A Novel Host Defense System of Airways Is Defective in Cystic Fibrosis

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Rationale: The respiratory tract is constantly exposed to airborne microorganisms. Nevertheless, normal airways remain sterile without recruiting phagocytes. This innate immune activity has been attributed to mucociliary clearance and antimicrobial polypeptides of airway surface liquid. Defective airway immunity characterizes cystic fibrosis (CF), a disease caused by mutations in the CF transmembrane conductance regulator, a chloride channel. The pathophysiology of defective immunity in CF remains to be elucidated. **Objective:** We investigated the ability of non-CF and CF airway epithelia to kill bacteria through the generation of reactive oxygen species (ROS).

Methods: ROS production and ROS-mediated bactericidal activity were determined on the apical surfaces of human and rat airway epithelia and on cow tracheal explants.

Measurements and Main Results: Dual oxidase enzyme of airway epithelial cells generated sufficient H_2O_2 to support production of bactericidal hypothiocyanite ($OSCN^-$) in the presence of airway surface liquid components lactoperoxidase and thiocyanate (SCN^-). This $OSCN^-$ formation eliminated *Staphylococcus aureus* and *Pseudomonas aeruginosa* on airway mucosal surfaces, whereas it was nontoxic to the host. In contrast to normal epithelia, CF epithelia failed to secrete SCN^- , thereby rendering the oxidative antimicrobial system inactive.

Conclusions: These data indicate a novel innate defense mechanism of airways that kills bacteria via ROS and suggest a new cellular and molecular basis for defective airway immunity in CF.

Keywords: innate immunity; airway infection; Duox; reactive oxygen species

Inhaled bacteria are trapped in a mucous gel above the airway epithelium and propelled toward the pharynx by concerted movements of the underlying cilia (1). The efficacy of this physical clearance is incomplete. It is unable to remove bacteria penetrating the mucous layer and adhering to the epithelium. Therefore, antibacterial polypeptides are secreted by the surface epithelium and submucosal glands (2). Although several potential airway pathogens are markedly resistant to a variety of these protein-based antimicrobials (3–6), they neither evoke inflam-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Lactoperoxidase and thiocyanate are secreted into the airway surface liquid. When H_2O_2 is artificially added (pipetted) to harvested airway surface liquid, lactoperoxidase catalyzes the formation of bactericidal hypothiocyanite by oxidizing thiocyanate.

What This Study Adds to the Field

Airway epithelia generate sufficient H_2O_2 to support hypothiocyanite production and hypothiocyanite-mediated bacterial killing. This oxidative host defense system is defective in the cystic fibrosis airway epithelium due to insufficient thiocyanate secretion.

matory responses in healthy airways nor colonize the respiratory tract, suggesting an important role for additional innate defense mechanisms in the lung.

Impaired airway host defense is characteristic of cystic fibrosis (CF) lung disease (7). How mutations in a chloride channel, the CF transmembrane conductance regulator (CFTR), lead to increased susceptibility to airway infections remains uncertain, although several mechanisms have been proposed (reviewed in References 8–11).

In addition to antimicrobial proteins and peptides, professional phagocytes possess a superoxide-producing enzyme complex, the phagocyte NADPH oxidase (*Phox*) (12), which plays a key role in the elimination of invading bacteria (13). Superoxide generated by *Phox* quickly dismutates to hydrogen peroxide (H_2O_2), which is used by myeloperoxidase to produce strongly bactericidal hypochlorous acid (14). Submucosal glands of large airways secrete the myeloperoxidase homolog lactoperoxidase (LPO) (15). LPO cannot generate hypochlorous acid (16), but the presence of H_2O_2 in the airway allows LPO to oxidize the airway surface liquid (ASL) component thiocyanate (SCN^-) (17), thereby generating antimicrobial hypothiocyanite ($OSCN^-$) (reviewed in Reference 18). However, it has not been demonstrated whether epithelial cells produce sufficient H_2O_2 to support oxidant-mediated bacterial killing in the airway.

Recently, six homologs of the catalytic *Phox* subunit gp91^{phox} were identified in humans (19), two of which, Duox1 and Duox2, are expressed in the airway epithelium (20). Duoxes produce H_2O_2 directly without releasing the intermediate molecule superoxide (21). Duox2 is also found in the thyroid gland, where it is required for thyroxine synthesis (22), and in the gastrointestinal tract (23), where its function is not known. In *Drosophila melanogaster*, Duox is involved in antimicrobial host defense of the gut

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(24), but the mechanism is unknown. Three distinct functions have been suggested for Duox in the airways, including regulation of mucin expression (25), regulation of proton secretion (26), and, as part of a Duox/LPO/SCN⁻ system, host defense (20). In accordance with the host defense hypothesis, it has been reported that the airway clearance of *Pasteurella haemolytica* is reduced in sheep when the animals inhale aerosolized dapsone, an antibiotic that also inhibits peroxidases, including LPO (27).

Here we show that Duox2, LPO, and SCN⁻ are components of a highly efficacious antimicrobial mechanism in the upper and lower airways that can eliminate gram-positive and gram-negative bacteria through reactive oxygen species (ROS). This oxidative host defense system is defective in the CF airway epithelium due to insufficient SCN⁻ secretion.

METHODS

Primary Culture of Airway Epithelial Cells

Airway epithelial cells (AECs) were isolated and cultured on collagen-coated Transwell inserts (0.3 cm²) as described previously (44). Human AECs (hAECs) were provided by the Cells and Tissue Culture Core of University of Iowa. CF donors were homozygous for the $\Delta F508$ CFTR mutation. This study was approved by the Institutional Review Board at the University of Iowa.

Transfection of Primary AECs

AECs were transfected in suspension with siRNA duplexes using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and immediately seeded on Transwell inserts.

Measurement of H₂O₂ Generation

H₂O₂ generation by AEC cultures was measured in phosphate-buffered saline (PBS) by monitoring the horseradish peroxidase (HRP)-dependent (2.5 U/ml) peroxidation of Amplex Red (50 μ M) at 550 nm and 37°C. The amount of H₂O₂ was calculated using an absorption coefficient of 54,000 M⁻¹ · cm⁻¹.

Measurement of OSCN⁻ Production by AEC and Tracheal Explants

OSCN⁻ production by AEC cultures was quantified by monitoring the oxidation of 5-thio-2-nitrobenzoic acid (TNB) (350 μ M) to 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in the upper chamber buffer (PBS plus 10 mM HEPES [pH 6.6] corresponding to the pH of ASL) (45) at 412 nm and 37°C as described (34). To measure OSCN⁻ production by tracheal explants, the mucosal and submucosal layers of freshly harvested cow trachea were dissected from the underlying cartilage, and 0.78 cm² mucosal surface areas were isolated by clipping a pair of rubber and metal rings on both sides of the explants. The submucosal side was submerged into Hanks' balanced salt solution (HBSS), and 400 μ l TNB solution (350 μ M) was pipetted on the isolated areas from which aliquots were removed for absorbance measurements. OSCN⁻ production was calculated based on the absorption coefficient of 14,100 M⁻¹ · cm⁻¹. Where indicated, the assay buffer contained the following compounds: LPO (6.5 μ g/ml), SCN⁻ (400 μ M), ATP (100 μ M), catalase (750 U/ml), diphenylene iodonium (DPI) (5 μ M), dapsone (100 μ M), and sodium azide (1 μ M).

Determination of the OSCN⁻ Content of Trachea ASL

The mucosal surfaces of freshly harvested cow trachea segments (~8 in) were washed with 1 ml PBS. The lavage samples were centrifuged (22,000 × g, 5 min, 4°C), supernatants were filtered through Centricon columns (molecular weight cutoff 3,000 Da), and aliquots of the flow through were used to measure OSCN⁻ concentration with the TNB method. OSCN⁻ content in other aliquots was reduced to SCN⁻ with glutathione (2 mM). The increase in SCN⁻ concentration was determined with the Konig reaction as described (34). Using an *in vitro* OSCN⁻-producing system (0–300 μ M H₂O₂, 400 μ M SCN⁻, and 6.5 μ g/ml LPO), the efficiency of the glutathione-dependent reduction of OSCN⁻ was found to be greater than 98%.

Immunoblot Detection of Duox Expression

Immunoblot analysis was performed as described (30) in the higher molecular weight region (> 80 kD) of blots using polyclonal anti-Duox antibody. The lower halves of blots were cut off and probed with anti-actin antibody (Abcam, Cambridge, CA).

Bacterial strains *Staphylococcus aureus* ALC1435 (46), *S. aureus*-Xen8.1 (derived from 8325-4; Xenogen Corporation, Hopkinton, MA), a clinical isolate of methicillin-resistant *S. aureus* (6), *Pseudomonas aeruginosa* PAO1 strain (47), and mucoid and nonmucoid clinical isolates of *P. aeruginosa* (6) were used for bacterial killing assays.

Bacterial Killing Assay Using Primary AECs

hAECs and rat AECs (rAECs) were incubated with antibiotic-free culture medium for several days before the bacterial killing assays. Indicated colony-forming units (CFU) of *S. aureus* or *P. aeruginosa* were resuspended in 25 μ l PBS (pH 6.6) containing various combinations of LPO (6.5 μ g/ml), SCN⁻ (400 μ M), catalase (1,500 U/ml), and ATP (100 μ M) and pipetted into the upper chamber of AEC cultures. Additional epithelia cultures were pretreated with DPI (5 μ M) for 5 min at 37°C and rinsed before inoculating bacteria. AECs were incubated with bacteria for the indicated periods of time at 37°C and placed on ice. Upper-chamber liquid was collected, and AECs were lysed with 1% saponin in distilled water. These two samples were pooled from each Transwell, and the number of surviving CFU was determined as described (29). Lower-chamber medium remained sterile in the course of assays.

Bacterial Killing Assay Using Tracheal Explants

Mucosal surface areas (0.78 cm²) were isolated on tracheal explants as described for the OSCN⁻ assay. PBS (50 μ l; pH 6.6) containing SCN⁻ (400 μ M) and 1,000 CFU of kanamycin-resistant *S. aureus* or ampicillin-resistant *P. aeruginosa* were added to the isolated areas. Where indicated, ATP (100 μ M) and catalase (1,500 U/ml) were included, or SCN⁻ was omitted from the assay buffer. After 3 h of incubation at 37°C, fluid was collected from the mucosal surface, and the mucosa was lysed with 1% saponin in distilled water. These two samples were pooled, and the number of surviving bacteria was determined in the presence of 25 μ g/ml kanamycin (for *S. aureus*) or 50 μ g/ml ampicillin (for *P. aeruginosa*) (29).

Relative Quantification of Duox1 and Duox2 mRNA Expression by Sequence Analysis

RNA was extracted from rat trachea and thyroid gland, DNase treated, and reverse transcribed as described (48). Primers annealing with rDuox1 (GenBank accession number: NM_153739) and rDuox2 (GenBank accession number: NM_024141) cDNA were 5'-GTAAGAAG ATCTACTTCATCTGGGTG-3' (forward) and 5'-GGCGACCAAA GTGGGTGACAGAG-3' (reverse). These regions (4139–4384 in rDuox2 and 4241–4486 in rDuox1) were amplified with polymerase chain reaction (PCR) and subcloned, and plasmids isolated from 52 randomly selected bacterial colonies were sequenced.

Assessment of Viability

AEC cultures were treated with the indicated combinations of LPO (19.5 μ g/ml), SCN⁻ (400 μ M), OSCN⁻ scavenger TNB (5.4 mM), and catalase (750 U/ml) in the upper chamber buffer (PBS, pH 6.6) for 24 h or with 1 mM H₂O₂ for 6 h. Single-cell suspension was generated with pronase digestion, and membrane permeability was determined with propidium iodide treatment (2 μ g/ml) and flow cytometry analysis.

Measurement of SCN⁻ Transport through Airway Epithelia

The basolateral-to-apical transport of SCN⁻ was measured by adding 40 μ M ¹⁴C-labeled SCN⁻ (50 mCi/mmol) to the lower chamber and by sampling the upper chamber buffer and the lower chamber culture medium for liquid scintillation counting.

Virus Preparation, Infection, and Transepithelial Ion Transport

Recombinant adenoviral vectors expressing CFTR (Ad2/CFTR-3) and green fluorescent protein (Ad2/GFP) were prepared and used to infect

hAECs as described (49). Restoration of CFTR function in CF hAECs by Ad2/CFTR-3 vector was confirmed by measuring transepithelial Cl^- current as previously described (49).

Statistical Analysis

All analyses were performed with SAS 9.1 software (SAS Institute, Inc., Cary, NC). The linear mixed model analysis for repeated measurements was performed to compare H_2O_2 production over time in siRNA-transfected rAECs with the fixed, within-culture, repeated measures effects being the siRNA constructs and time. The test of mean constant corresponding to the construct $\times$ time interaction was used to compare the time effect between scrambled siRNA (siSCR) and each of the siRNA constructs. A similar analysis was performed to compare SCN^- transport over time across stimulated and nonstimulated non-CF and CF hAECs. The fixed effects in the model included the two types of hAECs (non-CF and CF), which is the between-culture effect, and stimulation and time, which are the within-culture, repeated measures effects. The model included the two- and three-factor interactions. To test for differences in the time effect between stimulated and nonstimulated cultures, a test of mean contrast was used that corresponds to testing for stimulation $\times$ time interaction for each of the hAEC types.

RESULTS

Constitutive H_2O_2 Generation by Upper and Lower Airway Epithelia

Abundant ROS may eliminate microbes directly, as observed in professional phagocytes, whereas low rates of ROS production are associated with signaling functions (28). Therefore, to elucidate the role of ROS in the airway, we determined the rate of H_2O_2 generation of primary AECs of multiple species. Human, rat, cow, and mouse AECs were cultured at the air-liquid interface using Transwell inserts to allow differentiation of cells into pseudostratified mucociliary epithelia. H_2O_2 production was measured by HRP-catalyzed oxidation of Amplex Red in the upper (apical) chamber. Human, rat, and cow AECs exhibited constitutive H_2O_2 generation that was enhanced by Ca^{2+} ionophore ionomycin (data not shown) or apically added ATP (Figure 1A). However, mouse AECs were devoid of H_2O_2 generation (Figure 1A). Because the highest rate of H_2O_2 production was observed in rat epithelia (rAECs), we first characterized ROS production in rAECs.

Figure 1. H_2O_2 production and Duox expression in upper and lower airway epithelia. (A) Rates of H_2O_2 production by ATP-stimulated (100 μM , solid bars) and nonstimulated (open bars) human, rat, cow, and mouse airway epithelial cell (AEC) cultures measured with Amplex Red-horseradish peroxidase (HRP) (mean $\pm$ SEM; $n \geq 3$; paired t test, * $p < 0.05$). (B) Time course of H_2O_2 production by differentiated rat tracheal epithelium monitored by Amplex Red-HRP. Rat AECs were incubated with 100 μM ATP (closed triangles), no stimulus (open triangles), 100 μM ATP and 5 μM diphenylene iodonium (DPI) (closed circles), and 5 μM DPI (open circles). Results are normalized to 1 million cells. (C) H_2O_2 production by AEC primary cultures derived from the indicated regions of rat airway and stimulated with ATP (100 μM) or left nonstimulated (mean $\pm$ SEM; $n \geq 3$). (D) Immunoblot analysis of Duox expression in rat nasal, tracheal, and bronchial epithelia (left upper panel) and in HEK293 cells transfected with empty vector or rDuox2 and in human, rat, cow, and mouse AECs (right upper panel). Actin protein expression was detected as loading control (lower panels).

The constitutive and ATP-enhanced H_2O_2 generation of rAECs was strongly reduced by a flavoprotein inhibitor, DPI (Figure 1B), suggesting that a Nox/Duox enzyme might be responsible for the abundant ROS production of epithelial cells (8–17% that of formyl-methionyl-leucyl-phenylalanine-activated human neutrophil granulocytes [29]).

We hypothesized that the ROS-generating capacity might parallel the distribution of pseudostratified epithelium and extend to the upper airways and bronchi. Indeed, nasal and bronchial rAECs generated H_2O_2 , showing that the ROS-producing activity was not restricted to the tracheal epithelium (Figure 1C).

rAECs generated H_2O_2 without releasing superoxide intermediate, as demonstrated by the lack of signal when using a superoxide-specific detection method, the superoxide dismutase-sensitive ferricytochrome c reduction assay (data not shown). This suggested that a Duox enzyme, rather than a superoxide-generating Nox, was the source of ROS. Therefore, Duox protein expression was studied in upper and lower airway epithelia by immunoblotting protein extracts from nasal, tracheal, and bronchial rAECs using the only available anti-Duox antibody, which recognizes Duox1 and Duox2 (30). Upper and lower airway epithelia contained Duox protein (Duox1 or 2 or both), but the highest expression level was detected in tracheal rAECs (Figure 1D, *left panel*), which correlated well with the H_2O_2 production of rAECs of different airway regions.

Next, we asked whether the rate of H_2O_2 generation also correlated with Duox expression in the investigated species. Immunoblot experiments detected Duox protein in human, cow, and rat AECs and in the positive control rDuox2-transfected HEK293 cells but not in mouse airway epithelia (Figure 1D, *right panel*), although the anti-Duox antibody reacted with mouse Duox in the thyroid gland (data not shown). These results suggested that mouse airways have different ROS metabolism than that of the larger species and further supported the hypothesis that Duox1 or Duox2 (or both) was the source of ROS in airway epithelia.

Duox2 Is the Main Source of H_2O_2 in Rat Airway Epithelium

Duox1 and Duox2 mRNA have been detected in the airway (26); hence, either enzyme could be the H_2O_2 generator of airway epithelia. We assessed the relative levels of Duox1 and Duox2 transcripts in rat trachea by PCR amplification and sequencing of highly similar regions of the two mRNA. First, PCR oligos were designed to anneal with identical regions in rDuox1 and rDuox2 mRNA. Then, using these oligos and trachea cDNA template, 246-bp-long fragments of either transcript were amplified that differed by only two nucleotides over their entire lengths. After cloning and randomly sequencing 52 PCR products, we determined that the percentage of Duox2 transcript was 81%, suggesting that Duox2 might be the relevant source of ROS in rat trachea. The ratio of Duox1 and Duox2 transcripts was reversed in rat thyroid tissue (Duox1, 70%; Duox2, 30%), suggesting that rat Duox1 may correspond functionally to human Duox2, the H_2O_2 -generating enzyme involved in thyroid hormone synthesis.

We investigated further the H_2O_2 -producing enzyme of airway epithelia by selectively reducing Duox1 and Duox2 expression levels using RNA interference. Several short interfering RNA (siRNA) duplexes were designed to target dissimilar regions in the two Duox transcripts (Table E1 in the online supplement). We then tested the efficacy and specificity of these siRNA in a model system: HEK293 cell clones heterologously expressing rDuox1 or rDuox2 were transfected with the Duox1 and Duox2 siRNA constructs (siDuox1 and 2) or with a scrambled siRNA (siSCR, negative control), and Duox protein expression was

assessed by immunoblot assays using the pan-anti-Duox antibody (Figures 2A and 2B). We chose siDuox1-5 and siDuox2-2 for further experimentation because they reduced the amount of the corresponding Duox protein by more than 80%, as determined by band densitometry, without decreasing the expression of the other, nontargeted Duox enzyme (data not shown).

We made use of these siRNA duplexes in the primary epithelium. rAECs were transfected with siDuox1-5 and siDuox2-2 immediately after detaching cells from the tracheal mucosa. rAECs were seeded at high density and cultured for 4 d until epithelial layers with high transepithelial resistance developed. When AECs reached their maximum capacity for ROS production (data not shown), Duox protein expression was assessed by immunoblotting, and H_2O_2 generation was measured with Amplex Red–HRP in the presence of ATP stimulus. The pan-anti-Duox antibody detected reduced Duox expression levels only in siDuox2-2 transfected cultures, whereas the amount of Duox protein was unaltered by siDuox1-5 and siSCR (Figures 2C and 2D). H_2O_2 production was also reduced only by siDuox2-2, whereas siDuox1-5 had no effect compared with control transfectants (Figure 2E). These results indicate that Duox2 was the main source of H_2O_2 in rat airway epithelium.

OSCN[−] Production by Primary rAECs

Our data suggested that H_2O_2 was constitutively produced in the airways. Nevertheless, very little H_2O_2 is detected in the expired breath condensate of healthy individuals (~ 30 nM) (31). Peroxidases, however, may quickly consume H_2O_2 . Indeed, LPO is secreted by the submucosal glands of large airways and maintained at high level in ASL (~ 6.5 μg LPO/ml) (15). In addition, airway epithelia secrete the pseudohalide thiocyanate ($[\text{SCN}^-]_{\text{ASL}} \sim 400$ μM) (32), which can be readily oxidized by H_2O_2 in an LPO-catalyzed reaction generating OSCN[−].

To assess the ability of airway epithelia to produce OSCN[−], we included LPO (6.5 μg /ml) and SCN[−] (400 μM) in the upper chamber buffer of nonstimulated AECs and monitored OSCN[−] formation by measuring the oxidation of a sulfhydryl compound TNB. Continuous OSCN[−] generation was detected at a rate comparable with that of H_2O_2 production, suggesting that H_2O_2 efficiently oxidized SCN[−] in the presence of LPO (Figure 3A). In the absence of LPO or SCN[−], OSCN[−] could not be detected (Figure 3B). Although ATP treatment enhanced H_2O_2 generation (Figure 1B), it did not increase the rate of OSCN[−] formation under these experimental conditions, indicating that the availability of LPO or SCN[−] was rate limiting (Figure 3B). Indeed, threefold more LPO increased OSCN[−] formation when rAECs were stimulated with ATP (Figure 3B). Catalase and DPI each inhibited the accumulation of OSCN[−], confirming that it required enzymatic H_2O_2 production (Figure 3B). These data show that, in the presence of physiologic concentrations of LPO and SCN[−], airway epithelia support a high rate OSCN[−] generation.

OSCN[−]-dependent Killing of Airway Pathogens by the Tracheal Epithelium

Saliva contains LPO and SCN[−] and generates antimicrobial OSCN[−] if H_2O_2 is pipetted into this fluid or provided by H_2O_2 -producing bacterial strains (33). We tested whether airway epithelia could generate sufficient H_2O_2 to support an OSCN[−]-dependent bactericidal system. We used *S. aureus* (strain ALC1435) and *P. aeruginosa* (PAO1), a gram-positive and a gram-negative pathogen, respectively, for bacterial killing assays. Approximately 1,000 CFU of each strain were added to the apical chamber of differentiated, nonstimulated rAEC cultures in 25 μl assay buffer (PBS) together with various combinations of SCN[−], LPO, and catalase (Figures 4A and 4B). Additional

Figure 2. Duox2 is the main source of H₂O₂ in rat airway epithelium. (A) *Upper panel:* Immunoblot detection of Duox1 protein levels in an rDuox1-expressing HEK293 cell clone transfected with scrambled short interfering RNA (siSCR) and five siRNA duplexes targeting rDuox1 (siDuox1-1 to -5) or left nontransfected (NT). *Lower panel:* Immunoblot detection of actin expression as loading control. (B) *Upper panel:* Immunoblot detection of Duox2 protein levels in an rDuox2-expressing HEK293 cell clone transfected with siSCR and three siRNA duplexes targeting rDuox2 (siDuox2-1 to -3), or left nontransfected (NT). *Lower panel:* Actin was detected as loading control. (C) Quantification of Duox protein expression in siSCR transfected, NT, and siDuox2-2- and siDuox1-5-transfected primary rat AECs (rAECs) using immunoblot (upper panel). Amounts of loaded protein were verified by immunoblotting actin (lower panel). (D) Statistical analysis of three independent experiments performed as described in C. The intensities of Duox bands were normalized to actin and expressed as percentage of siSCR control (one-way analysis of variance [ANOVA], $p < 0.004$; *post hoc* Dunnett's test, control group "siSCR," * $p < 0.05$). (E) Time course of H₂O₂ production by ATP (100 μ M)-stimulated rAECs transfected with siSCR, siDuox2-2, or siDuox1-5 or left NT. Statistical analysis using linear mixed analysis model for repeated measure shows significantly reduced H₂O₂ generation only in siDuox2-2-transfected rAECs (siSCR vs. siDuox2-2; ** $p < 0.01$; mean $\pm$ SEM; $n = 3$).

negative control cultures were pretreated with the irreversible Nox/Duox inhibitor DPI and washed to preclude exposure of bacteria to DPI. After a 3-h incubation, the upper-chamber liquid was collected, and AECs were lysed with saponin to release adherent microbes. These two samples were pooled, and the number of surviving bacteria was determined using quantitative culture. *S. aureus* and *P. aeruginosa* were killed in the presence of LPO and SCN⁻ within 3 h (Figures 4A and 4B). Addition of catalase or DPI prevented bacterial killing, indicating that enzymatic H₂O₂ production was critical for the antimicrobial effect. In the absence of LPO or SCN⁻, the number of viable *S. aureus* remained stable, and *P. aeruginosa* grew despite continuous H₂O₂ production (Figures 4A and 4B), demonstrating that the concentration of H₂O₂ did not reach toxic levels for either organism. In the absence of rAECs, the number of surviving *P. aeruginosa* declined when incubated in the nutrient-free assay buffer for 3 h at 37°C (Figure 4B). Thus, under these conditions, primary rAEC cultures fostered the growth of *P. aeruginosa* if the OSCN⁻-generating system was inactive.

To explore further the bactericidal activity of rAEC-supported OSCN⁻ production, we expanded our investigation to clinical isolates of methicillin-resistant *S. aureus* as well as mucoid and nonmucoid *P. aeruginosa* strains collected from CF airways (6). Similar to the laboratory strains initially tested, the clinical isolates were eliminated by the OSCN⁻-generating

mechanism supported by the H₂O₂ production of airway epithelia (Figure E1). When any component of the OSCN⁻ generating system was missing, all three clinical isolates grew on the mucosal surface of rAECs.

To characterize the H₂O₂ dependence of bacterial killing, we determined the effect of various DPI concentrations on the H₂O₂ production and OSCN⁻-mediated antibacterial activity of rat airway epithelia (Figure 4C) using *S. aureus* (ALC1435) as a model organism. These experiments showed that H₂O₂ generation had to be reduced by more than 65% to observe an impairment of bactericidal activity, which highlighted the abundance of ROS generated by rAECs.

To estimate the bacterial killing capacity of the oxidative host defense system, we measured the survival of a range of *S. aureus* inocula (100–300,000 CFU) in the presence of SCN⁻ and LPO. Bacteria numbers were reduced by 2–3 log during the 3-h incubation, even with the largest inoculum tested (300,000 CFU corresponding to 1:1 multiplicity of infection; Figure 4D). Thus, the OSCN⁻-producing system has a robust antimicrobial capacity against *S. aureus*.

OSCN⁻ Production Is Not Toxic to the Airway Epithelium

ROS toxicity is usually not restricted to microbes and may cause collateral damage to the host. Thus, a constitutively active, high-capacity ROS generator seems disadvantageous to any organ.

Figure 3. Hypothiocyanite (OSCN⁻) production by rat airway epithelia. (A) Time course of OSCN⁻ production by nonstimulated (open triangles) and diphenylene iodonium (DPI)-treated (closed circles) rAECs in the presence of airway surface liquid components thiocyanate (SCN⁻) (400 μ M) and lactoperoxidase (LPO) (6.5 μ g/ml). (B) Summary of rates of OSCN⁻ production by rAECs incubated with the indicated combinations of ATP (100 μ M), LPO (6.5 μ g/ml), increased amount of LPO ($\times 3$ LPO, 20 μ g/ml), SCN⁻ (400 μ M), catalase (750 U/ml), and DPI (5 μ M) (mean $\pm$ SEM; $n \geq 3$; one-way ANOVA, $p < 0.0001$; five pairwise comparisons with Bonferroni's test; ** $p < 0.01$).

Therefore, we investigated the potential toxic effects of the OSCN⁻-producing system on rAECs. Primary cultures were incubated with the following compounds in the upper chamber for 24 h: assay buffer only to allow constitutive H₂O₂ production, catalase to scavenge H₂O₂, LPO-SCN⁻ to reconstitute the OSCN⁻ generator, and LPO-SCN⁻-TNB to scavenge OSCN⁻. Very high H₂O₂ concentrations are known to be toxic to AECs. Therefore, as a positive control for cell death, rAECs were incubated with 1 mM H₂O₂ for 6 h. Cell death was quantified by propidium iodide staining and flow cytometry. Neither OSCN⁻ generation nor constitutive H₂O₂ production increased cell death significantly compared with their respective scavenger controls, TNB and catalase. However, treatment of rAECs with 1 mM H₂O₂ markedly increased the percentage of propidium iodide-stained cells (Figure E2). Thus, the OSCN⁻-generating system did not damage the airway epithelium, whereas it was toxic to bacteria.

OSCN⁻ Production by Tracheal Explants

The rAEC culture model suggested that an OSCN⁻-producing system might be functional in the airway *in vivo*. However,

primary airway epithelium cultures lack the complexity of the *in vivo* tissue because they do not contain submucosal glands and thus have no LPO activity. Therefore, we investigated further the oxidative host defense system using freshly harvested trachea and short-term cultures of tracheal explants. For these experiments, we used cow trachea because of the large airway diameter and because primary AECs from cow trachea also expressed Duox protein (Figure 1D) and produced H₂O₂ at a rate similar to rAECs (Figure 1A).

If the oxidative system is functional *in vivo*, OSCN⁻, a relatively stable ROS, may accumulate in the ASL. To investigate this, mucosal surfaces of freshly harvested cow tracheas were rinsed with PBS, and the lavage was filtered before measuring the OSCN⁻ content with the TNB method. TNB detected $18.38 \pm 8.55 \mu$ M OSCN⁻ ($n = 3$) in the lavage despite the considerable dilution of the ASL (~ 20 -fold).

To verify the specificity of the TNB detection method, we used glutathione (GSH) to consume the OSCN⁻ content of the lavage (OSCN⁻ + 2GSH $\rightarrow$ SCN⁻ + GSSG) and measured the amount of newly generated SCN⁻ with the König reaction (34). The glutathione treatment elevated SCN⁻ concentration by $15.94 \pm 9.34 \mu$ M ($n = 3$), which corresponded well to the OSCN⁻ concentration measured in the lavage samples. These experiments suggested that OSCN⁻ accumulated at the airway mucosal surface *in vivo*.

To characterize the components of the oxidative system in the context of airway mucosa and submucosa, we established short-term cultures of tracheal explants in which we isolated 0.78 cm² mucosal surface areas by dissecting the mucosal and submucosal layers of cow trachea *en bloc* from the underlying cartilage and mounting them in small chambers (Figure E3). The submucosal side was immersed into HBSS, and OSCN⁻ production was measured on the mucosal surface with TNB solution by removing aliquots for absorbance measurement at each time point (Figure 5A). Because the assay buffer diluted ASL approximately 180-fold, SCN⁻ was added to the mucosal side to restore 400 μ M concentration. OSCN⁻ accumulated continuously in the mucosal chambers at a rate comparable with that of H₂O₂ production by primary cow AECs (Figure 1A; $\sim 10^6$ cAEC cover 1 cm²). Moreover, OSCN⁻ generation was enhanced by ATP and strongly reduced by DPI and catalase. OSCN⁻ generation was also reduced by dapsone (100 μ M) and azide (1 μ M) (Figure 5B), both of which inhibit LPO, but not MPO, at the chosen concentrations (35). These results strongly suggest that tracheal mucosa possesses endogenous H₂O₂-producing capacity and LPO activity and generates OSCN⁻.

Tracheal Explants Kill Bacteria through OSCN⁻ Production

In contrast to the primary AEC cultures, tracheal explants contain submucosal glands, which secrete LPO and a variety of other host defense proteins and peptides. Therefore, we assessed the relative importance and bactericidal capacity of the OSCN⁻-generating system using these explants. Approximately 1,000 CFU of *S. aureus* and *P. aeruginosa* were inoculated onto isolated mucosal surface areas (0.78 cm²) in 50 μ l assay buffer containing 400 μ M SCN⁻. Figure 6 illustrates that *S. aureus* and *P. aeruginosa* were largely eliminated within 3 h. The addition of catalase or the removal of SCN⁻ prevented bacterial killing (Figures 6A and 6B), whereas ATP stimulation enhanced the elimination of *S. aureus* (Figure 6A). Thus, in the presence of SCN⁻, tracheal explants exhibited a spontaneous, H₂O₂-dependent bactericidal activity against *S. aureus* and *P. aeruginosa*.

Defective SCN⁻ Secretion Renders the Oxidative Host Defense System Inactive in the CF Airway Epithelium

SCN⁻ secretion from the basolateral to apical side of airway epithelia is sensitive to the nonselective CFTR inhibitors (32).

Figure 5. Hypothiocyanite (OSCN^-) generation by cow tracheal explants. (A) Time course of OSCN^- production by isolated mucosal surface areas on cow trachea incubated with ATP (100 μM , closed triangles), no stimulus (open triangles), or ATP (100 μM) and DPI (5 μM) (closed circles) in the presence of thiocyanate (SCN^-) (400 μM). (B) Statistical analysis of rates of OSCN^- generation by cow tracheal explants incubated with the indicated combinations of ATP (100 μM), SCN^- (400 μM), peroxidase inhibitors dapsone (100 μM) and azide (1 μM), catalase (750 U/ml), and DPI (5 μM) (mean $\pm$ SEM; $n = 3$; one-way ANOVA, $p < 0.0001$; *post hoc* Dunnett's test, control group containing only ATP and SCN^- , $^{**}p < 0.01$).

Three components are required for OSCN^- production in the airway: (1) Duox activity to produce H_2O_2 , (2) SCN^- secretion to provide a reaction partner for H_2O_2 , and (3) LPO activity to catalyze the oxidation of SCN^- ($\text{H}_2\text{O}_2 + \text{SCN}^- \rightarrow \text{OSCN}^- + \text{H}_2\text{O}$). In CF airway epithelium, OSCN^- generation is diminished due to a CFTR-dependent defect in SCN^- secretion, which leads to a collapse of the oxidative antimicrobial mechanism.

Because SCN^- is critical for the function of this oxidative host defense system, a sustained supply of SCN^- from the circulation to the airway mucosal surface is essential. The SCN^- pool in the circulation is believed to arise from two sources: from the detoxification of cyanide (CN^-) catalyzed by the enzyme rhodanese and, more significantly, directly from the diet (37). Glucosinolates of plant tissue are especially rich sources of SCN^- . Indeed, 23% of the total body SCN^- content is replaced daily in rats, whereas the plasma SCN^- concentration is maintained at approximately 86 μM (37). Moreover, plasma SCN^- concentration does not decrease during fasting in rats (37), indicating that SCN^- homeostasis is carefully controlled. Although little is known about the regulatory mechanisms involved, increased renal retention of SCN^- and redistribution of SCN^- among body fluid compartments seem to play important roles.

Figure 6. Hypothiocyanite (OSCN^-)-dependent killing of *S. aureus* and *P. aeruginosa* by tracheal mucosa. Recovered CFU of (A) *S. aureus* (Xen8.1) and (B) *P. aeruginosa* (PAO1) after 3-h incubation of 1,000 CFU (dotted lines) of each strain on 0.78 cm^2 mucosal surface area of cow tracheal explants. The indicated combinations of SCN^- (400 μM), ATP (100 μM), and catalase (1,500 U/ml) were included in the assay buffer (mean $\pm$ SEM; $n \geq 3$; one-way ANOVA, $p < 0.0001$ [A], $p < 0.01$ [B]; *post hoc* Dunnett's test, control group containing catalase, $^{**}p < 0.01$).

The Na^+/I^- symporter (NIS) provides a pathway for SCN^- to enter the cells from the extracellular fluid (38). AECs express NIS in the basolateral membrane (32), which allows them to generate an outward concentration gradient for SCN^- . Propelled by this gradient, SCN^- may leave the cells through an apical plasma membrane conductance. Our results demonstrate for the first time that CFTR is critical for this transepithelial SCN^- secretion process. Because CFTR is localized to the apical plasma membrane of AECs, permeable to SCN^- and able to conduct SCN^- efflux (39), it might mediate the apical SCN^- current directly. NIS is also highly expressed in the thyroid gland (40), where it imports not only iodine but also SCN^- to the epithelial cells. However, the thyroid peroxidase catalyzing iodine organification in the thyroid follicles would be strongly inhibited by SCN^- (41). The thyroid epithelium lacks CFTR completely (42), suggesting a mechanism that could bar SCN^- from the peroxidase content of the follicles in this organ. Nevertheless, further studies and direct evidence are required to prove the identity of the SCN^- conductance.

Once SCN^- is secreted to the ASL, it is oxidized to OSCN^- by the concerted oxidase activities of Duox2 (in rat) and LPO. Our experiments using Duox1- and Duox2-selective RNAi indicated that Duox2 was the main source of H_2O_2 in rAECs, whereas a previous study using antisense RNA identified Duox1 as the

Figure 7. Defective thiocyanate (SCN^-) secretion and diminished hypothyocyanite (OSCN^-)-dependent bacteria killing by CF epithelia. (A) Changes in SCN^- concentration over time in the upper chamber of non-CF (triangles) and CF (circles) epithelia in the presence (filled symbols) or absence (open symbols) of db-cAMP after addition of $40 \mu\text{M}$ SCN^- to the lower chamber (linear mixed statistical analysis model for repeated measures, non-CF vs. CF hAECs; $**p < 0.01$). (B) Typical transepithelial short-circuit current profiles recorded with non-CF, CF ($\Delta\text{F508}/\Delta\text{F508}$), and Ad/CFTR-infected CF epithelia. Horizontal bars indicate the addition of amiloride ($100 \mu\text{M}$) to inhibit epithelial sodium conductance, IBMX ($100 \mu\text{M}$)/forskolin ($10 \mu\text{M}$) to stimulate CFTR-mediated transepithelial Cl^- current, and bumetanide ($100 \mu\text{M}$) to block all Cl^- movement into the cell from the basolateral membrane. (C) Rates of SCN^- transport across non-CF, CF, Ad/CFTR-, and Ad/GFP-infected polarized epithelia (mean $\pm$ SEM; $n = 3$ donors; one-way ANOVA, $p < 0.0001$; *post hoc* Dunnett's test, control group "non-CF," $**p < 0.01$). (D) Recovered CFU of *S. aureus* after incubating 1,000 CFU inocula (dotted line) on the apical side of ATP-stimulated non-CF and CF epithelia in the presence of LPO ($6.5 \mu\text{g}/\text{ml}$) for 3 h. SCN^- was added to the lower chamber ($40 \mu\text{M}$, basolateral) 10 h before the killing assay or to the upper chamber ($400 \mu\text{M}$, apical) as indicated. Catalase ($1,500 \text{ U}/\text{ml}$) was included into the upper chamber where indicated (mean $\pm$ SEM; $n = 3$ donors; paired *t* test, $**p < 0.01$).

H_2O_2 -generating enzyme in hAECs (20). Duox1 and Duox2 are similar to each other (72% amino acid identity) in both species, and the Duox1 and Duox2 nomenclature is based on the orientation of their respective genes rather than on a conserved structural difference. Thus, the discrepancy between the two sets of results could arise from a species difference.

In contrast to larger species (e.g., human, cow, and rat), mice lack Duox expression and H_2O_2 production in tracheal epithelia (Figure 1A). Thus, the host defense mechanisms of mouse airways may markedly differ from those of the conducting airways of humans. CFTR-deficient mice also fail to reproduce the "spontaneous" chronic bacterial airway infections and inflammations observed in patients with CF (43).

What is the importance of the diminished transepithelial SCN^- transport in the disease process of CF airways? Several mechanisms have been proposed to link inactivation of CFTR to impaired airway immunity. Nevertheless, existing data and the derived models do not fully explain the pathophysiology of

CF lung disease, hampering efforts to develop new therapies. Inactivation of CFTR is likely to cause multiple defects in the airway that together alter local innate immunity. The diminished SCN^- transport across the CF airway epithelium and the resultant collapse of the oxidative host defense system is one of these defects. However, the large capacity of the OSCN^- -dependent bactericidal mechanism and its efficacy against *S. aureus* and *P. aeruginosa*, two frequent CF pathogens, suggest that inactivation of the ROS-generating system may be a major factor in the disease process. Therefore, supplying SCN^- to the airway surfaces of people with CF may have important therapeutic implications.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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