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Bicarbonate Availability for Vocal Fold Epithelial Defense to Acidic Challenge

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Abstract

Objectives—Bicarbonate is critical for acid-base tissue homeostasis. In this study we investigated the role of bicarbonate ion transport in vocal fold epithelial defense to acid challenges. Acidic insults to the larynx are common in gastric reflux, carcinogenesis and metastasis, and acute inflammation.

Methods—Ion transport was measured in viable, porcine vocal fold epithelium. First, 18 vocal folds were exposed to either the carbonic anhydrase antagonist acetazolamide or to vehicle. Second, 32 vocal folds were exposed to either a control buffer or a bicarbonate-free buffer on their luminal or basolateral surface or both. Third, vocal folds were challenged with acid in the presence of bicarbonate-free or control buffer.

Results—The vocal fold transepithelial resistance was greater than $300 \Omega \cdot \text{cm}^2$, suggesting robust barrier integrity. Ion transport did not change after exposure to acetazolamide ($p > 0.05$). Exposure to bicarbonate-free buffer did not compromise vocal fold ion transport ($p > 0.05$). Ion transport increased after acid challenge. This increase approached statistical significance and was the greatest for the control buffer and for the bicarbonate-free buffer applied to the basolateral surface.

Conclusions—Bicarbonate secretion may contribute to vocal fold defense against acid challenge. Our data offer a potential novel role for bicarbonate as a therapeutic agent to reduce pH abnormalities in the larynx and prevent associated pathological changes.

Keywords

bicarbonate; ion transport; laryngopharyngeal reflux; vocal fold

INTRODUCTION

Squamous epithelial cells line the surface of the vocal folds and create a physical barrier that protects the underlying lamina propria and muscle. In addition, epithelial cells actively

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transport ions and water to regulate cell volume and vocal fold hydration.¹⁻³ It is known that environmental and systemic insults to the epithelium can disrupt vocal fold epithelial ion transport and barrier function.⁴⁻⁶ For example, porcine vocal fold epithelial barrier function is impaired by an acidic environment.⁴ Similarly, an ephemeral event of raised intensity phonation in rabbits leads to down-regulation of epithelial cell junction proteins.⁷ Because of their anatomic position and propensity for receiving airborne and systemic challenges, the vocal fold epithelial cells may serve as an important target tissue in the larynx for therapeutic interventions that strengthen both barrier and ion transport functions.

The exact mechanisms that regulate vocal fold epithelial ion transport are beginning to be better understood. Leydon et al³ described a vocal fold model of transcellular ion and water fluxes that incorporated a basolateral sodium-potassium ATPase pump and a sodium-potassium-chloride cotransporter. Epithelial sodium channel, cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, calcium-activated chloride channel, and aquaporins were immunolocalized to the luminal (air-facing) surface of the vocal fold epithelial cell. The role of bicarbonate in ion transport across vocal fold epithelia was not included in the model. Bicarbonate plays a critical biochemical role in the body's pH buffering system and is produced inside the cells by the interconversion of carbon dioxide and water into bicarbonate ion, carbonic acid, and protons by the enzyme carbonic anhydrase.^{8,9} Bicarbonate is charged and requires transport proteins to facilitate its movement across cell membranes in order to control cellular pH and regulate fluid movement.¹⁰ Esophageal and duodenal epithelia are both routinely subjected to acidic gastric contents, and epithelial bicarbonate secretion is believed to play a critical role in defense. Bicarbonate ion secretion from esophageal submucosal glands and duodenal columnar epithelium has been identified.^{11,12} Additionally, rat esophageal epithelium that was separated from the underlying submucosal glands also demonstrated bicarbonate secretion after an acid challenge, suggesting a role for the stratified epithelium alone in supplying the neutralizing bicarbonate.¹³ The role of bicarbonate as an acid buffer is especially relevant to vocal fold physiology, as low pH environments are routinely encountered during episodes of gastric reflux, carcinogenesis and metastasis, and acute inflammation.¹⁴⁻¹⁸

Isolating bicarbonate transport is challenging, as there are no specific pharmacologic antagonists of bicarbonate channels or transporters. Further, bicarbonate may be transported through anion exchange channels such as CFTR.^{19,20} Therefore, indirect methods are needed for elucidating the role of bicarbonate in vocal fold epithelial ion transport. These methods include blocking carbonic anhydrase in order to decrease the production of bicarbonate within the cell and decreasing bicarbonate in the surrounding environment to determine whether the presence of bicarbonate affects epithelial ion transport.

The objective of this study was to investigate the essential role of bicarbonate transport in vocal fold epithelia. First, we exposed excised, viable porcine vocal folds to a carbonic anhydrase inhibitor, acetazolamide, to determine whether blocking the production of bicarbonate affected epithelial ion transport. Second, the vocal folds were bathed in bicarbonate-free buffer to determine whether the presence of bicarbonate in the ambient environment affected vocal fold epithelial ion transport. This study also yielded data on

whether the location of bicarbonate (luminal versus basolateral versus bilateral) affected the ion transport across the excised vocal fold epithelia. Finally, hydrochloric acid was administered to the vocal folds in the “bicarbonate-free” study to simulate a physiologically relevant clinical condition. Previous research identified a consistent increase in vocal fold ion transport after a physiologic acidic challenge (pH 4), which was presumably a defense mechanism to buffer the acid-base imbalance.⁵ We set out to determine whether bicarbonate contributes to this observed increase in ion transport that follows a simulated bout of gastric reflux or other acid-base imbalance. We hypothesized that a lack of bicarbonate in the milieu surrounding the vocal folds would lead to a change in ion transport across the epithelium. In addition, we hypothesized that bicarbonate is a critical component of the increased ion transport observed after a vocal fold acidic challenge. This study sets the groundwork for quantifying the role of bicarbonate in vocal fold defense and as a potential therapeutic agent to treat clinical conditions of acid imbalance in the larynx.

MATERIALS AND METHODS

Tissue Preparation

Fresh adult male and female porcine larynges were obtained from commercial abattoirs in accordance with protocols approved by Purdue University's Animal Care and Use Committee. The animals were painlessly sacrificed via exsanguination, and the larynges were removed and immersed in cold saline solution for transport to the laboratory. A validated laryngeal dissection protocol was utilized to prepare each larynx for experiments immediately on arrival of the larynx to the laboratory.^{5,21} The midsagittal plane of the larynx was bisected to create hemilarynges in which the vocal fold could be visualized. The vocal fold epithelium, basal lamina, and superficial lamina propria were separated from the underlying connective tissue and muscle. The vocal folds were moistened throughout the dissection with Hanks' balanced salt solution (HBSS; NaCl, 136.8 mmol/L; dextrose, 5.6 mmol/L; KCl, 5.6 mmol/L; NaHCO₃, 4.2 mmol/L; CaCl₂, 1.3 mmol/L; MgSO₄, 0.8 mmol/L; KH₂PO₄, 0.4 mmol/L; and Na₂HPO₄, 0.3 mmol/L; pH 7.0) and prepared for electrophysiology experiments.

Solutions and Chemicals

All solutions and chemicals were obtained from Sigma Aldrich (St Louis, Missouri). In experiment 1, dimethyl sulfoxide was the solvent used to dissolve the carbonic anhydrase inhibitor acetazolamide and is hereinafter referred to as the vehicle. In experiments 2 and 3, the control was HBSS with sodium bicarbonate. Bicarbonate-free HBSS was prepared by not adding the sodium bicarbonate and supplementing the HBSS with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and HEPES sodium salt to maintain the osmolarity, isotonicity, and pH.

Electrophysiology Protocol

A calibrated Ussing system (model 15362; WPI) and voltage clamp (model DVC-1000) were used to record short-circuit current and transepithelial resistance across porcine vocal fold epithelia.⁴⁻⁶ Short-circuit current is a measure of ion transport, and transepithelial resistance reflects the integrity of the epithelial barrier. Vocal fold epithelia were mounted

on chambers that were subsequently installed in the Ussing apparatus. Both chambers were filled with 5 mL of HBSS buffer (Sigma Aldrich). The circulating HBSS was warmed to 37°C, and air composed of 95% oxygen and 5% carbon dioxide was bubbled into the media. Two voltage electrodes and 2 current electrodes (Ag +/AgCl electrodes with 3 mol/L KCl/agar salt bridges) were placed on either side of the vocal fold in the Ussing chamber. The electrodes measured the open circuit potential differences and short-circuit current across the vocal fold. Once mounted, the vocal folds reached baseline in approximately 1 hour (stable short-circuit current for approximately 5 to 10 minutes). Transepithelial resistance was measured according to Ohm's law once the tissues reached baseline. Epithelia that reached a baseline transepithelial resistance of 300 $\Omega \cdot \text{cm}^2$ were deemed acceptable to be used for the experiments.²²

Experiment 1: Carbonic Anhydrase Antagonism

Vocal folds were prepared as described above for the electrophysiology experiments, and the baseline short-circuit current was established. To determine the dose of acetazolamide in a preliminary experiment using 6 vocal folds, we tested various concentrations of acetazolamide (range, 0.1 to 6 mmol/L acetazolamide). On the basis of these data (results not shown), a 4 mmol/L acetazolamide dose was selected for further study, as this was the largest concentration that preserved vocal fold viability. Previous studies with human esophageal stratified squamous epithelium successfully inhibited carbonic anhydrase with an acetazolamide concentration of 0.1 mmol/L.²³

Eighteen vocal fold samples from the same larynx were mounted on Ussing chambers, and baseline short-circuit current was reached before administration of either 4 mmol/L acetazolamide or the same volume of vehicle alone. Ion transport was then recorded for 30 minutes. A transepithelial resistance of greater than 300 $\Omega \cdot \text{cm}^2$ was measured throughout the 30-minute experiment to ensure tissue viability.

Experiment 2: Bicarbonate-Free Ion Substitution

Once the tissues reached baseline, the luminal and basolateral chambers of the Ussing system were drained. Each chamber was flushed with the incoming buffer in order to eliminate remaining HBSS and then filled with 1 of 4 combinations of buffer: bilateral HBSS (8 samples); luminal bicarbonate-free HBSS and basolateral HBSS (8), luminal HBSS and basolateral bicarbonate-free HBSS (8), or bilateral bicarbonate-free HBSS (8). Ion transport was measured for 30 minutes after the solution exchange. Preliminary experiments (data not shown) in our laboratory indicated that a stable short-circuit current was reached in 30 minutes after a chamber exchange of fluid. A transepithelial resistance of greater than 300 $\Omega \cdot \text{cm}^2$ was measured throughout the 30-minute experiment to ensure tissue viability.

Experiment 3: Acid Challenge

Dissected vocal folds mounted on Ussing chambers were bathed in 1 of the 4 combinations of bicarbonate-free buffer described above for 30 minutes. After the 30 minutes, the pH of the solution in the luminal chamber was measured to ensure a physiologic pH of 7.²⁴ Thereafter, the luminal surface of each vocal fold was exposed to 2.5 μL hydrochloric acid

to cause an acidic environment (pH 3 to 4). The pH of 3 to 4 was selected because it is in the range of simulated acidic environments in the larynx.¹⁴ Ion transport was measured immediately after the acid challenge.

Data and Statistical Analysis

DataTrax software (WPI, Sarasota, Florida) was used to measure the short-circuit current in all electrophysiology experiments. Statistical analysis was completed with SPSS (version 20, Chicago, Illinois). The dependent variable in all experiments was the normalized short-circuit current (percentage of short-circuit current adjusted to baseline). For experiment 1, a *t*-test was performed to compare the effects of acetazolamide and vehicle on ion transport. For experiment 2, the short-circuit current value measured immediately before the systems were flushed was taken as the baseline measure. A second short-circuit current value was measured after the vocal fold was bathed in 1 of the 4 buffers. A univariate analysis of variance was used to investigate whether the changes in short-circuit current were different between the control and bicarbonate-free groups. For experiment 3, the baseline short-circuit current was the value measured immediately before we added hydrochloric acid. The post-acid challenge increase in short-circuit current was measured within 60 seconds of adding the hydrochloric acid. A univariate analysis of variance was used to investigate whether the increases in short-circuit current following acid challenge were different between the control and bicarbonate-free groups. An α level of 0.05 was considered statistically significant for all analyses.

RESULTS

Experiment 1

The vocal folds assigned to the acetazolamide group did not significantly differ in baseline short-circuit current from the vocal folds in the vehicle-alone group ($t = -0.851$; $p = 0.434$). Exposure to acetazolamide decreased the short-circuit current by 22%, in contrast to the 19% decrease in short-circuit current for exposure to vehicle alone. This effect on short-circuit current was nonsignificant ($t = -0.533$; $p = 0.617$; Fig 1).

Experiment 2

The baseline short-circuit current did not differ significantly across vocal folds in the 4 buffer groups ($F = 2.839$; $p = 0.06$). The availability of bicarbonate bilaterally (HBSS condition) reduced the short-circuit current by 27% as compared to baseline. Exposure to bicarbonate-free buffer reduced the short-circuit current by 30% (luminal), 15% (basolateral), and 8% (bilateral) as compared to baseline. Overall, the differential availability and location of bicarbonate did not significantly change the short-circuit current ($F = 1.890$; $p = 0.154$; Fig 2).

Experiment 3

Addition of acid increased the short-circuit current by 57% in the HBSS condition. The magnitude of the increase in short-circuit current was reduced in the bicarbonate-free condition. In the groups lacking bicarbonate, exposure to acid increased short-circuit current by 18% (luminal), 43% (basolateral), and 9% (bilateral) as compared to the pre-acid

baseline. The increase in short-circuit current was greatest for the HBSS and low-basolateral bicarbonate groups and approached statistical significance ($F = 2.886$; $p = 0.053$; Fig 3).

DISCUSSION

The epithelium of the vocal fold provides the interface between the environment and the underlying connective tissue. As such, the epithelium is the foremost defense mechanism against airborne and luminal environmental and systemic challenges.²⁵ In this study, we sought to investigate an epithelial defense mechanism (bicarbonate transport) against pH insults in the vocal fold environment. Bicarbonate plays a critical biochemical role in the body's pH buffering system. Our data show that exposure to an acidic environment increases ion transport and that the distribution of bicarbonate in the ambient environment of the vocal folds may influence the magnitude of the ion transport response.

The interconversion of carbon dioxide and water into bicarbonate is catalyzed by carbonic anhydrase.^{8,9} In experiment 1, we attempted to block the intracellular synthesis of bicarbonate by antagonizing carbonic anhydrase with acetazolamide. We predicted a drop in ion transport with carbonic anhydrase inhibition, signifying a reduction in bicarbonate ion synthesis and thus possibly a decrease in bicarbonate secretion. Acetazolamide, given at concentrations well above dosages previously reported to block carbonic anhydrase in esophageal stratified squamous epithelium, did not significantly reduce ion transport in the vocal fold epithelium as compared to vehicle. Potential reasons for this nonsignificant effect include the concentrations of acetazolamide used and/or its non-specificity as an inhibitor. It is possible that the concentration of acetazolamide was too low for it to accumulate sufficiently in the cytoplasm of the epithelial cells in which it was needed to antagonize carbonic anhydrase. It is also possible that the carbonic anhydrase isomer that is most prevalent and active in porcine vocal fold epithelium does not respond well to acetazolamide. Carbonic anhydrase has 16 isomers with different subcellular locations (cytosolic versus membranous versus extracellular), as well as different degrees of catalytic activity.²⁶ Minimal knowledge is available about the activity and distribution of carbonic anhydrases in species other than humans, mice, and rats. Future studies should also incorporate positive controls such as esophageal epithelium to more clearly define the vocal fold response to acetazolamide.

The presence and/or distribution of bicarbonate in the fluid environment of the vocal folds may influence the extent of the defense response to acidic insults. In esophageal epithelium, a bicarbonate-free fluid environment diminished the ion transport response to low pH.¹³ We first demonstrated that exposure to a low-bicarbonate environment did not adversely affect vocal fold epithelial viability (experiment 2). Reducing the pH in a low-bicarbonate environment increased the short-circuit current in vocal fold epithelium. These findings approached statistical significance. The increase was greatest when bicarbonate was present on the luminal and basolateral surfaces or on the basolateral surface alone. As expected, the bilateral bicarbonate-free buffer had the least amount of ion transport increase after an acidic challenge. Our findings suggest that bicarbonate in the environment may be crucial for buffering, as demonstrated by an increase in ion transport following an acidic encounter.⁵ The lack of statistical significance may be potentially attributed to our small sample size.

We chose to investigate an acidic environment (pH 3 to 4) because a multitude of disorders involve pH imbalances or insults. Possibly damaging acidic environments are encountered during generic cellular injuries such as ischemia, acute inflammation, and necrosis. Any perturbation to cellular respiration can also result in an increase in anaerobic glycolysis, followed by increased lactic acid and decreased pH.²⁷ In fact, anaerobic glycolysis is a common finding in neoplastic cells undergoing metastasis and is believed to be a mechanism of survival for these cells.^{16,17} Most notably in the laryngeal scientific literature, laryngopharyngeal reflux (LPR) involves the retrograde reflux of gastric contents into the pharynx and larynx.²⁸ The normal pH of the larynx is 7, whereas the pH of the gastric contents is between 1.5 and 2.²⁹ Consequently, the pH of the larynx and vocal fold can drop to less than 5 during an LPR episode. Non-acidic noxious substances in the reflux, such as pepsin, bile, and pancreatic enzymes, can also increase the damage to the vocal folds during a bout of LPR.^{4,24,28} Although pepsin is optimally active in an acidic environment, stable pepsin may remain in the larynx and be re-activated after another acidic reflux insult.²⁹ An acidic environment is therefore an important component of the pathophysiology of LPR, regardless of the primary causative agent in the refluxate.

In summary, the results of this study indicate a potential role for epithelial bicarbonate ion transport in vocal fold defense to acid challenges. The role of the extracellular matrix and muscle in supporting epithelial defense to acidic insults was not investigated in this study, and future studies will examine the interaction of these tissue layers in vocal fold defense. This investigation focused on the vocal fold epithelium, as this tissue is the primary recipient of airborne and systemic challenges. As such, the vocal fold epithelial cells may serve as an important target for therapeutic interventions that strengthen both barrier and ion transport functions. Future research will examine a role for aerosolized bicarbonate as a prophylactic treatment in reducing the clinical symptoms and pathologic changes associated with acidic challenges in laryngeal disease.

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Fig 1.

Average decrease in ion transport in vocal folds exposed to acetazolamide or vehicle (dimethyl sulfoxide). Error bars represent standard error of mean. Isc — short-circuit current.

Fig 2.

Average decrease in ion transport from baseline in 4 buffer groups. Error bars represent standard error of mean. HBSS — Hanks' balanced salt solution (see text).

Fig 3.

Average increase in ion transport from baseline after exposure to acid (pH range, 3 to 4). Error bars represent standard error of mean.