (Vanderbilt University, Nashville, TN), another leading expert in redox biology, expressed that studying “antioxidant” stress is of equal importance in the field of redox biology. Based, in part, on this scientific session, the definition of oxidative stress now alludes to imbalances in both pro- and antioxidants.

The innate limitations and benefits of methodological approaches and reagents commonly used in studying oxidative stress were also discussed during the symposium. Attendees discussed the drawbacks and reliability of 2',7'-dichlorofluorescein (DCF) as a reactive oxygen species indicator. This molecule is cell permeable and nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. The major drawback of DCF largely includes the fact that extracellular factors can enable the oxidation of DCF and that this molecule can autoexcite under 488-nm wavelengths. The former limitation can of course be circumvented by including cell-free media controls. The specificity and autoexcitation of DCF has been recently addressed by including control parameters such as superoxide dismutase, catalase, and DPI using a single excitation event, as described in Ref. 92.

An additional important point of discussion centered on the need to identify the regulatory factors that control NOX production of ROS. Through this dialogue, it was realized that another common challenge facing investigators is cross-reactivity of currently available antibodies for NOX isoforms and regulatory subunits. Without consensus on the reliability of antibodies, it is difficult to completely characterize NOX isoform protein distribution, track the subcellular translocation of regulatory domains, and to generalize relative abundance of protein expression across organ systems. Because of these challenges associated with NOX antibody specificity, change in transcript levels of NOX isoform expression was typically reported at the symposium. Optimizing NOX antibody specificity, generating specific NOX isoform inhibitors to be used in therapeutic compounds, and characterizing the (sub)cellular function of NOX enzyme activity are all future directions to be explored within this field.

Conclusion. Significant advancements have been made in our understanding of redox regulation of ion channels and transporters in lung epithelia. It is clear that cellular production of RS and environmental triggers of oxidative stress impact net salt and water transport in the lung. The role of antioxidants in ion transport remains unclear, and the nature and sequence of events that result from excessive RS accumulation in the lung also require additional investigation. However, in the past decade, significant advancement in the understanding of protein thiol modification by pro- and antioxidants has been made. Complex redox-sensitive signal transduction pathways leading to altered channel activity have also been postulated and require additional characterization to fully understand the mechanisms of redox regulation of ion channels in lung epithelium.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: C.A.D. prepared figures; C.A.D. and M.N.H. drafted manuscript; C.A.D. and M.N.H. edited and revised manuscript; C.A.D. and M.N.H. approved final version of manuscript.

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