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Vocal Fold Ion Transport and Mucin Expression Following Acrolein Exposure

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Abstract The vocal fold epithelium is exposed to inhaled particulates including pollutants during breathing in everyday environments. Yet, our understanding of the effects of pollutants on vocal fold epithelial function is extremely limited. The objective of this study was to investigate the effect of the pollutant acrolein on two vocal fold epithelial mechanisms: ion transport and mucin (MUC) synthesis. These mechanisms were chosen as each plays a critical role in vocal defense and in maintaining surface hydration which is necessary for optimal voice production. Healthy, native porcine vocal folds ($N = 85$) were excised and exposed to an acrolein or sham challenge. A 60-min acrolein, but not sham challenge significantly reduced ion transport and inhibited cyclic adenosine monophosphate-dependent, increases in ion transport. Decreases in ion transport were associated with reduced sodium absorption. Within the same timeline, no significant acrolein-induced changes in MUC gene or protein expression were observed. These results improve our understanding of the effects of acrolein on key vocal fold epithelial functions and inform the development of future investigations that seek to elucidate the impact of a wide range of pollutant exposures on vocal fold health.

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Keywords Vocal fold epithelium · Pollutants · Acrolein · Ion transport · Mucins

Introduction

As the outermost layer of the vocal folds, the epithelium is exposed to a multitude of inhaled particulates including pollutants (Thibeault et al. 2009). However, our understanding of the effects of pollutants on vocal fold epithelial function is extremely limited. Two mechanisms critical to homeostatic epithelial function include ion transport and mucin (MUC) glycoprotein synthesis. Epithelial cells actively transport Na^+ and Cl^- through ion channels to regulate the volume of salt and, consequently, water on the epithelial surface (Boucher 1999). MUC glycoproteins are synthesized and secreted onto the epithelial surface by specialized MUC-producing cells (Rose and Voynow 2006). Water combines with MUC to create a mucus barrier on the vocal fold epithelial surface that is important for vocal fold protection (Kutta et al. 2002, 2008) and vibration (Roy et al. 2003). It is unclear whether pollutant exposure alters these primary epithelial functions.

The pollutant acrolein is a low-molecular weight, α,β-unsaturated aldehyde pervasive in the ambient environment. Acrolein is a byproduct of mobile exhaust, industrial processes, and tobacco smoke (Bein and Leikauf 2011). In the nasal passages, trachea, and lungs, acrolein has a profound and rapid effect on epithelial ion transport and MUC synthesis (Alexander et al. 2012; Welsh 1983; Borchers et al. 1998, 1999). In excised canine tracheal epithelium and in cultures of sinonasal epithelial cells, exposure to acrolein significantly reduced ion transport within 30 min of initial exposure (Welsh 1983; Alexander et al. 2012). In cultured lung epithelial cells, both 60 and 240 min acrolein exposures

promotes increased synthesis of the secreted mucin, MUC5AC (Burcham et al. 2010; Borchers et al. 1999). These results suggest that acrolein exposure decreases the amount of liquid on the airway epithelial surface while simultaneously increasing the amount of MUC. These alterations result in a fundamental change in the physical properties of the mucus barrier (Knowles and Boucher 2002). Yet, our understanding of the effects of acrolein on vocal fold epithelial ion transport and MUC synthesis is unknown. The stratified squamous epithelium of the vocal folds is unique in structure and function as compared to other airway epithelia. Specifically, the epithelium undergoes immense mechanical stress during voice production. In addition to protecting the vocal folds during vibration, the mucus barrier overlying the vocal folds lubricates the epithelial surface and enables easy voice production (Roy et al. 2003). Consequently, investigating the distinct effect of pollutants, like acrolein, on vocal fold epithelial ion transport, and MUC synthesis is clinically important.

In an in vitro porcine model, we investigated the effects of acrolein on two key vocal fold epithelial mechanisms: (1) ion transport, and (2) MUC glycoprotein expression (MUC1, MUC4, and MUC5AC). In the first series of experiments, we tested the hypothesis that acrolein would inhibit cyclic adenosine monophosphate (cAMP)-dependent ion transport as measured by decreased short-circuit current (I_{sc}). We further hypothesized that reductions in I_{sc} would be associated with decreased transport of Na^+, and that the barrier function of epithelia would be maintained after acrolein exposure. In the second series of experiments, we tested the hypothesis that acrolein would increase MUC glycoprotein gene and protein expression. Data supporting the adverse effects of acrolein on ion transport and MUC expression will have implications for understanding the mechanisms underlying compromised voice production reported following pollutant exposures (Sataloff 1992). These data provide a foundation for future investigations that seek to expand our knowledge of the effects of various environmental challenges on vocal fold health.

Materials and Methods

Vocal Fold Acquisition and Preparation

Porcine larynges ($N = 43$) from approximately 6-month-old animals were obtained from local abattoirs and immediately immersed in cold saline following animal sacrifice for transport to the laboratory. Larynges were hemisected along the mid-sagittal plane and the vocal fold epithelium along with its basal lamina, and superficial lamina propria (hereafter referred to as vocal fold) was carefully separated as a sheet from the underlying connective tissue and muscle (Alper et al. 2011; Branski et al. 2011; Erickson and Sivasankar 2010) and used for the following experiments.

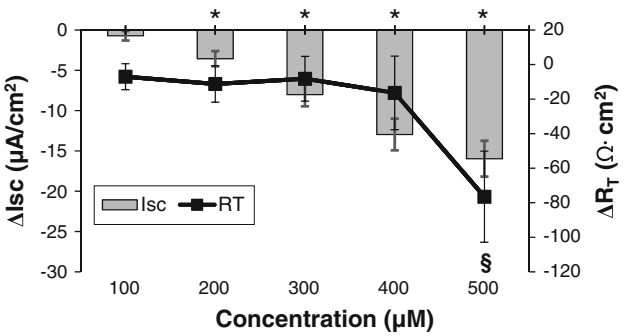

Fig. 1 Changes in I_{sc} (ΔI_{sc}) and R_T (ΔR_T) from baseline in vocal folds ($N = 10$) challenged with a series of acrolein concentrations. Negative ΔI_{sc} and ΔR_T indicates a decrease from baseline. Significant reductions in I_{sc} were observed at 200, 300, 400, and 500 μM (*). A significant reduction in R_T was only observed at 500 μM (§). *Error bars* represent standard error

Vocal Fold Electrophysiology

Short-circuit current (I_{sc}) and transepithelial resistance (R_T) were assessed using an Ussing system (model 15362, World Precision Instruments, WPI, Sarasota, FL) with associated voltage clamp (model DVC-1000; Sivasankar et al. 2010; Erickson-Levendoski and Sivasankar 2012). I_{sc} is a measure of net ion transport, and R_T is a marker of epithelial barrier function (Erickson and Sivasankar 2010). Vocal folds were mounted on lucite chambers (12 mm diameter, model CHM1, WPI) within 1.5–5 h of animal sacrifice and positioned in a calibrated Ussing system. Voltage-sensing and current-sensing electrodes (Ag^+/AgCl electrodes with 3 mol/L KCl/Agar salt bridges, WPI) were placed on either side of the vocal folds. Vocal folds were bathed in 5 mL of warm (37 °C), oxygenated (95 % O_2 and 5 % CO_2) Hank's balanced salt solution (HBSS). Mounted vocal folds were allowed approximately 60 min to reach baseline (I_{sc} ±1.0 μA for 10 min, $R_T \geq 300\ \Omega\ cm^2$).

To determine acrolein concentrations for the study, concentration–response experiments were conducted. I_{sc} and R_T were recorded after the luminal vocal fold epithelial surface ($N = 10$) was exposed sequentially to a range of physiologically relevant acrolein concentrations (100–500 μM; >99 % purity dissolved in dH_2O, Sigma Aldrich, Milwaukee, WI). The largest significant change in I_{sc} without reductions in R_T was seen at 400 μM (Fig. 1). 400 μM is within the range of acrolein concentrations (220–1,070 μM) found in automobile exhaust, cigarette smoke plumes, and wood and cotton smoke (Ayer and Yeager 1982; Faroon et al. 2008; Nishikawa et al. 1987).

Specifically, cigarette smoke plumes contain on average 890 μM acrolein (Ayer and Yeager 1982). When acrolein is inhaled directly such as in the case of cigarette smoke, retention in the respiratory tract is estimated between 97 and 99 % (Bein and Leikauf 2011; St. Charles et al. 2013) further supporting the concentration chosen for the current study.

Three experiments were conducted to investigate the effect of acrolein on ion transport. The investigator was not blinded to identity of the challenges during these experiments. In Experiment 1, the luminal vocal fold surface was exposed to the 400 μM acrolein challenge ($N = 15$). The contralateral vocal fold from the same larynx was exposed to the sham (dH_2O) challenge ($N = 15$). I_{sc} and R_T were monitored for 60 min. Acrolein and sham challenges were removed and replaced with fresh, warm HBSS. This entire process lasted less than 2 min. I_{sc} and R_T were monitored for 60 additional minutes. In Experiment 2, the luminal vocal fold surface was exposed to acrolein ($N = 5$) or sham ($N = 5$) challenge. After 60 min, forskolin (10 μM), an activator of adenylyl cyclase, was added to the luminal vocal fold surface, and I_{sc} was monitored for 30 additional minutes. In Experiment 3, vocal folds were exposed to ion channel blockers prior to addition of the acrolein challenge. The luminal vocal fold epithelial surface was exposed to either amiloride ($N = 5$) or diphenylamine-2-carboxylic acid, DPC ($N = 5$). Once I_{sc} stabilized (± 1 μA for 10 min), the 400 μM acrolein challenge was added to the vocal fold, and I_{sc} was monitored for 60 additional minutes. Amiloride (10 μM) was used to block Na^+ absorption, and DPC (100 μM) was used to block Cl^- secretion (Horisberger 1998; Smith and Benos 1991; Leydon et al. 2009).

DataTrax software (WPI) was used to measure I_{sc} and R_T. I_{sc} was recorded directly from software-displayed ion transport tracings. R_T was calculated by computing the current deflection to an imposed 2 mV pulse. Data that were not normally distributed were square root transformed to fulfill the assumptions required for parametric testing. Repeat Measures ANOVAs ($p < 0.05$) were used to investigate the effects of acrolein challenges on I_{sc} and R_T (*SPSS* Version 19.0, IBM®, Chicago, IL). Paired *t*-tests with Bonferroni-corrected α levels were used for post hoc analyses.

Vocal Fold MUC

Immunohistochemistry

For all vocal folds, MUC experiments vocal folds were tested within 1.5 h of animal sacrifice. Primary and secondary antibodies for MUC1, MUC4, and MUC5AC are listed in Table 1. Positive controls included porcine lung and intestine (MUC1, MUC4) and rat intestine (MUC5AC). Vocal folds ($N = 3$) were fixed in 10 % neutral buffered formalin, embedded in paraffin, and sectioned (5 μm). Sections were heat-retrieved using either Diva Decloaker solution (Biocare Medical, Concord, CA) or citrate buffer (pH 6). Blocking was performed at room temperature by incubating sections with 3 % H_2O_2 for 10 min, and then either 2 % horse serum for 20 min or 10 % bovine serum albumin for 60 min. Primary antibodies were applied for 60 min at room temperature with biotinylated secondary antibodies added for 30 min. Visualization was achieved using a 3′-diaminobenzidine horseradish peroxidase substrate kit for immunohistochemistry for 3–5 min (BD Bio-sciences, San Diego, CA). Sections were rated for positive and negative staining by a blinded observer familiar with vocal fold structure and immunohistochemical staining techniques. Sections were viewed and photographed using a Nikon Eclipse E600 microscope (Tokyo, Japan) with associated Olympus DP71 digital camera (Center Valley, PA).

Real-Time Polymerase Chain Reaction

Vocal folds were incubated in the 400 μM acrolein ($N = 8$) or sham ($N = 8$) challenge for 60 min at 37 °C. Vocal folds were immediately homogenized using the Tissue Tearor (Biospec Products, Bartlesville, OK), and total RNA isolated using the E.Z.N.A.™ Total RNA Midi Kit (OMEGA Bio-Tek, Norcoss, GA). RNA concentration and quantity were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL) and stored at −80 °C until use. One microgram of RNA from each vocal fold was converted to complementary DNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA).

Table 2 displays primer sequences and accession numbers. Porcine-specific primers for MUC1, MUC4, and MUC5AC were designed using publically available gene sequences through the National Center for Biotechnology Information (U.S. National Library of Medicine, Bethesda, MD) and synthesized by Integrated DNA technologies (Coralville, IA). Hypoxanthine phosphoribosyltransferase I (HPRT I) was selected as the endogenous reference gene. Real-time polymerase chain reaction (PCR) was performed using the FastStart Universal SYBR Green Kit (Roche Applied Science, Indianapolis, IN) in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was performed under the following conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 59 °C for 60 s, and 72 °C for 30 s. The specificity of PCR products for all tested genes was verified by DNA sequencing.

Relative quantitative analysis was performed using the standard comparative cycle threshold (C_t) method ($2^{-\Delta\Delta C_t}$(Livak and Schmittgen 2001; Schmittgen and Livak 2008). Raw C_t values were calculated using the ABI 7300 Real-Time PCR System software. To obtain normalized C_t values for each gene (ΔC_t), the raw C_t value of the

Table 1 Immunohistochemistry MUC antibodies

Proteins	Species	Concentration	Catalog numbers	Secondary antibody kit
MUC1	Mouse monoclonal	1:100	Invitrogen A14026	Vector polymer anti-mouse
MUC4	Mouse monoclonal	1:100	Invitrogen 182322	Vector polymer anti-mouse
MUC5AC	Mouse monoclonal	1:100	Abcam ab79082	ImPRESS anti-mouse

Table 2 Real-time PCR primer sequences

Genes	Forward	Reverse	Accession numbers
MUC1	agtgccgacgaaagaactgt	gctgccaggttcgagtaaga	AY243508
MUC4	ctgcaatgtcagttggctgt	acctaccaggccatcctttc	NM001206344
MUC5AC	gcaaatctcagcttccaagg	ggagcagacccttctgtgac	AF054854
HPRT I	aaggacccctcgaagtgttg	cacaaacatgattcaagtccctg	DQ845175

endogenous reference gene (HPRT I) was subtracted from the raw C_t value of the genes of interest (MUC1, MUC4, MUC5AC). For comparison between acrolein and sham-challenged vocal folds, the difference between the average ΔC_t value between groups was calculated ($\Delta\Delta C_t$), and the fold change was determined using the formula $2^{-\Delta\Delta C_t}$. Independent *t*-tests ($p < 0.01$) determined whether the average ΔC_t values were different between acrolein and sham-challenged vocal folds for each MUC gene.

Western Blot

Vocal folds were incubated in the 400 μM acrolein ($N = 3$) or sham ($N = 3$) challenge for 60 min at 37 °C. Vocal folds were homogenized in Triton X-100 protein lysis buffer [(20 mM HEPES, 150 mM NaCl, 1 % Triton X-100, 2 mM EDTA, and Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific)] using the PRO200 Homogenizer (*PRO* Scientific, Inc., Monroe, CT). Protein concentration was measuring using BCA protein assay (Thermo Scientific) and Nanodrop 1000 spectrophotometer. Protein aliquots were stored at −80 °C until use for western blot analysis.

For each vocal protein sample, concentrations of MUC1, MUC4, and MUC5AC were measured using western blots. Total protein (20 μg) was subjected to reduce denaturing precast gel electrophoresis (NuPAGE® Novex® 4–12 % Bis–Tris gels) using NuPAGE® MOPS SDS running buffer (Invitrogen, Carlsbad, CA). Proteins were transferred onto a nitrocellulose membrane using an XCell II™ Blot Module and blocked for 60 min at room temperature using membrane blocking solution (Invitrogen). Membranes were probed for MUC1, MUC4, and MUC5AC overnight at 4 °C (Table 3). β-Actin was selected as the loading control. Precision Plus Protein Standards (Kaleidoscope, Bio-Rad) were used to evaluate band size. Bound antibodies were detected using the WesternBreeze® Chemiluminescent Kit-Anti-Mouse kit (Invitrogen). Chemiluminescent signal was captured using an ImageQuant LAS 4000 Mini (GE Healthcare Bio-Sciences, Pittsburg, PA). Relative density of bands was measured using ImageJ analysis software (https://rsb.info.nih.gov/ij) and normalized to the respective band density of β-actin. Mann–Whitney *U*-tests ($p < 0.05$) were used to compare the effects of acrolein and sham challenges on MUC1, MUC4, and MUC5AC expression.

Table 3 Western blot MUC antibodies

Proteins	Species	Concentration	Catalog numbers
MUC1	Mouse monoclonal	1:1,000	Abcam ab70475
MUC4	Mouse monoclonal	1:500	Abcam ab60720
MUC5AC	Mouse monoclonal	1:500	Santa Cruz sc21701
β-Actin	Mouse monoclonal	1:1,000	Abcam ab8226

Results

Vocal Fold Electrophysiology

I_{sc}

Acrolein and sham challenges differentially affected I_{sc} across time as demonstrated by a significant interaction effect for challenge and time ($F_{(4,112)} = 26.89$, $p < 0.001$, Fig. 2). Acrolein challenge significantly reduced I_{sc} by 20 % at 30 min and by 33 % at 60 min ($p < 0.01$), but not immediately following challenge ($p > 0.01$). Acrolein-

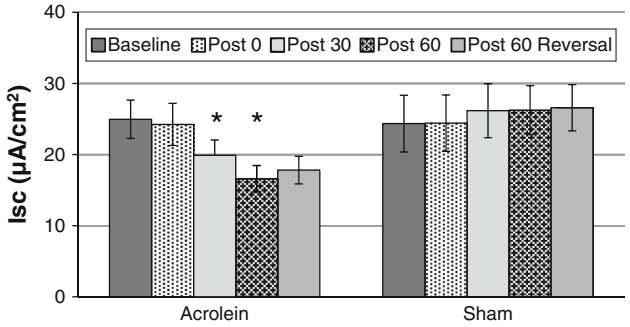

Fig. 2 I_{sc} in vocal folds exposed to an acrolein ($N = 15$) or sham ($N = 15$) challenge. I_{sc} significantly (*) decreased at 30 and 60 min following acrolein, but not sham challenge. Reductions in I_{sc} were irreversible. *Error bars* represent standard error

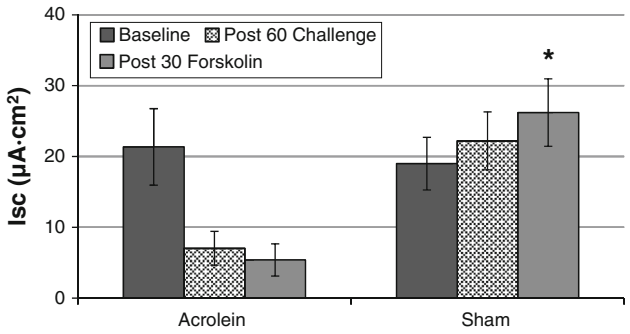

Fig. 3 I_{sc} in response to forskolin in vocal folds exposed to acrolein ($N = 5$) or sham ($N = 5$) challenge. cAMP-stimulated increases in I_{sc} were eliminated in vocal folds exposed to acrolein challenge. Significant (*) cAMP-induced increases in I_{sc} continued to be observed at 30 min following forskolin exposure in sham-challenged vocal folds. *Error bars* represent standard error

induced reductions in I_{sc} were irreversible ($p > 0.01$, Fig. 2). Conversely, sham challenges had no significant effect on I_{sc}. The reductions in ion transport were cAMP-dependent as demonstrated by a significant interaction effect for challenge and time on I_{sc} ($F_{(3,24)} = 12.28$, $p = 0.003$, Fig. 3). Specifically, acrolein, but not sham challenge, significantly inhibited cAMP-dependent I_{sc}. Amiloride and DPC differentially affected acrolein-induced reductions in I_{sc} as demonstrated by a significant interaction effect for ion channel blocker and time ($F_{(3,24)} = 38.50$, $p < 0.001$, Fig. 4). Blocking with DPC preserved acrolein-induced reductions in I_{sc}, while blocking with amiloride obliterated this response. Despite allowing I_{sc} values to stabilize for 10 min post amiloride and DPC addition, a gradual reduction in I_{sc} over 60 min was observed in vocal folds exposed to DPC alone (Fig. 5). However, the reduction in ion transport observed in vocal folds exposed to DPC followed by acrolein is of greater magnitude than when tissues were exposed to DPC alone at 30 min ($p = 0.007$) and 60 ($p = 0.048$) min.

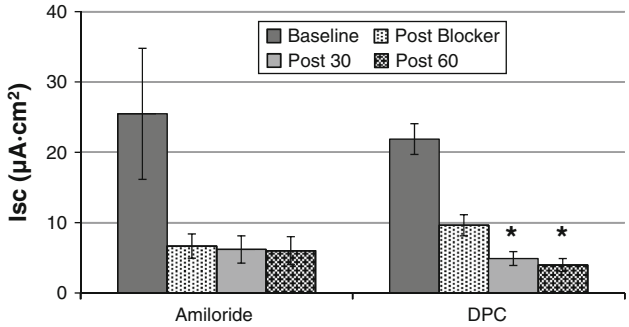

Fig. 4 I_{sc} in response to acrolein challenges in vocal folds exposed to amiloride ($N = 5$) or DPC ($N = 5$). Acrolein-induced reductions in I_{sc} were eliminated in vocal folds exposed to amiloride. Significant (*) acrolein-induced reductions in I_{sc} continued to be observed at 30 and 60 min in vocal folds exposed to DPC. *Error bars* represent standard error

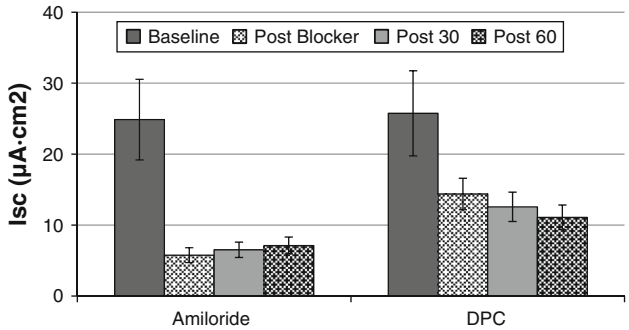

Fig. 5 I_{sc} in vocal folds exposed to amiloride ($N = 5$) or DPC ($N = 5$) alone. *Error bars* represent standard error

R_T

Acrolein significantly reduced R_T from baseline at 60 min ($p < 0.01$). However, mean R_T remained above 300 Ω cm^2 suggesting that the vocal fold epithelial barrier function was adequately maintained.

MUC Expression

Immunohistochemistry

Immunohistochemical staining of positive controls confirmed the specificity of MUC1, MUC4, and MUC5AC antibodies. Mild–moderate MUC1 staining and strong MUC4 staining were observed in both the stratified squamous cells of the vocal fold epithelium and the glands of the submucosa (Fig. 6). but positive staining of MUC5AC was localized only to submucosal glands.

Real-Time PCR

No significant differences were observed between acrolein and sham-challenged vocal folds for genes MUC1

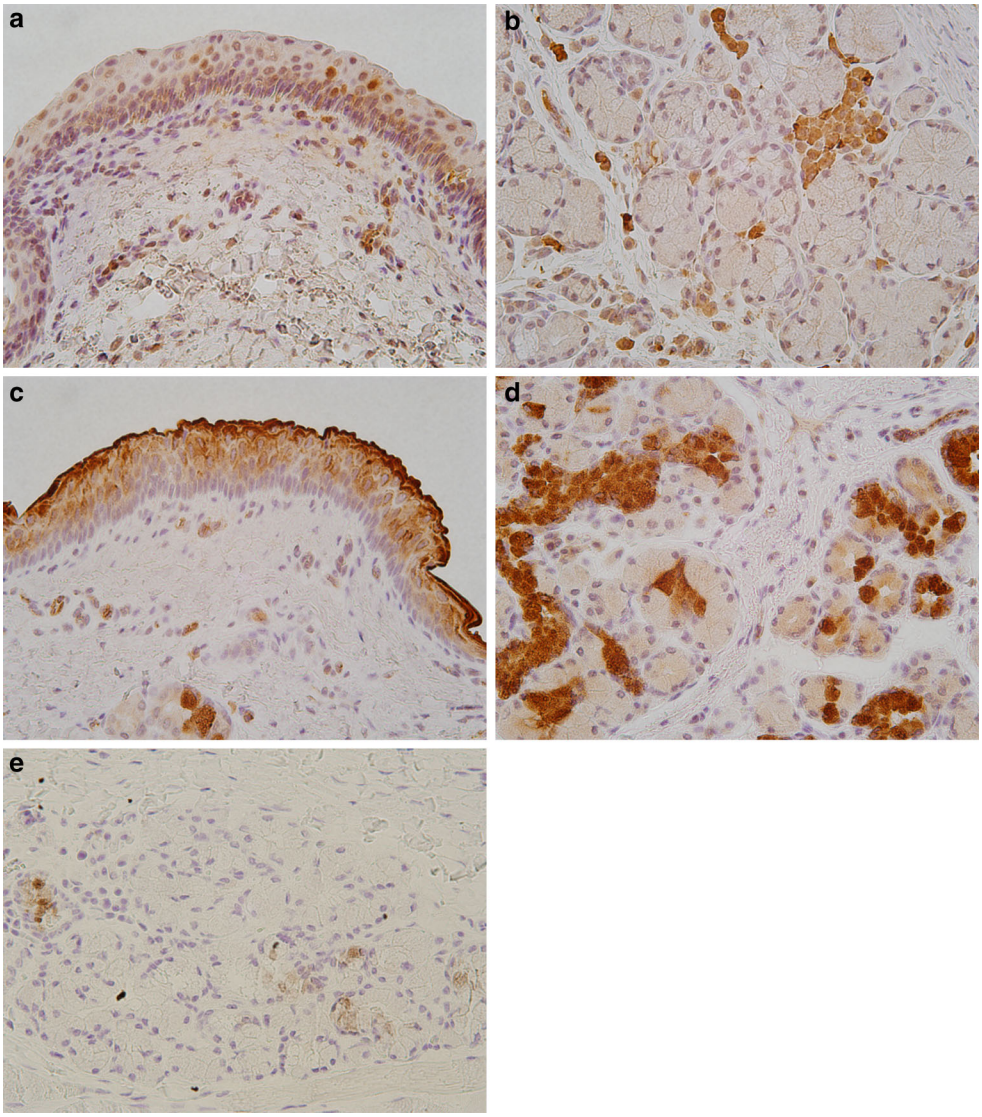

Fig. 6 Light micrographs of representative vocal folds demonstrating immunohistochemical staining of MUC1 (**a**, **b**), MUC4 (**c**, **d**), and MUC5AC (**e**). MUC1 and MUC4 stainings are observed in both cells of the epithelium (**a**, **c**) and submucosal glands (**b**, **d**). MUC5AC staining is only observed in cells of the submucosal glands (**e**)

($t_{(14)} = 1.03$, $p = 0.31$), MUC4 ($t_{(14)} = 1.30$, $p = 0.21$), and MUC5AC ($t_{(14)} = -0.49$, $p = 0.63$; Fig. 7).

Western Blot

No significant differences were observed between acrolein and sham-challenged vocal folds for proteins MUC1 ($U = 4.00$, $p = 0.82$), MUC4 ($U = 2.00$, $p = 0.27$), and MUC5AC ($U = 4.00$, $p = 0.27$; Fig. 8).

Discussion

Narrowing of the airway at the larynx creates a region of high turbulence that can facilitate the deposition of a wide range of pollutants onto the vocal fold epithelial surface (Mouadeb et al. 2009). Epithelial ion transport and MUC glycoprotein synthesis play a critical role in defense and maintain vocal fold surface hydration which is necessary for voice production. To date, the effect of pollutants on these mechanisms remains to be investigated in the vocal folds. In the current study, we investigated whether acrolein affects epithelial ion transport and MUC synthesis. In the distal airway, acrolein rapidly alters epithelial ion transport and MUC synthesis in a manner that may indicate reduced airway defenses (Borchers et al. 1998; Welsh 1983; Alexander et al. 2012). A 60 min acrolein challenge significantly inhibited cAMP-dependent ion transport. The decreased ion transport was associated with reduced Na^+ absorption. Within the same timeline, no significant changes in MUC gene and protein expression were observed.

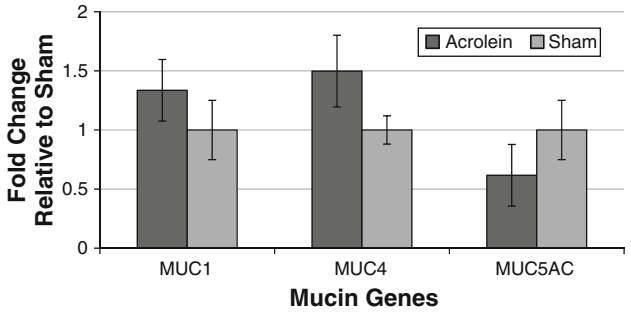

Fig. 7 Fold change in MUC1, MUC4, and MUC5AC gene expression in acrolein ($N = 8$) relative to sham-challenged ($N = 8$) vocal folds. Positive fold-change values indicate an increase in gene expression, while negative fold-change values indicate a decrease in gene expression. The expressions of MUC1, MUC4, and MUC5AC did not significantly change following the 60 min acrolein challenge. *Error bars* represent standard error

Acrolein-reduced vocal fold ion was transport by 20 %. This magnitude of reduction is smaller than the 54 % decrease observed in canine tracheal epithelia exposed to acrolein for 30 min (Welsh 1983). This difference in magnitude of ion transport reduction may be attributed to differences in epithelial cell type between the vocal folds and trachea. The vocal fold epithelium is stratified squamous (Gray 2000), while the tracheal epithelium is pseudostratified ciliated columnar (Knight and Holgate 2003). The density and distribution of ion transport proteins may differ between epithelial types and contribute to the different magnitudes of acrolein-induced reductions in ion transport. Forskolin, a potent cAMP agonist, was employed to determine the cellular mechanisms underlying reduced ion transport (Boucher 1994). Activation of the intracellular messenger cAMP regulates ion transport mechanisms responsible for fluid flow across many types of epithelia (Boucher 1994; Sivasankar et al. 2008; Mahmood et al. 2001). Forskolin increases cAMP concentrations and ion transport in excised porcine vocal folds (Sivasankar et al. 2008). In the present study, acrolein significantly inhibited cAMP-stimulated increases in ion transport. Similar findings have been reported in human bronchial epithelial cells (Raju et al. 2013; Milara et al. 2012).

The effects of acrolein were obliterated when Na^+ absorption was blocked using amiloride. This finding indicates that acrolein-induced decreases in ion transport are due to reduced Na^+ absorption. At the concentration used in the current study, amiloride is a specific inhibitor of the epithelial Na^+ channel (ENaC). Consequently, acrolein-induced changes in ion transport likely involve ENaC channels. It could be argued that since amiloride decreases ion transport, a subsequent acrolein challenge could not elicit further transport reductions. However, the magnitude of reduction in ion transport caused by amiloride was similar to that observed for DPC, a Cl^- channel blocker

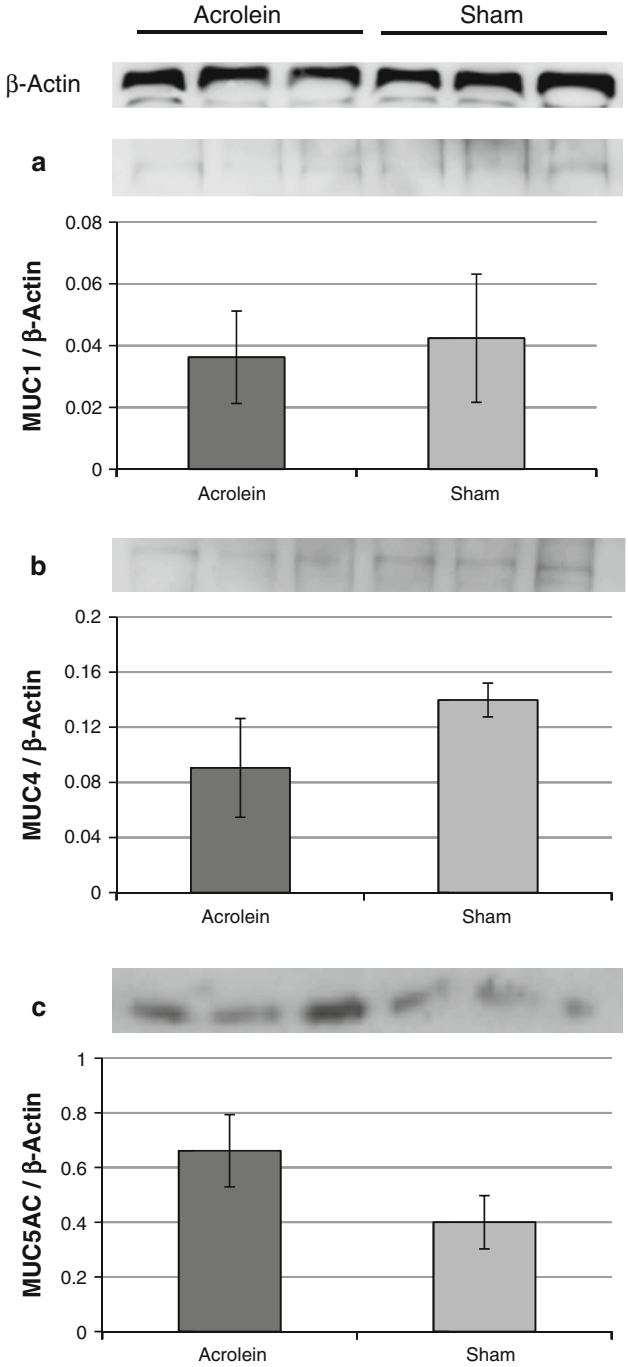

Fig. 8 Western blot analysis of MUC1 (**a**), MUC4 (**b**), and MUC5AC (**c**) in acrolein ($N = 3$) and sham-challenged ($N = 3$) vocal folds. Blots were analyzed by densitometry. Mucin protein expression was normalized to β-actin. The expressions of MUC1, MUC4, and MUC5AC did not significantly change following the 60 min acrolein challenge. *Error bars* represent standard error

($p > 0.05$). Acrolein-induced reductions in ion transport continued to be observed following exposure to DPC supporting the conclusion that acrolein selectively inhibits Na^+ absorption. Ion-driven water fluxes across epithelia regulate the depth of vocal fold surface liquid.

Consequently, by altering ion transport functions, acrolein may diminish the ability of the vocal folds to defend against inhaled particulates. Specifically, reduced Na^+ absorption may slow clearance of particulates. For example, in excised rabbit lung, carbon monoxide-induced reductions in Na^+ absorption result in impaired alveolar fluid clearance (Althaus et al. 2009). The long-term consequences of acrolein on vocal fold fluid clearance and epithelial cell toxicity are unknown, but should be a focus of future investigations.

We tested the reversibility of acrolein-induced reductions in Na^+ absorption by removing the acrolein challenge and monitoring ion transport for an additional 60 min. We found that acrolein-induced reductions in Na^+ absorption were irreversible. In excised canine trachea, reductions in ion transport following a 30 min acrolein challenge were also irreversible (Welsh 1983). Acrolein is a reactive electrophile that readily generates a variety of protein adducts and alters function (Burcham et al. 2010). It is possible that acrolein generates adducts with ENaC causing irreversible reductions in Na^+ absorption.

A 60 min timeline was used in the current investigation. Vocal fold R_T remained above 300 Ω cm^2 for the duration of the electrophysiology experiments. This indicates that effective vocal fold epithelial barrier function was maintained throughout the duration of the acrolein challenge. Vocal folds were exposed to forskolin at the conclusion of the sham challenge to demonstrate the maintenance of tissue responsiveness within our study timeline. Forskolin increased ion transport in sham-challenged vocal folds by an average of 3.99 $\mu A/cm^2$ which is smaller than the mean increase of 12 $\mu A/cm^2$ observed previously in excised porcine vocal folds (Sivasankar et al. 2008). The difference in magnitude between these studies may be associated with forskolin availability. As the luminal vocal fold surface is the likely target of pollutant deposition, in the current experiment we chose to only treat the luminal surface with forskolin. Sivasankar and colleagues, on the other hand, treated both the luminal and basal vocal fold surfaces with forskolin.

In addition to ion and water transport, synthesis and subsequent secretion of MUC glycoproteins are important contributors to vocal fold defense. MUC5AC is the major MUC secreted onto the epithelial surface (Rose and Voynow 2006). MUC1 and MUC4 are epithelial membrane-associated MUCs, but are also released into the mucus that covers the epithelial surface (Williams et al. 2006). All three MUCs have been identified in the surface epithelium and submucosal glands of human laryngeal tissue (Samuels et al. 2008; Kutta et al. 2008) but not in porcine vocal folds. The porcine animal model is an excellent model for laryngeal studies because of its structural similarities to the human vocal folds (Gill et al. 2005). We verified the presence and localization of MUC1, MUC4, and MUC5AC proteins in porcine vocal folds and investigated the effect of a 60 min acrolein challenge on MUC gene and protein expression. No significant changes in MUC1, MUC4, or MUC5AC gene or protein expression were observed following the 60 min acrolein challenge. Within 60 min, a 100 μM acrolein challenge increases MUC5AC gene expression in cultured monolayer of lung epithelial cells (Thompson and Burcham 2008). The fourfold increase in acrolein concentration in the current study (400 μM) may have been insufficient to increase MUC expression in excised stratified squamous epithelia. Our nonsignificant findings may also be attributed to the short challenge duration. It is difficult to compare our MUC1 and MUC4 expression data with the literature as previous studies have not investigated expression of the membrane-associated MUCs. The lack of significant change in expression levels in this study suggests that acrolein may not target these specific MUCs. However, our findings do not negate the need to examine the effects of other chronic pollutants on MUC gene and protein expression in the larynx. In rat lung and trachea, chronic exposure to acrolein increases MUC5AC gene expression and protein synthesis (Borchers et al. 1998). In various animal models, chronic exposure to calcium carbonate, ozone, and tobacco smoke, increase the accumulation of viscous mucus on the vocal fold surface (Leonard et al. 1995; Mouadeb et al. 2009; Marcelino and Oliveira 2005). Increased MUC synthesis may weaken vocal fold defenses by promoting pollutant accumulation and impaired pollutant clearance. Consequently, future investigations should be conducted that use both in vitro and in vivo techniques to quantify the effects of acute and chronic acrolein challenge on laryngeal MUC genes and proteins.

In the current study, we investigated the effect of a 60 min, 400 μM acrolein challenge on ion transport and MUC glycoprotein synthesis in excised porcine vocal folds. To increase generalizability, other pollutant challenges that vary in duration and concentration should be investigated in future studies. Inhalation is the principal route of pollutant exposure in humans. Due to the nature of this in vitro study, vocal folds were bathed in the acrolein challenge; consequently, we would benefit from in vivo animal studies that employ methods of inhaled pollutant delivery. However, the results of this in vitro study are likely indicative of how the vocal folds will respond to similar stresses that occur in vivo. This investigation is the first to demonstrate that acrolein alters a mechanism that is critical to vocal fold epithelial function. Pollutants like acrolein which alter important epithelial functions such as ion transport could hinder effective vocal fold defense. In addition to contributing epithelial defense, ion transport plays a critical role in maintaining vocal fold surface

hydration which is necessary for optimal voice production. However, it is less clear that how the changes in ion transport observed here may impact voice production. Overall, the results of the current study provide a foundation for future investigations that seek to expand our understanding of the effects of a wide range of pollutants on critical vocal fold functions.

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