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Airway acidification initiates host defense abnormalities in cystic fibrosis mice

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Abstract

Cystic fibrosis (CF) is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel. In humans and pigs, the loss of CFTR impairs respiratory host defenses, causing airway infection. But CF mice are spared. We found that in all three species, CFTR secreted bicarbonate into airway surface liquid. In humans and pigs lacking CFTR, unchecked H⁺ secretion by the nongastric H⁺/K⁺ adenosine triphosphatase (ATP12A) acidified airway surface liquid, which impaired airway host defenses. In contrast, mouse airways expressed little ATP12A and secreted minimal H⁺; consequently, airway surface liquid in CF and non-CF mice had similar pH. Inhibiting ATP12A reversed host defense abnormalities in human and pig airways. Conversely, expressing ATP12A in CF mouse airways acidified airway surface liquid, impaired defenses, and increased airway bacteria. These findings help explain why CF mice are protected from infection and nominate ATP12A as a potential therapeutic target for CF.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S20

References (34–67)

Athin layer of airway surface liquid (ASL) is the point of contact between an organism and potential pathogens from the environment. To maintain sterile lungs, ASL contains several innate defenses, including a complex mixture of antimicrobials that kill bacteria, mucociliary transport that carries pathogens out of the lung, and phagocytic cells (1–3). In the genetic disease cystic fibrosis (CF) (4, 5), the loss of cystic fibrosis transmembrane conductance regulator (CFTR) impairs airway host defenses, initiating a cascade of bacterial airway infection, inflammation, and progressive destruction (6). After the discovery that mutations in the human *CFTR* gene cause CF, mice were produced with a disrupted *Cftr* gene (7, 8). Unexpectedly, airways of CF mice cleared large bacterial inocula and did not develop the spontaneous bacterial infections typical of CF (7, 8). Speculation about why CF mice fail to develop airway infections has relied on correlations. Compared with humans, mice have only a few submucosal glands, have different airway epithelial cell types, express other anion channels, and are smaller—features that correlate with absence of CF-related infections (7–9).

The recent finding that CF pigs develop airway disease that mirrors that of CF in humans (10, 11) provided us with an opportunity to compare humans, pigs, and mice. We reasoned that a better understanding of why CF mice do not develop airway infections might offer new insights into the molecular basis of respiratory infections in humans with CF. A potential mechanism emerged with the discovery that a loss of CFTR-mediated HCO_3^- secretion and an acidic pH impair at least two airway host defense mechanisms. These defects inhibit the killing of bacteria in ASL (12, 13) (fig. S1). They also alter ASL and mucus viscosity and impede mucociliary transport (14, 15). In addition, they increase mucus viscoelasticity in other organs (16, 17). We therefore explored whether differences between the pH of ASL in humans, pigs, and mice might account for differences in host defense properties. We found that the loss of CFTR reduced ASL pH in differentiated cultures of pig airway epithelia and in vivo, consistent with earlier findings (Fig. 1, A and B) (12). Loss of CFTR also reduced ASL pH in cultures of human airway epithelia (Fig. 1A) (18). In vivo studies of human CF neonates also found a reduced ASL pH (19), although studies of older people with CF yielded variable results (19–21). In contrast, in mice, the loss of CFTR did not reduce ASL pH either in vitro or in vivo (Fig. 1, A and B) (22).

Ca^{2+} -activated Cl^- channels might compensate for the loss of CFTR-mediated HCO_3^- secretion and prevent ASL acidification in CF mice; Ca^{2+} -activated Cl^- channels are abundant in mouse but not in human airways (9, 23, 24). Therefore, we predicted that pig airways would exhibit few Ca^{2+} -activated anion channels. We found transcripts for the Ca^{2+} -activated anion channel TMEM16A (anoctamin-1) in CF airway epithelia in a human:pig:mouse ratio of 1:9:18 (Fig. 1C). CF epithelia exhibited Ca^{2+} -stimulated anion secretion in a human:pig:mouse ratio of 1:5:10 (Fig. 1D). Adding carbachol, a Ca^{2+} -mediated secretagogue, elevated ASL pH by 0.02 ± 0.01 units in human, 0.11 ± 0.02 units in pig, and 0.09 ± 0.03 units in mouse epithelia (Fig. 1E). Thus, pig airway epithelia exhibit substantial Ca^{2+} -activated anion secretion, yet they develop airway infections. Although these data do not disprove the proposal that Ca^{2+} -activated anion channels prevent infection in CF mice, they suggest that other factors may be important.

We also reasoned that CF mice might not have an abnormally acidic ASL pH if there was little CFTR in non-CF mouse airways (25). To test CFTR activity, we applied forskolin and IBMX (3-isobutyl-1-methylxanthine) to elevate intracellular cyclic adenosine monophosphate (cAMP) and phosphorylate CFTR. Increasing cAMP stimulated HCO_3^- secretion in non-CF epithelia of all three species (Fig. 1F) (18, 26, 27). Moreover, stimulating HCO_3^- secretion elevated ASL pH in non-CF epithelia of all three species (Fig. 1G). These data suggest that the lack of CFTR alone does not explain CF-versus-non-CF differences in ASL pH among species. We expected that without CFTR, cAMP would merely fail to alkalinize ASL; unexpectedly, in CF pig and human epithelia, cAMP stimulation reduced ASL pH (Fig. 1H). In mice, the loss of CFTR prevented cAMP-induced alkalization, but, in contrast to CF pigs and humans, it did not lower ASL pH.

These results suggest that pigs and humans have a mechanism that acidifies ASL and that this mechanism is absent or less efficient in mice. To focus selectively on H^+ secretion, we removed HCO_3^- and CO_2 from the basolateral solution and replaced ASL with a small amount of nonbuffered solution at pH 7. Non-CF pig and human epithelia rapidly acidified the apical solution, whereas non-CF mouse epithelia acidified at about one-sixth the rate (Fig. 2A). CF epithelia yielded similar results (Fig. 2B). Although several epithelial properties influence ASL pH, less H^+ secretion in mice is consistent with a higher ASL pH in CF mice than in CF pigs or humans (Fig. 1A).

To determine which transporters secrete H^+ in pig airways, we assessed several candidates (28–30). Of the transcripts evaluated, those for ATP12A [the α subunit of the nongastric H^+/K^+ adenosine triphosphatase (ATPase)] and the $\alpha 1$ and $\alpha 2$ subunits of V-type ATPase were the most abundant in airways, and levels were similar for CF and non-CF pigs (fig. S2). To test for function of H^+ secretory proteins, we applied pharmacological inhibitors to the apical surface and found that ouabain, which inhibits ATP12A, increased ASL pH (Fig. 2C and figs. S3 and S4). ASL contains 20 to 40 mM K^+ (12), which would be required for ATP12A activity; removing apical K^+ prevented H^+ secretion (Fig. 2D). Inhibition of ATP12A by small interfering RNA (siRNA) also increased ASL pH, further supporting a role for ATP12A (Fig. 2E and fig. S5A). Previous reports indicate that elevating cAMP stimulates ATP12A activity (31). Consistent with that, apical ouabain and ATP12A siRNA inhibited cAMP-stimulated ASL acidification (fig. S5, B to D). Although ouabain also inhibits the basolateral Na^+/K^+ ATPase, these effects of apical ouabain did not arise from Na^+/K^+ ATPase inhibition (fig. S6).

On the basis of these results, we hypothesized that in the absence of CFTR, ATP12A acidifies ASL in pigs and humans, which impairs factors associated with airway defense, but that this process does not occur or is minimal in CF mice. Consistent with these ideas, mouse airways had only 1 to 10% as many ATP12A transcripts (Fig. 2F).

Immunohistochemical staining revealed ATP12A at the apical surface of human and pig but not mouse airways (Fig. 2G). ATP12A associates with a β subunit, including the Na^+/K^+ ATPase β subunit (ATP1B1) (28). Pig and human epithelia showed apical ATP1B1 immunostaining in addition to basolateral staining, whereas in mouse airways, ATP1B1 immunostaining was primarily basolateral (fig. S8). Our hypotheses point to two predictions that can be tested experimentally. The first is that ATP12A is required for ASL acidification

and host defense abnormalities in CF pigs. The second is that expressing ATP12A in CF mouse airways would be sufficient to reduce ASL pH and create host defense abnormalities.

To test the first prediction, we inhibited ATP12A with apical ouabain and found that it raised ASL pH in primary cultures of pig airway epithelia (Fig. 3A). We assayed antibacterial activity by briefly touching the ASL with a gold grid coated with *Staphylococcus aureus* and then determining the percentage of bacteria killed (12). Apical ouabain increased the killing of *S. aureus* (Fig. 3B). We also assayed ASL viscosity, which is increased in CF and may contribute to impaired mucociliary transport (15, 32). We measured fluorescence recovery after photobleaching (32) and found that apical ouabain reduced viscosity (Fig. 3C). After we applied apical ouabain, the pH, antibacterial activity, and viscosity of CF ASL became similar to those of non-CF ASL.

To test the relevance of these findings in vivo, we studied pigs. We surgically created a small tracheal window and applied ouabain. As observed in vitro, ouabain increased ASL pH and the killing of *S. aureus* in CF pigs (Fig. 3, D and E). We also measured the effect of apical ouabain on the ASL pH of human epithelia; applying apical ouabain increased ASL pH (Fig. 3F). These data are consistent with a report implicating ATP12A in H⁺ secretion in human cultures (18). The increase in ASL pH enhanced bacterial killing and reduced ASL viscosity (Fig. 3, G and H). These results suggest that inhibiting ATP12A would also elevate ASL pH in non-CF pigs and humans and improve host defense; supporting this idea, ouabain had similar effects in non-CF as in CF pigs and humans, both in vitro and in vivo (Fig. 3, A to H).

We next tested the second prediction—i.e., that expressing ATP12A in airways of CF mice would decrease ASL pH and generate host defense abnormalities. We treated cultured mouse airway epithelia with an adenovirus encoding *ATP12A* and found that it acidified ASL (Fig. 4A and figs. S13 to S15). Apical ouabain reversed the effect of ATP12A expression, increasing ASL pH (fig. S16), a result that further supports the conclusion that ATP12A acidifies ASL. ATP12A expression also impaired bacterial killing and increased ASL viscosity in CF mouse epithelia (Fig. 4, B and C).

To test the effects of ATP12A in vivo, we instilled adenovirus expressing *ATP12A* into the tracheae of CF mice. ATP12A reduced ASL pH and impaired bacterial killing (Fig. 4, D and E). In parallel experiments, expressing *ATP12A* in non-CF mouse epithelia in vitro and in vivo did not significantly alter ASL pH, bacterial killing, or ASL viscosity (fig. S17, A to F). However, further increasing the amount of vector could reduce ASL pH (fig. S17G).

Finding acidic ASL in CF mice expressing *ATP12A* suggests that these mice would be predisposed to bacterial infection. The lungs of CF mice expressing *ATP12A* had 100 times as much bacteria as those of control mice (Fig. 4F). This result is similar to the spontaneous appearance of multiple different bacteria in the lungs of newborn CF pigs (fig. S18) (11, 33). CF mice expressing *ATP12A* also developed evidence of inflammation; bronchoalveolar lavage revealed elevated numbers of myeloid cells (Fig. 4G and fig. S19). There were also positive correlations between *ATP12A* mRNA levels and numbers of bacteria and airway macrophages (Fig. 4, H to J).

It has long been puzzling why mice with a disrupted *CFTR* gene have intact airway host defenses, and it has been hoped that understanding the reason might suggest a therapeutic strategy. Our data provide an explanation. In non-CF pigs and humans, CFTR mediates HCO_3^- secretion, and ATP12A mediates H^+ secretion. The balance between these two secretory processes influences ASL pH. In CF pigs and humans, the loss of CFTR leaves H^+ secretion unchecked by HCO_3^- secretion, ASL pH falls, and the acidic pH and/or the reduced HCO_3^- concentration impair at least two key ASL properties associated with host defense. Moreover, ASL antimicrobial activity and viscosity are very sensitive to small changes in pH. However, mouse airways express little *ATP12A* compared with those of pigs and humans. They use V-type ATPase for H^+ secretion, but the rate of H^+ secretion is low (Fig. 2, A and B, and fig. S20). As a result, the loss of CFTR has minimal effects on ASL pH, and two key ASL defense properties remain largely intact. Other differences between mice, humans, and pigs—such as Ca^{2+} -activated anion channels, the abundance of submucosal glands, and variations in cell types (7–9)—may also contribute to differences in host defense among these species.

Limited ASL acidification in CF mice provides an “experiment of nature” with implications for therapeutics. First, the knowledge that cAMP stimulates ATP12A-mediated H^+ secretion (31) and reduces ASL pH might have implications for CF treatments that elevate cAMP levels. For example, β -adrenergic agonists are often prescribed for people with CF to relax airway smooth muscle; although this approach can provide benefit, it seems possible that it might further impair respiratory host defenses. Second, inhibiting ATP12A might have therapeutic value in CF, regardless of *CFTR* genotype. The findings in mice and the results with apical ouabain in pigs and humans provide a proof of concept.

Supplementary Material

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Acknowledgments

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Fig. 1. ASL pH is abnormally acidic in CF pigs and humans, but not in CF mice

(A) ASL pH measured in differentiated primary airway epithelial cultures from humans, pigs, and mice using SNARF-dextran ($n = 6$). The basolateral solution contained 25 mM HCO₃⁻, and the atmosphere contained 5% CO₂. (B) ASL pH measured *in vivo* on tracheal surfaces of newborn pigs and mature mice using a pH-sensitive optode ($n = 6$ for non-CF and CF pigs, 18 for non-CF mice, and 9 for CF mice). (C) *TMEM16a* mRNA expression in CF human, pig, and mouse cultured airway epithelia ($n = 6$). (D) Change in the short-circuit current (I_{sc}) after adding 1 μ M ionomycin basolaterally to cultured CF airway epithelia studied in Ussing chambers ($n = 5$). The solution contained 140 mM Cl⁻ bilaterally without HCO₃⁻ and CO₂. (E) ASL pH before and after adding the Ca²⁺-mediated secretagogue carbachol (CCH, 200 μ M) to basolateral surfaces of CF epithelia in the presence of HCO₃⁻ and CO₂ ($n = 6$ pigs and humans and 5 mice). (F) I_{sc} induced by adding 10 μ M forskolin and 100 μ M IBMX to increase intracellular cAMP ($n = 5$ for CF pigs and mice, 6 for non-CF humans and mice, and 7 for non-CF pigs and CF humans). The solution was free of Cl⁻ and contained 25 mM HCO₃⁻ with a 5% CO₂ atmosphere. (G and H) ASL pH in non-CF and CF pig, human, and mouse cultured airway epithelia before and after basolateral addition of 10 μ M forskolin and 100 μ M IBMX to increase cAMP ($n = 6$). In all panels, each data point or pair of data points is from a different animal or human, and bars indicate means $\pm$ SEM. Analysis of variance (ANOVA) with Tukey's multiple comparison test [(C) and (D)] or paired [(E), (G), and (H)] or unpaired [(A), (B), and (F)] Student's *t* tests were used to assess statistical significance. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

Fig. 2. ATP12A secretes H⁺ in pig and human but not mouse airway epithelia

(A and B) The rate of apical acidification in cultured non-CF and CF airway epithelia ($n = 6$ for non-CF epithelia and 6 for CF human, 5 for CF pig, and 4 for CF mouse epithelia). 30 μ l of a pH 7.0 unbuffered solution was placed on the apical surface, and pH was measured immediately and after 5 min. The basolateral solution was free of HCO_3^- and CO_2 and buffered with 25 mM Hepes. (C) ASL pH of non-CF and CF pig cultured airway epithelia in media free of HCO_3^- and CO_2 ($n = 6$ or 7). Epithelia were treated with the following agents dissolved in solvent and added apically in 20 μ l of perfluorocarbon (target in parentheses): 10 μ M ouabain (nongastric H^+/K^+ ATPase), 100 μ M SCH28080 (gastric H^+/K^+ ATPase), 100 μ M bafilomycin (V-type H^+ -ATPase), 100 μ M dimethyl amiloride, dimethylamine (NHE3), 1 mM ZnCl_2 (HVCN1), or dimethyl sulfoxide (DMSO) vehicle. (D) The rate of apical acidification in the presence and absence of K^+ ($n = 4$ to 6). 30 mM Na^+ replaced K^+ . Conditions were same as described for (A) and (B). (E) Effect of siRNA directed against pig *ATP12A* or scrambled control (Scr) on ASL pH in cultured non-CF and CF airway epithelia ($n = 4$). Solutions contained HCO_3^- and CO_2 . (F) *ATP12A* mRNA levels in non-CF and CF human, pig, and mouse airway epithelia ($n = 5$ for non-CF pig and non-CF mouse and 6 for all other epithelia). Values for mouse epithelia were less than for human and pig epithelia for both genotypes. (G) ATP12A immunostaining of human, pig, and mouse tracheal epithelium. The surface epithelium showed immunostaining along the apical surface in humans and pigs, but this was absent in mice. Scale bar, 18 μ m. Figure S7 shows a positive control. In all panels, data points in each group are from epithelia from a different animal or human. Bars indicate means $\pm$ SEM. Data were analyzed using ANOVA with Tukey's multiple comparison test [(A) to (C) and (F)] or unpaired Student's *t* tests [(D) and (E)].

Fig. 3. Inhibiting ATP12A increases pig and human ASL pH and enhances host defense properties of ASL

(A to C) Non-CF and CF cultured pig airway epithelia were treated with 10 μ M apical ouabain or DMSO vehicle for 2 hours ($n = 6$). The data shown are ASL pH (A), bacterial killing by ASL (B), and the time constant (τ) for fluorescence recovery after photobleaching of ASL relative to saline (C). For testing antibacterial activity, *S. aureus*-coated grids were placed on ASL for 1 min and then evaluated with a live/dead stain (fig. S9 shows a negative control). A higher $\tau_{ASL} / \tau_{saline}$ indicates a greater viscosity (fig. S10 shows that ASL depth did not differ). (D and E) Effect of ouabain (dissolved in DMSO suspended in 100 μ l of perfluorocarbon; estimated final concentration, 10 μ M) applied to tracheal surfaces of non-CF and CF newborn pigs ($n = 6$). Thirty min later, we measured ASL pH (D) and bacterial killing [*S. aureus*-coated grids placed on the airway surface for 1 min (E)]. Figure S11 shows that aerosolized DMSO vehicle did not alter ASL pH, and fig. S12 shows a lack of histopathological changes after ouabain delivery. (F to H) Effect of 1 mM apical ouabain or vehicle on ASL pH (F), antibacterial activity (G), and ASL viscosity (H) in human non-CF and CF epithelia ($n = 6$ or 8). In all panels, each data point or pair of data points is from epithelia from a different pig [(A) to (E)] or human [(F) to (H)]. Bars indicate means $\pm$ SEM. Data were analyzed using unpaired [(A) to (C) and (F) to (H)] or paired [(D) and (E)] Student's *t* tests.

Fig. 4. Expressing ATP12A in CF mouse airways acidifies ASL, induces abnormalities in host defense processes, and increases the number of bacteria in lungs

(A) Effect of treating cultured mouse airway epithelia with adenovirus expressing *ATP12A* (Ad-ATP12A) or vehicle (control) on ASL pH, measured Three days later ($n = 7$). (B and C) Cultured mouse airway epithelia were treated with Ad-ATP12A or vehicle (control). Three days later, antimicrobial activity was measured by placing a *S. aureus*-coated grid on the apical surface for 1 min (B), and ASL viscosity was assessed by measuring the time constant (τ) for fluorescence recovery after photobleaching of ASL relative to saline (C) ($n = 5$). (D and E) Ad-ATP12A or Ad-GFP (control; GFP, green fluorescent protein) was administered to CF mice. ASL pH (D) and antimicrobial activity [*S. aureus*-coated grid placed on the apical surface for 1 min (E)] were measured in vivo 3 days later ($n = 6$ or 7). (F) Ad-ATP12A or Ad-GFP (control) were administered to CF mice. Three days later, lungs were removed, homogenized, and cultured. The data shown are colony-forming units (CFU) ($n = 8$; see also figs. S17H and S18). (G) Total cells/mL in bronchoalveolar lavage liquid from CF mice treated with Ad-ATP12A or Ad-GFP (control) 3 days earlier ($n = 5$ to 7). (H and I) Relationships between *ATP12A* mRNA and bacterial CFU from a partial lung homogenate

(H) and composite macrophage score [foamy macrophage plus Iba1 (ionized calcium binding adapter molecule 1) staining (I)]. (J) Histopathological sections from CF mice 3 days after administering Ad-ATP12A. The left panel is from a mouse that had a low level of *ATP12A* mRNA, and the right panel is from a mouse with greater *ATP12A* mRNA (log10 relative to Ad-GFP control). Arrows identify macrophages admixed with cell debris and inflammation [see (I)]. In all panels, each data point or pair of data points is from a different mouse, and bars indicate means $\pm$ SEM. Data were analyzed using unpaired Student's *t* tests [(A) to (E) and (G)] Mann-Whitney *U* rank sum test (F), or linear correlation [(H) and (I)].