Liquid secretion inhibitors reduce mucociliary transport in glandular airways

STEPHEN T. BALLARD,1 LAURA TROUT,1 ANIL MEHTA,2 AND SARAH K. INGLIS2

1Department of Physiology, College of Medicine, University of South Alabama, Mobile, Alabama 36688; and 2Department of Child Health, Ninewells Hospital, Dundee University, Dundee DD1 9SY, United Kingdom

Received 23 July 2001; accepted in final form 4 April 2002

Cystic fibrosis (CF), the most common lethal genetic disorder in Caucasian populations, is caused by mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR), which normally functions as an adenosine 3′,5′-cyclic monophosphate-activated anion channel (1). Mortality from CF typically results from advanced bronchiectasis and respiratory failure, a certain consequence of chronic obstruction of the airways with abnormally thickened mucus and chronic pulmonary infections. Ciliary clearance of mucus from the airways is reduced in CF patients (30, 41), but the beat frequency of airway cilia does not appear to be inherently affected in this disease (32). Together, these observations suggest that alterations in the mucus milieu at the airway surface somehow impair mucus transport in CF airways. Although CF mucus has been shown to be relatively dehydrated (10, 26, 27), alterations in mucus rheological properties sufficient to impair mucociliary clearance have not been demonstrated (23). Despite intense research efforts, it remains unclear exactly how CFTR dysfunction compromises mucociliary transport in CF airways.

Normally, CFTR is highly expressed in the serous epithelial cells of the submucosal glands (15, 20), where it is thought to mediate active transepithelial anion secretion by providing a conductive pathway for both Cl− and HCO3− efflux across the apical membrane (3, 13, 25, 39). It is the transepithelial secretion of Cl− and HCO3− that provides the driving force for liquid secretion by airway submucosal glands (3, 38). Therefore, deleterious mutations in the CFTR that occur in CF are expected to derange glandular mucus secretion by uncoupling the liquid component of gland secretion from the mucin glycoprotein component. Evidence for such uncoupling has been observed in vitro experiments with airways from pigs, a species whose lung morphology and expression of airway submucosal glands closely resemble those of humans (16, 28). When porcine bronchi were pretreated with inhibitors of Cl− and HCO3− secretion and then exposed to glandular secretogogues, changes occurred that parallel those observed in CF disease. First, the submucosal gland ducts became obstructed with mucin (17, 19), reproducing one of the earliest pathological signs of CF lung disease (44). Second, the airways secreted a thick, relatively dehydrated mucus that was more rigid and exhibited less recoil than normal mucus (39). Although these previous studies show that inhibition of anion secretion substantially alters the quantity and physical properties of airway mucus, no conclusive evidence to date has linked these changes directly to a reduction in mucociliary transport. In the present study, we tested the hypothesis that blocking anion and liquid secretion would also lead to impairment of mucociliary transport, thereby reproducing a key step in CF pathogenesis.
METHODS

Measurement of mucociliary transport. All procedures involving animal use complied with recommended United States and United Kingdom guidelines and were approved by institutional animal use committees. Young pigs (10–15 kg) were killed with intravenous pentobarbital sodium. The tracheas were removed intact, sectioned immediately distal to the larynx and just cranial to the first bronchial branch, and placed in HCO₃⁻-buffered Krebs-Ringer solution. Tissues were gradually warmed to 37°C and treated for 45 min with liquid secretion inhibitors or the drug vehicle. Three different inhibitor treatments were used. The combination of 100 μM bumetanide and 100 μM dimethylamiloride (DMA) was used to inhibit active Cl⁻ and HCO₃⁻ secretion by blocking Na⁺-K⁺-2Cl⁻ cotransport and Na⁺/H⁺ exchange, respectively (3). Either 100 μM diphenylamine-2-carboxylate (DPC) or 300 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), both of which are known to inhibit the CFTR (43), was used to block apical membrane anion channels. All three of these treatment strategies have been shown to be very effective to block apical membrane anion channels. All three of these treatment strategies have been shown to be very effective to block apical membrane anion channels.

Submucosal gland secretion inhibitors or vehicle, to a level that bathed the intralobular bronchi (3). The posterior membranous portion (trachealis muscle) of the trachea was removed from the tracheal surface area of the trachea to be viewed from above through the window created by resection of the trachealis muscle. The track, holding the trachea, was then placed in a Plexiglas box that was partially submerged in a heated (37°C) water bath. The box was filled with the same warm Krebs-Ringer incubation buffer containing the inhibitors or vehicle, to a level that bathed the ventrolateral adventitial surface but did not spilt over into the tracheal lumen. The Krebs-Ringer solution within the box was constantly bubbled with 95% O₂-5% CO₂ gas. A transparent lid was placed on the top of the box. To prevent fogging due to condensation, the lid was either coated on the inside surface with a surfactant or the lid was warmed with heating pads. In this manner, the adventitial surface of the trachea was bathed in physiological salt solution containing vehicle or anion transport inhibitors, while the mucosal lumens were exposed to a warm, humidified, O₂-rich, 5% CO₂ atmosphere. In some tissues, the luminal portion of the trachea was exposed to benzamil to block epithelial Na⁺ channels (ENaC) and inhibit Na⁺-dependent absorption of airway liquid across the surface epithelium. We accomplished this by filling the luminal space with warm Krebs-Ringer containing 10 μM benzamil immediately after mounting the tissue in the support rack and then draining off this solution after 5 min of exposure.

After being mounted, the tracheas were allowed to equilibrate in this configuration for 45 min. Then, 100 μM ACh was added to the Krebs bath to stimulate submucosal gland secretion, and another 45 min was allowed for the secretion response to develop fully. One group of tracheas was not exposed to ACh, but the timing of the protocol was otherwise the same.

To measure mucociliary transport, we sprinkled a few finely powdered flakes of dried India ink on the caudal end of the mucosal surface. The ink flakes typically coalesced in the airway surface mucus into several small particles, ~300–500 μm in diameter. Although individual particles sometimes started moving at different times, their rates were usually similar. To avoid unintended bias, we accepted the rate of transport of the leading edge, that is, the first clearly visible particle that moved in advance of all other particles, as the transport rate for each “run.” The migration of these particles toward the cranial end was observed through the transparent lid of the box with a charge-coupled device camera and video monitor and recorded with a VHS videotape recorder through a time-date generator. A calibrated scale, placed in the box beside the trachea, was used to track the particle position. The number of particle runs among all experiments was 6.1 ± 0.3. Later, the videotape was replayed, and the velocity of mucociliary transport was determined. All runs for each tissue were averaged to obtain a mean value for mucociliary transport in that tissue.

Measurement of ciliary beat frequency. Tracheas were excised, bisected longitudinally along their posterior axis, and pinned to a Sylgard-lined dissection dish with the ventral mucosal surface uppermost. Epithelial sheets were carefully dissected from underlying connective tissue and placed in warm (37°C) HEPES-buffered solution with either the inhibitor or drug vehicle for 45 min. An (100 μM) was then added to the bath, and the tissues were incubated for an additional 45 min. The timing of this incubation protocol therefore matched that of the mucociliary transport assay.

Once incubation was complete, each epithelial segment in turn was placed, submucosal side uppermost, in a rose chamber. To view the cilia, we folded the tissue in half diagonally so that a line of cilia protruded from the edge of the fold. The chamber was then filled with the bath solution, and the tissue was secured in place with a coverslip and a holding ring. The rose chamber was inverted and placed on the stage of a Nikon inverted microscope.

Ciliary beat frequency was measured at 37°C by a video-based, Hoffman contrast technique (Brian Reece Scientific, Newbury, UK) as previously described (14). This system allows alignment of selected fields of cilia by video projection on a monitor or through the eyepiece of the microscope. Ciliary beat frequency was determined from the rate of change in light intensity induced by ciliary motion. The video signal was relayed to a digitizer software package [Reece Scientific PCX Cilia Tracking System (V5), Grabber V3.92 Shareware Issue], which in turn displayed the individual ciliary waveforms over a 2-s period along with the mean frequency calculated from the peaks over this time. Ciliary beat frequency taken for each site was the mean of 10 consecutive measurements. Typically, six sites were sampled for each tissue. All measurements were made in a blind fashion to avoid viewer bias.

Quantitation of submucosal glands. Submucosal gland density was determined in selected tissues after measurement of mucociliary transport. The mucosal surface of the tissues was viewed with a Zeiss ACM videomicroscope using conventional transillumination. The airway surface was displayed on a video monitor that imaged 5.00 × 10⁻⁴ cm² of the tracheal surface area. Gland duct openings were counted in successive, adjacent images (between 63 and 91) of the mucosal surface in each of the cranial, middle, and caudal portions of the tracheas. These values were then averaged and normalized to the mucosal surface area.

Solutions and drugs. Bicarbonate-buffered Krebs-Ringer solution contained 112.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 2.4 mM MgSO₄, 1.2 mM KH₂PO₄, 25.0 mM NaHCO₃, and 11.6 mM glucose. The pH of all HCO₃⁻-buffered Krebs-Ringer solutions was maintained at 7.4 by constant gassing with 95% O₂-5% CO₂. HEPES-buffered solutions were similar in composition to HCO₃⁻-buffered Krebs-Ringer solution except that HEPES replaced HCO₃⁻ in an equimolar fashion. The
pH of HEPES-buffered solutions was initially adjusted to 7.4 with NaOH. DPC (as N-phenylanthranilic acid) was purchased from Aldrich Chemical, and NPPB was obtained from Calbiochem. All other drugs were purchased from Sigma. Stock solutions of all inhibitors were prepared in dimethyl sulfoxide. Equal volumes of the vehicle were added to the appropriate control tissues.

Data analysis. The data are presented as means ± SE. Statistical significance (P < 0.05) was assessed with either Student’s paired t-test or ANOVA when data passed normality requirements. When data were not normally distributed, the Kruskal-Wallis rank test was used. In all cases, n represents the number of tissues (each taken from a different animal) in each group.

RESULTS

Numerous submucosal gland duct openings were present in the ventrolateral mucosa of the porcine tracheas (Table 1). The greatest density of gland ducts was seen in the cranial region. There appeared to be a trend toward lower gland expression from cranial to caudal regions of the tracheas (Table 1).

In tracheas that received neither ACh nor inhibitor treatment, the mucociliary transport rate was 2.5 ± 0.5 mm/min (n = 8; Fig. 1), which is within the range of rates previously reported for other species (36). The mucosal surface of the airways gradually attained a drier appearance during the equilibration period beginning at the caudal end and progressing toward the cranial end. A small pool of mucus liquid typically accumulated at the cranial end of the tissues. It was assumed that this behavior reflected mucociliary transport activity. Exposure of the luminal surface of the tracheas to 10 μM benzamil to block ENaC-dependent absorption of surface liquid had no apparent effect on mucociliary transport rate (2.8 ± 0.9 mm/min, n = 12; Fig. 1). Pretreatment of the tissues with the combination of 100 μM bumetanide and 100 μM DMA to inhibit liquid secretion reduced mucociliary transport to 1.1 ± 0.3 mm/min (n = 6), but this decrease was not significantly different from untreated tissues.

In the presence of the glandular secretogogue ACh (100 μM), the rate of mucociliary transport (6.8 ± 0.8 mm/min, n = 19; Fig. 2) was substantially and significantly greater than the untreated controls, consistent with the well-known stimulatory actions of cholinergic agonists (5, 9, 34). After ACh addition, the airway surface became wet with the secretion of mucus liquid, and noticeably greater quantities of this fluid accumulated at the cranial end of the tracheas. When ACh-treated tracheas were pretreated with the combination of 100 μM bumetanide and 100 μM DMA to inhibit glandular anion and liquid secretion, the rate of mucociliary transport in the presence of ACh was profoundly and significantly reduced to 0.5 ± 0.1 mm/min (n = 17; Fig. 2). Under these conditions, the mucosal surface of the tracheas did not become noticeably wetter with ACh addition, and the mucus at the airway surface became tenaciously sticky, making removal of the ink

Table 1. Submucosal gland density in the ventrolateral mucosal surface of porcine tracheas

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Gland Density, ducts/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial trachea</td>
<td>5</td>
<td>594 ± 83</td>
</tr>
<tr>
<td>Medial trachea</td>
<td>5</td>
<td>438 ± 31</td>
</tr>
<tr>
<td>Caudal trachea</td>
<td>5</td>
<td>360 ± 55</td>
</tr>
<tr>
<td>All regions</td>
<td>5</td>
<td>464 ± 50</td>
</tr>
</tbody>
</table>

Values are means ± SE.

Fig. 1. Effect of ion and liquid transport inhibitors on baseline rates of mucociliary transport. Excised tracheas were treated with either the drug vehicle (Ctl), 100 μM bumetanide (Bum) + 100 μM dimethylamiloride (DMA), or 10 μM benzamil (Benz). No significant differences among the groups were observed. Number of observations (n) are shown in parentheses.

Fig. 2. Effect of ion and liquid transport inhibitors on ACh-stimulated mucociliary transport. Excised tracheas were pretreated with either the drug vehicle (Ctl), 100 μM bumetanide + 100 μM DMA, 300 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), or 100 μM diphenylamine-2-carboxylate (DPC). Tissues were subsequently treated with 100 μM ACh to induce submucosal gland secretion. One group received luminal exposure to 10 μM Benz after Bum and DMA pretreatment but before ACh addition. *Significant difference (P < 0.05) from ACh only controls; **significant difference from tissues treated with ACh, Bum, and DMA.
particles at the end of each run difficult. Similarly, pretreatment with the anion channel blocker NPPB (300 μM) essentially abolished mucociliary transport in ACh-treated tissues (0.2 ± 0.1 mm/min, n = 6; Fig. 2). DPC, however, was not as effective as NPPB or the combination of bumetanide and DMA, reducing mucociliary transport to only 3.1 ± 0.4 mm/min (n = 6). Luminal exposure to benzamil, intended to block liquid absorption and preserve airway surface liquid volume, caused a significant restoration of mucociliary transport in airways treated with ACh, bumetanide, and DMA (3.9 ± 1.0 mm/min, n = 11; Fig. 2). In all tissues that were pretreated with DPC, NPPB, or the combination of bumetanide and DMA (including tissues that were exposed to benzamil), noticeably less mucus liquid was present on the mucosal surface after ACh application than in ACh controls.

To confirm that the effects of the secretion inhibitors on mucociliary transport were not due to impairment of ciliary motility, we measured ciliary beat frequency in porcine tracheae. In the absence of inhibitors and ACh, ciliary beat frequency was 12.6 ± 2.4 Hz (n = 3), which compares favorably with published values for other species (36). The effects of liquid secretion inhibitors were assessed on paired segments of excised tissues in the presence of 100 μM ACh (Fig. 3). Ciliary beat frequency was not significantly affected by the combination of 100 μM bumetanide and 100 μM DMA (control tissues, 12.7 ± 1.3 Hz; treated tissues, 12.7 ± 1.7 Hz; n = 7) or 100 μM DPC (control tissues, 14.0 ± 2.1 Hz; treated tissues, 13.2 ± 1.0 Hz; n = 5). Surprisingly, NPPB (300 μM) pretreatment induced a significant increase in ciliary beat frequency (control tissues, 13.2 ± 1.0 Hz; treated tissues, 17.3 ± 0.8 Hz; n = 4) even though this agent had the greatest inhibitory effect on mucociliary transport. Benzamil (10 μM), which caused significant reversal of the inhibition of mucociliary transport seen with ACh, bumetanide, and DMA, had no effect on ciliary beat frequency in the presence of these same agents (control tissues, 13.9 ± 1.2 Hz; treated tissues, 13.3 ± 0.8 Hz; n = 5) (Fig. 3) or in their absence (control tissues, 12.7 ± 1.1 Hz; treated tissues, 14.3 ± 1.4 Hz; n = 7).

DISCUSSION

These data demonstrate that agents previously shown to inhibit liquid secretion from airway submucosal glands have a great inhibitory effect on mucociliary transport. Because these same transport blockers have no inhibitory effects on the rate of ciliary beating, it is likely that an alteration in the mucus and liquid milieu at the airway surface is responsible for disabling mucus transport. This notion is supported by findings that benzamil, a blocker of ENaC-mediated Na⁺ and liquid absorption, restored mucociliary transport in inhibitor-treated airways. We conclude that impairment of liquid secretion, either by pharmacological manipulation or disease, will substantially reduce mucociliary clearance from airways.

Several observations lead us to believe that inhibition of liquid secretion from glands accounts for the reduction in mucociliary transport observed in this study. First, our previous studies showed that the large majority of the ACh-induced liquid secretion in porcine bronchi arose from submucosal glands (3). This finding substantiated earlier studies by others showing that glandular canine airways responded to cholinergic stimulation by secreting ions, whereas aglandular rabbit airways did not (7, 21). Second, when anion secretion inhibitors were applied to porcine bronchi, densely stained mucin accumulated within the gland ducts, demonstrating the uncoupling of mucin and liquid secretion by glands (17, 19). Third, the nonvolatile solids in the mucus liquid that were secreted by glands in the presence of the inhibitors were concentrated threefold and had altered rheological properties consistent with dehydration (39). Consequently, we believe that the inhibition of glandular liquid secretion is key to the impairment of mucociliary transport in this model. We admit the possibility, however, that fluid secreted from the surface epithelium, which theoretically could contribute small but important quantities of fluid to airway surface liquid, would be subject to inhibition by the same inhibitors used in the present study. We feel that this scenario is unlikely to explain our results because numerous studies demonstrate that, in the absence of secretagogues and Na⁺ transport inhibitors, net anion and liquid secretion is not typically observed across large airway epithelium (for review, see Ref. 6). Despite this rationale, anion and liquid secretion across surface epithelium must logically occur at least under certain conditions because the airways of some species, notably rabbits and mice, do not have submucosal glands beyond the perilaryngeal trachea, yet mucociliary transport functions normally and airway surface liquid is present. Recently, Tarran and coworkers...
able to account for this difference in behavior because inhibiting mucociliary transport. We are currently un-
NPPB or the combination of bumetanide and DMA at blocking mucus strands.

ume at the airway surface to promote hydration of this ENaC blocker could maintain suf-
reason that preservation of airway surface liquid with inhibitors supports this notion, although one could also transport in the presence of anion and liquid secretion fi-
cement in the presence of DPC was related to spillover inhibition of ENaC, an effect of this inhibitor that has been documented in nasal epithelium (31). This latter possibility could explain the similar levels of mucociliary transport observed between DPC and benzamil-bumetanide-DMA treatments in Fig. 2. Submucosal glands are abundant in the ventrolat-
portion of porcine tracheas with a trend toward greater density in the cranial regions. The mean values for gland density in these young, 4- to 8-wk-old pigs are about eightfold greater than the 0.62 ducts/mm² (62 ducts/cm²) reported for the tracheas of adult pigs (11). Similar proportions of gland density between the young and adults are also seen in human airways. Tos (37) demonstrated that the expression of tracheal submu-
cosal gland ducts decreased from 7–10 ducts/mm² (700–1,000 ducts/cm²) at birth to 0.7–0.9 ducts/mm² (70–90 ducts/cm²) at adulthood. This 8- to 10-fold decrease in gland density during development probably reflects the relative stability in absolute gland numbers as the surface area of the airways increases during growth and maturation. It should also be noted that the density of gland expression in the tracheas of these young pigs is somewhat lower than previously reported for the intralobular bronchi of similar-aged pigs (916 ± 84 gland ducts/cm²)(2).

Because of the likely role that CFTR plays in the normal secretion of liquid from submucosal glands, the results of the present study have important relevance to CF, a disease caused by deleterious mutations in the gene that codes for this protein. An impairment of liquid secretion, similar to that produced in the present study with anion secretion inhibitors, would also be expected to occur in CF airways. Mucociliary transport would therefore be similarly crippled, probably leading to the luminal accumulation of thickened mucus that typifies this disease. Inability to clear mucus from the airways would certainly lead to mucus retention as well as retention of inhaled pathogens that become imbedded in this material, thereby predisposing the airways to pathogen colonization. Bronchiectasis would be the inevitable consequence of chronic airway obstruction and infection. This scenario does not ac-
count, however, for observations that mucociliary transport is reportedly normal in some CF patients, particularly patients who have mild lung disease (33, 42). At least two explanations are possible for this paradox. First, these early studies were performed before the availability of genotype information; therefore, the CF patients that had high mucociliary rates

AJP-Lung Cell Mol Physiol • VOL 283 • AUGUST 2002 • www.ajplung.org
could have had mild lung disease due to less severe CFTR mutations that permit partial anion channel and liquid secretory function. However, more recent findings that a few CF patients homozygous for the severe ΔF508 mutation clearly inhaled colloidal particles at near normal rates argue against this notion (30). Second, mucociliary transport may function adequately in CF patients when submucosal gland secretion is minimal. But, when significant irritation or inflammation of the mucosal surface occurs, as would accompany episodes of airway infection, secretion of thickened mucus from glands greatly increases, impeding ciliary and cough clearance and trapping of infectious pathogens in the distal lung. When infections are severe, these events could induce an endless cycle of irritation, mucus secretion, reduced clearance, and pathogen trapping that could explain the progression of the airway disease in CF.

The beneficial effects of benzamil in this model of mucociliary transport impairment have important implications for treatment of CF lung disease. It suggests that inhibition of liquid absorption with an ENaC blocker could prevent the depletion of airway surface liquid and maintain mucociliary transport function even when liquid secretion is greatly impaired, a condition that is likely to occur in CF lung disease. This treatment strategy has been used in the past in several clinical trials with mixed results. Knowles and coworkers (24) reported improvements in pulmonary function, mucociliary and cough clearance, and sputum viscoelasticity in CF patients after aerosolized amiloride treatments. However, neither Pons et al. (29) nor Bowler et al. (8) saw significant improvements in pulmonary function in CF patients after amiloride treatment. Although we saw increased mucociliary transport with benzamil in the present in vitro model, this may have occurred because the airway surface was well hydrated at the beginning of the experiment, and the benzamil prevented depletion of this liquid by blocking its absorption. If liquid secretion is indeed chronically impaired in the CF condition, we suspect that the strategy of blocking liquid absorption alone would be of less potential benefit, because the mucus liquid at the airway surface would be chronically depleted and there would be little luminal liquid volume present.

We acknowledge that numerous diverse theories of CF lung pathogenesis currently exist and that other complications linked to CFTR dysfunction could contribute to the pulmonary complications of the disease. However, the results of the present study, when taken in the context of our previous findings, support the role of an abnormal secretion product from submucosal glands of the airways as an important factor in the etiology of CF lung disease. This notion of CF pathogenesis is not unique. After observations of submucosal gland hypertrophy, gland duct obstruction, and mucus plugging of distal airways in CF infants, Zuelzer and Newton (44) proposed in 1949 that an abnormality of mucus secretion from bronchial and tracheal submucosal glands was the root cause of the airway disease.

Now, over 50 years later, we present new findings that provide significant support for this “old” hypothesis.

The authors acknowledge the excellent technical assistance of Mita Patel and Maree Constable as well as the diligent work of students Stephen Laurendine and Robert Southard, whose preliminary studies were helpful in the development of the methodology for measurement of mucociliary transport. We also thank Dr. Stuart Wilson for useful comments and suggestions.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-48622 and HL-63302 (S. T. Ballard) and a Wellcome Research Career Development Fellowship (S. K. Inglis).

REFERENCES


