Inhibition of airway liquid secretion and its effect on the physical properties of airway mucus

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Trout, Laura, Malcolm King, Wei Feng, Sarah K. Inglis, and Stephen T. Ballard. Inhibition of airway liquid secretion and its effect on the physical properties of airway mucus. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L258–L263, 1998.—The combination of both Cl– and HCO3– secretion inhibitors causes an accumulation of mucins within the submucosal gland ducts of acetylcholine (ACH)-treated bronchi [S. K. Inglis, M. R. Corboz, A. E. Taylor, and S. T. Ballard. Am. J. Physiol. 272 (Lung Cell. Mol. Physiol. 16): L372–L377, 1997], suggesting indirectly that these agents block airway gland liquid secretion. The present study tested the hypotheses that ACh-stimulated liquid secretion is driven by Cl– and HCO3– secretion and that inhibition of this process leads to secretion of a dehydrated mucus with altered rheological properties. Excised distal bronchi from pigs were pretreated with either a combination of Cl– and HCO3– secretion inhibitors (bumetanide, acetazolamide, dimethylamiloride, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) or the dimethyl sulfoxide vehicle and were then treated with ACh to induce secretion. The rate of mucus liquid secretion was substantially reduced when the airways were pretreated with the anion secretion inhibitors. Mucus liquid from inhibitor-pretreated airways contained almost threefold more nonvolatile solids than the control liquid. Rheological analysis revealed that mucus liquid from inhibitor-pretreated airways expressed a significantly greater log G* (rigidity factor), whereas tangent δ (recoil factor) was significantly reduced. These results suggest that 1) ACh-induced liquid secretion in bronchi is driven by both Cl– and HCO3– secretion and 2) inhibition of ACh-induced liquid secretion results in the secretion of mucus with a reduced water content and altered rheological properties.

Cystic fibrosis; bicarbonate secretion; chloride secretion; mucus rheology; distal bronchi

Cystic fibrosis (CF) is a fatal inherited disease that affects the exocrine function of many organs including the lung, pancreas, small intestine, genitourinary tract, and sweat glands (9). Patients typically succumb from an accumulation within the airways of an abnormally thick, purulent mucus and repeated bouts of severe, chronic pulmonary infection. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) protein, which functions in respiratory epithelia as a Cl– channel (1, 22). Although currently the object of intense study and speculation, the link between a defective CFTR and the production of abnormal airway mucus in CF has not been conclusively demonstrated.

The CFTR may play several important roles in the maintenance of cellular homeostasis. It has been variously suggested that CFTR facilitates intracellular vesicular acidification (5), modulates Na+/channel conductance (41), mediates cellular efflux of ATP (37), and participates in the clearance of inhaled bacteria (33). However, the function of CFTR that has received the most attention in recent years relative to CF disease is its role in adenosine 3′,5′-cyclic monophosphate (cAMP)-mediated Cl– secretion. Indeed, Cl– secretion is either present or inducible in airway epithelia (3, 8) and serves as the likely driving force for transepithelial liquid secretion (20). CFTR is also permeable to HCO3– (34) and could support active secretion of this anion as well (39). Recent evidence suggests that the Cl– and HCO3– secretion response to acetylcholine in the airways is localized, at least in part, to the bronchial submucosal glands, where it probably functions to drive liquid secretion needed to flush glycoprotein secretions from the ducts (17). These observations are provocative because both CFTR and carbonic anhydrase are localized to the serous cells of the airway submucosal glands (11, 19, 40) and because gland obstruction and hypertrophy are among the earliest pathological findings in CF disease (32, 38). These observations support the notion that the uncoupling of liquid and mucin secretions from the submucosal glands could be the central event in the development of CF airway disease.

Mucus from the airways of CF patients differs in many respects from the mucus obtained from disease-free persons. CF mucus contains products of the omnipresent bacterial infections, including mucoid secretions, lipids, DNA, and actin, as well as proteases and other enzymes. Some of these substances exert significant effects on the physical properties of airway mucus that could substantially alter the efficiency of ciliary clearance (12, 13, 35). Consequently, it is very difficult to determine with certainty whether the physical differences between CF and normal mucus are due to a defective primary secretion product or the result of postsecretion modification by pathogenic and inflammatory processes. If anion and liquid secretions from airway submucosal glands are indeed defective in CF, the primary mucus secretion product could have a reduced water content compared with normal mucus, leading to altered rheological properties and a decreased ciliary or cough clearance.

Inglis et al. (17) recently reported that blockade of both Cl– and HCO3– secretion causes significant mucin accumulation within the submucosal gland ducts of bronchi that were treated with acetylcholine (ACH), a glandular liquid and mucin secretogogue. These find-
ings were taken as indirect evidence that liquid secretion from submucosal glands was driven by secretion of Cl\(^-\) and HCO\(_3\)\(^-\). If this hypothesis is correct, inhibitors of Cl\(^-\) and HCO\(_3\)\(^-\) secretion uncouple liquid secretion from mucin secretion and thereby create an ion and liquid transport deficiency that resembles CF. The aims of the present study were 1) to confirm that ACh-induced liquid secretion in distal bronchi is blocked by inhibitors of Cl\(^-\) and HCO\(_3\)\(^-\) secretion and 2) to determine whether such an intervention alters the physical properties of normal airway mucus.

**METHODS**

Airway excision. Young pigs (10–15 kg, 7–8 wk old) of either sex were obtained from a local vendor. The animals were sedated with an intramuscular injection of ketamine (80 mg) and xylazine (4 mg) and euthanized with an intravenous overdose of pentobarbital sodium. Portions of either the right or left lung were quickly excised and placed in Krebs-Ringer bicarbonate solution (KRB) at room temperature. Distal bronchi (external diameter 2–3 mm, length 25–35 mm) were dissected from the surrounding parenchyma. Airway segments typically had 8–10 side branches. These branches were ligated with 6-0 sutures as close as possible to the central airway trunk. Isolated bronchi were then warmed slowly (−0.2°C/min) from room temperature to 37°C.

Collection of airway mucus liquid. Bronchi were pretreated with a combination of four drugs to inhibit Cl\(^-\) and HCO\(_3\)\(^-\) secretion: bumetanide (0.01 mM) was used to inhibit Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport, acetazolamide (1 mM) was used to block carbonic anhydrase, 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS; 1 mM) was used to block Cl\(^-\)/HCO\(_3\)\(^-\) exchange, and dimethylamiloride (DMA; 0.1 mM) was used to inhibit Na\(^+\)/H\(^+\) exchange. This drug combination has been shown to block the electrogenic anion secretion response to ACh and to cause mucin accumulation within the gland ducts of ACh-treated tissues (16, 17). Control tissues were treated with an equal volume of the dimethyl sulfoxide (DMSO) vehicle. After an incubation period of 45 min, the bronchi were removed from the KRB bath and cleared of all luminal liquid and mucus. The bronchi were then cannulated with polyethylene tubing and returned to their respective solutions. To stimulate gland secretion, 0.01 mM ACh was then added to the KRB bath, which contained the drug pretreatments, and the tissues were incubated in the solutions for an additional 2 h. When the incubation was complete, the bronchi were removed from the cannulas and cut open lengthwise. Because the mucus liquid was often too thick to be adequately sampled with pipettes, this fluid was collected from the airway lumen with a 1- to 2-mm-diameter glass rod and forceps. This collection method was possible because the non-Newtonian properties of mucus permit this fluid to be picked up and manipulated as a semisolid. If present in the cannulas, the mucus liquid was gently blown out of the tubes with forced air. The mucus liquid was rapidly transferred to a tared piece of aluminum foil and immediately weighed with either a Mettler H20 balance or a Cahn C-22 microbalance. The weight was corrected to wet weight (in g).

Measurement of nonvolatile mucus solids. After wet weight determination, the mucus liquid samples were dried overnight at 80°C. The dried samples were then removed from the oven, cooled to room temperature in a desicator, and reweighed. Mucus dry weights could be reproducibly determined with a Mettler H20 balance when the liquid volumes exceeded 3 µl. However, reproducible measurements of the dry weights for 0.5- to 3.0-µl mucus samples required the use of a Cahn C-22 microbalance.

Measurement of mucus rheological properties. Mucus liquid was collected as described in Collection of airway mucus liquid and placed in tared 0.5-ml Eppendorf polypropylene centrifuge tubes. The tubes were immediately sealed, weighed to determine sample wet weight, and then quickly frozen. The samples were shipped on dry ice to the rheology laboratory, where they were allowed to warm to room temperature before they were tested.

The magnetic microrheometer technique was used to measure the viscosity and elasticity of the mucus samples (26). Each mucus sample was placed in a Plexiglas container and covered with light paraffin oil to minimize evaporation. A 70- to 100-µm steel ball was inserted into the mucus sample, and the container was positioned in the gap of a toroidal electromagnet mounted on the stage of a projecting microscope. The displacement of the ball, under the influence of a sinusoidally oscillating magnetic field gradient, was measured. This ball acts as a rheological probe because its movement is opposed by viscous and elastic forces within the mucus gel fluid. The image of the steel ball was projected onto a pair of photocells that provided an electrical output in proportion to the displacement of the moving ball. The toroid current (proportional to magnetic force) and photocell outputs were transmitted to a digital oscilloscope connected to a plotter for off-line processing. From the resulting measurements of driving force and displacement, mucus viscoelasticity was determined at 1, 10, and 100 rad/s of applied frequency, and the results are expressed as log *G*\(^*\) (dyn/cm\(^2\)) and tangent δ (loss tangent), where *G*\(^*\) is the vectorial sum of elasticity and viscosity, also considered as a rigidity factor, and tangent δ is a recoil factor, the viscosity-to-elasticity ratio.

Solution composition and drugs. The KRB contained (in mM) 112 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 11.6 glucose in an aqueous solution. Solution pH was maintained at 7.4 by continuous gassing with 5% CO\(_2\) in O\(_2\). All drugs and chemicals were purchased from Sigma.

Statistics. Within each experimental group, one control and one treated airway were obtained from each animal. Results were compared with either dependent or independent t-tests, with *P* < 0.05 as the level of significance. Data are reported as means ± SE.

**RESULTS**

Mucus liquid secretion. Because the mucus liquid volumes from bronchi pretreated with transport inhibitors were very small (2.4–26 µl), evaporation of water from these samples during collection could have led to a significant error in the measurement of mucus hydration. To test this notion, mucus liquid samples of varying volumes were collected from ACh-treated bronchi. As shown in Fig. 1, the percentage of nonvolatile solids in the control mucus samples was consistent and not correlated to the sample volume (r\(^2\) = 0.0006). The solids content of mucus collected from bronchi pretreated with anion secretion inhibitors was consistently
greater than that in control samples of comparable volume (Fig. 1).

When bronchi were pretreated with the DMSO vehicle and then treated with 0.01 mM ACh, mucus liquid was secreted at the rate of 0.24 ± 0.04 µl·cm⁻²·min⁻¹ (n = 12 bronchi; Fig. 2). Nonvolatile mucus solids were 1.55 ± 0.01% (n = 10 bronchi) of the total mucus liquid weight (Fig. 3), which corresponds to 98.45% hydration. After pretreatment with anion secretion inhibitors (0.01 mM bumetanide, 1.0 mM acetazolamide, 0.1 mM dimethylamiloride, and 1.0 mM DIDS), ACh-induced mucus liquid secretion was significantly reduced to 0.02 ± 0.01 µl·cm⁻²·min⁻¹ (n = 12 bronchi; Fig. 2). Anion secretion inhibition resulted in a significant threefold increase in nonvolatile mucus solids to 4.31 ± 0.49% (n = 10 bronchi; Fig. 3), equivalent to 95.69% hydration.

Mucus rheological properties. Four control samples from bronchi pretreated with DMSO vehicle and six samples from bronchi pretreated with the combination of anion secretion inhibitors were analyzed for rheological (viscoelastic) properties by magnetic microrheometry. Log G* (rigidity factor) was determined at driving frequencies of 1, 10, and 100 rad/s. Tangent δ (recoil factor) was determined only at 1 and 10 rad/s because the rigidity and small size of some of the samples resulted in insufficient tangent δ data at 100 rad/s. Mean mucus rigidity (log G*) was higher with anion secretion inhibitor treatment at all three measurement frequencies (Table 1), and the differences were statistically significant for the two lower frequencies (1 and 10 rad/s). Tangent δ (recoil) was significantly lower for the anion secretion inhibitor group at 10 rad/s.

**DISCUSSION**

These results confirm the previous suggestion by Inglis et al. (17) that ACh-induced liquid secretion in distal bronchi is blocked by inhibitors of Cl⁻ and HCO₃⁻ secretion. Additionally, we show that inhibition of liquid secretion results in the production of a mucus with increased nonvolatile solids content, increased rigidity, and decreased recoil factor. These findings demonstrate that a deficiency in anion transport alone is sufficient to alter the physical properties of airway mucus in a manner that predicts reduced airway clearance.

Much debate has centered on the etiology of the abnormal airway mucus in CF patients. Some early studies using sputum failed to detect an overall difference in mucus rheological properties compared with
The results of the present study indicate that abolition of lung secretion product is defective in these persons. The determine with certainty whether the primary glandular level of airway infection, it is not possible to proteases, which are capable of reducing network elements (10). Because virtually all CF patients exhibit abnormalities in the rheological properties of CF mucus are known to be related to the severity of non-CF lung disease (31) or normal animals (24), although abnormalities in both the water and ion content of CF sputum have been well documented (7, 42). Abnormalities in the rheological properties of CF mucus are known to be related to the severity of CF lung disease (31, 35). The interrelationships between mucus rheology and water and ion content are made complex by the influence of the by-products of infection such as leukocytic DNA and filamentous actin, which can add to the gel cross-link density, and neutrophilic proteases, which are capable of reducing network elements (10). Because virtually all CF patients exhibit some level of airway infection, it is not possible to determine with certainty whether the primary glandular secretion product is defective in these persons. The results of the present study indicate that abolition of Cl- and HCO3- secretion, which likely corresponds to the situation in CF, is sufficient to decrease mucus hydration, increase mucus rigidity, and decrease mucus recoil. Such changes in the rheological properties should decrease mucus transportability (28) and could possibly lead to mucus accumulation and eventual obstruction of the bronchial airways. Furthermore, the inability to remove inhaled pathogens from the airways could facilitate establishment of the chronic airway infections that typify CF.

The relationship between mucus viscoelasticity and clearance is complex. Clearability is impaired not only by increased viscosity and elasticity but also by an increased ratio of viscosity to elasticity (23). Furthermore, increases in solids content leading to correspondingly large increases in viscosity and elasticity, as seen in this study, may exert a relatively minor effect on clearability as long as the mucus retains its elastic character because it is the transfer of momentum from cilia to mucus that is critical in moving secretions by this mechanism. The dehydration of periciliary fluid may be much more consequential for mucus clearance because small changes in the depth of periciliary fluid could greatly alter the efficiency of mucus-cilia interaction. (6, 27). Although not directly testable in our model, the same processes that lead to the reduced water content of the mucus layer would likely also lead to dehydration of the periciliary fluid. The increased elasticity of the mucus would have a major retarding effect in clearance by airflow-related mechanisms such as cough and physiotherapy. Although not relevant to pig bronchi, similar dehydration mechanisms in CF airways would lead to mucus with a greatly reduced cough clearability (25).

The site of ACh-induced liquid secretion in distal bronchi cannot be precisely determined from the results of the present study. However, a significant fraction of the secreted liquid likely originates from the submucosal glands. The same combination of anion transport inhibitors used in the present study has been shown to cause occlusion of submucosal gland ducts in porcine bronchi, suggesting that gland liquid secretion is uncoupled from mucus secretion by these agents (17). Additionally, individual submucosal glands in the cat trachea have been shown to secrete liquid in response to atropine-sensitive vagal stimulation (44) and methacholine application (36). We cannot, however, discount the possibility that some fraction of the secretion response to ACh originates from the bronchial surface epithelium.

In this study, three drugs were used in combination to inhibit HCO3- secretion. Each agent targeted a specific site that could potentially support HCO3- secretion. The model of Smith and Welsh (39) for secretion of HCO3- from airway epithelia suggests the presence of intracellular carbonic anhydrase and basolateral Na+/H+ exchange. In the present study, these two sites were inhibited with acetazolamide and DMA, respectively. Because of the possible existence of basolateral Cl-/HCO3- exchange in these tissues, DIDS was added to block Cl-dependent HCO3- secretion. Although we believe that these agents selectively target these sites, we cannot completely discount possible nonelective actions of these agents. For instance, DMA could also block conductive Na+ channels in the apical membrane of airway epithelial cells. However, this unintended effect would not affect secretion but would block active absorption of Na+ and liquid by surface epithelial cells and should result in an increased rather than a decreased net liquid production. DIDS is known to block non-CFTR protein Cl- channels such as Ca2+-activated, outwardly rectifying, and volume-regulated Cl- channels (2). However, the combination of all three HCO3- secretion inhibitors, including DIDS, had no effect on the electrogenic anion secretion response to ACh in the absence of bumetanide (16), suggesting that the Cl- secretory pathway remains intact. It is unlikely that the combination of anion transport inhibitors is overtly toxic because mucus occlusion of gland ducts can be reversed by removal of the inhibitors and reaplication of ACh (Inglis, unpublished observations).

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<th>Pretreatment</th>
<th>Log G′</th>
<th>Tangent δ</th>
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<tr>
<td></td>
<td>1 rad/s</td>
<td>10 rad/s</td>
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<tr>
<td>Vehicle</td>
<td>2.24 ± 0.22</td>
<td>2.42 ± 0.22</td>
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<tr>
<td>Anion secretion inhibitors</td>
<td>2.75 ± 0.08†</td>
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Values are means ± SE. Bronchi were pretreated with either DMSO vehicle (n = 4) or anion secretion inhibitors (0.01 mM bumetanide, 1 mM acetazolamide, 1 mM DIDS, and 0.1 mM dimethylamiloride; n = 6). All tissues were then treated with 0.01 mM acetylcholine to induce secretion. Log G′, rigidity factor; tangent δ, recoil factor (both measured at indicated driving frequencies). †Significant difference from vehicle-pretreated tissues, P < 0.05.
The combination of drugs used in the present study was intended to block HCO₃⁻ secretion, but the same drug cocktail could potentially block HCO₃⁻-dependent Cl⁻ secretion as well. However, recent findings suggest that HCO₃⁻-dependent Cl⁻ secretion plays no significant role in ACh-induced liquid secretion in pig bronchi. Preliminary studies show that of the three putative bicarbonate secretion inhibitors, only DMA in combination with bumetanide is required to block liquid secretions (4) and to cause mucin accumulation within the submucosal gland ducts (15, 18). These data implicate basolateral Na⁺/H⁺ exchange as the critical site for inhibition and suggest that basolateral Cl⁻/HCO₃⁻ exchange, which would be necessary for HCO₃⁻-dependent Cl⁻ secretion to occur, does not play a role in this process. Additional preliminary results show that the concentration of HCO₃⁻ in the secreted fluid as well as the total secreted HCO₃⁻ is reduced in tissues that have been pretreated with DMA only (4; Ballard, unpublished observations).

The results show that inhibitor pretreatment reduced mucus liquid volume by 90% but caused only a threefold increase in mucus solids. Consequently, the total quantity of mucus solids that were recovered from the airway lumen was reduced approximately fivefold by inhibitor pretreatment. The first and most plausible explanation for this observation is that the missing mucus solids are trapped in the gland ducts after Cl⁻ and HCO₃⁻ secretion inhibitor treatment. Inglis et al. (17) confirmed histologically that mucus fills the ducts after inhibitor treatment. But because we cannot quantitatively measure the total mass of mucus solids contained within the lumen of gland ducts, this notion is difficult to prove beyond a doubt. A second possibility is that we cannot collect all of the luminal mucus liquid from the inhibitor-pretreated airways. This could contribute, in part, to the missing mass, especially because the liquid volumes after inhibitor pretreatment are so small. However, visual inspection of the lumens of airways after mucus liquid collection revealed little residual material, certainly not enough to account for a fivefold difference in mucus solids. The third possibility is that the Cl⁻ and HCO₃⁻ transport inhibitors also inhibit mucin secretion. The presence of large amounts of mucin in gland ducts after inhibitor pretreatment argues against this notion, although it is possible that fractional inhibition of this process occurs.

For these results to have direct relevance to CF, it is presumed that CFTR, a cAMP-activated Cl⁻ channel, participates in active anion secretion induced by ACh. Because ACh typically transduces its actions through Ca²⁺, not cAMP, a role for CFTR in this process could be questioned. Evidence is accumulating, however, that CFTR is indeed involved in cholinergically stimulated anion and liquid transport. Jiang et al. (21) and Yamaya et al. (45) showed that CFTR most likely participates in cholinergically stimulated ion secretion from cultures of airway gland cells and suggested that CFTR is nearly maximally activated under baseline conditions. Additionally, Hogan et al. (14) demonstrated that both cAMP-activated and Ca²⁺-activated duodenal HCO₃⁻ secretion is mediated through CFTR. The most likely explanation for these findings is that ACh increases the intracellular Ca²⁺ concentration, which opens basolateral Ca²⁺-activated K⁺ channels and hyperpolarizes the cell membrane. This action increases the driving force for anion efflux across the apical membrane, and, if CFTR is constitutively active, anions could exit across apical cell membrane through this channel.

Similar CF-related defects in exocrine function have been reported in the pancreas. Pancreatic liquid secretion in CF patients is only one-third that of non-CF patients, and the concentration of protein in the pancreatic liquid is almost three times higher in CF patients than control subjects (30). This reduction in pancreatic liquid secretion in CF patients is concomitant with impaired Cl⁻ and HCO₃⁻ secretion in this organ (29). These observations in the pancreas mirror our own findings in the bronchi where a block of Cl⁻ and HCO₃⁻ transport reduces liquid secretion, most likely from submucosal glands, resulting in a concentrated acinar secretion product.

In conclusion, ACh-induced liquid secretion appears to be driven by Cl⁻ and HCO₃⁻ secretion. Inhibition of ACh-induced liquid secretion leads to the production of a relatively dehydrated mucus, the rheological properties of which are altered in a manner that predicts decreased mucus transportability and alveolar clearance. These results are directly relevant to the pathogenesis of CF in that they provide a physiological link between anion transport and alterations in the rheological properties of airway mucus.

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Portions of this work have been published in abstract form (43).

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