Mechanisms of Airway Responses to Esophageal Acidification in Cats

by

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Running head: Mechanisms of airway responses to esophageal acidification

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ABSTRACT

Acid in the esophagus causes airway constriction, tracheobronchial mucous secretion, and a decrease in tracheal mucociliary transport rate. This study was designed to investigate the neuropharmacological mechanisms controlling these responses. In chloralose anesthetized cats (N=72), we investigated the effects of vagotomy or atropine (100 μg/kg/30minutes, IV), on airway responses to esophageal infusion of 0.1M PBS or 0.1N HCl at 1ml/min. We quantified: 1) diameter of bronchi, 2) tracheobronchial mucociliary transport rate, 3) tracheobronchial mucous secretion, and 4) the mucous content of the tracheal epithelium and submucosa. We found that vagotomy or atropine blocked the airway constriction response, but only atropine blocked the increase in mucous output and decrease in mucociliary transport rate caused by esophageal acidification. The mucous cells of the mucosa produced more AB (Alcian Blue) than PAS (Periodic Acid Schiff) stained mucosubstances and the mucous cells of the submucosa produce more PAS than AB stained mucosubstances. Selective perfusion of the different segments of esophagus with HCl or PBS resulted in significantly more PAS stained mucus produced in the submucosa of the trachea adjacent to the esophagus receiving HCl than PBS. In conclusion, airway constriction caused by esophageal acidification is mediated by a vagal cholinergic pathway and the tracheobronchial transport response is mediated by cholinergic receptors. Acid perfusion of the esophagus selectively increases production of neutral mucosubstances of the apocrine glands by a local mechanism. We hypothesize that the airway responses to esophageal acid exposure are part of the innate rather than acute emergency airway defense system.

Key words: airway, esophagus, acid, vagus nerve, mucus
New & Noteworthy

This manuscript provides the neurochemical mechanisms for activation of the airway responses to acid exposure of the esophagus only. The airway constrictive response, which does not produce a change in airway resistance, is mediated by vagal cholinergics. The increased mucous secretion is mediated by a local neural pathway that primarily affects the airway apocrine glands. The low amplitude of these responses suggests they are part of the innate rather than acute emergency defense system.
I. Introduction

Prior studies have found that gastroesophageal reflux is sometimes associated with asthmatic symptoms (13, 23), but it was unknown whether these symptoms were caused by stimulation of the esophagus or supra-esophageal structures, because no study of this issue (28, 34, 58) had prevented supra-esophageal reflux, and acid exposure of the larynx (28) can cause significant changes in airway function. We (31) were the first to examine the respiratory effects of exposure of the esophagus to acid while preventing supra-esophageal reflux of acid. We found that esophageal acid exposure significantly decreased the diameter of the smaller airways without causing a change in airway resistance suggesting that this reflex response does not cause asthmatic symptoms. Therefore, we concluded (31) that the airway changes reflexly activated by esophageal acid exposure likely serve a physiological function rather than an emergency protective function or cause a pathological condition.

Our other findings suggested the possible physiological function of the mild bronchoconstriction caused by the esophageal reflex activated by acid. We found that esophageal acid exposure caused a decrease in tracheal mucociliary transport rate, and an increase in tracheobronchial mucus output. Tracheal mucociliary transport rate is indirectly related to the amount of epithelial mucus secreted (43, 49), therefore, it is likely that the decrease in rate of mucociliary transport caused by esophageal acidification was due in part to the increase in mucus output. Considering that mucus is an excellent buffer of acid (15), we hypothesized that an acid-induced esophageal reflex caused mild constriction of small airways, without increasing airway resistance, which assists the expulsion of mucus from the airway mucous glands in preparation for microaspiration of refluxed acid.
Prior studies (34) found that cutting of the vagus nerves blocked the fall in airway conductance caused by esophageal acidification. In these studies the subjects, i.e. dogs, had prior esophagitis and no effort was made to prevent supra-esophageal reflux of the acid infused into the esophagus. In these studies (34), one cannot exclude the possibility that the airway response to the infusion of acid was due to supra-esophageal reflux of acid rather than activation of an esophageal reflex. Therefore, the role of the vagus nerves in the reflex activation of the airway responses to stimulation of the esophagus with acid is unknown.

The role of cholinergic receptors in the airway motor responses to esophageal acidification is unclear. In humans (24), but not in rabbits (19), atropine inhibited the increase in airway resistance caused by esophageal acidification. However, these human studies (24) cannot be considered specific to the esophagus, because no attempt was made to prevent supra-esophageal reflux of acid, and laryngeal acidification-induced airway constriction is mediated by the vagus nerves (58). In the rabbit study (19), very high concentrations (up to 4N) of HCl were used such that the acid may have had a direct and/or non-physiological effect on the airways. Therefore, the role of acetylcholine receptors in the airway responses to esophageal acidification is unknown.

Airway mucus secretion is under vagal control (16, 18, 21, 50), but the mechanism of increased airway mucus secretion in response to reflex stimulation of the esophagus by acid is unknown. Cholinergic receptors have been found to mediate airway mucus secretion stimulated reflexly (21, 50) or by vagal stimulation (16, 18, 47), but the neuropharmacological receptors involved in the airway mucus response to reflex stimulation of the esophagus by acid is unknown.
The airway is not only under extrinsic long arc neural control, but also under extrinsic direct neural control from the esophagus. Two types of direct neural pathways from the esophagus to the airways have been identified. One pathway, which causes a decrease in tone (6, 14), is connected with airway smooth muscle, and a separate pathway is connected with the tracheal ganglia (35). Considering that the tracheal ganglia supply innervation to all effector cells, this esophago-tracheal connection could mediate glandular, i.e. mucous, as well as muscular responses. The role of this direct neural pathway in the mucous response to esophageal acidification has not been investigated.

There are two sources and histochemical types of mucus in the airway (17, 18, 60): acid mucins of the mucosal goblet cells, and neutral mucins of the submucosal apocrine glands. However, the source and type of mucus produced and secreted by the airway in response to esophageal acidification is unknown.

The aims of this study were to investigate whether the vagus nerves, muscarinic cholinergic receptors, or the local connections between the esophagus and trachea mediate any of the airway responses to esophageal acidification and to determine the source of the mucus secreted in response to esophageal acidification. We hypothesize that vagus nerves, local connections between esophagus and trachea, and muscarinic receptors play roles in the airway responses to acid exposure of the esophagus. In addition, we hypothesize that the primary source of secreted mucins in response to esophageal acid exposure is the submucosal apocrine glands.
II. Materials and Methods

A. Animal Preparation

1. General Preparation - We used 72 cats of either sex weighing from 2.2 to 4.7 kg. The cats were anesthetized using alpha-chloralose. Alpha-chloralose was chosen because we have found in over 20 years of research (37) that this is the best anesthetic available to provide a surgical level of anesthesia while preserving reflex responsiveness in cats. Alpha-chloralose was used in a specific manner and in conjunction with other anesthetics, as described below, to obtain a surgical level of anesthesia in all cases and different protocols were used based on the extent of surgical preparation. The specific anesthetic protocols were selected in consultation with and approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin.

For the studies on airway diameter, mucociliary transport, and mucus secretion alpha-chloralose was titrated to effect to ensure that all animals reached the same surgical level of anesthesia. Alpha-chloralose was initially given at 55 mg/kg IP and if the animals had not attained a surgical level of anesthesia in one hour, they were supplemented with 25% of the original dose. If after 45 minutes, they were still not at a surgical level of anesthesia they were administered pentobarbital (3 mg/kg, IV). Fewer than 5% of the animals required pentobarbital. For the experiments involving histology, the cats were placed in an anesthetizing box and anesthetized using isoflurane (5%). Once anesthetized, the cats were removed, placed on their back, and anesthesia maintained with isoflurane (3%) using a mask. After the appropriate surgeries described below were performed, alpha chloralose (60 mg/kg) was injected slowly
intravenously as the concentration of isoflurane was reduced to maintain anesthesia during the experiments. Experiments were begun at least one hour after isoflurane was discontinued.

The femoral vein was cannulated for infusion of 0.9% NaCl for hydration. In all cats the esophagus was cannulated just distal to the upper esophageal sphincter (UES) and just proximal to the lower esophageal sphincter (LES) using 0.5cm diameter Tygon tubing. The opposite end of the UES cannula exited the oral cavity. The opposite end of the LES cannula exited the digestive tract through the stomach wall and the abdomen through the abdominal incision. A gastric fistula was created in the fundus to allow continuous evacuation of gastric secretions. The animals were killed after the study using a pentobarbital based euthanasia solution (600 mg, IV)

2. Specific preparations

a. Vagotomy - The vagus nerves were carefully separated from the carotid sheaths and a thread placed, but not tied, around the vagus nerves. These ties were exited through the neck wound and used during the study to pull the vagi out of the neck when appropriate to allow sectioning them without disturbing the trachea or esophagus.

b. Bronchial diameter – Tantalum bronchograms were obtained as described previously (31). The trachea was intubated and the cats were placed supine onto the micro-focal x-ray system stage for imaging the bronchi. The bronchi were dusted with fine tantalum powder injected in the air inflow from a ventilator set at 2-3 times tidal volume for 3-5 breaths. The goal was to finely and evenly coat the airway with tantalum dust. After airway dusting, the airway was imaged and the study begun. Image data was saved on computer for post-acquisition analysis.
Tantalum has been found to be chemically inert (39) causing no inflammation of the airways or lungs and no significant changes in blood gases or measures of lung function, and in our prior study (31), we found that lung mechanics did not change in response to esophageal acidification whether exposed to tantalum or not.

c. **Tracheobronchial mucociliary transport** – Tracheobronchial mucociliary transport was measured as described previously (31). Cats were placed onto the micro-focal x-ray imaging system stage and a small hole was made in a tracheal cartilage ring using a cautery. Care was taken to avoid bleeding into the trachea as bleeding retarded mucociliary transport. A tantalum disc injection device was inserted into the hole in the tracheal cartilage and tantalum discs (1mm diameter, 0.3ug) were injected as far distally in the airway as possible. The discs usually landed near the carina. The disc movement through the trachea by mucociliary transport was recorded using micro focal x-ray system and stored on computer. At a later date the velocity of orad travel of the tantalum discs was measured. Between injections of discs a saline soaked gauze pad was placed over the hole in the tracheal cartilage. After transiting through the trachea, the tantalum discs deposited on the hard palate. An endotracheal tube was not placed in these studies in order to disturb the trachea as little as possible.

d. **Tracheal mucus output.** - The trachea was transected just distal to the cricoid cartilage and a Y-shaped endotracheal tube inserted. The cat was then placed at 10° angle head down to promote outflow by gravity. Polyethylene (PE)-50 tubing attached to a syringe pump was inserted in the non-dependent arm of the endotracheal tube until resistance was met. The catheter was then withdrawn 0.5 cm and taped to the endotracheal tube. On average the tube was inserted
about 10 cm from the larynx. Saline (0.9% NaCl) was injected at 0.25 ml/min, and the perfusate collected every 30 minutes over ice. The volumes of the samples were determined, and the hexosamine concentrations quantified.

e. Effect of selective perfusion of the esophagus - The esophagus was ligated 4 cm from the cricoid cartilage forming two esophageal sections, proximal and distal. Tubing (PE50) was inserted into the ends of the larger catheters already implanted, as described above, and advanced to the point of the esophageal ligation. This arrangement allowed for selective perfusion of each esophageal segment as fluid entered the esophageal sections through the smaller catheters and exited through the larger catheters.

B. Protocols

In all studies the esophagus was perfused with either 0.1M PBS (PBS) or 0.1N HCl (HCl) at 1 ml/min. When atropine was given, it was administered at 100 μg/kg IV 30 minutes until the end of the experiment.

1. Role of vagus nerves or cholinergic receptors

a. Bronchial Diameter - The esophagus was perfused with PBS or HCl throughout the experiment and microfocal x-ray images of the bronchus were taken every 30 minutes to quantify bronchial diameter. The esophagus was first perfused for one hour with PBS and the vagus nerves were transected or atropine administered after the first 30 minutes of PBS. The perfusate was then changed to HCl for 30 minutes and then changed back to PBS for 30 minutes.
b. Tracheal mucociliary transport - While taking microfocal x-rays of the trachea, the esophagus was perfused with PBS. Tantalum discs were then injected into the trachea one at a time and their position captured and recorded at intervals during their movement orad from the digitized radiographic images of the trachea. The vagus nerves were then transected or atropine administered, and tracheobronchial transport tested again. The esophageal perfusate was then changed to HCl for 30 minutes and tantalum discs were again injected into the trachea one at a time and their rate of movement recorded over the next hour.

c. Broncho-tracheal mucous output - We perfused the airway with 0.9% NaCl at 0.25ml/min for the entire experiment and collected mucous output every 30 minutes. For the first 60 minutes the esophagus was perfused with PBS. The first 30 minutes allowed for the system to stabilize and the second 30 minutes was used as the control period for statistical comparison. The vagus nerves were then transected or atropine administered and the esophagus perfused with PBS for another 30 minutes. The esophageal perfusate was then changed to HCl for 30 minutes and then back to PBS for another hour.

2. Control of mucous production

Selective perfusion - Either the proximal or distal esophageal segment was perfused with HCl for 30 minutes followed by PBS for another 30 minutes. At the end of the experiment the trachea adjacent to the separate esophageal segments was removed and placed in 10% formalin for later histological analysis. The two separate tracheal segments were 1 to 3 cm and 5 to 7 cm from the cricoid cartilage.
C. Techniques

1. X-ray image system: The micro-focal x-ray system is composed of a Fein Focus-100 x-ray source (3 mm focal spot), a North American Imaging AI-5830-HP image intensifier coupled to a Silicon Mountain Design (SMD) CCD, and a New England Affiliated Technologies specimen micromanipulator stage all mounted on a precision rail with position information provided by Mitutoyo linear encoders. The geometry of the system allows magnification of the specimen to be adjusted by changing the specimen's proximity to the x-ray source. The image data is sent from the CCD to a frame grabber board mounted in a Dell 610 workstation running Windows NT operating system.

2. Bronchial diameter measurement.
   The lungs of the cat were visualized using the micro focal x-ray system as described previously (31). Appropriate lung field was selected that contained airways of many different diameters and in which the tantalum had dusted the airways well enough to measure the diameters and evenly enough so that clumping of tantalum did not occur. The x-ray stage and image intensifier were then fixed at this position and radiographic images of the lung field were obtained every two minutes throughout the study. The diameters of various airways were quantified at a later date as described below in section C. Analysis Techniques. The control values were obtained during the last 5 minutes of the initial 0.1M PBS perfusion, because the preparation is well stabilized by this time.
3. Quantification of bronchial diameters.

The diameter of the airways was determined in an objective fashion as described previously (31) using mathematical models originally developed for the quantification of pulmonary vasculature (9) and later adapted for the airways (55). One of two methods was used depending on the size and location of the airway. For small and intermediate diameter airways (< 2.1 mm), we used the mathematical model. This method is optimal when there is sufficient distance between airway segments of interest and there is a region of background on both sides of the airway. This is commonly the case for the intermediate and smaller airways. For large (> 2.1 mm) airways the diameter can be measured by manually placing a line Region of Interest (RO) perpendicular to the axis from edge-to-edge of the airway.

The radiographic image is a two-dimensional projection of a three-dimensional object, i.e. the lungs, and objects closer to the x-ray source appear larger in the image. Therefore, each measurement of diameter in the radiographic image was calibrated separately. Distance within the plane of the radiographic image was calibrated by moving the cat a known distance perpendicular to the x-ray beam while recording the image. The distance moved in pixels of a shadow of an airway of interest in the image per μm of movement was the distance calibration factor for that airway.

4. Quantification of tracheal mucociliary transport rate

Tracheal mucociliary transport rate was determined by acquiring images of the trachea while the injected tantalum disc traverses the field of view as described previously (31). This technique was adapted from similar techniques used by others (8, 62). We calculated the
transport rate by allowing the disc to traverse as much of the field of view as possible and
determining the time and distance traveled. The average velocity was calculated as the slope of
the regression line of the distance vs time of travel of each disc. The velocity of at least three
discs for each experimental condition was determined and averaged to obtain the representative
average value for that experimental condition.

5. Quantification of mucous output.

We quantified mucous secretion based on concentration or a major constituent of mucus, rather than volume of fluid collected from the trachea because in preliminary experiments we
found that the volume of fluid collected from the trachea changed little during the experiments.
Hexosamines are major constituents of mucus (26, 29) and hexosamine concentration of the
perfusates was determined using a modification of a standard technique (59) we developed
previously (32). The hexosamine output varied considerably amongst animals and across time,
therefore, we quantified the output every 30 minutes and expressed the output as a percent of the
initial hexosamine output for each animal.

6. Quantification of tracheal mucous content

Prior studies have suggested that quantitative histochemistry of the trachea should be
done using longitudinal rather than cross sections, because of the significant variation in the
amount of submucosal glands in longitudinal direction (26). The majority of the tracheal
submucosal glands are found between the cartilage rings (7). However, not only is there
variability in the glands in cross section, but also longitudinally. Most of the glands are located in
the dorsolateral portion of the cross section (7), i.e. at 4 and 8 o'clock positions with 12 o'clock
being the most ventral point. Because our goal was to compare tracheal regions along the longitudinal axis we took into consideration the variability in location of the submucosal glands in all planes. We sliced the trachea in cross section primarily in between the cartilages, and we selected those cross sections to quantify which had the largest extent of submucosal glands at their 4 and 8 o'clock positions. We quantified mucous content as a percentage of the total area of the epithelial layer, i.e. mucosa and submucosa. Therefore, our measure of mucous content optimized the sections with the largest amount of submucosal glands in all planes, but also quantified mucus as a percentage of the total area of the epithelial layer in order to minimize variability among animals.

The fixed tracheal segments were imbedded in paraffin and sliced at 5 μm and stained with AB (Alcian Blue at pH 1.0) and PAS (Periodic Acid Schiff reaction). The sections were then magnified 10x (Olympus BH-2) and micrographs (Spot RT digital microscope camera) of the entire sections taken (Fig. 1). It required from 14 to 18 pictures to cover the entire tracheal section and we micrographed two complete tracheal sections, selected as described above, for each experimental group. In the longitudinal direction of the trachea the number of mucous cells is greatest between cartilages, therefore, we targeted this region. Because the number of mucous cells varied longitudinally we quantified all the mucous cells of two tracheal sections to control for sample bias. The results of the two sections were averaged.

There are two types of mucous glands differentiated by anatomical position (Fig. 1): mucosal and submucosal, and two types of mucus differentiated by staining properties (Fig. 1): acidic, stained by AB (blue color) and neutral, stained by PAS (pink color). The areas of total,
neutral, and acidic mucus were quantified for both mucosal and submucosal mucous glands using Image J software.

a. Total Mucus - Using Image J software the RGB color space of the image was first auto color balanced and then split such that green was used for quantifying mucus within the submucosa, red for quantifying mucus in the mucosa, and blue was discarded. The areas of mucus in each epithelial layer, i.e. mucosa and submucosa, were quantified using the area function such that the area threshold just filled the mucous cells to capacity. These threshold values differed for each image and were saved for quantification of type of mucus below. The percent mucus in each layer was calculated by dividing the area of mucus in each layer by the total area of that layer, i.e. mucosa or submucosa.

b. Type of Mucus - The area of tissue containing neutral (PAS stained) and acidic (AB stained) mucosubstances were separated by adjusting the threshold of the hue of the RGB color space of the color balanced image using the color threshold function. The threshold that maximally separated these areas was selected, and it averaged 187±3 (N=26) pixel intensity value (out of a maximum of 256). The thresholded image was stop filtered to create the AB stained image (Fig. 1), and pass filtered to create the PAS stained image (Fig. 1). The areas of the mucosa and submucosa containing mucus of the thresholded images, i.e., Alcian Blue and PAS stained, were then quantified as described above for total mucus using the same area thresholds. The percent of each type of mucus, neutral (PAS stained) and acidic (AB stained), in each epithelial layer, i.e., mucosal or submucosal, was calculated.
C. Statistics

For studies of multiple experimental means, ANOVA followed by a multiple comparison test was used. When the comparison was made over time the repeated measures ANOVA was used, and when the comparison was across time a one-way ANOVA was used. When the objective was to compare all groups with each other Tukey's multiple comparison's test was used. For the histological studies, differences between the control and experimental areas were tested using Wilcoxon matched-pair signed rank test. The data is listed as mean ± SE and a P value of 0.05 or less was considered statistically significant.
III. Results

A. Effect of Vagotomy

1. Airway Diameter - After cutting the vagus nerves (N=7) the airways of all diameters significantly dilated (Figs 2 and 3A) on average 35% (Fig 3A), while the esophagus was perfused with PBS. Changing of the perfusate to HCl had no significant effects on the airways of any diameter for up to 60 minutes after HCl administration (Figs 2 and 3A).

2. Mucociliary transport - After cutting the vagus nerves (N=5) there was no significant change in tracheal mucociliary transport rate (Fig 4A) while the esophagus was perfused with PBS. However, after the perfusate was changed to HCl the tracheal mucociliary transport rate was significantly reduced by about 75% during the first 30 minutes and 50% for up to 1 hour after the start of HCl perfusion (Fig 4A).

3. Mucous output - We found that compared with the control period of the first 30 minutes of PBS perfusion of the esophagus (N=7), the basal level of hexosamine output continually declined over time (Fig 5, PBS graph). When we statistically compared the effects over (Fig 5) or across (Fig 6) time, we found that hexosamine output significantly increased after esophageal acidification (Fig 5, HCl graph; Fig 6, 60 to 90, 90 to 120 and 120 to 150 min graphs; (N=7)). Vagotomy (N=8) did not significantly alter the hexosamine output response to HCl during the first 30 minutes of HCl administration (Fig 6: 60-90 min), but did significantly reduce the response to esophageal acidification from 30 to 90 minutes after HCl administration (Fig 6: 90-120 min and 120-150 min graphs).
B. Effect of Atropine

1. Airway diameter – After the administration of atropine began (N = 7) the airways of all diameters significantly dilated (Fig 3B) on average 32% while the esophagus was perfused with PBS. Changing of the perfusate to HCl had no significant effects on the airways of any diameter for up to 60 minutes after HCl administration (3B).

2. Mucociliary transport - After the beginning of atropine administration (N = 5), there was no significant change in tracheal mucociliary transport rate (Fig 4B), while the esophagus was perfused with PBS. In addition, after the perfusate was changed to HCl there was no significant change in the tracheal mucociliary transport rate for up to 1 hour after the start of HCl perfusion (Fig 4B).

3. Mucus output - Atropine (N=10) caused no significant change in basal mucous output (Figs 5; HCl-Atropine graph), but it significantly reduced the increase in mucous output caused by esophageal acid exposure across all time periods (Fig 6; 60 to 90, 90 to 120, 120 to 150 min after control).

C. Control of Mucous Production

1. Selective perfusion - We found that selective perfusion of a segment of the esophagus (N=11), i.e. proximal or distal cervical esophagus, with HCl (pH=1.2) or PBS (pH=7.4) resulted in significantly more total mucous produced in the submucosal glands of the trachea adjacent to the segment that received HCl than received PBS (Fig. 7). This relationship of tracheal mucous production related to adjacent esophageal pH was also observed in the mucosa when HCl was
perfused into the distal esophageal segment. When HCl was perfused into the proximal segment no relationship with mucous production of the mucosa in adjacent tracheal segment was observed (Fig 7).

2. Type of mucus - We found that the primary type of mucus produced by the submucosal glands of the trachea is stained by PAS, and produced by the mucosal glands is stained with AB (Fig 7). Perfusion of either segment, proximal or distal cervical, of the esophagus with HCl or PBS resulted in significantly more PAS-stained mucus produced in the submucosal glands of the trachea adjacent to the esophageal segment that received HCl than received PBS (Fig 7). The same relationship of tracheal mucus production related to adjacent esophageal pH was also observed for the PAS-stained mucous production of the mucosal glands when HCl was perfused through the distal esophageal segment. The production of AB-stained mucus was not related to the pH of either segment for either the mucosal or submucosal glands except for one situation. In the case where HCl was perfused through the distal cervical esophageal segment and PBS perfused through the proximal esophageal segment, the PAS-stained mucous production of the mucosa was significantly more in the trachea adjacent to the HCL-perfused distal segment than the PBS-perfused segment (Fig 7).
IV. Discussion

We found that there was no airway diameter response to esophageal acidification after vagotomy or atropine, but our prior studies found (31) that esophageal acidification significantly decreased the diameter of the airways. Therefore, the airway constriction response to esophageal acidification is mediated by vagal cholinergic fibers.

This finding is consistent with prior studies which found that the primary reflex pathway controlling airway smooth muscle (5, 6, 60) as well as mediating airway resistance (60) and airway diameter (60) is composed of vagal cholinergic fibers. In addition, vagal stimulation has been found to primarily constrict the smaller airways (4), and these are the airways reflexly constricted by exposure of the esophagus to acid (31).

We also found that there was no mucociliary transport response to esophageal acidification after atropine administration, but our prior study (31) found that esophageal acidification significantly decreased mucociliary transport. Therefore, cholinergic receptors mediate the mucociliary transport response to esophageal acid exposure. In addition, we found that atropine blocked the mucous output response to esophageal acid exposure indicating that the mucous response is also mediated by cholinergic receptors. These findings are consistent and support each other as mucociliary transport rate is inversely related to mucous output (49, 54).

This finding is supported by prior studies which found that tracheal mucous secretion activated reflexly (21, 25, 44), neurally (2, 3, 53) or by vagal (16, 16, 42, 47, 57) stimulation are mediated by cholinergic receptors.
We found that vagotomy did not block the initial effects of esophageal acidification on increasing mucous output or prevent a significant decrease in tracheal mucociliary transport rate caused by esophageal acid exposure. These findings are consistent with each other as there is an inverse relationship between mucous output and mucociliary transport rate (49, 54). On the other hand, vagotomy significantly reduced mucous output after 30 minutes of esophageal acidification at a time when the mucociliary transport rate was still significantly reduced. This suggests that mucociliary transport rate and mucous output are not always inversely related, and that the vagus nerves may affect this relationship. There are many possible explanations for these results as there are many factors that control mucociliary transport rate and mucous output.

Mucociliary transport rate is dependent upon the viscosity and elasticity of the mucus lining the airway (30, 51), depth of mucous layer (51), depth of periciliary fluid layer (51), and ciliary beat frequency and velocity (10, 49, 51, 63). Mucous output depends on the source of the mucus, goblet vs apocrine gland cells, as the different sources have different constituents (17, 18, 29, 52), and how the mucus is measured (2, 3, 11, 16-18, 29-31, 45, 47, 48, 52, 57), as there are many different constituents of mucus which can be neurochemically altered (16-18, 29).

Considering that we have not recorded all of these factors, we cannot make conclusions about this observation, therefore, more studies are needed to resolve this issue.

Our finding that the vagal innervation had no role in the initial effects of esophageal acidification on mucous output seems to contradict prior studies that tracheal mucous output is mediated by the vagus nerves (2, 3, 16, 18, 21, 25, 42, 47, 50). However, our results do not contradict other studies they show that there are more ways to activate mucous secretion than through the vagus nerves, as described below.
There are two sources of mucus in the airways: goblet cells of the mucosa and apocrine gland cells of the submucosa, but the apocrine gland cells outnumber the goblet cells (48). We found similar or higher percentage area of mucus in the mucosa than submucosa, but this was because the total area of submucosa was larger than the mucosa. Studies in cats have found that most secretions collected from the trachea in response to neural or chemical stimulation come primarily from the submucosal rather than goblet cells (17, 18, 29, 52). In addition, studies in cat trachea (52) have found that cholinergic agonists do not stimulate secretion from goblet cells. Therefore, it is likely that most of the mucus collected in our studies came from the submucosal apocrine glands rather than the mucosal goblet cells.

We found that the amount of mucus produced by the trachea was related to the pH of the adjacent esophagus. Therefore, it is likely that there is a local connection between the esophagus and adjacent trachea controlling this effect. We hypothesize that this local connection is mediated by the previously defined direct neural pathway from the esophagus to the trachea ganglia (35). Further studies are needed to confirm this relationship and to identify the local mediator.

We also found that the majority of the mucosubstances within the submucosal apocrine glands and activated by esophageal acid exposure are neutral as they are mostly stained by PAS (20, 26, 36). On the other hand, the majority of mucosubstances of the mucosal goblet cells are acid mucosubstances as they stain primarily by AB (20, 26, 36). Therefore, the esophageal acid exposure is associated with the production of neutral mucosubstances of the submucosal apocrine glands of the adjacent trachea. Acid mucosubstances are produced and secreted by
mucosal cells in position to provide defense against bacteria, e.g. colon (45), whereas neutral mucosubstances are produced and secreted primarily by submucosal glands in position to defend against acid, e.g. duodenum (38, 46). Therefore, the increased production of PAS-stained mucus from submucosal glands of the trachea with acid exposure of the adjacent esophagus is the appropriate response to the possible threat of acid exposure of the airway.

Acid perfusion of the distal segment of the esophagus seemed to have a greater and more widespread, i.e. both mucosal and submucosal glands were effected, effect on mucous production of the adjacent trachea than the proximal segment. This larger effect may have been due to the larger stimulus as the distal segment encompassed three times longer length of esophagus than the proximal segment.

A significant difference between our studies and those of others which investigated the role of extrinsic reflexes or vagal stimulation in the control of tracheal mucous output was the magnitude and duration of the responses. In our study the magnitude of the mucous response was relatively low, at most 150% of control, but the duration was long, at least 60 minutes after the stimulus. However, others stimulating extrinsic reflexes (25, 50) or the vagus nerves (16, 18, 42, 47) recorded mucous responses many fold greater than the control response which lasted not much longer than the duration of the stimulus. This dichotomy suggests that there may be two types of tracheal mucous secretions that are controlled differently.

Prior studies have found that there are two basic types of responses of the airway submucosal glands (61): an innate mucosal defense system and an emergency airway defense
system. The innate system primarily regulates housekeeping functions by the secretion of mucus onto the airway surface to aid clearance and protection from physiological levels and types of offending substances like bacteria. Studies (1, 18) have found that the resting tracheal mucous output is not vagally dependent, and because this is the resting mucous output it is likely to be part of the innate defense system. The innate system is largely locally controlled and its responses are moderate. On the other hand, the emergency system reacts to remove and neutralize non-physiological destructive substances like foreign bodies. The emergency response is large and extrinsically controlled. Therefore, based on our findings in this study, we conclude that the tracheal mucous response to acid exposure of the esophagus is primarily part of the airway innate defense system controlling housekeeping functions and not part of an extrinsic emergency response. This conclusion is also consistent with our finding that esophageal acid exposure caused reflex contraction of the smaller, rather than larger airways, causing no change in airway resistance.

The nature of the airway response to esophageal acid exposure as part of the innate defense system is not only consistent with our results, but consistent with the nature of esophageal acid exposure. In normal individuals the esophagus is often exposed to gastric reflux (41) and microaspiration of gastric juice occurs in normal individuals (40). In addition, microaspiration has been associated with a number of airway symptoms (27) and diseases (21, 56). Therefore, just as the airway must provide a constant defense system to bacteria it must also provide a constant defense system to acid. While mucosubstances produced and secreted by the mucosal cells are particularly effective against bacteria (33, 45), mucosubstances produced and secreted by submucosal glands are particularly effective against luminal acid (12, 22).
Limitations

In the current study we did not conduct control studies on the effects of esophageal acid exposure on airway diameter or mucociliary transport, because these studies were conducted in our prior study (31) and repeating them would have violated the principle of the Animal Welfare Act which calls for minimization of the number of animals used for experimentation. However, we did conduct control studies in experiments which were significantly different from our prior study, i.e. mucous output, or new, i.e. mucous production. The mucous output study was different in that the cats were placed at 10° head down to assist in the collection of mucus. However, this resulted in a stronger stimulation because gravity caused the acid to remain longer in the cervical region of the esophagus and the result was a longer lasting mucous response.

In summary, esophageal acidification causes four airway responses: airway constriction primarily of the smaller diameter airways, increased mucus, i.e. hexosamine, output, deceased broncho-tracheal mucociliary transport rate, and increased mucous production primarily by the apocrine glands. Our data suggests that acid exposure of the esophagus causes reflex constriction of the airway through a vagal cholinergic pathway, and reflex inhibition of tracheal mucociliary transport through a non-vagal cholinergic pathway. The tracheal mucous output response to esophageal acid exposure is partly mediated by an immediate non-vagal cholinergic pathway and a delayed vagal cholinergic pathway. There also exists a delayed stimulation of production of neutral mucosubstances by the apocrine gland cells possibly through a local neural pathway. Considering the delayed nature and the low magnitude of the mucus responses, the acid buffering capacity of apocrine gland secretion, and the local neural control of the apocrine gland response, we hypothesize that the airway responses to esophageal acidification are part of the innate rather than acute emergency defense system of the airway.
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References:


Figure Legends

Figure 1. Technique for separation of PAS and AB stained sections. The original image is a gray scale image of the original PAS/AB stained tracheal section. The PAS and AB images are the appropriately filtered (as described in the Methods) original images that illustrate the type of images used to quantify the relative amounts of PAS and AB stained mucous in the tracheal sections. PAS, Periodic Acid Schiff; AB, Alcian Blue.

Figure 2. Radiographic evidence of the effects of vagotomy on the airway diameter responses to acid exposure of the esophagus. This figure is a series of radiographs of the airways of a cat with tantalum-dusted airways. Note, by comparing regions pointed out by the arrows, that vagotomy resulted in an increase in diameter of airways, but blocked the airway diameter response to esophageal acidification.

Figure 3. Effects of vagotomy or atropine on the airway diameter responses to acid exposure of the esophagus. The airways were grouped into three categories based on diameter and compared over time during response to different esophageal perfusates, i.e. PBS or HCl. Two groups of cats were used to test the effects of vagotomy (A) or atropine (B) on the airway diameter response to esophageal HCl exposure. Note that either vagotomy (A) or atropine (B) significantly increased the diameter of all three sizes of airways, but that exposure of the esophagus to acid under either experimental treatment had no significant effect on airway diameters. *, P<0.05 for a difference between each period after vagotomy or atropine and the corresponding control value using ANOVA with repeated measure and Tukey’s multiple comparison test, N=7 for each study.
Figure 4. Effects of vagotomy or atropine on the tracheal mucociliary transport rate response to acid exposure of the esophagus. This graph depicts the results from two groups of cats used to test the effects of vagotomy (A) or atropine (B) on the mucociliary transport response to esophageal HCl exposure. Note that vagotomy or atropine had no significant effect on tracheal mucociliary transport rate. On the other hand, esophageal acid exposure significantly decreased mucociliary transport after vagotomy but not after atropine administration. * or #, P<0.05 for ANOVA with repeated measures with Tukey's multiple comparison test; *, compared to PBS before vagotomy; #, compared to PBS after vagotomy, N=5 for each study.

Figure 5. Effects of vagotomy and atropine on tracheal mucus output stimulated by acid exposure of the esophagus: comparison within treatment. These graphs depict the change in mucous output over four time periods during four different experimental treatments. All values are the percentage of the control measure obtained during the initial 0-30 minutes of perfusion with PBS of the esophagus. In those studies that received an experimental alteration, it was begun at the end of the 30 minute control period. In those groups that received HCl, it was administered between 60-90 minutes of the experiment. Note that HCl significantly increased mucus output for the first hour, and that vagotomy blocked this response at all time periods while atropine block at all time periods but the first caused by acid exposure of the esophagus. *, P<0.05 for difference among the different time periods during each experimental treatment using ANOVA with repeated measure and Tukey's multiple comparison test.
Figure 6. Effects of vagotomy and atropine on airway mucus output stimulated by acid exposure of the esophagus: comparison across treatment. These graphs depict the change in mucous output across experimental treatments during the same time period. All values are the percentage of the control measure obtained during the initial 0-30 minutes of perfusion with PBS of the esophagus. In those studies that received an experimental alteration, it was begun at the end of the 30 minute control period. In those groups that received HCl, it was administered between 60-90 minutes of the experiment. Note that HCl significantly increased mucus output, and that atropine blocked this response at all time periods. On the other hand, vagotomy did not block the initial mucus output caused by esophageal acidification, but did block the delayed increase in mucus output. * or #, P<0.05 for ANOVA with Tukey’s multiple comparison test; *, compared to PBS; #, compared to HCl. N=5, 7, 8 and 10 for groups PBS, HCl, HCl-vagx and HCl-atr

Figure 7. Effect of selective perfusion of the proximal and distal esophageal segments on mucous production of the adjacent trachea. Graphs of the percent areas of the submucosa and mucosa of tracheal transections containing material stained with AB, PAS, or both. These tracheal transections were adjacent to the esophageal segments that contained either PBS (pH=7.4) or HCl (pH=1.2) and were labelled according to which fluid the adjacent esophageal segment had been exposed. Note that whether proximal or distal esophageal segment was perfused with HCl, the trachea adjacent to the esophagus which received HCl had significantly more mucous production than the trachea adjacent to the esophageal segment that received PBS. The only exception was the mucous production of the mucosa when the proximal esophageal segment was perfused with HCl. Therefore, mucous production of the trachea, especially of the submucosal glands, is highly related to the acid level of the corresponding adjacent esophagus. Prox,
proximal, Dist, distal; AB, Alcian Blue stained area; PAS, Periodic Acid Schiff stained area; Total, total area. *, P<0.05 in a Wilcoxin matched-pair signed rank test comparing adjacent regions of the trachea, each containing fluid of different pH (N=11).
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6

![Graph showing % Change from Control Period in Hexosamine Output for different experimental groups at 30-60 min, 60-90 min, 90-120 min, and 120-150 min.](image-url)
Figure 7