Published in final edited form as:
FEBS Lett. 2007 January 23; 581(2): 271–278.

The Lactoperoxidase System Links Anion Transport To Host Defense in Cystic Fibrosis

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Abstract

Chronic respiratory infections in cystic fibrosis result from CFTR channel mutations but how these impair antibacterial defense is less clear. Airway host defense depends on lactoperoxidase (LPO) that requires thiocyanate (SCN^-) to function and epithelia use CFTR to concentrate SCN^- at the apical surface. To test whether CFTR mutations result in impaired LPO-mediated host defense, CF epithelial SCN^- transport was measured. CF epithelia had significantly lower transport rates, did not accumulate SCN^- in the apical compartment. The lower CF [SCN^-] did not support LPO antibacterial activity. Modeling of airway LPO activity suggested that reduced transport impairs LPO-mediated defense and cannot be compensated by LPO or H_2O_2 upregulation.

Keywords

Lactoperoxidase; CFTR; Host Defense; thiocyanate; hydrogen peroxide; cystic fibrosis

Cystic fibrosis (CF) is one of the most common genetic disorders among Caucasians. The disease is characterized by recurrent respiratory infections caused primarily by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [1]. Lifelong respiratory infections result in progressive loss of lung function and shortened life expectancy. Chronic infection implies failure of airway host defenses against bacterial colonization. However, the exact defects in CF host defense that are primarily responsible chronic infection in this disease remain unclear. The normal airway barrier to infection is complex and multifactorial. One possible view is that mucus delays growth of pathogens and attachment of bacteria and other particles to epithelia cells until cilia mechanically clear them. The complex composition of mucus must provide the proper viscoelastic properties to permit ciliary clearance but also must contain broad-spectrum antibiotic activities in order to be effective.

CFTR, the gene product responsible for CF, contains an anion channel that has been extensively characterized [2]. CFTR mutations result in loss of functional anion channel activity on the apical cell surface, although, it is not fully understood how a loss of anion channel activity

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completely explains defective antibacterial defenses. Several hypotheses have been proposed to relate these two phenomena and focus on: a) the effects of airway surface liquid (ASL) dehydration that leads to impaired ciliary and cough clearance of mucus [3]; b) the effects of elevated ASL salt concentration on the activity of antimicrobials in mucus [4–6]; or c) CFTR mediated defective bacterial-epithelial interactions [for reviews, see 7,8–11].

Current views of CF pathogenesis may be limited by incomplete knowledge regarding the array of mechanisms at work in airway defense. For example, the lactoperoxidase (LPO) system has recently been shown to be present in airways, and to be important for airway antibacterial activity and bacterial clearance in both humans and sheep [12,13]. The LPO system works to preserve sterility of secretions [14],[15] in several species including human. Similar to myeloperoxidase (MPO) and eosinophil peroxidase (EPO), LPO uses H_2O_2 to catalyze oxidation of the anion SCN^- to the antibiotic $OSCN^-$ [16] via an oxidized enzyme intermediate called compound I (LPO-O).

$$H_2O_2 + \text{LPO} \rightarrow \text{LPO} - O + H_2O$$

$$\text{LPO} - O + SCN^- \rightarrow OSCN^- + \text{LPO}$$

Both SCN^- and H_2O_2 are required for LPO to function in the airway lumen. Normal human airway epithelia transport SCN^- from the basolateral to the apical surface and concentrate it in airway secretions [17]. This transport utilizes the sodium/iodide symporter at the basolateral surface to mediate uptake of SCN^- or I^- into the cells followed by release to the apical surface that is regulated by CFTR [17]. Normal human airway epithelia also actively produce H_2O_2 at the apical surface using Duox and release it into the apical surface liquid [18]. This airway H_2O_2 production is increased through stimulation of purinergic receptors [18] that also act to increase mucus secretion [19,20] and ciliary beating [21–23] all through intracellular Ca^{2+} increases. Thus, a complete antibacterial LPO system is present in the airway lumen and can be regulated in concert with other facets of the airway host defense.

Since the LPO system is effective against staphylococci [24], *E. coli* and pseudomonads [24–26] and since SCN^- is a requisite substrate for LPO, defects in SCN^- transport and resulting loss of LPO activity could contribute, at least partially, to the chronic respiratory infections seen in CF patients. Although MPO was previously thought to primarily use chloride as a substrate, it is now recognized that MPO also uses SCN^- and that SCN^- maybe the physiological substrate for MPO [27]. In addition, hypochlorite (OCl^-), the product of MPO oxidation of chloride, reacts almost instantaneously with SCN^- at physiological concentrations [28] to give $OSCN^-$ suggesting that SCN^- may have a central role not only in LPO antibacterial activity but also MPO antibacterial activity.

The described experiments test the hypothesis that CF airway epithelia are defective in SCN^- transport to the airway surface and that this defect may be reflected by impaired or altered peroxidase antibiotic activity in CF epithelial cell secretions.

Methods

Cell Culture and Thiocyanate Transport Experiments

Human airways were obtained following IRB approved protocols from CF patients at the time of transplant or from organ donors whose lungs were not used for transplant. Epithelial cells were isolated, grown and differentiated on either 6.5 mm or 24 mm collagen-coated T-clear membranes (Costar #3450) at an air-liquid interface as previously described [29,30]. All experiments comparing non-CF and CF cells were performed with cultures grown simultaneously and matched in passage number, the number of cells plated, and days in culture.

All cultures had a resistivity ≥ 300 Ω.cm. Prior to initiating experiments, surfaces of ALI cultures were washed with PBS, returned to the air interface and incubated 18–24 h with 70 μM ^{14}C-SCN^- (50 μCi/mM) in the basolateral media. To start the experiments, apical surfaces of the cultures were rapidly washed three times with Dulbecco's PBS (50 μL for 6.5mm filters or 500 μL for 24 mm filters). Following the third wash, additional aliquots were placed on the apical surface for sequential 2 min incubations at 37° in humidified 5% CO_2. Basolateral media were sampled after the last wash. ^{14}C-SCN^- in media and apical washes was determined by liquid scintillation counting. All of the collected ^{14}C-SCN^- was soluble following 10% TCA precipitation showing that radiolabel was not covalently attached to protein.

PKA stimulation was accomplished by adding 500 μM dibutyryl cAMP and 10 μM forskolin to the PBS washes. To inhibit CFTR during PKA stimulation, glibenclamide (500 μM) was added to washes containing dibutyryl cAMP and forskolin.

Assay of Antibacterial Activity

Antibacterial activity assays were performed essentially as described previously [12]. *Pseudomonas aeruginosa* (ATCC 27853) were grown in LB broth overnight, collected in the stationary phase, diluted to 5 – 6 x 10^4 / ml in LB broth with 15% glycerol and stored at −80ºC. At least 24h prior to the experiments, basolateral media of matched non-CF and CF ALI cultures (except controls) were supplemented with 100 μM SCN^-. The apical surfaces of the cultures were washed with 0.25 ml PBS and washes were pooled and stored at −20ºC. SCN^- dependent antibiotic activity was assayed using 560 μl of apical culture washes, adjusted to pH 5.7, and containing 2400–3600 *P. aeruginosa*. Since LPO was not secreted by the cells under these culture conditions, washes were supplemented with 1.1 μg/ml LPO and 10^{-5} M H_2O_2. Control experiments were performed in the absence of basolateral addition of SCN^- or using only PBS never added to the cultures and adjusted to pH 5.7. Growth of bacteria was not affected by addition of 1.125 μg/ml LPO, 10^{-5} M H_2O_2, or 5 x 10^{-4} M SCN^- singly or in pairs. However, addition of all three reconstituted a functional LPO system that was bactericidal. To show SCN^- dependence, apical CF culture washes were also supplemented to 5 x 10^{-4} M SCN^- in some experiments. Mixtures were sampled immediately and 4 h after incubation at room temperature and CFU were determined by plating on LB Agar. Antibacterial activity was expressed as a ratio of CFU after 4 h to the starting CFU in the sample to control for slight differences in the starting number of bacteria.

Computational Modeling of LPO Activity

Differential equations describing LPO enzymatic activity were reiteratively integrated over 0.1 sec intervals using IgorPro (WaveMetrics, Portland, OR). Kinetic constants (k1 and k4) were those published for human salivary lactoperoxidase [31] at pH 6.8 with the normal steady state assumptions. Initial values for reactants, products and LPO are shown in table 1. The used equations were:

$$dH_2O_2 / dt = -k1 * [H_2O_2] * [LPO] + k2 \quad (1)$$

$$dLPO / dt = -k1 * [H_2O_2] * [LPO] - k4 * [LPO - O] * [SCN] \quad (2)$$

$$dLPO - O / dt = k1 * [H_2O_2] * [LPO] - k4 * [LPO - O] * [SCN] \quad (3)$$

$$dSCN / dt = -k4 * [LPO - O] * [SCN] + k3 \quad (4)$$

$$dOSCN / dt = k4 * [LPO - O] * [SCN] \quad (5)$$

where k2 and k3 are the H_2O_2 production rate and SCN^- transport rates respectively. The Igor Pro procedure code is available on request to the authors.

Results

Thiocyanate Transport

We have previously shown that normal human bronchial epithelial cells actively transport SCN^- from the basolateral to apical surfaces and that the transport was regulated by CFTR as it was stimulated by cAMP analogs and by forskolin, and inhibited by apical glibenclamide, diphenylamine-2-carboxylic acid and 5-nitro-2- (3-phenylpropylamino)-benzoate [17]. Since a loss of SCN^- in the airway lumen might result in a loss of LPO antibacterial activity and perhaps also affect neutrophil MPO antibacterial activity, we assessed whether SCN^- transport was defective in CF bronchial epithelial cells compared to normal epithelia. Airway epithelial cells, obtained from CF lungs or non-CF lungs at the time of transplant or organ donation, were passaged twice (thereby de-differentiated) and then cultured at an air-liquid interface (ALI) for re-differentiation [29,30]. As expected CF cell cultures showed no change in short circuit current in Ussing chambers following stimulation with forskolin

To measure the transport of SCN^- across the epithelium, ^{14}C-SCN^- was added to the basolateral media and, after overnight incubation to allow an approach to steady state, its rate of appearance in the apical surface liquid was monitored. Airway epithelial cell cultures from three separate CF patients were compared to non-CF control cultures from three individuals. CF cells showed 4 fold reduced SCN^- transport rates when compared to normal (CF, 2.3 ± 0.5 nmol/h/cm^2 vs. non-CF, 9.3 ± 1.7 nmol/h/cm^2; n=18; 3 donors, $p < 0.0002$, one way ANOVA) (Fig. 1A). All three individuals had one ΔF508 allele, one had a second W1282X allele (nonsense mutation), while the second alleles in the other two patients were unidentified unknown. However, all were diagnosed with CF and underwent lung transplant. CFTR function as diagnosed by an abnormal sweat test. Transport rates for cultures from each CF individual were: ΔF508/W1282X, 1.69 ± 0.32 (n=8) and 2.41 ± 0.72 (n=5) as well as 3.65 ± 2.34 (n=3) for the other two. The differences in SCN^- transport between non-CF and CF cultures were independent of the age of the cultures (repeated measurements from 2 – 8 week old cultures), suggesting that a possible difference in time to reach full differentiation between CF and non-CF cells was not responsible for these observations.

Exact determination of apical SCN^- concentration was not possible in these experiments because the small apical surface volumes naturally present on ALI cultures prevented accurate sampling of CF cultures. However, comparison of the total amounts of SCN^-, recovered in the initial 0.5 ml PBS wash of the apical surface after overnight incubations with ^{14}C-SCN^- in the basolateral media, showed 11-fold higher levels in non-CF compared to CF cultures (non-CF, 11.2 ± 3.0 nmol/cm^2 vs. CF, 0.8 ± 0.2 nmol/cm^2, n = 18, $p < 0.0008$, one way ANOVA; Fig. 1B). Even though CF cultures are reported to have about one half the amount of apical surface fluid when compared to non-CF [3], the difference in accumulated apical ^{14}C-SCN^- between CF and non-CF cultures would remain significant, even if this volume difference is taken into account (5.5 fold). Thus, it appears that non-CF epithelial layers accumulate SCN^- to significantly higher levels than CF. On the occasions when apical surface liquid was visible on non-CF cultures that had been pre-incubated with basolateral ^{14}C-SCN^-, direct sampling (2–5 μl) allowed measurement of the [SCN^-] and showed that it was concentrated 6 fold over the 0.07 mM in the basolateral media to 0.43 ± 0.13 mM, *n* = 3. This concentration of SCN^-, measured in undiluted non-CF apical surface liquid, is close to levels measured in undiluted airway secretions collected from patients, where we measured 0.5 mM [12]. Liquid was never observed on the apical surface of CF cultures, as expected from a previous report [3] and therefore, direct measurement of [SCN^-] in CF apical surface fluid could not be made. However, by estimating the values for CF cells using the difference to non-CF cells measured

above (5.5 fold), the apical SCN^- concentration appears likely to be close to that in the basolateral media (0.4 mM divided by 5.5 would equal 0.072 mM).

Since previous studies showed that CFTR played a role in SCN^- transport from the serosal to mucosal compartments [17], we evaluated the possible contribution of CFTR anion channel activity to the SCN^- transport observed in cultures. Non-CF cultures showed the expected increase in SCN^- transport after stimulation of PKA by apical addition of dibutyryl cAMP (0.5 mM) and forskolin (10 μM), while CF cultures did not (Fig. 2, A–C). In non-CF airway epithelia, both the PKA-stimulated and baseline SCN^- transport are completely and reversibly blocked by glibenclamide [17]. Previous studies showed that SCN^- transport in normal airway epithelia was also sensitive to diphenylamine-2-carboxylic acid and 5-nitro-2- (3-phenylpropylamino)-benzoate but not affected by DIDS and DNDS [17]. Thus the lack of cAMP stimulated SCN^- transport by CF epithelia is consistent with the idea that SCN^- is delivered to the apical surface through CFTR.

Thiocyanate Transport Defects and *In Vitro* Antibacterial Activity

To assess the effects of reduced SCN^- transport on LPO-mediated antibacterial activity, the apical surfaces of cultures were washed with PBS and these washes were used in antibacterial assays previously developed to assess LPO activity in human tracheal secretions [12]. The day prior to washing, SCN^- (100 μM) was added to the basolateral media of some cultures to allow transport to the apical surface. Since culture conditions did not result in LPO synthesis and secretion in these ALI cultures, washes were supplemented with exogenous bovine milk LPO and H_2O_2 (10^{-5} M). Supplementing washes from cells cultured in the absence of basolateral SCN^-, with this concentration of H_2O_2 alone or in combination with LPO did not give rise to any antibacterial activity (Fig. 3, compare panel A and B). However, incubation of *P. aeruginosa* with LPO/H_2O_2 supplemented washes from non-CF cultures grown with basolateral SCN^- resulted in LPO-dependent killing of bacteria (Fig. 3, panel C). In contrast, incubation of *P. aeruginosa* with LPO/H_2O_2 supplemented washes from CF cultures grown with basolateral SCN^- had no detectable LPO-dependent killing activity. Addition of exogenous SCN^- (0.5 mM) to LPO/H_2O_2 supplemented culture washes generated antibacterial activity in CF samples grown with basolateral SCN^-, demonstrating that the lack of antibacterial activity was the result of defective SCN^- transport by the CF epithelial cells (see below). Addition of the same amount of SCN^- to the non-CF culture washes grown with basolateral SCN^- increased the LPO-dependent activity, already present, due to the further increase in [SCN^-]. Thus, the lack of SCN^- transport by CF cells, compromised an *in vitro* LPO system reconstituted in apical washes of the ALI cultures [32]. Although small numbers of bacteria were used in the assays, substrates were not replenished and were limiting.

Computational Modeling of LPO activity in Airway Surface Liquid (ASL)

Since LPO activity depends on adequate [SCN^-], it is important to understand whether lower LPO activity due to lower [SCN^-], could be up-regulated by compensatory changes in enzyme concentration or H_2O_2 production. To visualize the effects of decreased [SCN^-] on LPO enzyme activity, we estimated LPO activity as a function of [SCN^-] by calculating reaction rates based on kinetic constants and the velocity equation published by Pruitt et al. [31].

$$V = \frac{[E_0][H_2O_2][SCN^-]}{\frac{[H_2O_2]}{k_4} + \frac{[SCN^-]}{k_1} + K_i\frac{[SCN^-]^2}{k_1}}$$

As expected, the lower SCN^- concentrations found in apical CF culture washes resulted in lower enzymatic activity Fig 4A and B). Interestingly, LPO has increased activity with lower

[SCN^-] at lower pH that might be encountered during infection or with reduced bicarbonate transport seen in CF (Fig. 4A and B). Increased initial LPO activity due to lower initial pH resulted in more rapid depletion of SCN^- and was not able to compensate for the loss of SCN^- transport (not shown).

To approximate the effects of lower SCN^- transport, differential equations describing changes in reactants were used to model LPO activity in ASL at pH 6.8. These computations allowed assessment of the possible effects of increasing [LPO] and [H_2O_2] on LPO activity in an otherwise normal airway except for a sharply reduced [SCN^-] transport ass measured here in CF airway epithelial cultures. The model used LPO kinetic constants at pH 6.8 [31], the measured amounts of LPO in tracheal secretions [12], the measured rates of SCN^- transport by non-CF [17] and CF airway epithelia (above), as well as estimates of ASL volume [3] per unit surface area. The concentration of H_2O_2 in ASL is not known. Therefore initial values of [H_2O_2] were set to zero and H_2O_2 production rates were set to balance the transport of SCN^- and its consumption by LPO in normal airways. The CF model utilized an ASL volume equal to half that of the normal model [3] to account for a possible increase in [SCN^-] due to a reduced volume. To account for equivalent synthesis and secretion of LPO to into this reduced volume of ASL in CF, initial [LPO] was 2-fold normal in the reduced transport (CF) model.

The simulation was carried out for 600 sec. At 300 sec, LPO was increased 10 fold over the initial concentration in both the CF and in the non-CF model to simulate possible compensatory increases in enzyme concentration. Figure 5 shows the calculated concentrations of the substrates and the rate. Figure 5A depicts [SCN^-] changes in the normal and CF models. In the low SCN^- transport CF model, SCN^- is rapidly consumed resulting in decreased rate of product formation (Fig. 5C) and a corresponding increase in [H_2O_2] (Fig. 5B) as catalytic consumption depletes SCN^- faster than it is replenished by diminished transport and thus, leading to decreased use of H_2O_2. To demonstrate that an increase of LPO could not offset the loss of SCN^- transport, [LPO] was increased at 300 sec, and only resulted in a short transient increase in reaction velocity (Fig. 5C).

Similar upregulation of H_2O_2 production did not restore LPO activity in the CF model, however, increased H_2O_2 production did stimulate LPO activity in the normal model until [SCN^-] fell due to inability of SCN^- transport to sustain the increased catalysis (not shown). Interestingly, increasing SCN^- transport rate under normal conditions made little difference in LPO activity as the enzyme is nearly saturated with substrate at normal transport rates. Computation using LPO rate constants at pH 5.6 that could be encountered locally during infection accelerated the loss of SCN^- in the low transport CF model. Thus, this computational model supports the idea that the reduced [SCN^-] predicted by the above transport studies cannot be compensated by upregulation of LPO or H_2O_2 or changes in pH. The model also predicted that decreased SCN^- transport may cause CF ASL to be deficient in SCN^- rendering the LPO system unable to generate $OSCN^-$, therefore impairing the antibacterial properties of mucus secretions.

Discussion

The experiments presented here showed that CF airway epithelia, cultured at the ALI, were defective in transporting SCN^- from the basolateral to the apical compartment. The absence of PKA stimulated SCN^- transport in CF cultures is consistent with a role for CFTR in SCN^- transport suggested by our previous studies [17]. The measured defect in SCN^- transport by CF ALI cultures was reflected in a reduction of SCN^- accumulation on the apical surface and this reduction resulted in a loss of reconstituted LPO antibacterial activity that relied on substrate transported by the cultured epithelia. Finally, a computational model of LPO activity supported the concept that the effect of the measured decrease in SCN^- transport on LPO

enzymatic activity could not be compensated by up-regulating either LPO or H_2O_2 and thus predicted a loss of LPO-mediated host defense in CF airways. If the measured difference in apical SCN^- between CF and non-CF airway epithelial cells is reflected *in vivo*, it is likely to be functionally significant since $OSCN^-$ production by LPO at the relevant SCN^- concentrations are significantly different [33] and bacterial growth *in vitro* is inversely proportional to the [$OSCN^-$] [34]. Although certain changes in CF epithelial phenotype in culture have been noted by others, these changes were seen in primary cultures and disappeared after 6–11 days in culture [35]. Our re-differentiated cultures (passaged once) have been in culture for much longer and are not expected to exhibit these changes noted in primary cultures.

Exact determination of SCN^- concentration in normal and CF airway secretions is not currently possible. Thiocyanate content in normal secretions is near the limit of detection of colorimetric assays [12] and dilutions associated with bronchoalveolar lavage will require development of more sensitive methods. Our model predicted complete depletion of SCN^- in CF secretions and the presence of large amounts of MPO due to chronic infection of CF airways virtually assures no SCN^- to be measurable in secretions. Thus, accurate comparison of SCN^- levels in CF and normal airways will require development of a highly sensitive assay method and will be best done in patients early in life prior to establishment of chronic infection and neutrophilic inflammation.

In lieu of direct measurement of ASL [SCN^-], the computational model presented here predicted that decrease SCN^- transport of the magnitude measured results in complete depletion of this LPO substrate and that upregulation of LPO enzyme activity cannot compensate for lower SCN^- transport measured in the *in vitro* system. The model also suggested that H_2O_2 normally produced by airway epithelia [18,36,37] and consumed by LPO [38] will increase if SCN^- transport is not able to provide sufficient substrate and might perhaps lead to increased oxidative damage in the CF airway. Thus the model predicted that changes in the LPO and H_2O_2 concentrations in CF ASL cannot offset the measured loss of SCN^- transport.

It is important to note that the computational model used empirically determined rate constants for purified human LPO from saliva and thus the model does not take into account the presence of other components normally found in normal and CF airways. The model therefore cannot accurately predict the effects of other potential antibacterial peroxidase substrates such as nitrite. Nor does the model take into account the formation of LPO compound II that can generate SCN radicals and is now thought to be an important part of the normal peroxidase catalytic cycle [39]. The model also does not address MPO activities and cannot predict a complete loss of peroxidase mediated antibacterial activity but instead primarily serves to predict that upregulation of LPO expression cannot compensate for the observed reduction in SCN^- transport.

Although most studies of MPO-mediated bacterial killing have concentrated on its use of Cl^-, SCN^- has recently been shown to be the preferred substrate of MPO [27,40] and normally OCl^- immediately reacts with SCN^- to produce $OSCN^-$ [28]. The presence of high levels of MPO in CF airways, that also consumes SCN^- [e.g. 27], virtually ensures the full consumption of SCN^- in the presence of an impaired transport system and suggests an increased use of chloride as a substrate and generation of OCl^-. Since OCl^- is believed to very rapidly react with SCN^- in physiological conditions, increased production of OCl^- that might also contribute to increased oxidative damage in the airway. Although hypochlorite itself is a potent antibacterial, CF airway secretions are reported to have high levels of taurine (> 3 mM) [41] that reacts extremely rapidly with hypochlorite and thus is expected to buffer the antibacterial effects [42,43]. Nevertheless mono and dichlorotaurine are also antibacterial, although much less so than HOCl [44,45]. The mechanisms by with chlorotaurines are antibacterial are not

known. Clearly, a full understanding of the effects of SCN^- transport changes in CF airways will require more investigation including studies of the phenotypic changes in bacteria that occur after chronic colonization. Thus, impaired SCN^- transport to the airway lumen could potentially cause significant alteration of both the epithelial-derived LPO system as well as the neutrophil-derived MPO antibacterial activity.

The SCN^- permeability and conductance of the CFTR anion channel is known [e.g. 46,47,48] and suggested that a SCN^- transport deficiency might be relevant to increased infection in CF. Our previous studies on SCN^- transport by airway epithelia support a role for CFTR in SCN^- transport [17] and the decreased SCN^- transport and lack of PKA stimulation in the CF cultures shown here are also consistent with a role for CFTR. We have not ruled out the possibility that NaI symporter in the basolateral membrane is also reduced in CF although such a reduction is not expected to be relevant in the absence of functional CFTR.

Since LPO has antimicrobial activity against a broad spectrum of pathogens including respiratory pathogens relevant in CF [e.g. 12], a CFTR dependent defect in LPO-mediated host defense might be relevant to cystic fibrosis. In our studies shown here, and in previous studies [12], small numbers of bacteria were used in an *in vitro* system that was not replenished with either SCN^- or H_2O_2 during the assay. *In vivo*, continuous production of H_2O_2 [18,36,49] and SCN^- transport in the presence of LPO are expected to continuously produce larger quantities of $OSCN^-$ for antibacterial host defense that are expected to prevent colonization of the airway but may not be able to eradicate an established infection.

There may be several reasons that the role of LPO in airway host defense, and a possible loss of its activity due to the anion transport defect in CF, have not previously attracted attention. First, studies that focus on antibacterial activity of airway secretions do not typically detect LPO activity since LPO requires H_2O_2 as a substrate and H_2O_2 is rapidly consumed and not present in secretions after collection and storage [38]. Thus, to detect LPO activity requires replenishing secretions with the estimated low physiological [H_2O_2] something not generally added in studies of antibacterial activity in airway secretions [e.g. 50]. These levels of H_2O_2, added to *in vitro* peroxidase antibacterial systems, are well below the levels needed for killing of bacteria by H_2O_2 alone [e.g. 12]. Second, broad screens of antibacterial substances have often focused on components with molecular masses below that of LPO [e.g. 51]. Finally LPO has an acidic pH optimum (~ pH 5.5) and shows reduced activity above pH 7.0. Thus, LPO's characteristics and substrate needs may have slowed appreciation of its role in airway host defense.

Chronic infection in CF appears to arise from multiple defects in airway host defense. For example, inadequate ASL leads to dehydrated mucus, reduced mucociliary clearance [3], and decreased neutrophil migration into mucus [52]. Since LPO appears to be constitutively present in normal airway secretions, loss of its activity due to a SCN^- transport defect may be an additional defect in CF airway host defense. Thus the data presented here support further studies of SCN^- levels in CF airways.

Acknowledgements

We thank Dr. Adam Wanner for his thoughtful advice and strong support throughout this work. This work also acknowledges the memory of James R. Haft M.D. who inspired us by his lifelong determination and his dedication to medicine before his death from cystic fibrosis. This work was supported in part by the grants from NHLBI (GEC and MS) and The Cystic Fibrosis Foundation (SHR). Correspondence and requests for materials should be addressed to GEC. (Email: gconner@miami.edu)

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Abbreviations

ALI
: air-liquid interface

ASL
: airway surface liquid

CF
: cystic fibrosis

CFTR
: cystic fibrosis transmembrane regulator

LPO
: lactoperoxidase

MPO
: myeloperoxidase

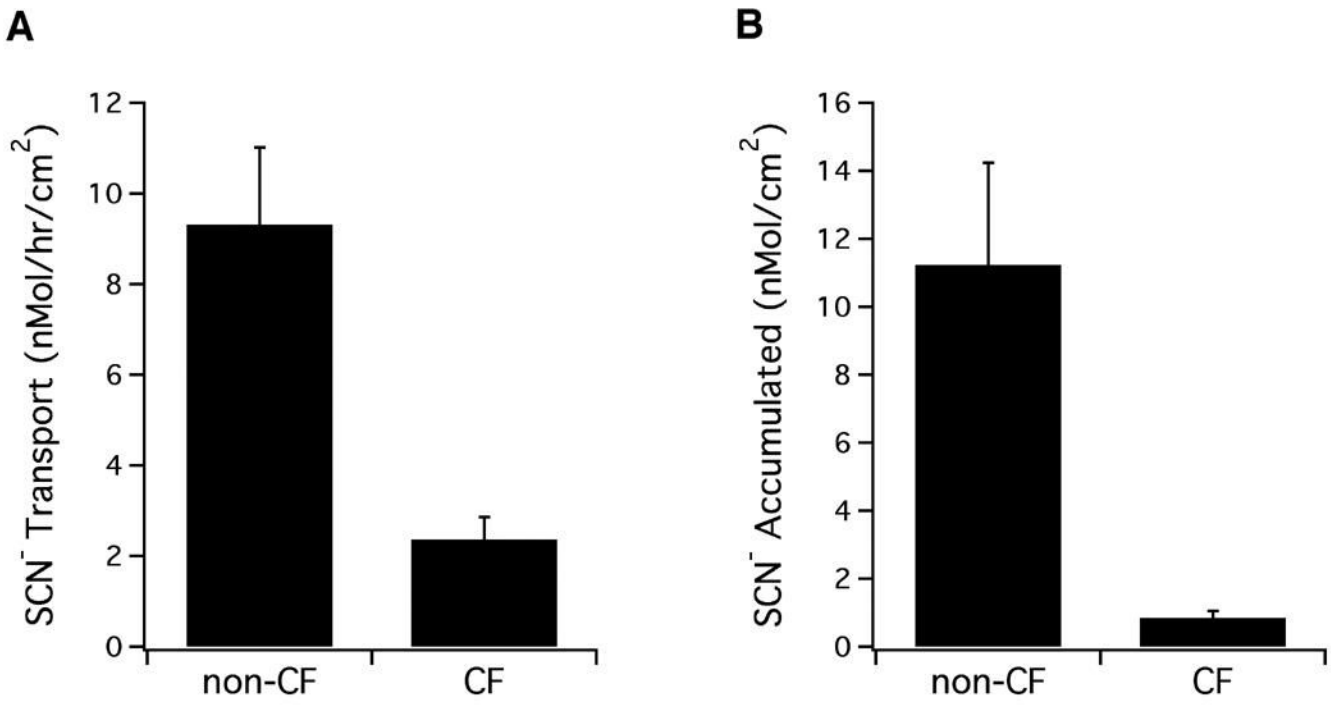

Figure 1. Thiocyanate transport in CF airway epithelium is defective

Panel A. ^{14}C-SCN^{-} (70 μM) was added to the basolateral media of non-CF and CF cultures for 18 – 24 h and stable unstimulated transport rates measured as described previously [17]. Non-CF cultures showed a 4-fold higher rate of transport using 18 from three non-CF and three CF individuals (n= 18, $p < 0.0001$). Panel B. ^{14}C-SCN^{-} (70 μM) was added to the basolateral media of cultures for 18 – 24 h. The ^{14}C -SCN^{-} accumulated on the apical surface was collected in a PBS wash and measured by liquid scintillation counting (n = 18 cultures each from three non-CF and three CF, $p < 0.007$).

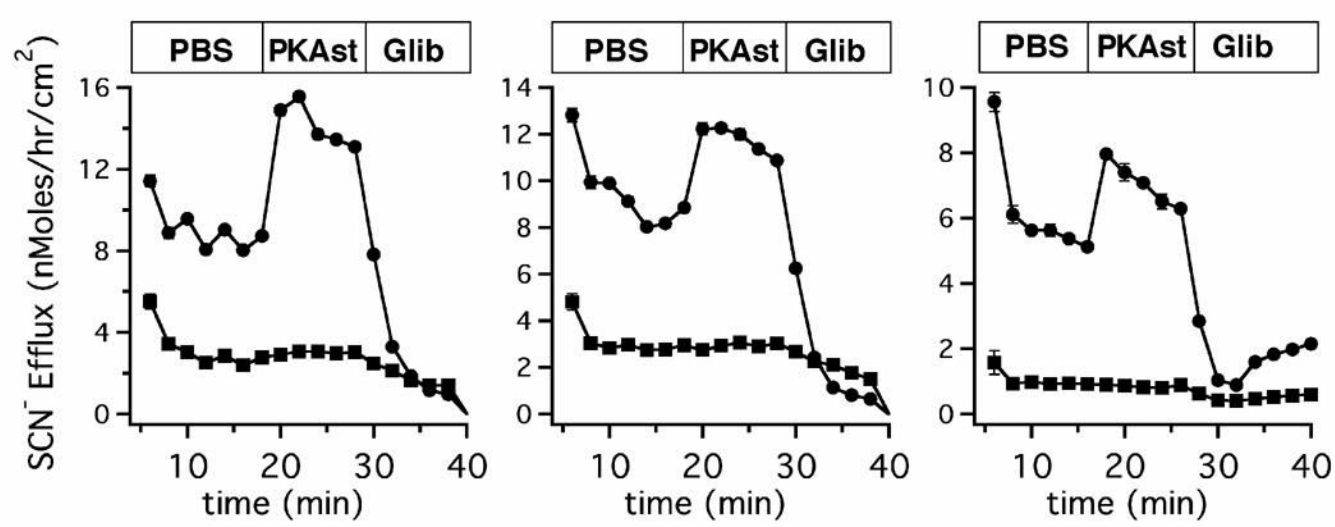

Figure 2. PKA stimulation increases thiocyanate transport in non-CF but not in CF airway epithelia

To initiate the experiments, apical surfaces of the cultures incubated overnight in ^{14}C-SCN^{-} were rapidly washed three times. Additional PBS aliquots were placed on the apical surface for sequential 2 min washes and ^{14}C-SCN^{-} was determined by liquid scintillation counting. cAMP stimulation of SCN^{-} efflux was demonstrated by adding dibutyryl cAMP and forskolin to the PBS washes (18 min, indicated by the bar at top). The stimulated transport was inhibited by addition of glibenclamide (28 min) to washes containing dibutyryl cAMP and forskolin. SCN^{-} flux is expressed in nMoles of SCN^{-}/h normalized to 1 cm^2 filter area. In the CF culture, SCN^{-} transport was greatly diminished and not stimulated by cAMP. Bars at the top of graphs indicate the presence of PBS alone, dibutyryl cAMP and forskolin (PKAst), or dibutyryl cAMP, forskolin and glibenclamide (Glib). Panels A–C show single experiments comparing pairs of cultures obtained from three different CF patients and three different non-CF patients.

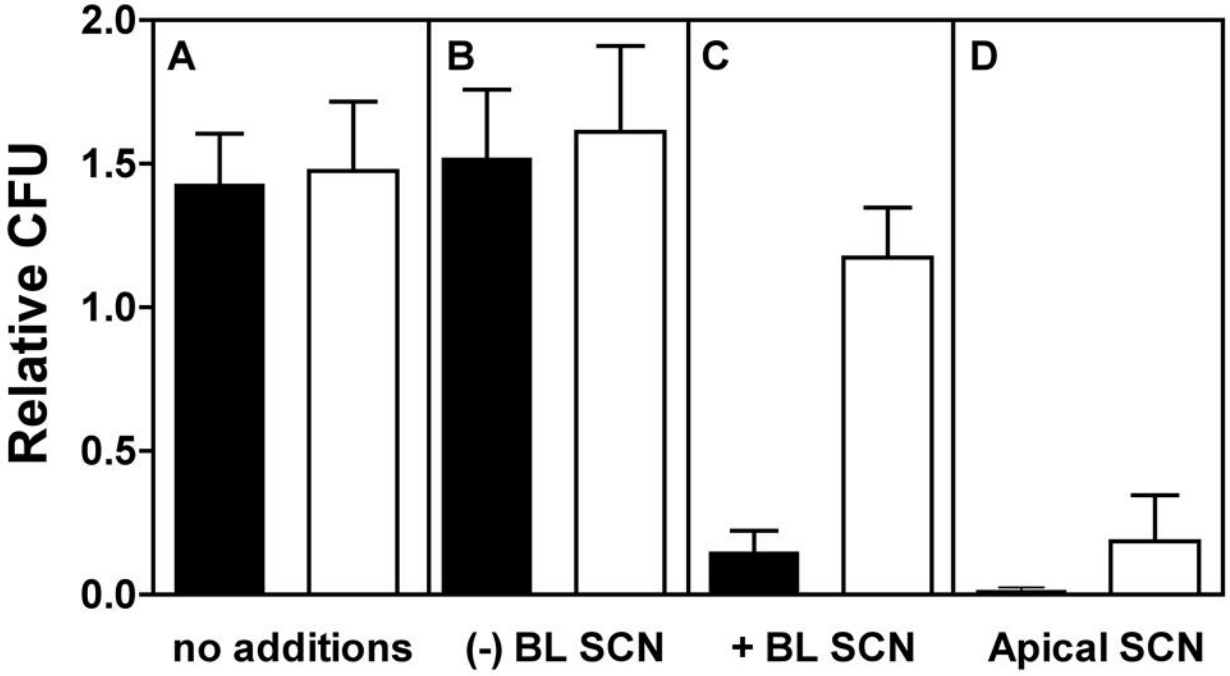

Figure 3. Thiocyanate-mediated bacterial killing in ALI culture secretions

Non-CF (solid bars, $n = 6$ cultures on separate days) and CF (open bars, $n = 4$ cultures on separate days) ALI cultures were incubated in the presence or absence of SCN^- in the basolateral media. Apical surfaces were washed with PBS and combined for assay of antibacterial activity as described in Methods. Assays were performed with no additions (panel A) or with LPO and H_2O_2 added to the washes (panel B). LPO and H_2O_2 did not increase the antibacterial activity of the washes in the absence of SCN^-, however in the presence of basolateral SCN^- (panel C), only non-CF culture washes showed antibacterial activity. Addition of SCN^- to washes of CF cultures restored the antibacterial activity and increased the activity of non-CF culture washes (panel D).

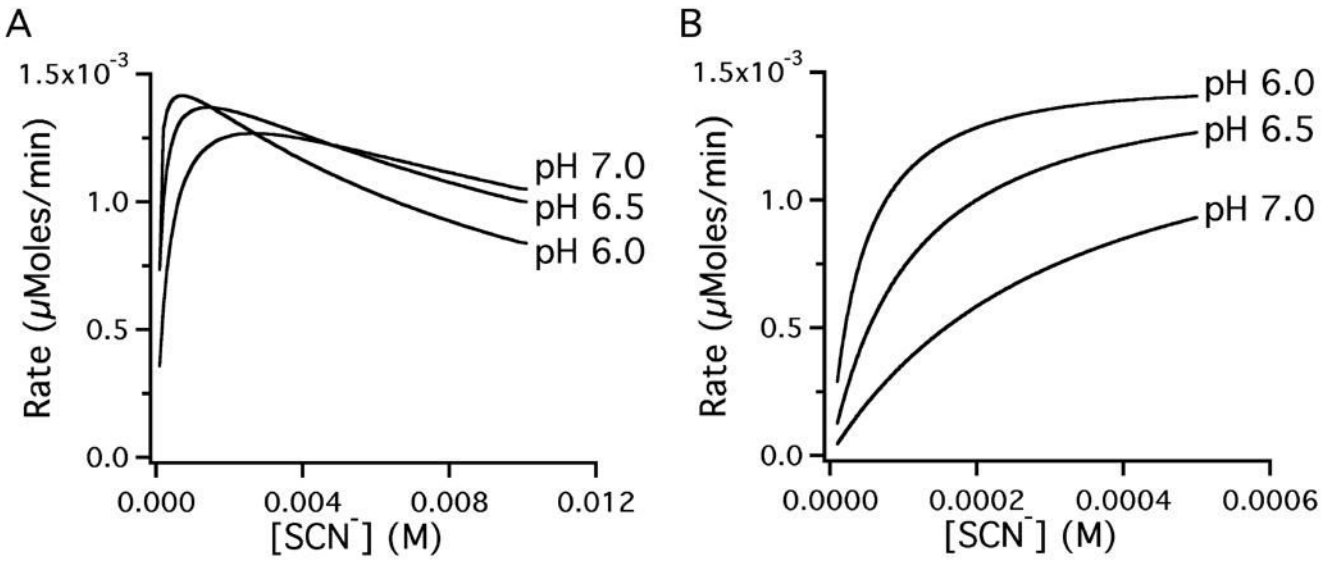

Figure 4. LPO activity varies with thiocyanate concentration and with pH

An arithmetic model of LPO activity using the kinetic constants published by Pruitt et al. [31] calculated the expected LPO activity with [SCN^-] between 0.0001 M and 0.01 M (left panel) at pH 7.0, pH 6.5 and pH 6.0. The right panel shows an expanded view between 0 and 0.5 mM (0.4 mM being the concentration found in human airway secretions).

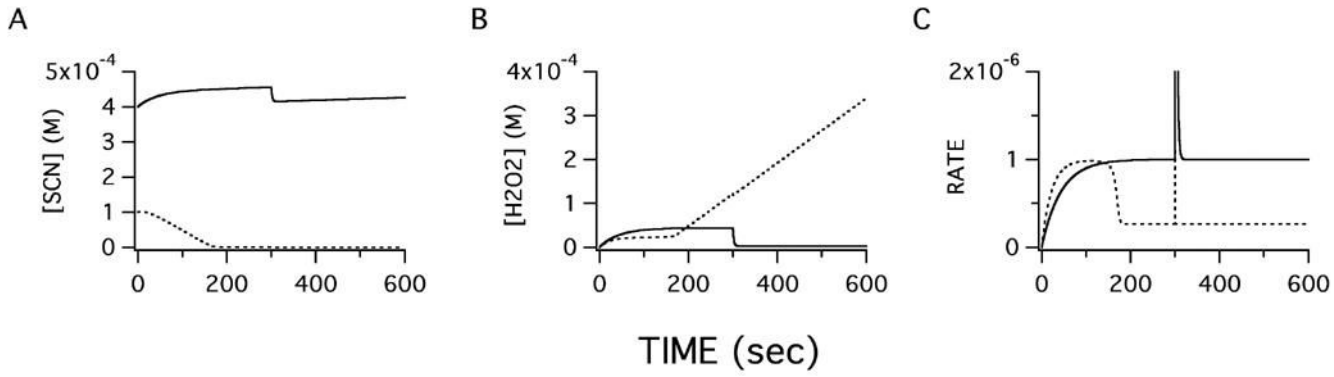

Figure 5. Defective thiocyanate transport predicts a loss of LPO antibacterial activity

Computational modeling of LPO enzymatic activity in airway surface liquid shows predicted changes in response to altered conditions in CF airways, i.e. decreased volume and decreased SCN^- transport rates. Initial conditions (shown in Table 1) were chosen based on published values, except [H_2O_2] that was arbitrarily set to zero. The H_2O_2 production rate was set to balance SCN^- transport rates in normal cultures. Integration steps were 0.1 sec. Shown below are values of non-CF (solid line) and CF (dotted line) conditions. Changes in [SCN^-], (panel A) and [H_2O_2], (panel B) include consumption by LPO catalysis and appearance due to production or transport. Differentiation of $OSCN^-$ product concentration with respect to time is shown in panel C. After 300 sec, LPO concentration was reset to a level 10 fold higher than the CF initial [LPO] to mimic possible upregulation of the enzyme concentration in response to lower SCN^- transport. Despite a higher initial CF [LPO] to accommodate decreased CF ASL volume, computation showed that SCN^- could not be replaced fast enough given the transport defect in CF. An increase in [LPO] (300 sec) was unable to restore LPO reaction rate in CF due to faster consumption of SCN^-. The model predicts a loss of antibacterial activity in CF airways that cannot be compensated by increased LPO or H_2O_2.

Table 1

Initial conditions for computations shown in Figure 5 were derived from estimates or measured amounts of enzyme and substrates [12], transport rates (preliminary data, above), and volumes [3]. Transport rates described in the text (shown in Figure 1) are recalculated to reflect the estimated ASL volume on 1 cm of epithelial surface. CF LPO was increased 2 fold over normal to accommodate the reduced ASL volume in CF.

	Normal	CF	units
$[H_2O_2]$	0	0	M
$[SCN^-]$	4×10^{-4}	1×10^{-4}	M
[LPO]	7.2×10^{-8}	14.4×10^{-8}	M
SCN^- Transport	1.04×10^{-6}	0.263×10^{-6}	$M \cdot sec^{-1}$
H_2O_2 production	1×10^{-6}	1×10^{-6}	$M \cdot sec^{-1}$
ASL Volume ($1\ cm^2$)	2.5×10^{-6}	1.25×10^{-6}	1