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Role for Ion Transport in Porcine Vocal Fold Epithelial Defense to Acid Challenge

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Abstract

Objective—The vocal fold epithelium is routinely exposed to gastric contents, including acid and pepsin, during laryngopharyngeal reflux events. The epithelium may possess intrinsic defenses to reflux. The first objective of the current study was to examine whether vocal fold epithelial ion transport is one potential mechanism of defense to gastric contents. The second objective was to determine whether ion transport in response to gastric contents is associated with the secretion of bicarbonate.

Study Design—Prospective design in excised porcine larynges.

Setting—Laboratory.

Subjects and Methods—Porcine vocal folds (N = 56) were exposed on the luminal surface to acid, pepsin, or sham challenges. Ion transport at baseline and following challenge exposure was measured using electrophysiological techniques. To examine specific ion transport mechanisms, vocal folds were pretreated with either a sodium channel blocker or bicarbonate channel blocker.

Results—Within 60 seconds of acid but not pepsin exposure, there was a significant increase in ion transport. This rapid increase in ion transport was transient and related to bicarbonate secretion.

Conclusion—The current data suggest that porcine vocal folds immediately increase bicarbonate secretion following exposure to acid. Bicarbonate secretion may act to neutralize acid. These findings contribute to the identification of the mechanisms underlying vocal fold defense to reflux and offer implications for the development of treatments for reflux-induced vocal fold injury.

Keywords

vocal fold epithelium; acid; pepsin; bicarbonate ion transport

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Author Contributions

Elizabeth Erickson-Levendoski, substantial contribution to the research process and manuscript via conception and design, acquisition and analysis of data, interpretation of data, drafting and revising manuscript, and providing final approval; **M. Preeti Sivasankar**, substantial contribution to the research process and manuscript via conception and design, interpretation of data, drafting and revising manuscript, and providing final approval.

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Exposure of the laryngeal epithelium to refluxed gastric contents is implicated in the development of laryngeal disease.^{1,2} An aggressive component of refluxed gastric contents is hydrochloric acid.³ In the vocal folds, hydrochloric acid induces epithelial leakiness and some gross structural damage⁴ that could potentially heighten vocal fold susceptibility to damage from further reflux events or environmental pollutants. However, the epithelium may exhibit some intrinsic defenses to protect against the damaging effects of acid. This study seeks to test the hypothesis that bicarbonate (HCO_3^-) ion secretion could function as one such defense mechanism. In the epithelium of the esophagus and stomach, HCO_3^- is rapidly secreted in response to acid exposure.⁵⁻⁷ HCO_3^- functions to neutralize acid,⁸ and its secretion may help protect gastric and esophageal epithelia from acid-induced injury. Active ion transport contributes to the health and viability of the vocal fold epithelium.^{9,10} However, the potential role of ion transport, particularly HCO_3^- secretion, in vocal fold defense to acid, remains to be investigated. Findings that the vocal folds rapidly secrete HCO_3^- in response to acid would suggest that in the vocal folds, ion transport is one intrinsic defense mechanism to reflux.

The primary goal of the current study was to examine vocal fold epithelial ion transport in response to an acid challenge. Given that other gastric contents, including pepsin, have been implicated in the development of reflux-related laryngeal disease,¹ the secondary goal of the present study was to examine vocal fold epithelial ion transport in response to a pepsin challenge. The distinct contributions of acid and pepsin to ion transport were investigated to characterize the mechanisms underlying vocal fold epithelial defense to reflux. To address these goals, 2 experiments were conducted using a porcine model and electrophysiological techniques. Experiment 1 sought to investigate whether acid and pepsin challenges increase ion transport in porcine vocal folds. Based on similar research in the esophagus,⁷ the hypothesis was that an acidic environment would immediately increase vocal fold epithelial ion transport. Experiment 2 then sought to determine if increases in ion transport were a function of secretion of HCO_3^- , an acid neutralizer. To address this question, ion channel blockers were used to selectively inhibit ion transport. Specifically, vocal fold epithelia were exposed to either an epithelial Na^+ channel blocker (amiloride) or HCO_3^- channel blocker (NPPB: 5-nitro-2-(3-phenylpropylamino) benzoic acid) prior to acid challenges. The hypothesis was that NPPB would inhibit acid-induced increases in ion transport. NPPB inhibition of ion transport suggests that acid-induced increases in ion transport are a function of HCO_3^- secretion. Findings from this investigation will contribute to the identification of mechanisms underlying vocal fold epithelial defense to reflux. Through a better understanding of these mechanisms, we can develop targeted treatments for reflux-induced vocal fold injury.

Methods

Tissue Preparation

Fresh porcine larynges (ages 9–12 months) were obtained from commercial abattoirs in accordance with approved protocols from the Animal Care and Use Committee at Purdue University. Within 15 minutes of animal sacrifice, larynges were immersed in cold saline and transported to the laboratory. All dissection techniques followed validated procedures routinely used by our laboratory.^{4,9,11} Each larynx was bisected into hemilarynges along the mid-sagittal plane. The vocal fold epithelium, basal lamina, and superficial layer of the lamina propria, hereafter referred to as the vocal fold, were carefully dissected as a sheet from the underlying muscle. Throughout dissection, the vocal fold was kept moist with Hank balanced salt solution (HBSS; mM: NaCl, 136.8; dextrose, 5.6; KCl, 5.6; NaHCO_3 , 4.2; CaCl_2 , 1.3; MgSO_4 , 0.8; KH_2PO_4 , 0.4; Na_2HPO_4 , 0.3; pH 7.0). Dissected vocal folds

were immediately mounted on removable lucite Ussing chambers (World Precision Instruments [WPI], Sarasota, Florida) for electrophysiology experiments as detailed below.

Acid Challenges

The acid challenges were hydrochloric acid (HCl) at pH 3 and sulfuric acid (H₂SO₄) at pH 3. The pepsin challenge was 1.0 mg/mL of porcine nonacidic pepsin. The sham challenge was HBSS at pH 7. HCl was selected because it is the primary acidic component of gastric contents.¹² H₂SO₄ was included to ensure that any acid-induced changes in ion transport are independent of the presence of specific anions (Cl⁻, SO₄²⁻) in the luminal fluid. The pH selected here is within the range of pH values used previously to simulate acidic environments.¹ The concentration of pepsin selected here has also been used previously in an experimental model of laryngopharyngeal reflux (LPR).¹ pH values were verified using pH indicator strips (EMD Chemicals, Gibbstown, New Jersey). All chemicals were obtained from Sigma Aldrich (St Louis, Missouri).

Protocol

An Ussing system (model 15362; WPI) with associated voltage clamp (model DVC-1000) was used for electrophysiology experiments.^{4,9,13} The dissected vocal fold was placed in the calibrated Ussing system. Voltage and current electrode (Ag⁺/AgCl electrodes with 3 mol/L KCl/agar salt bridges) were placed on either side of the vocal fold, and warm, oxygenated HBSS was used to fill the luminal and basal reservoirs of the Ussing system. Reservoir temperature was maintained at 37°C with the use of a circulating water bath. To monitor vocal fold viability, transepithelial resistance (R_T) was measured during the experiment. A baseline R_T value of at least 300 Ω·cm² was required for the vocal folds to be included in experiments 1 and 2 as detailed below.

Experiment 1—Experiment 1 sought to investigate whether acid and pepsin challenges would immediately increase ion transport in porcine vocal folds. Short-circuit current (I_{sc}) is a measure of net ion transport. I_{sc} was the dependent variable in this investigation. Vocal folds were allowed 45 to 60 minutes to reach baseline. Once vocal folds reached baseline (stable I_{sc} (± 0.5 μA) for at least 10 minutes), the luminal surface of the vocal fold was exposed to HCl acid (n = 6), H₂SO₄ acid (n = 5), or pepsin (n = 6) challenge. The contralateral vocal fold from the same larynx was exposed to the sham challenge (n = 17). Immediate changes in I_{sc} postchallenge were monitored. Immediate was defined as the change in I_{sc} within 90 seconds postchallenge. A typical LPR event lasts for an average of 90 seconds,¹⁴ with brief “flashes” of reflux reaching the laryngopharynx,¹² providing the basis for the timeline in the current study. To ensure continued tissue viability following acid and pepsin challenge, vocal folds where R_T decreased to below 100 Ω·cm² were excluded from analyses as this may be suggestive of vocal fold damage.

Experiment 2—Findings from experiment 1 indicated that acid but not pepsin challenge immediately increases ion transport. Consequently, experiment 2 sought to determine if acid-induced increases in ion transport were a function of HCO₃⁻ secretion. Because pepsin did not increase ion transport (see Results), experiment 2 was not conducted for the pepsin challenge. To examine the nature of acid-induced increases in ion transport, ion channel blockers, amiloride (to block Na⁺ absorption) and NPPB (to block HCO₃⁻ secretion), were used to selectively inhibit ion transport. These ion species were targeted because they are 2 primary ions actively transported across the vocal fold epithelium. Once vocal folds reached baseline I_{sc}, the luminal surface was exposed to either amiloride pretreatment (10 μM) or NPPB pretreatment (100 μM). Following pretreatment, the I_{sc} was allowed to stabilize prior to the luminal acid challenge. Specific blocker-challenge combinations included the following: amiloride-HCl (n = 6), NPPB-HCl (n = 6), amiloride-H₂SO₄ (n = 5), and NPPB-

H₂SO₄ (n = 5). Immediate changes in I_{sc} post-acid challenge were monitored. To ensure continued tissue viability following acid challenge, vocal folds where R_T decreased to below 100 Ω·cm² were excluded from analyses as this may be suggestive of vocal fold damage.

Data and Statistical Analysis

DataTrax software (WPI) was used to measure I_{sc} at baseline and postchallenge. For both experiments 1 and 2, the I_{sc} value immediately prior to challenge was taken as the baseline measure. Because a typical LPR event lasts for an average of 90 seconds,¹⁴ the point of maximum I_{sc} increase within 90 seconds postchallenge was taken as the postchallenge measure. I_{sc} data that were not normally distributed were transformed to fulfill assumptions of normality. A mixed, repeated-measure analysis of variance (ANOVA) was used in experiment 1 to investigate whether HCl, H₂SO₄, or pepsin as compared with sham challenges would immediately increase I_{sc}. One-way ANOVA with Tukey tests were used for post hoc testing. Results from experiment 1 demonstrated that HCl and H₂SO₄ but not pepsin challenges immediately increased ion transport. Consequently, an additional mixed, repeated-measure ANOVA was used in experiment 2 to investigate the differential effect of amiloride and NPPB pretreatments on acid-induced increases in I_{sc}. An α level of 0.05 was considered statistically significant for all analyses.

Results

Experiment 1: Acid Challenges Increase Ion Transport

Figure 1A–D demonstrates representative traces of the effects of HCl, H₂SO₄, pepsin, and sham challenges on I_{sc}. I_{sc} significantly increased following HCl and H₂SO₄ but not pepsin or sham challenge, $F(2, 28) = 9.70$, $P = .001$ (Figure 2A–C). Specifically, I_{sc} increased by an average of 18 μA/cm² following HCl challenge and by 22 μA/cm² following H₂SO₄ challenge. On the other hand, the average I_{sc} increase was only 2 μA/cm² for pepsin challenge and 0 μA/cm² for sham challenge. Maximal I_{sc} increase with HCl and H₂SO₄ was observed within 60 seconds. These increases were transient, with I_{sc} values returning toward baseline or below baseline I_{sc}. When comparing HCl and H₂SO₄, increases in I_{sc} were similar across acid challenges $P = .72$; (Figure 3).

Experiment 2: Acid Challenges Increase Bicarbonate Secretion

Findings from experiment 1 indicated that acid but not pepsin challenges immediately increase I_{sc}. Consequently, experiment 2 used ion channel blockers to determine if acid-induced increases in ion transport were a function of HCO₃[−] secretion. Figure 4A–D demonstrates representative traces of the effects of acid on I_{sc} in vocal folds pretreated with amiloride (Figure 4A,C) or NPPB (Figure 4B,D). The acid-stimulated increase in I_{sc} in tissues pretreated with NPPB is significantly attenuated as compared with the increase in I_{sc} in tissues pretreated with amiloride, $F(1, 18) = 29.38$, $P < .001$ (Figure 5A,B). Specifically, average increases in I_{sc} for vocal folds pretreated with NPPB were 11 μA/cm² and 12 μA/cm² for HCl and H₂SO₄ challenges, respectively. Average increases in I_{sc} for vocal folds pretreated with amiloride were 27 μA/cm² and 30 μA/cm² for HCl and H₂SO₄ challenges, respectively. NPPB inhibition of I_{sc} suggests that acid-stimulated increases in ion transport are, in part, a function of HCO₃[−] secretion. When comparing HCl and H₂SO₄, the effects of pretreatments on acid-stimulated increases in I_{sc} were similar across acid challenges, $F(1, 18) = 0.17$, $P = .66$ (Figure 6).

Discussion

The reflux of gastric contents into the laryngopharynx is estimated to occur in up to 50% of individuals presenting with voice problems.¹⁵ Potentially damaging components of gastric

contents include acid, pepsin, bile salts, and pancreatic enzymes. These agents may act separately or in combination; however, research suggests that acid and pepsin exert some of the most significant detrimental effects on vocal folds.³ As the outermost layer of the vocal folds, the epithelium is the first layer of the vocal folds exposed to reflux and may act as an early line of defense. By better understanding how the epithelium protects against reflux, the treatment of vocal fold injury may be improved. The present study explored epithelial ion transport as one potential mechanism of vocal fold defense to gastric contents. We demonstrated a rapid, transient increase in porcine vocal fold ion transport following luminal HCl and H₂SO₄ acid challenges but not pepsin challenge. Acid-induced increases in ion transport were independent of the presence of a specific anion (Cl⁻, SO₄²⁻) and were attenuated when vocal folds were pretreated with NPPB (an HCO₃⁻ blocker). The ion transport increases were not attenuated after amiloride pretreatment (a Na⁺ blocker), suggesting that HCO₃⁻ is secreted in response to acidic challenges.

Exposure to acid immediately increases HCO₃⁻ secretion in esophageal and gastric epithelia.⁵⁻⁷ HCO₃⁻ secretion in the stomach is mediated by multiple factors, including endogenous prostaglandins, nitric oxide, and neural pathways.^{5,6} Our data suggest that vocal fold epithelia also secrete bicarbonate in response to acidic challenge. The molecular and neuronal pathways regulating HCO₃⁻ secretion in the vocal folds remain to be investigated. Rapidly secreted HCO₃⁻ may neutralize luminal acid and play a role in the protection of the vocal folds from acid-induced injury. The H⁺/K⁺-ATPase (proton) pump has been localized to the laryngeal submucosal glands.¹⁶⁻¹⁸ Although the specific functions of this pump are currently unknown, it is hypothesized to also play a role in the protection of the laryngeal epithelium from acidic environments.¹⁷ Ion transport mechanisms such as HCO₃⁻ secretion and the H⁺/K⁺-ATPase pump would be critical defenses for the laryngeal epithelium as this structure is considered particularly susceptible to gastric contents.¹

To examine if acid-stimulated increases in ion transport were associated with HCO₃⁻ secretion, vocal folds were exposed to the ion channel blocker NPPB prior to the acid challenge. NPPB is a blocker of the cystic fibrosis transmembrane regulator (CFTR) channel.¹⁹ NPPB was chosen for the current study because it has been used previously in other tissues, such as duodenum, to inhibit epithelial HCO₃⁻ secretion.²⁰ However, NPPB is a nonspecific blocker and may also inhibit epithelial chloride secretion.⁹ Although the current findings do suggest that HCO₃⁻ is being secreted by the vocal fold epithelium in response to acid, additional studies are needed to confirm the specific ion channel underlying acid-stimulated increases in ion transport. Consequently, future studies will include ion substitution experiments and immunolocalization to verify the location of HCO₃⁻ channels.

Results from the current study suggest that in porcine vocal folds, HCO₃⁻ is rapidly secreted in response to luminal acid exposure. Although the source of secreted HCO₃⁻ is currently unknown, it may be produced by the intracellular enzyme, carbonic anhydrase. In a reversible reaction, carbonic anhydrase hydrolyzes carbon dioxide to produce HCO₃⁻. Believed to be an important laryngeal defense to LPR, carbonic anhydrase is present in normal laryngeal epithelium²¹ but depleted in laryngeal epithelium from patients with LPR.²² The rapid epithelial transport of HCO₃⁻ observed in the current investigation may, in part, defend the tissue against acid. This finding raises an interesting question as to whether, in some individuals, impaired HCO₃⁻ production or HCO₃⁻ secretion may contribute to acid-related vocal fold injury. If so, there may be implications for the development of pharmacologic treatments that target enhanced vocal fold HCO₃⁻ production or secretion in these individuals.

A nonacidic pepsin challenge did not significantly increase vocal fold epithelial ion transport. Published data from gastric epithelium²³ support our finding that nonacidic pepsin does not increase epithelial ion transport. This is also consistent with findings from the esophageal literature⁷ that indicate the increased secretion of HCO_3^- is induced by an acidic environment. The present study served to quantify the distinct effects of acid and pepsin on vocal fold epithelial ion transport. Characterizing these distinct effects is critical as acid-induced HCO_3^- secretion may function to reduce peptic activity by neutralizing acid. Our previous work demonstrates that acid and acidified pepsin induce similar changes to the integrity of the epithelial barrier.⁴ However, we also recognize that this does not preclude an effect of acidified pepsin on epithelial ion transport. The effect of these agents in combination on vocal fold ion transport awaits investigation. A porcine model was used to investigate the effects of acid and pepsin challenges on vocal fold ion transport. Although porcine tissue has been validated for use in studies investigating the effects of LPR on the vocal folds,²⁴ the use of an animal model may limit the ability to generalize data, and the current findings need to be confirmed in human vocal fold tissues.

In summary, we found that acid challenges induced a rapid, transient increase in ion transport that appears to be related to HCO_3^- secretion. Secretion of HCO_3^- , an acid neutralizer, may help protect the vocal folds from reflux-induced injury. The current study is the first to investigate epithelial ion transport as a potential defense mechanism to reflux and suggests that vocal fold epithelial cells actively sense and respond to changes in the environment.

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Figure 1.

Representative data traces of ion transport (Isc) in vocal folds exposed to HCl (A), H_2SO_4 (B), pepsin (C), and sham (D) challenges. Challenges were added to the luminal surface of the vocal folds at time zero. HCl and H_2SO_4 but not pepsin and sham challenges induced a rapid, transient increase in Isc.

Figure 2. Ion transport (Isc) at baseline and in response to HCl (A), H_2SO_4 (B), and pepsin (C) challenges. Isc significantly increased following HCl and H_2SO_4 challenges but not pepsin challenge ($P < .05$). Error bars represent standard deviation.

Figure 3. Comparison of the effects of HCl and H_2SO_4 acid challenges on ion transport (Isc). HCl and H_2SO_4 challenges induced similar increases in Isc ($P > .05$). Error bars represent standard deviation.

Figure 4. Representative data traces of the effects of HCl (A, B) and H_2SO_4 (C, D) acid challenges on ion transport (Isc) in vocal folds pretreated with amiloride or NPPB. Acid challenges were added to the luminal surface of the vocal folds at time zero. Acid-induced increases in Isc are attenuated in vocal folds pretreated with NPPB as compared with vocal folds pretreated with amiloride.

Figure 5.

Ion transport (Isc) at baseline and in response to HCl (A) and H₂SO₄ (B) acid challenges in vocal folds pretreated with amiloride or NPPB. Acid-stimulated increases in Isc were significantly attenuated in vocal folds pretreated with NPPB as compared with vocal folds pretreated with amiloride ($P < .05$). Error bars represent standard deviation.

Figure 6.

Comparison of the effects of HCl and H₂SO₄ acid challenges on ion transport (Isc) in vocal folds pretreated with amiloride (A) and NPPB (B). HCl and H₂SO₄ challenges induced similar increases in Isc for vocal folds pretreated with amiloride and NPPB ($P > .05$). Error bars represent standard deviation.