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Original Contribution

Concentration of the antibacterial precursor thiocyanate in cystic fibrosis airway secretions

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A B S T R A C T

A recently discovered enzyme system produces antibacterial hypothiocyanite ($OSCN^-$) in the airway lumen by oxidizing the secreted precursor thiocyanate (SCN^-). Airway epithelial cultures have been shown to secrete SCN^- in a CFTR-dependent manner. Thus, reduced SCN^- availability in the airway might contribute to the pathogenesis of cystic fibrosis (CF), a disease caused by mutations in the *CFTR* gene and characterized by an airway host defense defect. We tested this hypothesis by analyzing the SCN^- concentration in the nasal airway surface liquid (ASL) of CF patients and non-CF subjects and in the tracheobronchial ASL of CFTR-ΔF508 homozygous pigs and control littermates. In the nasal ASL, the SCN^- concentration was ~30-fold higher than in serum independent of the CFTR mutation status of the human subject. In the tracheobronchial ASL of CF pigs, the SCN^- concentration was somewhat reduced. Among human subjects, SCN^- concentrations in the ASL varied from person to person independent of CFTR expression, and CF patients with high SCN^- levels had better lung function than those with low SCN^- levels. Thus, although CFTR can contribute to SCN^- transport, it is not indispensable for the high SCN^- concentration in ASL. The correlation between lung function and SCN^- concentration in CF patients may reflect a beneficial role for SCN^-.

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Introduction

Most inhaled bacteria become entrapped in a mucus layer that covers the conducting airways. This mucus layer is constantly cleared from the healthy respiratory tract by the concerted movement of airway cilia. During the mucociliary clearance process, bacterial growth and survival are limited by the antimicrobial proteins of the airway surface liquid (ASL)[1]. Recent studies suggest that in addition to the antimicrobial proteins of airway secretions, an oxidative host defense mechanism of airway epithelia may also contribute to the antibacterial activity in the ASL [1–3].

Airway epithelial cells express two plasma membrane-embedded cytochromes—dual oxidase 1 (Duox1) and Duox2—that generate H_2O_2 on the extracellular side of the apical membrane [4–7]. Within the extracellular space, H_2O_2 is metabolized by the secretory protein lactoperoxidase (LPO) [8,9], which uses H_2O_2 to oxidize the physiological ASL component thiocyanate (SCN^-) to the potent antibacterial molecule hypothiocyanite ($OSCN^-$). Cultured airway epithelia produce sufficient H_2O_2 to support $OSCN^-$ generation at levels toxic to bacteria [10–12]. Furthermore, inhibiting LPO activity in vivo hinders bacterial clearance from the lower airways [1].

Although the airway epithelium does not synthesize SCN^-, the concentration of SCN^- in the ASL (~460 μM) is far higher than that in the serum (5–50 μM) [3]. Cell-culture experiments indicated that SCN^- is imported into the airway epithelium basolaterally, via the Na^+–I^- symporter NIS [13]. SCN^- subsequently leaves the cells apically, through

Abbreviations: ASL, airway surface liquid; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CFU, colony-forming unit; Duox, dual oxidase; FEV_1, forced expiratory volume in 1 s; H_2O_2, hyrogen peroxide; LPO, lactoperoxidase; MPO, myeloperoxidase; NIS, Na^+–I^- symporter; $OSCN^-$, hypothiocyanite; SCN^-, thiocyanate.

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the CFTR anion channel [10,11,14], which is permeable to SCN^- as well as to chloride (Cl^-) and bicarbonate. Mutations in the gene encoding CFTR lead to cystic fibrosis (CF) disease, which in the airway is characterized by recurrent and chronic infections [15]. Notably, primary cultures of CF airway epithelia are defective for $OSCN^-$-dependent bacterial killing, because of a reduction in SCN^- secretion [10,11]. These findings are consistent with the notion that insufficient SCN^- secretion in CF airways might contribute to the pathogenesis of CF lung disease. However, the SCN^- concentration in the ASL of CF patients has not been determined.

We used a recently developed porcine model of CF to evaluate the effect of CFTR inactivation on the SCN^- concentration in tracheobronchial secretions. We also evaluated SCN^- levels in the nasal secretions from CF patients and non-CF subjects. Contrary to our expectations, we found that the SCN^- concentration was similar in the nasal secretions of CF and non-CF subjects, whereas a moderate reduction in SCN^- concentration was detected in the tracheobronchial secretions of CF pigs compared to control littermates. Furthermore, in humans CFTR-independent factors led to significant person-to-person variability in ASL SCN^- concentrations, and CF patients with high SCN^- levels exhibited better lung function than those with low SCN^-.

Materials and methods

Human subjects

Twenty-three CF patients and 21 non-CF subjects participated in this study. Nasal ASL was collected from all participants. Fourteen CF subjects and 18 non-CF subjects also provided blood samples. Both CF and non-CF volunteers were nonsmokers and experienced no symptoms of upper airway infection or allergic rhinitis during the 3 weeks before recruitment. For all recruited patients, the diagnosis of CF had been previously confirmed by genotyping. The pulmonary function of CF patients was evaluated based on the spirometric measurement of forced expiratory volume in 1 s (FEV_1). Spirometry was done according to the American Thoracic Society guidelines [16]. Additional subject information is summarized in Table 1. This study was approved by the Institutional Review Board of the University of Iowa.

CFTR mutant and control pigs

Production of heterozygous CFTR-ΔF508 pigs was previously reported [17]. These animals were intercrossed to generate homozygous CFTR-ΔF508 pigs and wild-type littermates. The lung phenotypes of homozygous CFTR-ΔF508 pigs and CFTR-null pigs [18–20] are indistinguishable (unpublished observation). Newborn pigs were genotyped immediately, and homozygous CFTR-ΔF508 pigs ($n = 6$) and wild-type pigs ($n = 14$) were used for this study within 12 h of birth. This study was approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Table 1
Study subject information.

Characteristic	CF ($n = 23$)	Non-CF ($n = 21$)
Age in years (SD)	34.7 (9.7)	28.4 (7.1)
Age range in years	23–60	21–52
Gender ratio (M/F)	14/9	13/8
ΔF508 homozygous	70%	0
ΔF508 compound heterozygous	30%	0
Inpatient	48%	0
Outpatient	52%	0
Intravenous antibiotics	36%	0
Oral antibiotics	83%	0
Inhaled tobramycin and/or colistin	61%	0
No antibiotics	9%	100%
Pseudomonas aeruginosa in sputum	74%	ND[a]

[a] Not determined.

Blood and ASL collection

Venous blood of human subjects was collected from an arm vein. Blood of CFTR-ΔF508 homozygous and wild-type pigs was collected under propofol anesthesia. Before the analysis of anion composition, the serum fraction was filtered (3 kDa cut-off Ultracel filter; Millipore) to remove the majority of serum proteins.

Nasal ASL was harvested from human subjects using microsampling probes (Olympus BC-402C) [21,22]. Before sample collection, nostrils were kept closed with a diver's clip for 5 min to minimize evaporation. The probes were then introduced deep into the nose and held gently to the nasal turbinates. After 30–60 s, the probes were removed from the nose and placed onto filters in microcentrifuge tubes (Costar Spin-X filter). Undiluted ASL was recovered from the probes by centrifugation.

Lower airway secretions were collected from pigs under propofol anesthesia. The tracheas of pigs were surgically exposed and opened horizontally using electrocauterization. Microsampling probes were introduced into the respiratory tract through the surgical opening and were held gently to the surface of the trachea and bronchi at multiple points. Our initial experiments indicated that the volume of ASL collected from the lower airways was not always sufficient for analysis when dry probes were used and that the efficiency of collection could be improved by prewetting the probes with 2 μl isosmotic mannitol solution containing 300 μM Evans blue dye. After ASL collection was completed, fluid was extracted from the probes by centrifugation, and the ASL content of the harvested fluid was calculated based on the extent to which Evans blue dye was diluted. The dilution factor was determined by measuring the optical density of the collected fluid at 600 nm, using a NanoDrop ND-1000 spectrophotometer.

Ion-exchange chromatography

Ultrapure water was used to dilute the serum (4-fold) and ASL samples (50- and 100-fold) before measuring ion concentration using a Metrohm advanced ion chromatography system (MIC-2; Metrohm USA, Inc.) and a Metrosep A Supp 5–150 column. The mobile phase was composed of 1 mM sodium carbonate and 3.2 mM sodium bicarbonate. Anions were detected based on changes in conductivity, and the conductivity detector was calibrated with standard solutions.

Bacterial killing assay

Well-differentiated primary cultures of human airway epithelia were obtained from the *In Vitro* Models and Cell Culture Core at the University of Iowa [23]. These cultures were maintained at an air–liquid interface and incubated in the absence of antibiotics for 5 days before the bacterial killing assays. The bacterial killing activity of airway epithelial cultures was measured as previously described [10]. In brief, mid-log phase liquid cultures of *Staphylococcus aureus* (strain Xen8.1; Xenogen Corp., Hokinton, MA, USA) were pelleted and resuspended in PBS. Bacterial density was estimated by measuring optical density at 550 nm. Approximately 3000 and 1000 colony-forming units (CFU) of bacteria were inoculated onto the apical surface of airway epithelial cultures in PBS (60 μl inoculum/cm^2 surface area) supplemented with LPO (7 μg/ml), SCN^- (0–700 μM), and Hepes (10 mM, pH 6.6). Epithelial H_2O_2 production was maximized with the apical addition of ATP (100 μM) [6,24,25]. After a 3-h incubation at 37 °C, liquid was collected from the apical surface. Epithelial cultures were then lysed with 1% saponin in distilled water, and lysates were pooled with the previously collected apical fluid. The

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