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The *In Vitro* Effect of Nebulised Hypertonic Saline on Human Bronchial Epithelium

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Title: The *In Vitro* Effect of Nebulized Hypertonic Saline on Human Bronchial Epithelium

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Take Home Message:
This study provides insight into the magnitude of effect of hypertonic saline on airway surface hydration in muco-obstructed diseases.

Abstract

RATIONALE: Inhaled hypertonic saline (HS) is an effective therapy for muco-obstructive lung diseases. However, the mechanism of action and principles pertinent to HS administration remain unclear.

METHODS: An *in vitro* system aerosolized HS to epithelial cells at rates comparable to *in vivo* conditions. Airway surface liquid (ASL) volume and cell height responses were measured by confocal microscopy under normal and hyperconcentrated mucus states.

MAIN RESULTS: Aerosolized HS produced a rapid increase in ASL height and decrease in cell height. Added ASL volume was quickly reabsorbed following termination of nebulization, though cell height did not recover within the same time frame. ASL volume responses to repeated HS administrations were blunted but could be restored by a hypotonic saline bolus interposed between HS administrations. HS-induced ASL hydration was prolonged with hyperconcentrated mucus on the airway surface, with more modest reductions in cell volume.

CONCLUSIONS: Aerosolized HS produced osmotically-induced increases in ASL height that were limited by active Na^+ absorption and cell volume-induced reductions in cell water permeability. Mucus on airway surfaces prolonged the effect of HS via mucus dependent osmotic forces, suggesting that the duration of action of HS is increased in patients with hyperconcentrated mucus.

Key Words: aerosolization, ASL, epithelium, hypertonic saline, cell volume

Abbreviation List

ALI – air-liquid interface

AQP - aquaporin

ASL – airway surface liquid

AUC – area under the ASL height curve

CF – cystic fibrosis

CFTR – cystic fibrosis transmembrane conductance regulator

CO_2 – carbon dioxide

ENaC – epithelial sodium channel

FEV_1 – forced expiratory volume in 1 second

HBE – human bronchial epithelium

HS – hypertonic saline

IL-8 - interleukin 8

mOsm/L – milli-osmoles per liter

MCC – mucociliary clearance

NaCl – sodium chloride

PBS – phosphate buffered saline

TRD – Texas Red Dextran

UNC – University of North Carolina at Chapel Hill

Introduction

The effectiveness of mucociliary clearance is heavily dependent on adequate hydration of mucus. Under normal circumstances, airway epithelial cells have the capacity to absorb and secrete ions via active ion transport, with movement of water governed by transepithelial osmotic gradients. The net effect of this balance between secretion and absorption is to maintain mucus layer hydration at levels adequate to promote efficient mucus clearance [1]. Hyperconcentrated mucus, with failed mucus clearance, is characteristic of many muco-obstructive diseases, including cystic fibrosis (CF) [2], primary ciliary dyskinesia [3], and non-CF bronchiectasis [4].

Aerosolized hypertonic saline (HS) is an effective therapy in adults with CF [5,6] and non-CF bronchiectasis [7,8], producing improvements in MCC, forced expiratory volume in one second (FEV_1), and quality of life.. Despite its clinical utility, the mechanism of action of HS remains speculative. Accelerating MCC via electrostatic interactions with mucins [9], expanding ASL hydration [10], or inhibiting epithelial sodium channels (ENaC) (9) have been proposed as mechanisms. Most mechanistic studies deposited large volumes of HS onto HBE surfaces and have not mimicked *in vivo* aerosol deliveries. Further, most studies using cultured HBE were performed without endogenous normal (2% solids) or hyperconcentrated (~8% solids) mucus on HBE surfaces [5,10].

As prescribed for clinical use, HS is aerosolized to the lower airway surfaces in small (nanoliter/cm^2/min) volumes over protracted intervals (~15 minutes). In this study, an *in vitro* aerosolization system was developed to mimic *in vivo* aerosol delivery rates to airway epithelia that exhibited a range of mucus concentrations, spanning “normal” to muco-obstructive conditions. The primary goal was to characterize the kinetics of HS effects on airway surface hydration. The ancillary goal was to identify strategies to improve the efficacy of HS in the treatment of lung disease. Because of the great

diversity of nebulizers and HS strengths used in clinical practice, and the fact that the osmotic effectiveness of HS depends on the rate of NaCl delivery to airway surfaces, our studies are described in µg NaCl deposited per cm^2 per min, rather than % HS at a given deposition rate. Some of these data have been previously reported in the form of an abstract [11-15].

Methods:

A full detail of the methods is provided in the Supplement.

HBE Cultures and Apical Mucus Content: Primary HBE cells generated from cells isolated from explanted lungs were maintained at an air-liquid interface (ALI) until fully differentiated. Culture surfaces were washed daily to produce HBE cultures with small amounts of residual mucus that mimicked normal epithelia, whereas HBE preparations with the mucus layer left unwashed for two weeks were generated to produce higher mucus compositions.

Confocal Measurements of ASL: Primary HBE cells were labeled with 2.0 µM calcein-AM (Invitrogen) basolaterally. ASL was visualized by adding a 5µl of Texas Red Dextran (Invitrogen, 70,000 MW, 5mg/ml) luminally. HS (7%) solution was aerosolized utilizing a vibrating mesh nebulizer (Aeroneb Lab; Aerogen), modified to deliver small volumes (nanoliters per minute). Nebulizers were cleaned daily in accordance with Cystic Fibrosis Foundation guidelines and tested periodically for functionality. The system was mounted in an environmental chamber that controlled air currents and regulated temperature to 37°C, humidity at >50%, and CO_2 at 5%, interfaced to a scanning confocal microscope (SP5, Leica). Cell and ASL heights were measured by high-speed X-Z confocal scanning. Following baseline imaging, 7% HS was nebulized to apical surfaces of HBE cells at the prescribed rate and effects were measured at 30-second intervals.

ELISA determination IL-8 Secretion: Production of IL-8 in HBE was measured 24 hours after nebulizing either 3, 8, or 18 µg NaCl/cm^2/min or sham nebulization of PBS (control). For comparison, parallel cultures were treated with a bulk addition of hypertonic saline (100 µl of a 7% solution). Concentrations of IL-8 in the culture medium were measured

by using a human IL-8 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacture's protocol. [Briefly, after incubation, the culture medium was removed. Samples and standards were added to wells of microplates precoated with an anti-human IL-8 monoclonal antibody and incubated for 2 hours. Each well was washed and incubated with the enzyme-linked polyclonal antibody specific for human IL-8 for 2 hours. The wells were washed to remove unbound antibody-enzyme reagent, substrate solution added to each well for 20 min at room temperature, the enzyme reaction was stopped, and IL-8 concentrations determined by comparison of the optical density results with the standard curve.

Mathematical Modeling: The airway fluid transport model of Warren et al. [16] was utilized in the construction of an integrative model of human airway ion and fluid transport [17,18]. This model solves an ordinary differential equation system for membrane potentials (V_a, V_b), intracellular ion concentrations ($[Na^+]_i$, $[Cl^-]_i$, $[K^+]_i$), extracellular ion concentrations ($[Na^+]_e$, $[Cl^-]_e$, $[K^+]_e$), and extracellular and intracellular fluid volumes (W_e, W_i) [18]. For the HS simulations, water with a high concentration of NaCl was "added" to the apical surface for designated volumes.

Statistical Analysis: All data analyses and graphing were performed in SigmaPlot (Systat). Mean values were compared via one-way ANOVA, with Holm-Sidak method for pairwise multiple comparisons in statistically significant findings. For non-normal data, the Kruskal-Wallis one-way ANOVA was used, with multiple groups compared via the Dunn Method. Paired t-tests or Mann-Whitney Rank Sum test were used where appropriate.

Results:

The first series of studies focused on the effect of HS on HBE cultures with "normal" mucus concentrations (1.9 ± 1.7% solids) on apical surfaces. HS was delivered to HBE cultures for 15 minutes at a rate of 8µg NaCl/cm^2/min (7% HS delivered at 110 nl/cm^2/min) to mimic a standard jet nebulizer delivery rate for human subjects (see **Supplement**). A rapid and significant increase in ASL height was observed, reflecting the osmotic-driven transepithelial fluid flow in response to deposited HS [19] (**Figure 1**).

Net reabsorption of the osmotically expanded ASL began immediately after nebulization was terminated, and ASL height returned to baseline height within 60 minutes after aerosol initiation.

In clinical practice, jet nebulizers deliver HS in typically a 4 ml dose over approximately 15 minutes, whereas vibrating mesh nebulizers deliver the same volume in about 5 minutes. To address whether HS delivery rates affect ASL volume responses, a constant total mass of salt was nebulized onto the HBE surface at varying nebulization rates and, hence, durations of nebulization. The rates selected for study were designed to a mimic delivery of 7% HS via jet nebulizer (8 µg NaCl/cm^2/min), a vibrating mesh nebulizer (18 µg NaCl /cm^2/min), and an arbitrarily defined "slow" nebulizer (3 µg/cm^2/min) (**Figure 2A**). Both the 8 and 18 µg NaCl/cm^2/min rates produced rapid and significant increases in ASL height. The response of cultures to the 3 µg NaCl/cm^2/min rate was considerably smaller with respect to ASL volume expansion, but longer due to the extended nebulization duration. All cultures, irrespective of nebulization rate, rapidly absorbed the added volume from the ASL once nebulization was terminated.

Area under the curves (AUCs) of the ASL height data were calculated as an index of "hydration activity" of the nebulized solution. Interestingly, faster salt deposition (18 µg/cm^2/min) produced a lower, though not statistically different, total ASL hydration compared to standard jet nebulizer rates (8 µg/cm^2/min). Slower aerosol deposition (3 µg/cm^2/min) produced trends towards lower peak and AUC values compared to the faster rates (**Figure 2B**).

Assuming no active sodium-mediated volume absorption or change in apical membrane water permeability during aerosolization, deposition of a constantly accumulating mass of salt to the surface of airway epithelia is predicted to produce a linear increase in ASL volume. For all rates studied, the measured ASL height deviated consistently from the predicted height (**Figures 2C-E**). The deviation of measured ASL height from predicted values could reflect active Na^+ absorption, reductions in HBE water permeabilities that govern rates of osmotically driven water flow to the HBE surface, or both.

With respect to the role of Na^+ transport on ASL volume responses to HS administration, we speculated that Na^+ transport would modify the magnitude of HS-induced ASL volume expansion immediately after initiation of HS administration. To experimentally investigate this possibility, ASL volume responses to the low rate of HS administration (3 µg NaCl/cm^2/min) were measured in the presence and absence of a selective ENaC blocker (VX-371, Vertex Pharmaceuticals). As compared to HS alone, the co-administration of VX-371 (50 µg/mL) produced a more rapid and sustained ASL response during nebulization (**Figure 3A**). A mathematical model of airway epithelial ion and water transport quantitatively analyzed the relationship between HS-mediated changes to ASL height and active Na+ absorption in the presence and absence of ENaC blockade (**Figure 3B**). The fit of the model to experimental data describing ASL response for HS administration in the absence of ENaC inhibition was achieved by maintaining Na^+ transport at basal rates during HS aerosolization and increasing Na^+ transport roughly five-fold during the re-absorptive phase. The mathematical model replicated the effect of ASL kinetics with ENaC inhibition, with a more rapid rate of accumulation and overshoot of ASL height, consistent with inhibition of active Na^+ absorption during HS administration.

Previous studies have demonstrated that HS can produce a reduction in cell volume [20,21], which can then inhibit cell water permeabilities [21-23]. Additionally, studies suggest that administration of hypertonic saline also will result in inflammatory changes within the airway epithelia, particularly interleukin-8 (IL-8), a known neutrophil chemoattractant [24-26]. Accordingly, we measured HBE cell volume responses and IL-8 production to HS aerosol administration to test whether reductions in cell water permeabilities blunted ASL responses to aerosolized HS and/or induced pro-inflammatory cytokines. **Figure 4A** shows that cell height (a surrogate for cell volume) was reduced during and after HS nebulization by ~ 20% from initial cell heights, ($p<0.01$). In contrast, cell volume was not affected by nebulization of isotonic saline. These data suggest that deposition of luminal HS osmotically draws water from cells onto the surface via apical membrane water channels. **Figure 4B** demonstrates cumulative IL-8 production by HBE 24 hours after nebulizing HS or PBS control to

airway surfaces. A stepwise increase in IL-8 production was noted with increasing doses of HS compared with PBS control (statistically different from control at 18µg dose and bulk dosing.

The contribution of cellular water permeability to ASL volume responses was investigated further using a pharmacologic approach. Because mercury-sensitive water channels (aquaporins; AQPs) have been identified in the apical and basolateral membranes of HBE epithelia [27,28], the role of these channels on HS-mediated changes in ASL volume responses was evaluated experimentally. Because mercury has off-target toxic effects with long exposure, the protocols involved acute exposures to mercury and HS [29]. As shown in **Figure 5A**, both apical and basolateral $HgCl_2$ administration significantly reduced ASL volume responses to aerosolized HS. Inhibition of apical AQPs resulted in a smaller decrease in cell height compared to basolateral AQP inhibition. These data suggest that in the setting of aerosolized HS, cell volume homeostasis is dominated by the apical membrane water permeability (**Figure 5B)**. Modeling studies mimicked the experimental data **(Figure 5C)**.

We hypothesized that persistent reduction in cell volumes observed after HS administration would limit the ability of HBEs to respond to repeated administrations of HS. This hypothesis was tested by exposing HBE cultures to sequential HS administrations (8 µg NaCl/cm^2/min), with the second administration delivered 15 minutes after ASL height had returned to baseline following the first HS dose. ASL height and AUC of the second administration was significantly reduced compared to initial HS administration (**Figure 6A)**.

We next directly tested the hypothesis that the reduction in the effectiveness of the second HS administration reflected reduced HBE cell water permeabilities consequent with cell volume reduction, rather than persistent acceleration of Na^+ transport. To test this notion, a small volume of hypotonic saline (0.63% NaCl, 10 µl) was transiently added (3 minutes) to the apical surface of HBE's following the first aerosolized HS administration. Application of the hypotonic saline bolus resulted in cell swelling (increase in height 32 ± 9 µm) that restored cell height to baseline levels. The interposition of the hypotonic bolus was associated with a statistically larger response in

ASL and AUC to the second HS administration (**Figure 6B**). In contrast, applying a bolus dose of isotonic saline (0.9% NaCl, 10 µl) produced neither cell swelling nor a recovery in responsiveness to the subsequent HS challenge (**Figure 6C**). Modeling this phenomenon predicted that reduction in apical water permeability to about 10% of baseline levels would produce the AUC decrement with the second HS administration observed experimentally **(Figure 6D).**

We next investigated whether the presence of hyperconcentrated mucus associated with muco-obstructive diseases [30] alters the kinetics of HS-induced hydration, utilizing HBE cultures exhibiting hyperconcentrated mucus (12 ± 4.3% solids). Cystic fibrosis (CF) is the disorder best characterized by hyperconcentrated mucus. However, because other muco-obstructive diseases, *e.g.*, primary ciliary dyskinesia [3] and non-CF bronchiectasis [4] exhibit hyperconcentrated mucus, and we have previously shown that CF cells and normal HBE in the presence of hyperconcentrated mucus behave similarly in response to nebulized hypertonic saline [13]. Accordingly, we focused our studies of muco-obstructive disease mucus on normal HBE with hyperconcentrated mucus to make the data relevant to other possible muco-obstrcutive lung diseases. Pre-HS administration, ASL height in the hyperconcentrated mucus cultures was ~ 3-fold higher than the normal mucus cultures. Notably, the changes in ASL height with HS administration were substantially increased in hyperconcentrated mucus cultures as compared to normal mucus cultures **(Figure 1, 7A).** Although both normal and hyperconcentrated mucus cultures absorbed the increased ASL volume immediately after cessation of nebulization, the rate of reabsorption was slower in hyperconcentrated versus normal mucus cultures (5.5 vs. 2.8 µm/min, p=0.052, **Figure 7B**). In the hyperconcentrated mucus cultures, the total duration of HS-induced ASL volume expansion was approximately double that of normal mucus cultures. Further, in repetitive HS administration protocols, the second administration of HS produced an ASL response similar to the first administration, **Figure 7C**. Pertinent to this observation, cell heights in HBEs covered by hyperconcentrated mucus decreased less in response to HS (15 ± 4.7% vs. 23 ± 5.7%) than in normal cultures, though this difference did not achieve statistical significance.

The larger peak ASL response and slower absorption of NaCl and water in the hyperconcentrated versus normal mucus cultures suggested an additional force was governing water flux in the hyperconcentrated mucus system. To generate an index of the magnitude of this effect, the predicted versus measured ASL heights during HS administration were compared (**Figure 7D**). The measured early administration values were significantly closer to the predicted value for hyperconcentrated compared with normal mucus cultures (see **Figures 2C-E**), suggesting that the presence of concentrated mucus generated additional mucus-related osmotic forces that retarded fluid absorption [30].

Discussion:

Our *in vitro* studies of clinically-relevant rates of aerosolized HS delivered to HBE cultures with 2% mucus solids revealed a rapid HS-induced expansion of ASL volume. This ASL expansion in response to HS aerosols is similar to that reported *in vivo* in mice measured by synchrotron-based tomography and in HBE cultures by optical coherence tomography [31,32]. Further, acute aerosolization of HS has been reported to reduce airway mucus concentrations in COPD subjects [33]. Thus, the ASL expansion findings, juxtaposed to our findings that the osmolarity of ASL during HS nebulization does not likely exceed 370 mOsm/L (see below), argues that the major effect of HS on mucus clearance is via ASL volume expansion and mucus dilution.

The HS-induced ASL volume response was mediated by water flux in response to the deposition of osmotically active NaCI on HBE surfaces. Two observations suggest that aquaporin-mediated transepithelial water fluxes dominated the ASL response to aerosolized HS. First, the flux of water to the lumen in response to HS was accompanied by a reduction in cell height/volume, suggesting intracellular water moved into ASL. This notion is consistent with previous studies [21] that reported the aquaporin-dominated apical membrane water permeability of HBE was ~ 10 fold higher than the basolateral membrane. Note, this configuration permits the use of changes in cell volume as an “osmometer” to detect ASL osmolality. The cell volume responses to HS aerosols suggest that ASL achieved osmolalities of ~370 mOsm during HS administration (7% HS given at 7.7 µg/NaCl/cm^2/min), far less than achieved by direct

HS additions. The second observation was that the ASL responses to aerosolized HS were blocked by mercury chloride, an inhibitor of AQP 3-5 known to be expressed in HBEs. These data, along with the cell modeling data presented, strongly suggest that aquaporin-mediated cellular water permeabilities participate in the ASL volume responses to aerosolized HS (**Figure 4**).

The effects of aerosolized HS on cell volume reduction and transepithelial Na^+ transport likely explain the discrepancy between the predicted ASL responses to deposition of HS on HBE surfaces and measured responses. First, reductions in cell volume were associated with reductions in cell water permeabilities, limiting water fluxes toward a hypertonic lumen. Second, as evidenced by a greater maximal response to HS administration in the presence of an ENaC blocker, active Na^+ transport removed a component of the deposited NaCl during aerosol administration. An interesting observation that emerged from the modeling of active Na^+ transport responses to HS was that an increase in Na^+ transport rates was required to mimic the rate of ASL volume absorption post cessation of aerosol. This effect likely reflects dilution of local extra-cellular inhibitors of ENaC, e.g. ATP, that accelerated the rate of absorption during HS administration [34].

A key issue with respect to HS therapeutic responses relates to the concentration of mucus on airway surfaces. The presence of hyperconcentrated mucus on HBE surfaces was associated with increased ASL heights, a longer duration of ASL hydration to a single HS administration, and larger responses to repetitive doses of HS. We postulate that mucus acts as a “sponge”, providing a concentration-dependent polymer gel-mediated osmotic driving force, added to that of HS-induced osmotic gradients, which modulates maximal mucus/ASL heights on the HBE surface. The increased durability of the ASL expansion in response to aerosolized HS in hyperconcentrated mucus cultures parallels the longer duration of action of HS in CF as compared to normal subjects observed in *in vivo* MCC studies [5,35].

Our studies were also designed to identify strategies to increase the effectiveness of aerosolized HS to expand ASL hydration, including studies of HS delivery rates and repetitive dosing. We found very slow rates of HS delivery were relatively ineffective.

We speculate that this finding reflects the fact that NaCl deposition rates were similar to rates of endogenous active transepithelial Na^+ absorption [36]. Delivery of HS at rates approximating a jet nebulizer were effective in increasing ASL hydration but a further increase in efficacy was not observed with faster rates mimicking vibrating mesh nebulizers. The absolute decrease in AUC and the slowing of ASL volume expansion observed towards the end of rapid HS administration (18 µg NaCl/cm^2/min) are consistent with large reductions in transepithelial water permeabilities. Based on our IL-8 measurements and the pro-inflammatory nature of this cytokine, it is possible that faster rates of nebulization could actually be detrimental.

An important observation pertinent to HS dosing frequency was that HBE cell heights did not return to baseline levels for 4 hours following HS administration. We speculate that the absence of cell volume regulation mechanisms reflected decreased water permeabilities. The delay in return of apical water permeability after HS likely accounted for the blunted response to second HS administrations on HBE cultures with normal mucus (**Figure 5**). Importantly, epithelial cell swelling induced by hypotonic saline restored second HS responses. These data suggest that novel strategies may be required to optimize the effectiveness of repetitive HS dosing in patients with milder lung disease. In contrast, repetitive HS dosing in subjects with severe (12% mucus solids) disease may be an effective strategy.

In conclusion, ASL volume of HBE cultures with normal mucus concentrations increased in response to nebulized HS delivered at rates similar to those delivered clinically, but reabsorption began immediately with termination of nebulization. Neither nebulizing faster nor slower improved ASL hydration. However, the effects of aerosolized HS were more pronounced and prolonged in HBE cultures with hyper-concentrated mucus, likely reflecting the increased osmotic forces generated by concentrated mucus. These data predict a prolonged duration of HS action in patients with muco-obstructive diseases.

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Figure Legends:

Figure 1: (A) XZ-confocal image of a cross section of airway epithelial cells stained with Calcein, a fluorescent green dye, and ASL stained with Texas Red Dextran, a cell impermeable red dye. **(B)** After administration of an osmotic stimulus (HS), there is an increase in ASL height along with a decline in cell height. **(C)** HS aerosolized to the surface of HBE cultures results in a significant but transient increase in ASL height, lasting under an hour. n=8 for each experiment. Rectangle represents duration of HS administration (8µg NaCl/cm^2/min for 15 min).

Figure 2: Effects of variation in HS delivery rates on ASL volumes responses. **(A)** Comparison of ASL heights achieved with three rates of nebulized HS (low, 3µg NaCl/cm^2/min; moderate, 8 µg NaCl/cm^2/min; and high, 18 µg NaCl/cm^2/min) with duration varied to hold constant total delivered mass of salt (demonstrated by length of bar at top). Isotonic saline showed for comparison. $p<0.05$ for all groups compared to isotonic saline control. (**B**) Both absolute height change and total hydration (integrated area over time) favored the mid-range dosing regimen but did not reach statistical significance (**A**; $p=0.373$). **(C,D,E)** Dashed lines indicates the expected rise in ASL height if all salt deposited resulted in an equimolar flux of water into the ASL. Solid black lines indicate the actual change in ASL height observed during experiments. The failure of these two lines to overlay indicates an inhibition of the transport of water from the epithelial cells into the ASL. **(C)** Low dose nebulization (3 µg NaCl/cm^2/min). Theoretical slope 4.72 µm/min versus experimental slope of 0.45 µm/min, $p<0.001$. **(D)** Medium dose nebulization (8 µg NaCl/cm^2/min). Theoretical slope 14.92 µm/min versus experimental slope of 8.66 µm/min, $p<0.001$. **(E)** High dose nebulization (18 µg NaCl/cm^2/min). Theoretical slope 51.49 µm/min versus experimental slope of 20.78 µm/min, $p<0.001$. N=4 per experimental condition.

Figure 3: Effect of sodium reabsorption on HS-mediated ASL height. **(A)** Experimental data of hypertonic saline (at 8 µg NaCl/cm^2/min) in the absence and presence of a potent sodium channel blocker (VX-371 at 50 µg/ml). **(B)** Mathematical model predictions of the HS effect with normal ENaC conductance (solid line, consistent with experimental data) and 15% of ENaC channel normal conductance (dashed line).

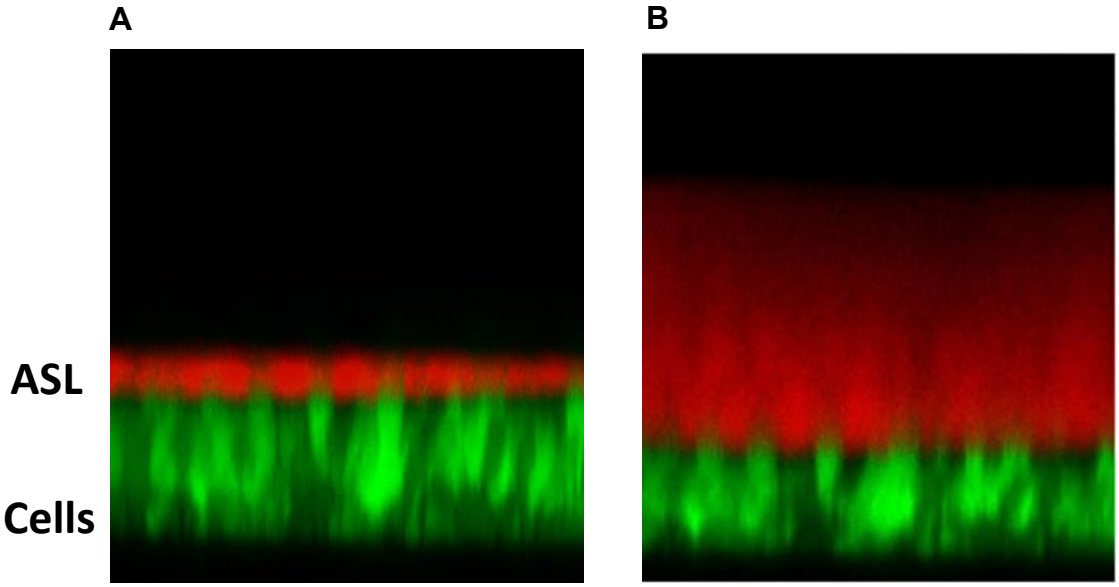
Figure 4: (A) Cell volume changes in response to osmotic stimulus of HS at varying HS deposition rates. A 20% decrease in cell height in response to the hypertonic stimulus was observed at all rates, which was significantly different from baseline and isotonic (PBS) saline administration ($p<0.01$ by Holm-Sidak method). Cell height did not recover during the time frame of the experimentation (60 minutes). Cell volume reduction was a consistent finding regardless of rate of HS administration. (n=4 for each condition). (B) Production of IL-8 in response to increasing doses of nebulized or bulk HS. Statistically different from control (PBS) at the 18 µg dose ($p<0.002$ by Holm-Sidak method) and bulk dosing (100µl of 7% HS) ($p<0.001$).

Figure 5: Incubation with mercury chloride apically or basolaterally resulted in a diminished ASL response to aerosolized HS (at 8µg NaCl/cm^2/min) compared with native cultures. (**A)** Experimental data. p<0.001 via Holm-Sidak method for comparisons to control. **(B)** Mathematical model of experimental data. N=8 for each condition. **(C)** Cell height change to aerosolized HS in the absence (control) or presence of selective block of the apical or basolateral cell membrane with mercury chloride (p<0.001 for all comparisons).

Figure 6: Sequential ASL volume responses to aerosolized 7% HS for 15 min (8µg NaCl/cm^2/min). **(A)** Sequential ASL volume responses to aerosolized HS. A smaller ASL response to a second administration of aerosolized HS was observed when two identical HS doses were separated by 15 minutes. Break in x-axis indicate time between two doses. For each dose, HS was nebulized at 8 µg NaCl/cm^2/min, administered continuously for 15 minutes. The ASL height of the second peak was about 60% of the first peak. P<0.001 by Mann-Whitney Rank Sum test. **(B)** A hypotonic saline rinse was interposed between HS administration. The hypotonic solution administration produced cellular swelling associated with improved ASL volume responses to a subsequent administration of HS (~140% of first dose). The difference in response was statistically significant (p=0.002 via Mann-Whitney Rank Sum test) in favor of the post-hypotonic peak. **(C)** Interposition of an isotonic rinse between the first and second HS administration. The same second dose effect is not seen when doses of HS were separated by an isotonic saline bolus. (p=0.78 by Mann-Whitney Rank Sum Test). ASL height of second peak persisted ~50% of the first peak. **(D)** In model simulation, the water permeability of the HBE apical membrane during the second dose was reduced to 10% of the basal water permeability. N=4 for each condition.

Figure 7: The effects of hyperconcentrated mucus on HBE surfaces on nebulized HS (at 8µg NaCl/cm^2/min) in inducing ASL volume responses. **(A)** In the normal (2% mucus) state (solid line), there was a 3-fold increase in ASL volume during nebulization. HBE cultures with hyperconcentrated mucus (12%) (Dashed line) exhibited a 7-fold increase in ASL above baseline ASL. The duration of ASL being increased above basal levels was also increased in hyperconcentrated mucus HBE cultures. **(B)** Rates of reabsorption (time to baseline ASL, in µm/min) were significantly different between the normal and the hyperconcentrated mucus cultures. P=0.052 by Mann-Whitney Rank Sum Testing. **(C)** Two sequential doses of HS administered to hyperconcentrated cultures. ASL height difference favors the second dose (p<0.001). **(D)** Dashed line indicates the expected rise in ASL height if all salt deposited remained on the surface and resulted in an equimolar flux of water into the ASL. Solid line indicates the actual change in ASL height observed during experiments. Compare to **Figures 2C-E**: In the presence of an intact mucus layer (12% solids), actual and expected ASL height more closely approximated each other early in the HS delivery interval**.**

Figure 1:


A
B
ASL
Cells

C

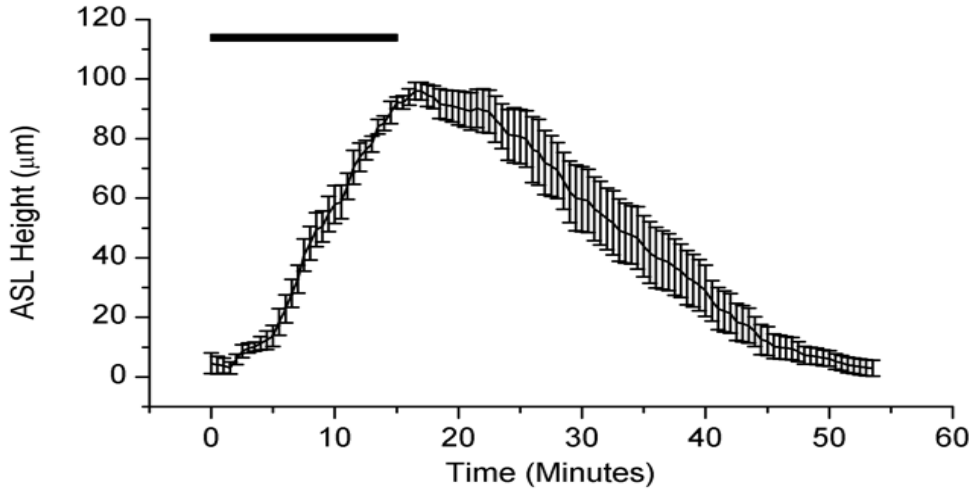
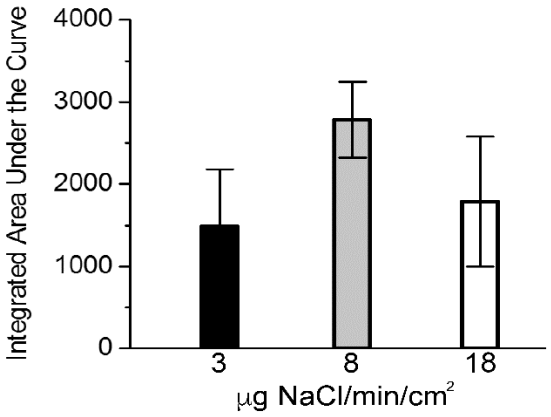
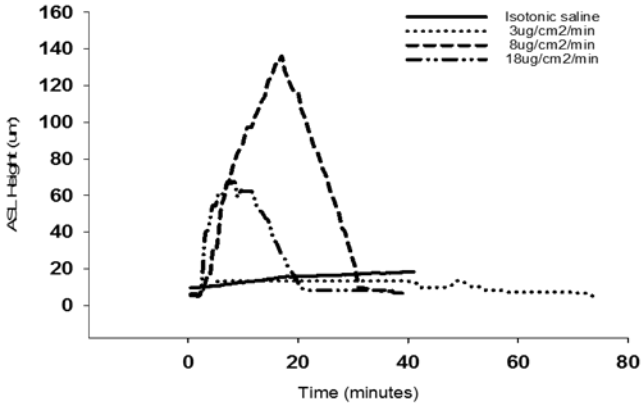

ASL Height (μm)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (Minutes)

Figure 2:

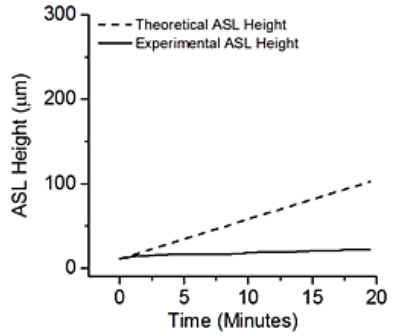
A



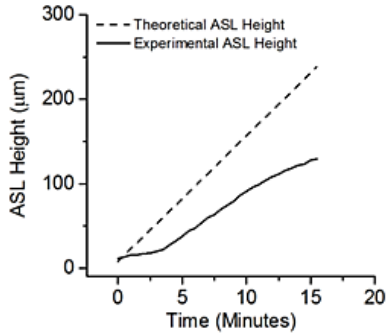
B



C



D



E

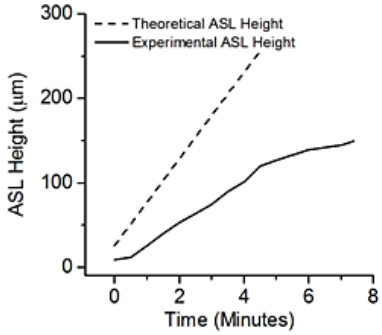
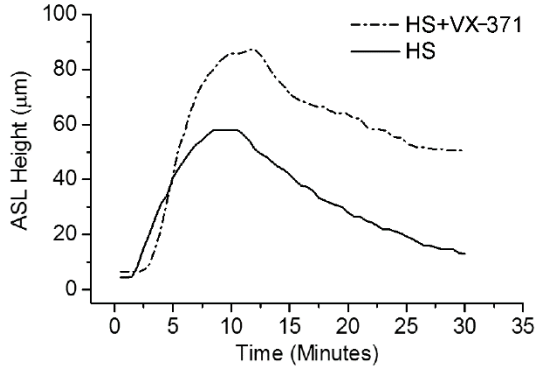


Figure 3:

A


HS+VX-371
HS
ASL Height (µm)
Time (Minutes)
0
20
40
60
80
100
0
5
10
15
20
25
30
35

B

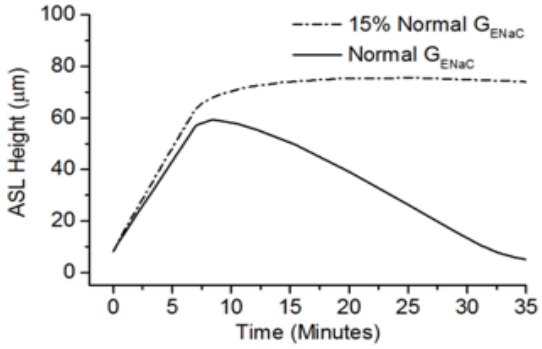
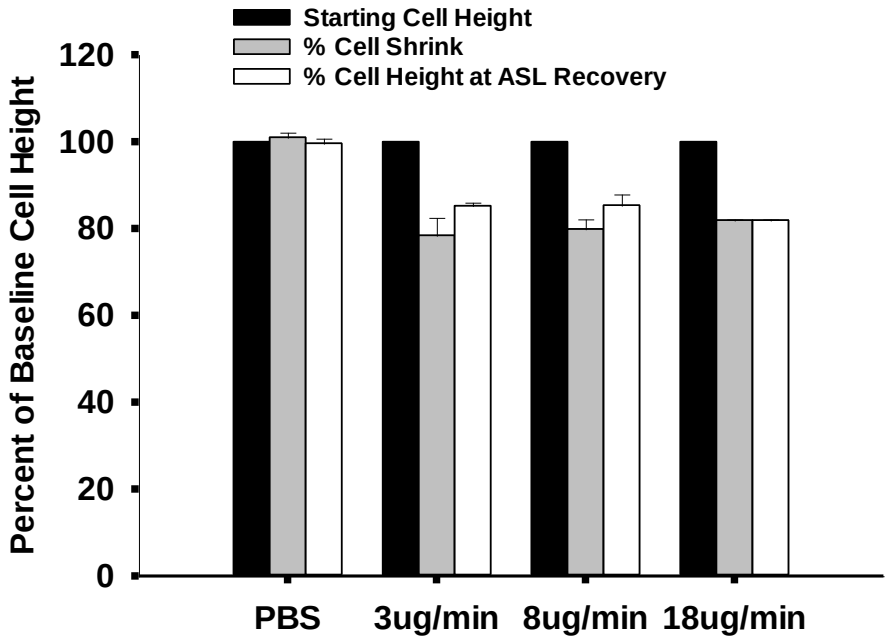

15% Normal G ENaC
Normal G ENaC
ASL Height (µm)
Time (Minutes)
0
20
40
60
80
100
0
5
10
15
20
25
30
35

Figure 4:

A


Starting Cell Height
% Cell Shrink
% Cell Height at ASL Recovery
Percent of Baseline Cell Height
120
100
80
60
40
20
0
PBS
3ug/min
8ug/min
18ug/min

B

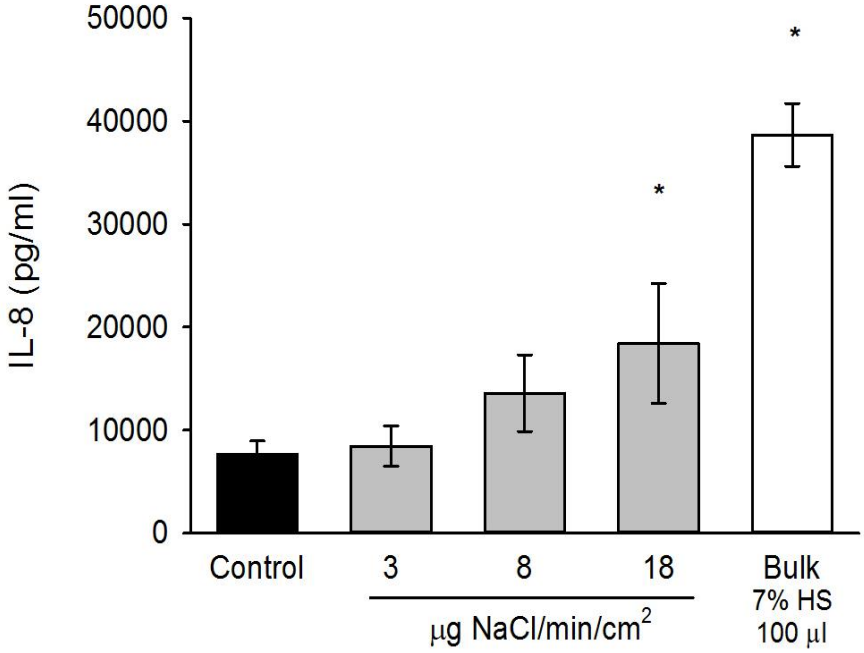

IL-8 (pg/ml)
50000
40000
30000
20000
10000
0
*
*
Control
3
8
18
Bulk
7% HS
100 µl
µg NaCl/min/cm2

Figure 5:

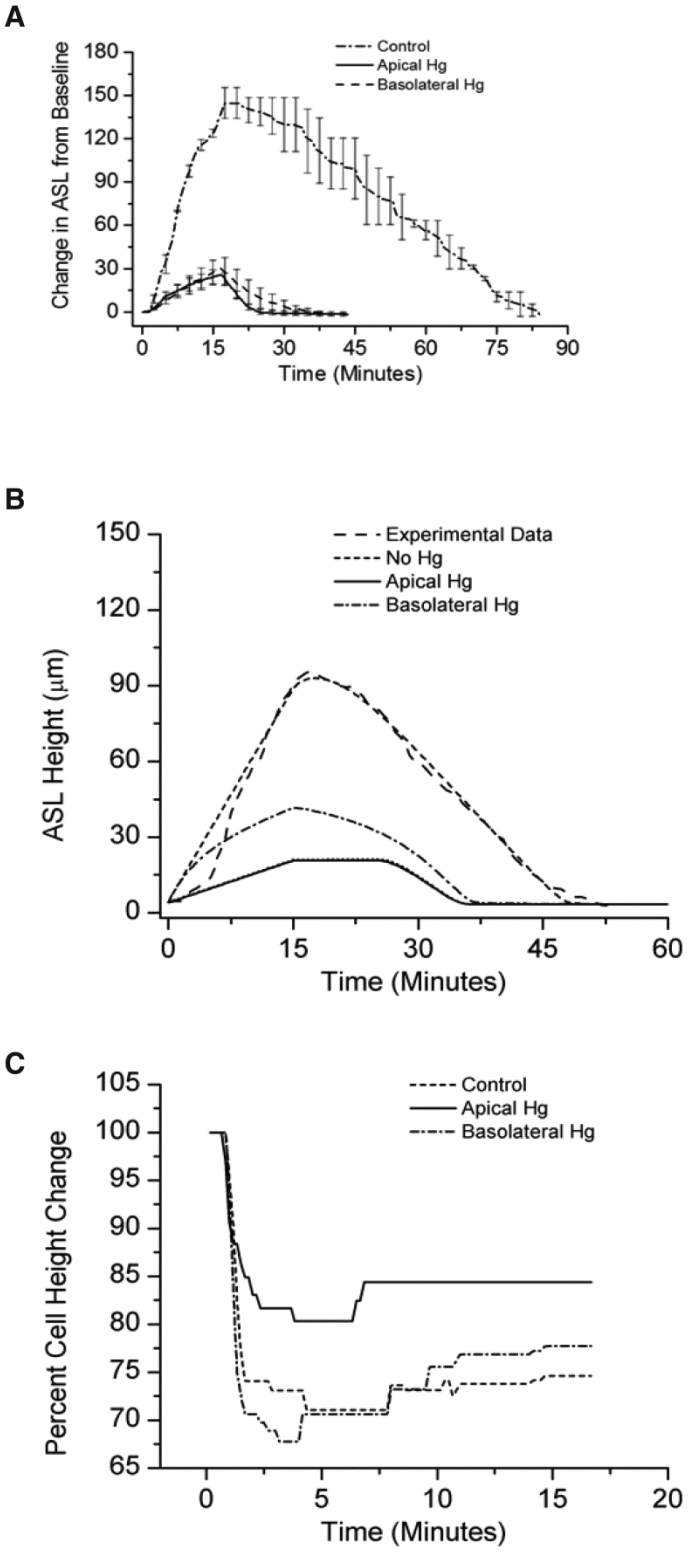

A
Control
Apical Hg
Basolateral Hg
Change in ASL from Baseline
180
150
120
90
60
30
0
0
15
30
45
60
75
90
Time (Minutes)
B
Experimental Data
No Hg
Apical Hg
Basolateral Hg
ASL Height (μm)
150
120
90
60
30
0
0
15
30
45
60
Time (Minutes)
C
Control
Apical Hg
Basolateral Hg
Percent Cell Height Change
105
100
95
90
85
80
75
70
65
0
5
10
15
20
Time (Minutes)

Figure 6:

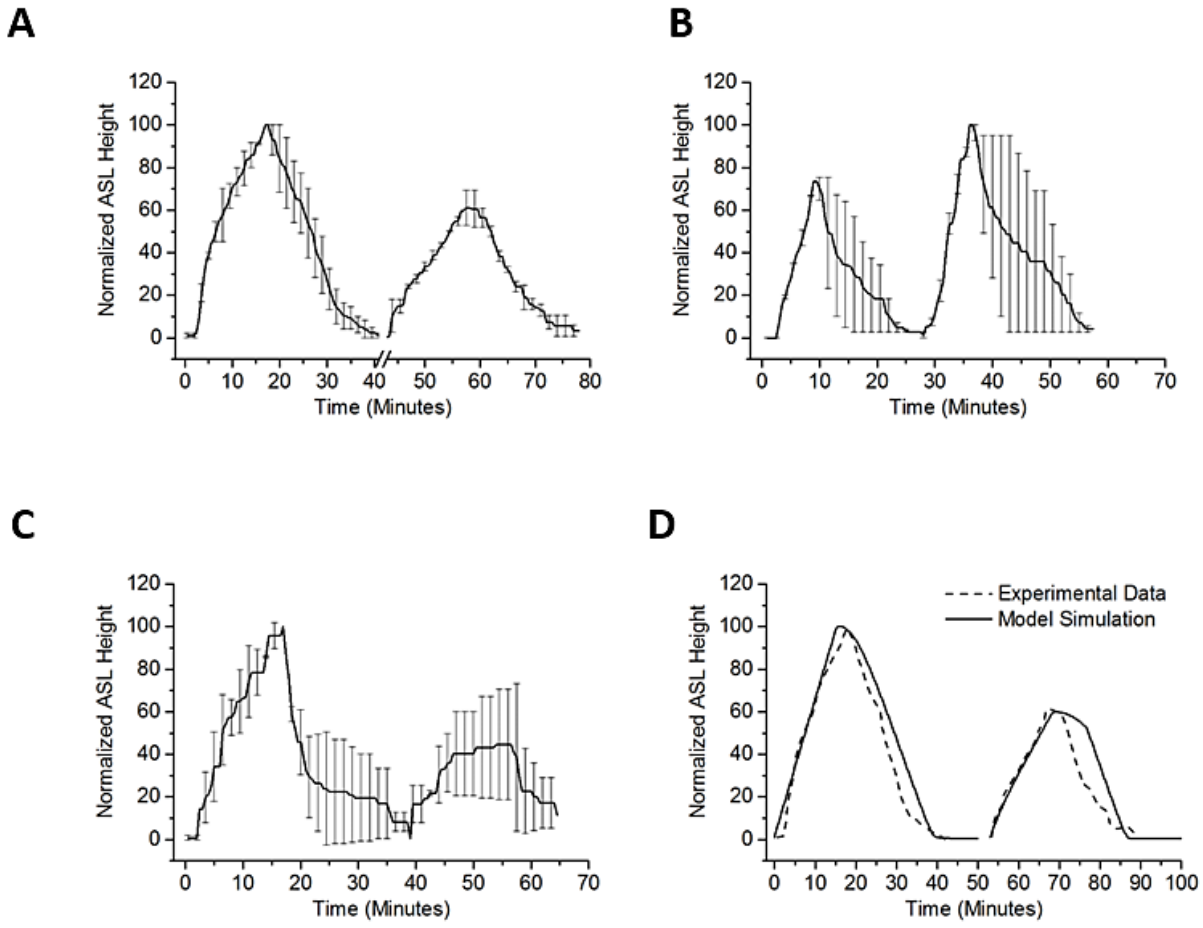
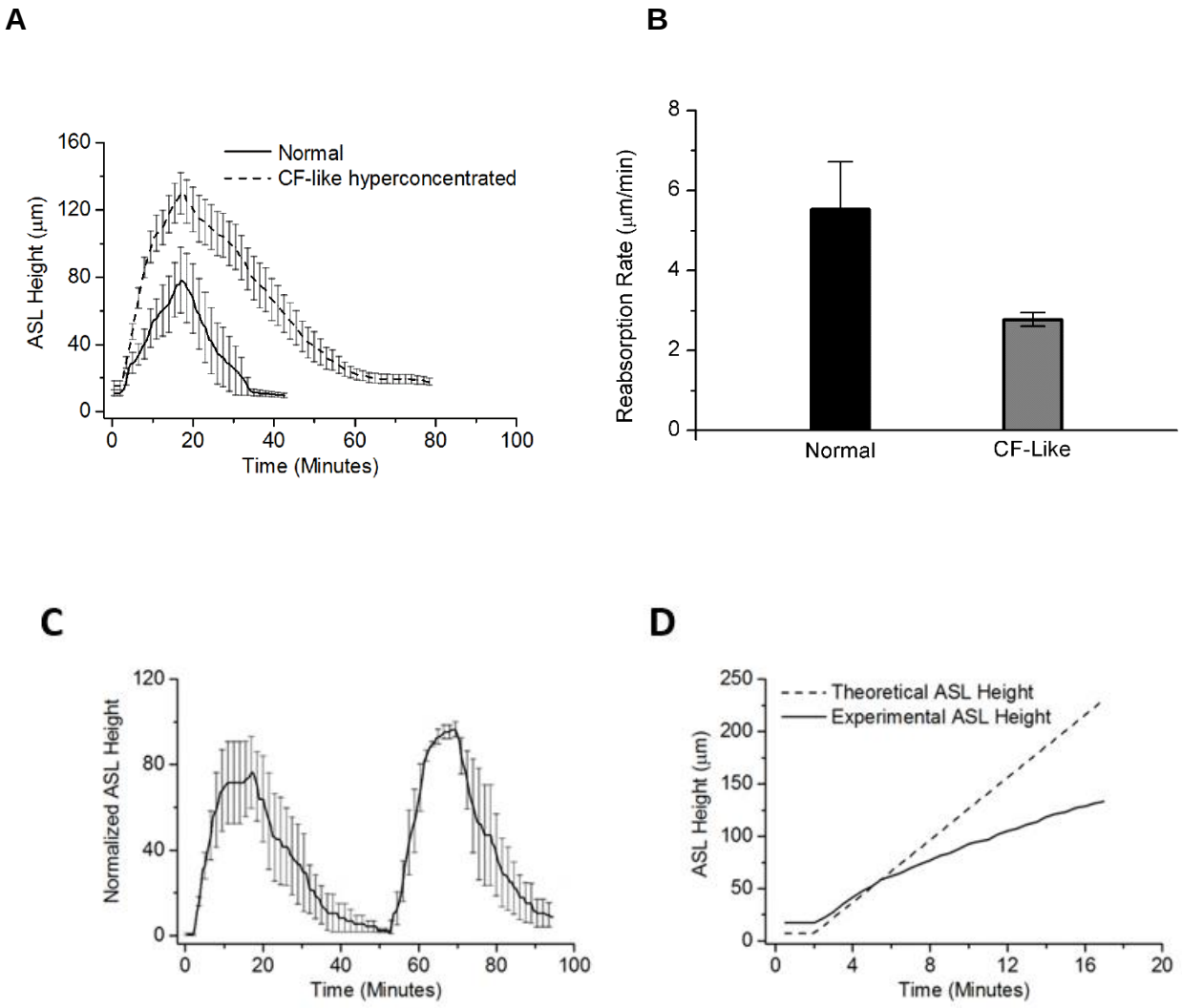

A
B
C
D
Normalized ASL Height
Time (Minutes)
Experimental Data
Model Simulation

Figure 7:


A
B
C
D
Normal
CF-like hyperconcentrated
ASL Height (μm)
Time (Minutes)
Reabsorption Rate (μm/min)
Normal
CF-Like
Normalized ASL Height
Theoretical ASL Height
Experimental ASL Height

Supplemental Material

Cell Culture: Primary human bronchial epithelial cells were obtained per established protocols from the UNC Tissue Core Facility.(11) Non-CF human lung tissue was obtained from lungs donated for research after being deemed unsuitable for transplantation. Cells were seeded as passage 0 (P0) cultures onto 12mm Transwell polycarbonate inserts that were previously coated with a thin layer of human placental collagen. Plated cells were then incubated at 37°C for at least 3 weeks until an air-liquid interface (ALI) was achieved prior to experiments. Cells were not apically washed unless otherwise specified per protocol.

Because mucus is not cleared from airway surfaces in culture, airway surface mucus concentrations can be controlled by varying culture-washing intervals. Washed cultures exhibited 1.9% mucus solids content, similar to that reported for normal subjects [1]. The unwashed mucus cultures exhibited mucus concentrations that mimicked the mucus extracted from explanted CF lungs (12.2%). Mucus mass (weight) [1] for the normal cultures was 1.84 $^{+}$/- 0.03mg, whereas the mass for the CF-like mucus was 2.74 +/- 0.18mg, measured in the absence of HS.

HBE Preparation:

For confocal experiments, epithelia were first labeled with calcein-AM (Invitrogen) basolaterally for 30 minutes. For HBE preparations that were to be used in “normal” mucus experiments, visible mucus was washed from the surface of the cell culture at least 30 minutes prior to study using three serial washes with PBS followed by removal with gentle suction after 5 minutes of incubation. The concentration of the “normal” (non-CF) vs CF-like hyperconcentrated mucus culture surfaces was measured using a mesh sampling technique coupled to a computer controlled microbalance to measure % solids [2,3]. To visualize the ASL, the luminal surface was labeled with Texas Red Dextran (TRD) (Invitrogen, 70,000 MW; 10mg/ml PBS), a cell impermeable fluorescent dye. For washed culture experiments, 10µl TRD was applied 30 minutes before the study and after the third wash. For experiments with undisturbed mucus on the HBE

surface, 10 µl of 70,000 MW TRD was applied apically at least 12 hours before experimentation to allow the dye to distribute in concentrated mucus. In all cases, to eliminate edge/meniscus effects on ASL volume, the Transwell membrane was excised from its support and affixed via a hydrophobic gel to the surface of a polycarbonate ring embedded on a cell culture dish. On the basolateral surface, 10µl of PBS was placed under the culture to maintain continued hydration of the cell culture during the experiments.

Aerosol Delivery: A 7% HS solution was aerosolized utilizing an Aerogen Lab Pro Vibrating Mesh Nebulizer modified to deliver clinically relevant small volumes (nl/cm^2/min) directly onto HBE surfaces. The volumes deposited were designed to simulate the deposition of nebulized HS generated by two clinically available nebulizers (jet and vibrating mesh) onto the airway epithelial surfaces of the large and mid-sized airways *in vivo*. (**Figure S1**) This system is mounted on a confocal microscope, allowing for direct visualization of HS aerosolization to the HBE surface.

Confocal Microscopy: Following HBE preparation and labeling with TRD, cultures were housed in an environmental chamber that regulated temperature to 37°C, humidity at 50%, and CO_2 at 5%, interfaced to a scanning confocal microscope (SP5, Leica). ASL and HBE heights were measured simultaneously by X-Z confocal microscopy. Following 2 minutes of baseline imaging (t0), 7% HS was nebulized via the nebulizer system to deposit HS onto apical surface of HBE cells at 8µg NaCl/cm^2/min. Serosal, cell, and ASL heights were measured before nebulization, every 30 seconds during the nebulization period, and until ASL volume had returned to baseline. This protocol utilized both normal and CF-like hyperconcentrated mucus HBE cultures. In another set of experiments, the total delivered mass of sodium chloride was held constant, but the rate of nebulization was varied (3µg NaCl/cm^2/min for 47.5 minutes, 8µg NaCl/cm^2/min for 15 minutes, or 18µg NaCl/cm^2/min for 6.5 minutes) to compare rate of delivery of HS on ASL volume. In a third protocol, HS was nebulized onto the apical surface of HBS at a rate of 8 µg NaCl/cm^2/min for 15 min, with administrations repeated at intervals of 15 min to examine the effectiveness of repeated dosing. These experiments were then modified to include a bolus challenge 10 µl of hypotonic (0.63%) or (isotonic, 0.9%)

saline applied to HBE apical surfaces between doses. Finally, to assess the role of apical membrane water channels on ASL volume responses to HS, mercury chloride, a non-selective aquaporin inhibitor, was incubated with HBE cells, followed by nebulized HS dosing.

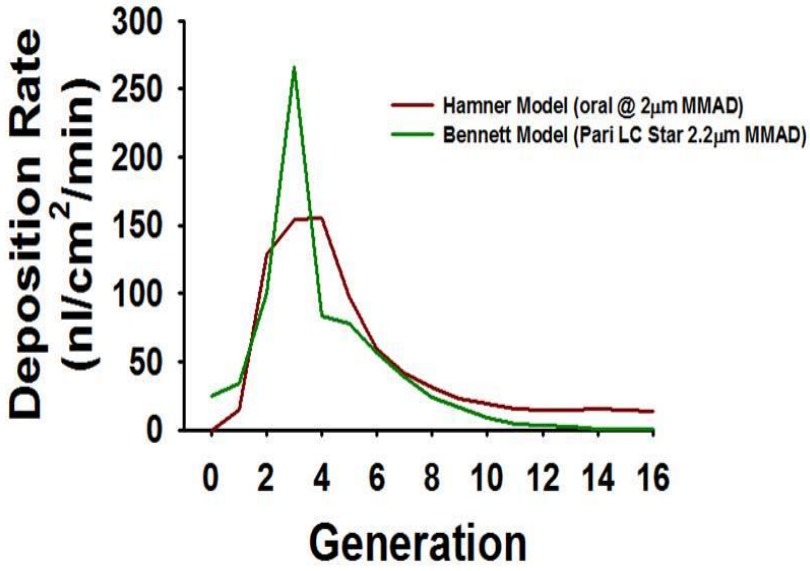


Figure S1: The deposition rate (in nl/min/cm2 of airway) is shown for the first 16 generations, based on 2 separate models of deposition calculated for the Pari LC Star (which nebulizers 0.2 ml/min). Only about 15% of the delivered dose is deposited in the airways/lungs.

Uncategorized References

1. Anderson WH, Coakley RD, Button B *et al.* The relationship of mucus concentration (hydration) to mucus osmotic pressure and transport in chronic bronchitis. *Am J Respir Crit Care Med* 192(2), 182-190 (2015).
2. Matsui H, Wagner VE, Hill DB *et al.* A physical linkage between cystic fibrosis airway surface dehydration and Pseudomonas aeruginosa biofilms. *Proc Natl Acad Sci U S A* 103(48), 18131-18136 (2006).
3. Button B, Cai LH, Ehre C *et al.* A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. *Science* 337(6097), 937-941 (2012).