Hypertonic Saline Is Effective in the Prevention and Treatment of Mucus Obstruction, but Not Airway Inflammation, in Mice with Chronic Obstructive Lung Disease

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Recent evidence suggests that inadequate hydration of airway surfaces is a common mechanism in the pathogenesis of airway mucus obstruction. Inhaled hypertonic saline (HS) induces osmotic water flux, improving hydration of airway surfaces. However, trials in patients with obstructive lung diseases are limited. The aim of this study was to investigate effects of HS on mucus obstruction and airway inflammation in the prevention and treatment of obstructive lung disease *in vivo*. We, therefore, used the β-epithelial Na^+ channel (βENaC)–overexpressing mouse as a model of chronic obstructive lung disease and determined effects of preventive and late therapy with 3% HS and 7% HS on pulmonary mortality, airway mucus obstruction, and inflammation. We found that preventive treatment with 3% HS and 7% HS improved growth, reduced mortality, and reduced mucus obstruction in neonatal βENaC-overexpressing mice. In adult βENaC-overexpressing mice with chronic lung disease, mucus obstruction was significantly reduced by 7% HS, but not by 3% HS. Treatment with HS triggered airway inflammation with elevated keratinocyte chemoattractant levels and neutrophils in airways from wild-type mice, but reduced keratinocyte chemoattractant in chronic neutrophilic inflammation in adult βENaC-overexpressing mice. Our data demonstrate that airway surface rehydration with HS provides an effective preventive and late therapy of mucus obstruction with no consistent effects on inflammation in chronic lung disease. These results suggest that, through mucokinetic effects, HS may be beneficial for patients with a spectrum of obstructive lung diseases, and that additional strategies are required for effective treatment of associated airway inflammation.

Keywords: airway surface liquid; cystic fibrosis; chronic obstructive pulmonary disease; mucus; inflammation

CLINICAL RELEVANCE

Airway mucus obstruction contributes significantly to the morbidity and mortality of cystic fibrosis (CF) and other chronic obstructive lung diseases. However, therapies to clear mucus are limited, and their *in vivo* effects on airway mucus obstruction unknown. This study demonstrates that improving airway surface hydration with the osmotically active inhaled hypertonic saline provides an effective mucus clearance (mucokinetic) therapy in a murine disease model, suggesting that hydration strategies may be beneficial for the prevention and treatment of mucus obstruction in patients with CF and potentially other obstructive lung diseases.

Airway surface dehydration caused by abnormal transport of ions and fluid across airway epithelia due to mutations in the cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene is an important disease mechanism, producing increased mucus concentration, reduced mucociliary clearance, and airway mucus obstruction in patients with CF (1–6). Recent results suggest that relative dehydration of airway surfaces, either caused by reduced availability of airway surface liquid due to dysregulated epithelial ion transport, or increased mucin secretion triggered by airway inflammation, generates an abnormally concentrated mucus that impairs mucus clearance and produces airway mucus obstruction in a spectrum of other acute and chronic obstructive lung diseases, including asthma and chronic obstructive pulmonary disease (COPD) (7–12).

Evidence from these studies predicts that airway surface rehydration provides a rational therapeutic approach to restore normal mucus concentrations and reduce mucus obstruction in CF and potentially other mucostatic airway diseases. Therapeutic strategies currently available in the clinic to clear mucus (i.e., "mucokinetic agents") are limited to hyperosmolar agents, such as inhaled hypertonic saline (HS) and mannitol. These agents, when inhaled, create osmotic gradients that draw water into the airway lumen and produce dose-dependent, transient improvements in airway surface liquid volume and mucociliary clearance (13–16). In patients with CF, the prototypical osmolyte, HS, improved mucus clearance and lung function after short-term inhalation (17). Furthermore, long-term inhalation of 7% HS produced modest improvements in lung function and reduced pulmonary exacerbations in patients older than 6 years (18). However, in infants and younger children with CF, the assessment of therapeutic benefits of HS has been hampered by the lack of established clinical trial endpoints, and produced more mixed results. In this

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age group, HS had no effects on pulmonary exacerbations, but, in substudies, had positive effects on $FEV_{0.5}$ and lung clearance index (19, 20).

Based on these mixed results, the therapeutic potential of inhaled HS for the prevention and treatment of mucus obstruction remains poorly defined. The dose–effect relationships between the frequency of daily HS administration and FEV_1 (17, 18), and the mixed data in the Infant Study of Inhaled Saline in CF (ISIS) (19), raise the question of whether the optimal dose of deposited HS for efficacy has been defined. Moreover, previous studies indicated that HS can induce airway inflammation (21–25) in healthy individuals, consistent with dose-dependent offsetting effects in the treatment of airway disease. However, the interpretation of results from these clinical trials is hampered by the lack of endpoints that directly measure dose-dependent effects of HS on airway mucus obstruction and inflammation (17–19).

The aim of our study was, therefore, to measure the dose-dependent effects of HS on airway mucus obstruction and inflammation *in vivo*. To achieve this goal, we used the β-epithelial Na^+ channel (βENaC)–overexpressing mouse as a model of chronic mucostatic lung disease that shares key features with CF and COPD (26–28), and determined the effects of preventive HS treatment versus late HS intervention on growth, survival, airway mucus obstruction, and airway inflammation. Furthermore, we compared effects of different concentrations of HS (3 and 7%) on these endpoints. Some of the results of these studies have been previously reported in the form of an abstract (29).

MATERIALS AND METHODS

Experimental Animals

All animal studies were approved by the Regierungspräsidium Karlsruhe, Germany. βENaC-overexpressing mice (line 6608) were bred to C3H/HeN × C57BL/6N F1 wild-type mice to maintain the colony on a mixed genetic background, and were identified by PCR as previously described (26). Wild-type littermates served as control animals in all experiments. Mice were housed in a specific pathogen-free animal facility and had free access to chow and water.

HS Treatment Studies

NaCl (10%; B. Braun Melsungen AG, Melsungen, Germany) was diluted with sterile distilled water (ddH_2O) to obtain 3% HS or 7% HS solutions. To test preventive effects of increasing doses of HS, newborn βENaC-overexpressing mice and wild-type littermates were treated with intranasal instillation of 3% HS or 7% HS (1 μl/g body weight, three times per d) or equal volumes of vehicle (ddH_2O) alone from the first day of life for a period of 2 weeks. To test effects of HS in chronic obstructive lung disease, 4-week-old adult βENaC-overexpressing mice and wild-type littermates were treated with intratracheal instillation of 3% HS or 7% HS (1 μl/g body weight, three times per d) or equal volumes of vehicle (ddH_2O) alone for a period of 2 weeks. During HS treatment, growth and survival were monitored. At 12 hours after the last treatment, bronchoalveolar lavage (BAL) was performed and lungs were removed for histology and morphometry, as previously described (30). Furthermore, adult wild-type mice were treated with intratracheal instillation of 7% HS or 18% mannitol (D-Mannitol [Sigma-Aldrich Co., St. Louis, MO] dissolved in ddH_2O) for a period of 3 days, and BAL was performed 12 hours after the last treatment and assessed for signs of airway inflammation. All endpoint studies were performed by investigators blinded to genotype and treatment of the mice.

BAL Cell Counts and Cytokine Measurements

Mice were deeply anesthetized by intraperitoneal injection with ketamine/xylazine (120 mg/kg and 16 mg/kg), and the right lobes were lavaged with PBS. BAL cell counts were determined on cytospin preparations, as previously described (27). Cytokine concentrations were measured in cell-free BAL fluid, stored at −80°C, by ELISA (R&D Systems, Minneapolis, MN), according to manufacturer's instructions.

Histology and Airway Morphometry

The left lobe was immersion fixed in 4% formalin, paraffin embedded and sectioned at the level of the proximal intrapulmonary main axial airway near the hilus and the distal intrapulmonary main axial airway (27). Sections were cut at 5 μm and stained with Alcian blue periodic acid-Schiff. Airway mucus obstruction was assessed by determining mucus volume density on images of airway sections taken with an Olympus IX-71 microscope (Olympus, Hamburg, Germany), as previously described (27). Images were analyzed using Cell^F imaging software (OSIS, Münster, Germany), and mucus volume density, obtained for each experimental group, was normalized to vehicle-treated wild-type mice.

Statistical Analysis

Data were analyzed with SigmaStat version 3.5 (Systat Software, Erkrath, Germany) and are reported as means (±SEM). Statistical analyses were performed using unpaired Student's *t* test, Mann-Whitney rank sum test, and Kaplan-Meier survival analysis, as appropriate, and a P value less than 0.05 was accepted to indicate statistical significance.

RESULTS

Preventive HS Therapy Reduces Mortality and Growth Retardation Associated with Lung Disease in βENaC-Overexpressing Mice

Airway surface dehydration in βENaC-overexpressing mice caused early onset mucus obstruction associated with substantial pulmonary mortality and reduced weight gain in the first weeks of life (Figure 1) (26, 27). To determine if preventive therapy with HS has benefits on growth and survival, and if therapeutic effects are dose dependent, we treated βENaC-overexpressing mice and wild-type littermates from the first day of life with intranasal instillation (1 μl/g body weight) of 3% HS, 7% HS, or vehicle alone (ddH_2O), three times per day for a period of 2 weeks. Vehicle-treated βENaC-overexpressing mice showed a mortality rate of roughly 60% and growth retardation with a reduction in body weight of approximately 10% (Figure 1) at the age of 2 weeks. Preventive treatment with both 3% HS and 7% HS reduced pulmonary mortality significantly in βENaC-overexpressing mice (Figures 1A and 1B). Furthermore, impaired growth associated with obstructive lung disease in surviving βENaC-overexpressing mice was restored by treatment with both concentrations of HS (Figures 1C and 1D). In wild-type mice, HS treatment was well tolerated and had no effect on growth or survival (Figures 1A–1D). These results suggest that preventive HS therapy, at concentrations ranging from 3 to 7%, reduced mucus obstruction–induced mortality in βENaC-overexpressing mice.

Preventive HS Therapy Reduces Airway Mucus Obstruction in βENaC-Overexpressing Mice

To evaluate effects of preventive HS treatment on airway mucus obstruction more directly, we next assessed the mucus content in distal and proximal airways in βENaC-overexpressing mice and wild-type littermates after 2 weeks of preventive therapy with either 3% HS, 7% HS, or vehicle alone. Morphometric analyses of lung sections from vehicle-treated βENaC-overexpressing mice exhibited mucus obstruction in proximal and distal airways (Figures 2A–2C). Treatment with HS had no effect on mucus content in wild-type mice. Both 3% HS and 7% HS resulted in a significant reduction of airway mucus in distal airways of βENaC-overexpressing mice (Figures 2A and 2C). Furthermore, preventive treatment with 7% HS, but not 3% HS, reduced mucus

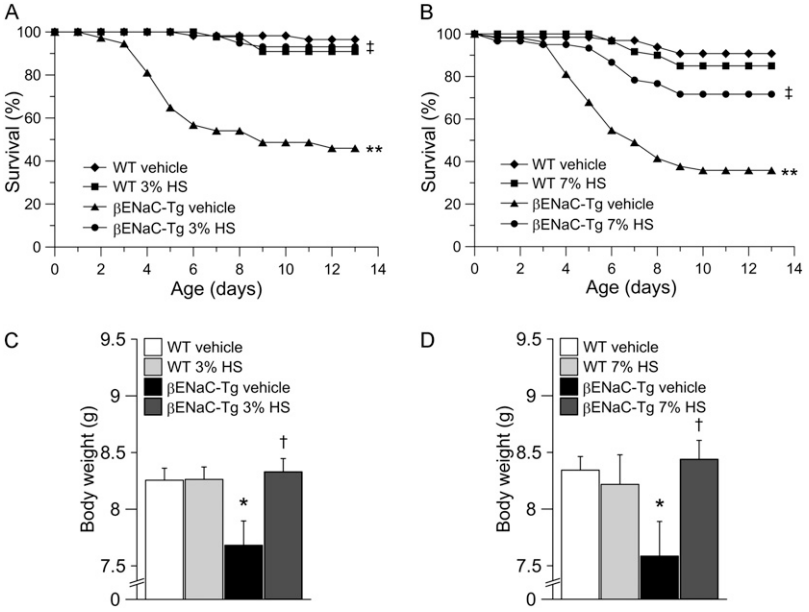

Figure 1. Preventive treatment with 3% and 7% hypertonic saline (HS) reduces mortality and improves growth in neonatal β-epithelial Na^+ channel (βENaC)–overexpressing mice. (*A* and *B*) Survival curves for βENaC-overexpressing (βENaC-Tg) mice and wild-type (WT) littermates treated with 3% HS (*A*), 7% HS (*B*), or vehicle alone, administered from the first day of life for a period of 2 weeks. (*C* and *D*) Body weight of βENaC-overexpressing mice and WT littermates on the last day of the 2-week treatment with 3% HS (*C*), 7% HS (*D*), or vehicle alone (n = 17–65 mice per group). *$P < 0.05$ and **$P < 0.001$ compared with vehicle-treated WT mice; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ compared with vehicle-treated βENaC-overexpressing mice.

to near-normal levels in proximal airways of βENaC-overexpressing mice (Figure 2B). These results demonstrate that preventive HS has potent mucokinetic effects in the early pathogenesis of chronic obstructive lung disease in βENaC-overexpressing mice, and suggest that 7% HS may be more effective for clearance of mucus from central airways.

Treatment with 7% HS Is Required to Reduce Airway Mucus Obstruction in Adult βENaC-Overexpressing Mice with Chronic Obstructive Lung Disease

Adult βENaC-overexpressing mice exhibit characteristic features of chronic obstructive lung disease in humans, including chronic airway inflammation with goblet cell metaplasia and mucus hypersecretion (26, 27). To evaluate effects of HS therapy on mucus obstruction in chronic lung disease, we extended our studies to adult βENaC-overexpressing mice. As for the preventive HS studies, adult βENaC-overexpressing mice and wild-type littermates were treated with 3% HS, 7% HS, or vehicle alone for a period of 2 weeks. Similar to the preventive studies, HS had no effect on mucus content in wild-type mice, and vehicle-treated βENaC-overexpressing mice showed mucus obstruction in proximal and distal main axial airways (Figure 3). Late therapy with 3% HS failed to reduce airway mucus obstruction in small (or large) airways in adult βENaC-overexpressing mice with chronic lung disease (Figure 3). However, when the concentration

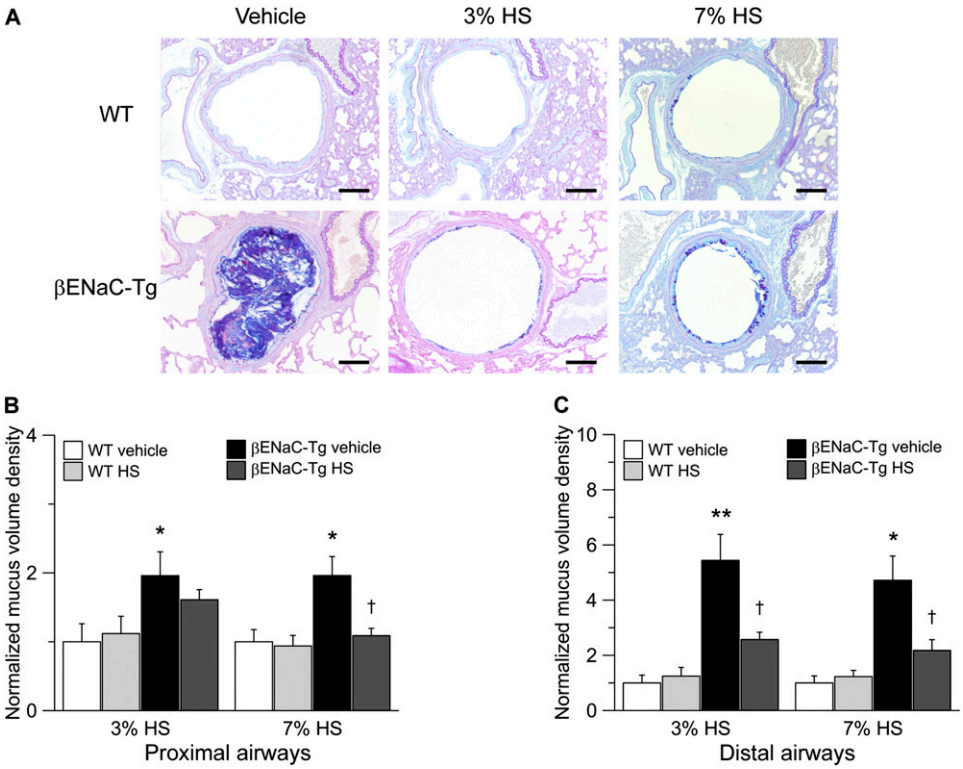

Figure 2. Preventive treatment with 3% and 7% HS reduces mucus obstruction in neonatal βENaC-overexpressing mice. (*A*) Morphology of distal main axial airways from neonatal βENaC-overexpressing (βENaC-Tg) mice and WT littermates after 2 weeks of preventive treatment with 3% HS, 7% HS, or vehicle alone. Sections were stained with Alcian blue periodic acid-Schiff (AB-PAS) to determine the presence of airway mucus. *Scale bars,* 100 μm. (*B* and *C*) Airway mucus content was determined by measuring the volume density of AB-PAS–positive material in proximal (*B*) and distal (*C*) main axial airways (n = 14–52 mice for each group). *$P \leq 0.01$ and **$P < 0.001$ compared with vehicle-treated wild-type mice; $^{\dagger}P < 0.05$ compared with vehicle-treated βENaC-overexpressing mice.

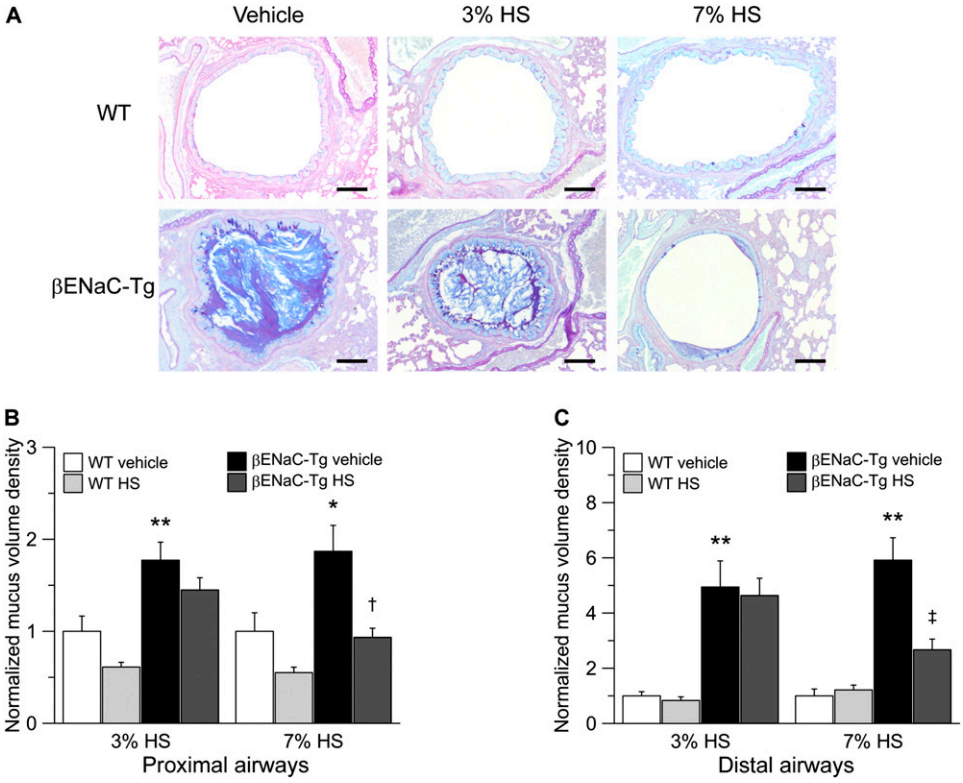

Figure 3. Therapeutic effects of late HS treatment on mucus obstruction in adult βENaC-overexpressing mice with chronic lung disease are dose dependent. (*A*) Morphology of distal main axial airways from 6-week-old βENaC-overexpressing (βENaC-Tg) mice and WT littermates after 2 weeks of treatment with 3% HS, 7% HS or vehicle alone. Sections were stained with Alcian blue periodic acid-Schiff (AB-PAS) to determine the presence of airway mucus. *Scale bars*, 100 μm. (*B* and *C*) Airway mucus content was determined by measuring the volume density of AB-PAS–positive material in proximal (*B*) and distal (*C*) main axial airways (n = 24–35 mice for each group). *$P < 0.05$ and **$P < 0.001$ compared with vehicle-treated wild-type mice; $^{\dagger}P < 0.01$ and $^{\ddagger}P < 0.001$ compared with vehicle-treated βENaC-overexpressing mice.

was increased to 7% HS, therapeutic effects were observed and airway mucus content was reduced to near-normal levels in both proximal and distal airway regions in βENaC-overexpressing mice (Figure 3). These data show that 7% HS is an effective mucokinetic agent in chronic lung disease *in vivo*.

HS and Airway Inflammation in Wild-Type and βENaC-Overexpressing Mice

Airway inflammation is an important feature of chronic obstructive lung disease that can cause progressive damage and remodeling of the lung. Because of contrasting studies reporting proinflammatory effects of HS in human airway epithelial cells *in vitro* (21, 22) and in healthy individuals *in vivo* (23–25), versus studies reporting anti-inflammatory effects in patients with CF *in vivo* (31), we evaluated the effects of preventive and late therapy with 3% HS and 7% HS on BAL cell counts and levels of keratinocyte chemoattractant (KC; CXCL1), the mouse homolog of human IL-8, in wild-type and βENaC-overexpressing mice (Figures 4 and 5).

As previously reported and consistent with a T helper type 2–biased immune system in the neonatal period (27, 32), BAL from 2-week-old wild-type mice contained substantial numbers of eosinophils. βENaC-overexpressing mice were characterized by increased inflammation, as evidenced by an increase in eosinophils and neutrophils in BAL (Figures 4A, 4D, and 4E). In the preventive HS studies, both 3% HS and 7% HS triggered a mild and dose-dependent inflammatory response, with increased macrophages, eosinophils, and neutrophils in BAL from HS-treated compared with vehicle-treated wild-type mice (Figures 4A–4E). Furthermore, KC levels were slightly but significantly (~2-fold) higher in wild-type mice treated with 7% HS compared with vehicle-treated control animals (Figure 4F). In βENaC-overexpressing mice, preventive therapy with 3% HS and 7% HS had no effects on elevated BAL macrophages and eosinophils (Figures 4A–4D). Treatment with 7% HS, but not 3% HS, increased BAL neutrophils compared with vehicle-treated βENaC-overexpressing littermates (Figure 4E). Lymphocyte numbers in BAL were low, and did not differ between experimental groups (data not shown). KC levels were substantially increased in BAL fluid from vehicle-treated βENaC-overexpressing mice compared with wild-type littermates, and preventive HS had no effect on elevated KC levels in βENaC-overexpressing mice (Figure 4F).

In the late intervention studies, treatment of wild-type mice with 3% HS and 7% HS had no effect on BAL macrophages and eosinophils, but both doses induced neutrophilia (Figures 5A–5D). Furthermore, HS also caused a dose-dependent increase in KC levels in wild-type mice (Figure 5E). In adult βENaC-overexpressing mice, late therapy with 3% HS and 7% HS reduced the low-grade eosinophilia, but had no effects on BAL macrophages and neutrophils that dominate chronic airway inflammation at this age (Figures 5A–5D) (27). Neutrophilic inflammation was associated with increased KC levels in BAL fluid from vehicle-treated βENaC-overexpressing mice. Late treatment with 7% HS, but not 3% HS, reduced KC levels significantly in βENaC-overexpressing mice.

To determine if the proinflammatory response to HS therapy was specific for HS or mediated by hypertonicity as previously described for cultured airway epithelial cells (21, 22), we compared effects of 7% HS and 18% mannitol on airway inflammation in adult wild-type mice. Similar to 7% HS, treatment with 18% mannitol caused a significant increase in neutrophils and increased KC levels in wild-type mice (*see* Figure E1 in the online supplement). These results show that HS therapy causes dose-dependent inflammation in healthy airways, and indicate that this proinflammatory effect was mediated by an osmotic stress response. In βENaC-overexpressing mice with chronic lung disease, HS treatment did not aggravate chronic airway inflammation, but was associated with a dose-dependent reduction of KC levels in the lung.

DISCUSSION

Airway mucus obstruction causes airflow limitation and is a nidus for inflammation and infection in CF and other chronic obstructive

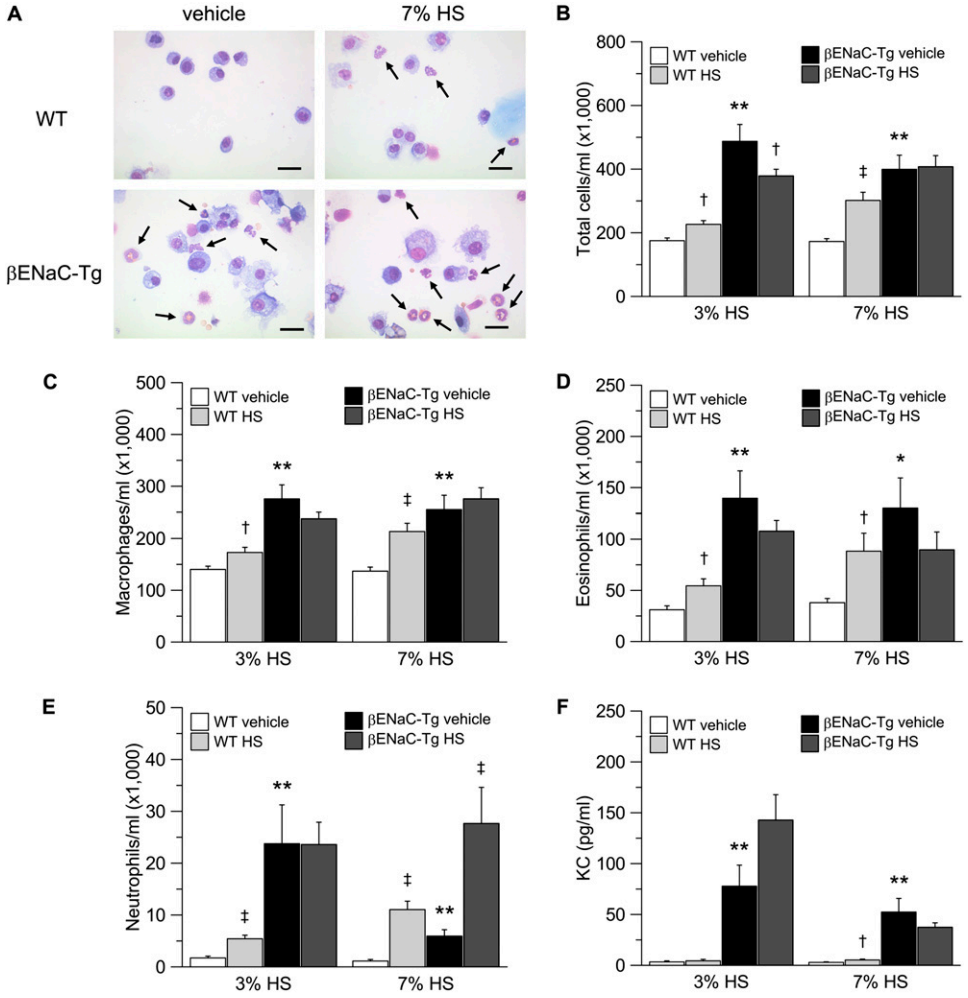

Figure 4. Preventive treatment with 3% and 7% HS triggers airway inflammation. (*A–F*) Neonatal βENaC-overexpressing (βENaC-Tg) mice and WT littermates were treated with 3% HS, 7% HS, or vehicle alone from birth for a period of 2 weeks. (*A*) Effects of preventive treatment on cellularity of cytospin preparations (stained with May-Grünwald-Giemsa; neutrophilic and eosinophilic granulocytes are indicated by *arrows*; *scale bars*, 20 μm), and summary of effects on total cells (*B*), macrophages (*C*), eosinophils (*D*), neutrophils (*E*), and keratinocyte chemoattractant (KC) concentrations (*F*) in bronchoalveolar lavage (BAL) ($n = 13$–55 mice for each group). $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with vehicle-treated wild-type mice; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ compared with vehicle-treated mice of the same genotype.

lung diseases (6, 12, 32, 33). However, currently available therapies to clear mucus are limited, and the assessment of their effectiveness relies on surrogate endpoints, such as lung function and pulmonary exacerbations (17, 18, 34, 35). Consequently, their effects on clearing obstructive mucus from airways *in vivo* remain unknown. In the present study, we used the βENaC-overexpressing mouse as a model of chronic obstructive lung disease that shares key features with CF and COPD (26–28) to determine therapeutic effects of HS in the prevention and treatment of mucus obstruction and associated airway inflammation. Our results show, for the first time, that inhaled HS is effective as a preventive treatment as well as a rescue therapy in chronic obstructive lung disease *in vivo* (Figures 1–3). Previous studies in cultured human primary airway epithelia demonstrated that HS, similar to nonionic osmolytes, produces a transient increase in airway surface hydration (13, 17). Although we did not compare HS with other osmotic agents, such as mannitol, in our study, we predict that therapeutic benefits of HS in mobilizing mucus in βENaC-overexpressing mice were mediated by this mechanism of action.

Our studies in neonatal mice show that preventive HS treatment reduced airway mucus content to near-normal levels, and that this therapeutic effect was associated with significant reduction in pulmonary mortality and growth retardation in βENaC-overexpressing mice (Figures 1 and 2). In a previous study, we demonstrated that preventive inhibition of epithelial Na^{+} absorption with the ENaC blocker amiloride is also an effective approach to reduce mucus obstruction and mortality in this murine disease model (30). However, although early diagnosis has become feasible for a substantial number of patients with CF by widespread implementation of newborn screening, clinical testing of preventive amiloride inhalation therapy has been impeded by lack of toxicity data for amiloride in neonatal development and regulatory requirements for clinical testing of pharmacological compounds in infants (36). The potential and requirement for preventive mucokinetic therapies for CF is highlighted by recent studies demonstrating that signs of obstructive airway disease, such as air trapping and bronchiectasis, are frequently present on chest CT and lung function testing in the first months of life, even in asymptomatic infants diagnosed by CF newborn screening (37–40). In a recent randomized controlled trial (ISIS) in infants and children younger than 6 years with CF, inhaled HS did not reduce the rate of pulmonary exacerbations. However, substudy outcome measures that may reflect airway mucus obstruction (e.g., $FEV_{0.5}$ and lung clearance index) suggested benefit from HS (19, 20). Our data from neonatal βENaC-overexpressing mice demonstrate directly that airway surface rehydration with HS is effective in preventing the development of mucus obstruction *in vivo*. These results, together with safety data in infants and young children (19, 41), suggest inhaled HS as an inexpensive and safe preventive treatment for infants with CF. Consequently, the potential benefits of this preventive strategy should be further explored in clinical trials that include morphological and functional surrogate endpoints that are more sensitive and more specific in capturing treatment responses in early obstructive airways disease (36, 38–40, 42, 43).

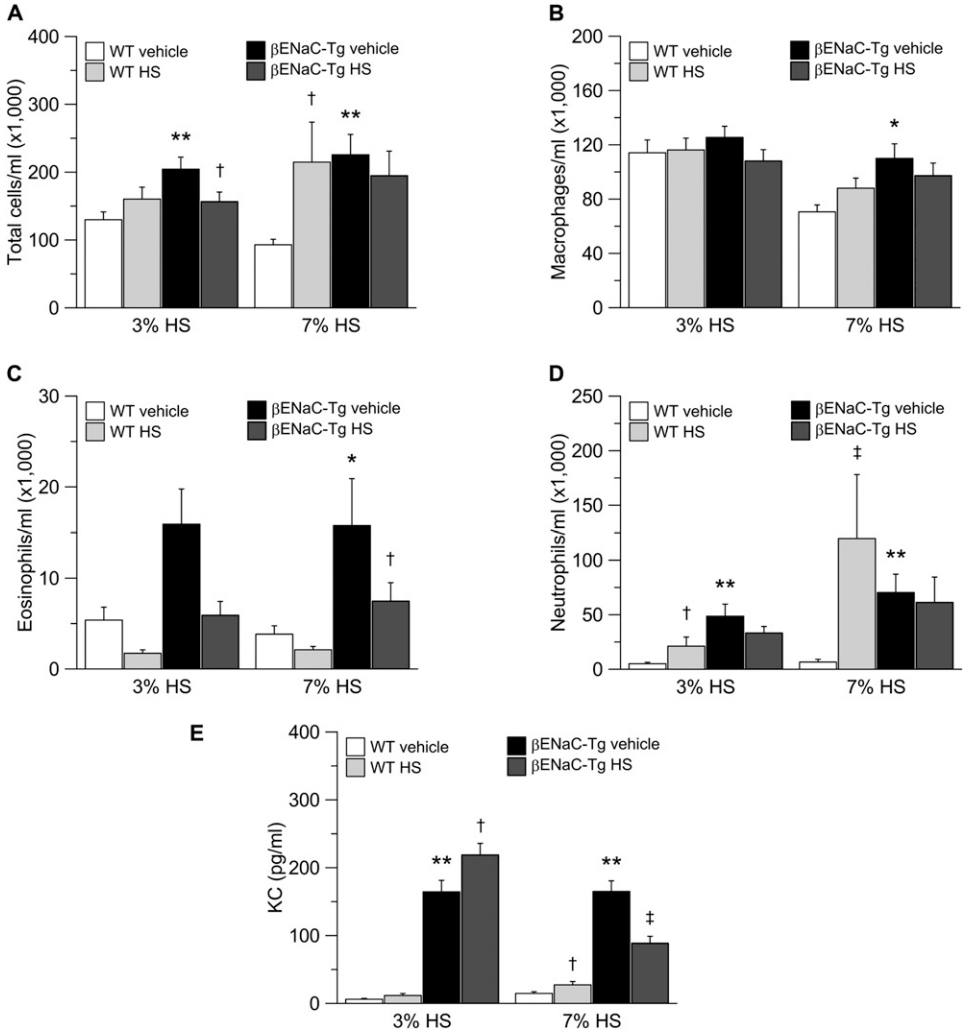

Figure 5. Late treatment with 3% and 7% HS causes neutrophilic inflammation in WT mice, but does not increase airway neutrophilia in adult βENaC-overexpressing mice with chronic lung disease. (*A–E*) Adult βENaC-overexpressing mice (βENaC-Tg) and WT littermates were treated with 3% HS, 7% HS, or vehicle alone from birth for a period of 2 weeks. Summary of effects on total cells (*A*), macrophages (*B*), eosinophils (*C*), neutrophils (*D*), and KC concentrations (*E*) in BAL (n = 24–35 mice for each group). *$P < 0.01$ and **$P \leq 0.001$ compared with vehicle-treated wild-type mice; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ compared with vehicle-treated mice of the same genotype.

In a previous study, we also demonstrated that treatment with amiloride, probably because of limited potency and short half-life on airway surfaces, was ineffective when treatment was started after the onset of lung disease in βENaC-overexpressing mice (30). In contrast, 7% HS exhibited significant mucokinetic effects in adult βENaC-overexpressing mice with chronic lung disease (Figure 3). However, treatment with 3% HS was ineffective, indicating that higher osmotic gradients were required for sufficient mucus hydration and clearance in the context of chronic airway disease associated with goblet cell metaplasia and mucus hypersecretion (Figure 3) (27). Our observations, that mucokinetic effects of HS in chronic lung disease were dose-dependent, are consistent with results from human studies, where more intensive inhalation of 7% HS (i.e., 5 ml four times daily) was more effective in improving lung function in patients with established CF lung disease than a less-intensive inhalation regimen of 4 ml twice daily (17, 18). These results indicate that therapeutic benefits of 7% HS may be enhanced by increasing its overall dose via more frequent inhalations in patients with CF with chronic lung disease.

Interestingly, evidence from recent biophysical studies suggests that airway surface dehydration and increased mucus concentration may not only be critical in CF, but also in the pathogenesis of other airway diseases (7, 44). These studies demonstrate that an increase in mucus concentration leads to an increase of the osmotic modulus of the mucus gel that covers airway surfaces. Above a critical threshold, the osmotic modulus of the mucus layer exceeds the osmotic modulus of the subjacent periciliary layer, causing compression of the periciliary layer and cilia, and mucociliary dysfunction (7). Because increased mucus concentration cannot only result from reduced amounts of airway surface fluid due to primary defects in epithelial ion transport as in CF (1, 2, 6), but also from an increased amount of secreted mucins triggered by airway inflammation and infection (11, 12, 45), the study by Button and colleagues (7) supports the concept that insufficient hydration of airway surfaces is a common disease mechanism contributing to mucociliary dysfunction and mucus obstruction in a broad spectrum of obstructive lung diseases (44). This concept is supported by the airway phenotype of the βENaC-overexpressing mouse, which develops spontaneous mucus obstruction caused by constitutive hyperabsorption of airway surface fluid due to an increased activity of ENaC (9, 26, 27). The concept is also supported by the phenotype of mice lacking the SLC26A9 Cl^- channel, which show normal ion transport and airway morphology under physiological conditions, but develop airway mucus obstruction due to a reduced capacity to increase Cl^- and fluid secretion in parallel to mucin hypersecretion in allergic airway disease (8, 46).

In this context, the results from our studies in βENaC-overexpressing mice, demonstrating that HS has potent mucokinetic effects *in vivo*, provide a proof of concept that airway surface rehydration may be an effective therapy in a spectrum of acute and chronic obstructive airway diseases beyond CF. So far, this notion is supported by a limited number of clinical trials demonstrating that nebulized HS reduces the length of hospital stay and improves

clinical symptoms in infants with acute viral bronchiolitis (47), and improves lung function and quality of life in patients with non-CF bronchiectasis (48). Of note, therapeutic effects of HS were superior to treatment with bronchodilators or anti-inflammatory therapy with glucocorticoids in acute viral bronchiolitis (47, 49, 50). However, further clinical trials are required to determine the therapeutic potential of rehydration therapies in common chronic lung diseases, such as asthma and COPD, including patients with subphenotypes that are accompanied by substantial mucus obstruction and respond poorly to bronchodilators and anti-inflammatory drugs (12, 33).

Consistent with previous *in vitro* studies demonstrating that hyperosmolarity induces production of IL-8 in human airway epithelial cells (21, 22), treatment with HS induced a dose-dependent elevation of KC, the mouse homolog of IL-8, in BAL fluid from neonatal and adult wild-type mice (Figures 4 and 5). Increased KC concentrations were associated with induction of mild neutrophilic airway inflammation in HS-treated wild-type mice. Similar responses with elevated KC and neutrophils in BAL were observed when mice were treated with mannitol, demonstrating that this proinflammatory response was indeed induced by hyperosmolarity (Figure E1). Repeated inhalation of HS has also been shown to induce transient neutrophilic airway inflammation due to mediator release from epithelial and/or mast cells in healthy individuals (23–25). Taken together, these results suggest that proinflammatory effects triggered by hypertonic stress may limit the therapeutic benefits of inhaled HS when used in patients before the onset of airway inflammation. Importantly, our studies in adult βENaC-overexpressing mice demonstrate that HS treatment did not aggravate chronic airway inflammation associated with chronic mucus obstruction (Figure 5). In this context, neither 3% HS nor 7% HS increased KC expression or airway neutrophilia, supporting the safety of inhaled HS in the treatment of chronic obstructive lung disease. Of note, KC levels were significantly reduced by 7% HS in the context of chronic lung disease in adult βENaC-overexpressing mice (Figure 5). These results are consistent with a recent study showing that inhaled HS decreased IL-8 in the sputum of patients with CF (31). This study demonstrated that HS disrupts the interaction between IL-8 and protective glycosaminoglycans, rendering IL-8 susceptible to proteolytic degradation that may result in a decrease in neutrophil chemotaxis, and thus limit neutrophilic inflammation in chronic CF lung disease (16, 31).

In summary, our results suggest that airway surface rehydration by inhalation of the osmotically active agent HS constitutes an effective rehydration strategy for the prevention and treatment of mucus obstruction in CF and, potentially, other obstructive lung diseases. Limited anti-inflammatory effects of HS suggest that additional anti-inflammatory strategies are necessary for effective treatment of airway inflammation associated with mucus obstruction in chronic obstructive lung diseases.

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