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The Impact of Bilateral Vagotomy on the Physostigmine-induced Airway Constriction in Ferrets

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Introduction

The respiratory airways are innervated abundantly by autonomic sensory and motor nerves (Undem and Potenzieri, 2012). The autonomic nervous system controls airway diameter and bronchial tone (Scott and Fryer, 2012). Even though both sympathetic and parasympathetic components innervate the airway, the parasympathetic branch dominates throughout control of airway smooth muscle tone and secretions (Undem and Potenzieri, 2012). In general, a complex neural network functions through airway-related, medullary vagal preganglionic neurons to control the airway. These input pre-pre-ganglionic neurons originate from several discrete brainstem nuclei (Haxhiu *et al.*, 1993; Jordan, 2001). In turn, the medullary pre-ganglionic vagal neurons transmit signals to the intrinsic tracheobronchial ganglia and the postganglionic neurons regulate the functions of specific targets (Baker *et al.*, 1986).

The parasympathetic cholinergic network is the primary neural pathway responsible for bronchoconstriction in various mammal species, including humans (Scott and Fryer, 2012). Acetylcholine (ACh), which is released from cholinergic nerve endings, increases airway tone and mucus secretion via muscarinic receptors (Dehkordi *et al.*, 2004)(Coulson and Fryer, 2003). The muscarinic receptor subtypes (M1, M2, & M3), which are coupled to G

Sadly Musa Haxhiu died before this work was completed for publication.

Conflict of Interest

We hereby declare that this manuscript is original and the work has not been published elsewhere.

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proteins, modulate the airway contractile response and acting via varying complexity among different species (Zaagsma *et al.*, 1997).

The M1 subtype muscarinic receptors are located in the ganglia through-out the tracheobronchial smooth muscle and mediate during excitatory neurotransmission between pre-and post-ganglionic neurons (Abraham *et al.*, 2007; Brown *et al.*, 2013). Activation of the M3 subtype cause, at least partially, tracheobronchial smooth muscle contraction. However, the M2 subtype is the primary receptor type involved in the regulation of tracheobronchial smooth muscle, because it regulates the release of ACh from parasympathetic nerves. Activation of presynaptic M2 receptors limits the release of ACh by inhibiting adenylyl cyclase and by activating G protein-gated potassium channels (Ishii and Kurachi, 2006). Both of these mechanisms attenuate tracheobronchial smooth muscle contraction (Fryer and Jacoby, 1998; Haddad *et al.*, 1991; Mizuta *et al.*, 2012).

Airway hyper-reactivity is associated with dysfunction of airway caliber and diseases of the airways, including asthma and chronic obstructive bronchitis. Increased vagal tone is due to reflex mechanisms, *i.e.*, sustained sensory/receptor stimulation by inflammatory mediators, excessive acetylcholine release from nerve terminals, and increased airway smooth muscle (ASM) responsiveness to acetylcholine (Barnes, 1996; Zanini *et al.*, 2015). Indeed, anticholinergic agents exert a greater bronchodilatory effect in patients suffering from airway diseases than in healthy subjects (Barnes, 1996; Zanini *et al.*, 2015). Thus, heightened peripheral function leads to the observed airway hyperactivity.

However, central mechanisms regulate ongoing activity of neuronal inputs to the tracheobronchial smooth muscle. While this input sets basal bronchomotor tone, it may be directly related to the control of breathing frequency (Mazzone and Canning, 2002). The complementary roles of central and local regulatory mechanisms is not elucidated clearly in airway function in healthy and pathological conditions (Barnes, 1996; Brannan *et al.*, 2012; Undem and Carr, 2002).

The vagus links central control to peripheral effectors. Ironically, the role of airway mechanisms after vagotomy is a gap in our knowledge, despite our knowledge of their role with the vagus intact. We hypothesize that vagotomy alters the peripheral control of the airways. With intact vagi, the inhibition of cholinesterase activity by systemic administration of physostigmine evokes bronchoconstriction. This effect is diminished by atropine, a muscarinic receptor antagonist (Jooste *et al.*, 2007; Mitchell *et al.*, 1985). The purpose of our study was to elucidate a plausible explanation for the central mechanisms related to descending vagal neural pathways and the cholinergic outflow in the control of tracheobronchial smooth muscle tone and lung mechanics by examining the reflex responses tracheal smooth muscle before and after vagotomy.

Materials and methods

We conducted experiments on European ferrets (n=7), *Mustella putorius furo* (700–900 g). Experimental protocols were approved by the Case Western Reserve University Institutional Animal Care Unit and Use Committee and were in full accordance with the *Guide for the*

Care and Use of Laboratory Animals (National Research Council). We limited our experiments to male animals to minimize the possible impact of physiological variations from hormonal changes on the measured parameters in our protocol. We chose the ferret as the subject for our experiments, because they are considered to be the best-suited model for the study of different respiratory pathophysiological conditions in humans (Belser *et al.*, 2011). The animals were purchased from Marshall Farms (North Rose, NY, USA), and housed in a room equipped with an air-filtering system in the Animal Resource Center for Case Western Reserve University with free access to water and food.

We conducted non-survival invasive surgery on the ferrets to prepare them for experiment. We used alpha-chloralose (70 mg/kg i.p.) as an anesthetic (Ueki *et al.*, 1992). The anesthetic level was monitored to ensure that a nociceptive stimulus did not elicit movement or a cardiorespiratory response. We administered additional alpha-chloralose (5mg/kg, i.v.) as needed to maintain an adequate state of general anesthesia.

Once the ferrets achieved a surgical plane of anesthesia, we inserted a polyethylene catheter into the common carotid artery for blood pressure recording, and another into the jugular vein for intravenous access and administration of drugs. We inserted another cannula in the femoral artery to sample (0.2 mL) arterial blood for blood gas measurements and analysis (Type ABLTM5/BPHTM5, Radiometer Medical A/S, Copenhagen, DK).

We formed a tracheal window in the cervical trachea. We inserted a tracheostomy tube in the caudal part of the cervical trachea. The tube was connected to a mechanical ventilator (Rodent Ventilator Model 683, Harvard Apparatus) that delivered a tidal volume of 8 mL/kg at a respiratory rate of 55 to 60 per minute. Ventilator rate rather than tidal volume was adjusted to maintain PaCO₂ 32 – 35 mmHg. The rostral end of the tracheal window remained unobstructed to allow later placement of our instruments.

The ferrets were paralyzed with gallamine triethiodide (4 mg/kg, *i.v.*) and the same dose was administered hourly thereafter. Hyperoxic gas (100% O₂) supplied the mechanical ventilator to reduce chemosensory drive and cholinergic tone to the airways, as well as to minimize the influence of peripheral receptors and chest wall ventilatory reflexes into airway responses (Ferguson *et al.*, 2000; Haxhiu *et al.*, 1986). An esophageal thermistor probe was inserted to monitor body temperature continuously, and the ferret was placed on a heating pad to maintain body temperatures between 38 and 39 °C.

During surgery, we opened the carotid sheath and separated the common carotid artery and the internal jugular vein. We isolated the vagi bilaterally and avoided injury to the plexus of ganglia in the posterior wall of the trachea, and interruption of its blood supply. For the success of the experiment, cholinergic outflow to the airways could not be affected. To verify integrity of this system, we compared measurements of tracheal smooth muscle tone evoked by lung deflation and hyperoxic hypocapnia before and after isolating the vagi.

After the instrumentation, the ferret was placed in a head-out plethysmograph in the supine position. Latex collars on the plethysmograph and gel around the ferret's neck provided an adequate seal. A custom fitted pneumotachograph assembly was attached connected to a differential low-pressure transducer with scale range ± 1.4 cm H₂O (DP250, Validyne

Engineering, Northridge, CA). In a side port of the plethysmograph, we placed a second differential pressure transducer (TRD 5700, Buxco Electronics, Wilmington, NC) to measure inflation pressure (P). The plethysmograph was calibrated by injecting and withdrawing 10 mL of ambient air. The assembly flow was calibrated by directly measuring flow (dv/dt) using an electronic flow meter designed and fabricated at Case Western Reserve University.

The recordings from these devices were displayed and stored on computer. We used an acquisition interface unit (ACQ-7700, Buxco Electronics) and carrier signal conditioner (Universal XE, Buxco Electronics) to acquire the data. Tidal volume and lung mechanics were quantified by integrating the flow and pressure signals using Biosystem XA software (version 2.7 r2, Buxco Electronics, Wilmington, NC). Both signals (v, P) were used to compute lung resistance (RL) and dynamic compliance (C_{dyn}).

We inserted a balloon-tipped catheter (3 cm length) in the rostral segment of the trachea through the rostral end of the tracheal window. The balloon was distended with 1.0–1.2 mL of saline. The catheter was connected to a pressure transducer to measure tracheal pressure. The transducer was calibrated by manometer from 0 to 45 cm H₂O. To define a basal tracheal tone, the pressure in the balloon (Ptseg) was measured after withdrawal of cholinergic outflow to the airways induced by hyperoxic hypocapnia by gradually increasing the rate of the ventilator to lower PaCO₂, based on previous studies (Haxhiu *et al.*, 2003). We considered 10.1 cm H₂O to be an optimal basal tracheal tone in the upper tracheal segment for our experiments, and was our reference value based on a previous studies in cats (Mitchell *et al.*, 1985).

Research Protocol

To define lung mechanics and tracheal tone, we measured the following variables: RL, C_{dyn}, and Ptseg, before and after lung deflation and transient asphyxia induced by interrupting ventilation for 30–45 s. After obtaining the baseline measurements, we followed the research protocol as shown in Figure 1(A). The reflex responses of tracheal smooth muscle provoked by lung deflation and hyperoxic hypocapnia were repeated after bilateral vagotomy.

Data Analysis

For data analysis we recorded and analyzed five respiratory cycles during a control period and at peak responses to cholinergic outflow before and after bilateral vagotomy. The values for each variable were averaged and means $\pm$ SEM are reported here. Statistical comparisons were made using *SigmaStat* software (SPSS, Chicago, IL) and the Student's t-test or two-way repeated measures ANOVA followed by *post-hoc* Bonferroni correction. Confidence Interval set at 95%. The criterion for statistical significance was $P < 0.05$.

Results

Effect of physostigmine on Ptseg before and after vagotomy

Stimulation of preganglionic neurons due to blocking acetylcholinesterase, which increases cholinergic outflow, was reflected by a progressive increase in tracheal smooth muscle tone. Figure 2 presents the effects of different doses of physostigmine on Ptseg before and after vagotomy. Specifically, the baseline values of Ptseg were not significantly different; 16.3 ± 3.4 and 8.5 ± 1.8 cm H₂O before and after vagotomy respectively ($p > 0.05$). After administrating 10 µg/kg of physostigmine, Ptseg became significantly different; 18.6 ± 3.0 before and 9.3 ± 2.1 cm H₂O after vagotomy ($p < 0.03$). The difference in measured Ptseg before and after vagotomy remained significant at higher doses of physostigmine. At 40 µg/kg dose of physostigmine, the values of Ptseg reached 23.8 ± 3.9 cm H₂O before and 10.1 ± 3.3 cm H₂O after vagotomy ($p < 0.02$); and at 100 µg/kg, Ptseg was 32.5 ± 3.3 cm H₂O before, and 10.6 ± 1.5 cm H₂O after vagotomy ($p < 0.0001$). In summary, physostigmine produced dose-dependent differences of the absolute pressures of the tracheal segment before, and after vagotomy. The significant differences in the values of Ptseg before and after vagotomy evoked by physostigmine treatment were due to a progressive increase in Ptseg elicited by physostigmine with the vagi intact. After bilateral vagotomy, physostigmine did not elicit an increase in Ptseg.

Effect of physostigmine in lung resistance (RL) before and after vagotomy

Intravenous administration of physostigmine also evoked a progressive increase in RL (Figure 3). Baseline RL was not significantly different before and after vagotomy (0.08 ± 0.007 cm H₂O/mL/s, and 0.072 ± 0.004 cm H₂O/mL/s, respectively, $p > 0.05$). After administering 10 µg/kg of physostigmine, even though mean RL increased slightly before vagotomy (0.09 ± 0.01 cm H₂O/mL/s), it remained not significantly different from that after vagotomy (0.075 ± 0.004 cm H₂O/mL/s). However, significant differences in RL before and after vagotomy were apparent after administering higher doses of physostigmine. After administering 40 µg/kg and 100 µg/kg physostigmine, RL was 0.10 ± 0.02 and 0.16 ± 0.04 cm H₂O/mL/s, respectively, before vagotomy and only 0.06 ± 0.006 and 0.067 ± 0.006 cmH₂O/mL/s respectively after vagotomy. It was apparent that physostigmine produced a dose-dependent increase in RL due to its effect on RL with the vagi intact but not after vagotomy.

Effect of physostigmine in dynamic compliance (Cdyn) before and after vagotomy

Baseline Cdyn was significantly greater before (0.8 mL/cm H₂O) compared to that after (0.56 ± 0.05 mL/cm H₂O) vagotomy, ($P < 0.004$). The stimulation of cholinergic outflow also affected dynamic compliance (Figure 4). Physostigmine (10, 40 and 100 µg/kg) had a dose dependent on Cdyn before but not after vagotomy. After administration of 10 µg/kg of physostigmine, Cdyn decreased slightly to 0.75 ± 0.05 mL/cm H₂O before vagotomy but remained the same (0.53 ± 0.04 mL/cm H₂O) after vagotomy and Cdyn remained significantly different before and after vagotomy ($P < 0.004$). After administration of physostigmine at dose of 40 µg/kg, the values of Cdyn were 0.77 ± 0.08 mL/cm H₂O before vagotomy, and 0.65 ± 0.1 mL/cm H₂O after vagotomy and were not significantly different ($P > 0.05$). After 100 µg/kg bw of physostigmine, Cdyn was 0.65 ± 0.1 mL/cm H₂O before

vagotomy, and 0.64 ± 0.07 mL/cm H₂O after vagotomy also not significantly different ($P > 0.05$). In summary, the significant difference in Cdyn before and after vagotomy that was present at baseline remained different only at the lowest dose of physostigmine.

Discussion

The cholinergic component of airway innervation has a mainly excitatory effect on the tracheobronchial smooth muscle tone of ferrets, which produces contraction via the vagal cholinergic efferent axons. Blocking cholinesterase activity by the systemic administration of physostigmine affects both central network excitation of preganglionic vagal neurons as well as efficacy of acetylcholine at the neuromuscular junctions in the airway. Specifically, physostigmine facilitates inspiration which in turn facilitates inspiratory-modulated excitatory input to vagal preganglionic neurons. Thus, physostigmine acts at the level of neural circuits affecting a network that is stimulated by neuronal and non-neuronal factors (Miller, 1949).

The effects of different doses of physostigmine before vagotomy are within the expected range for all three of our measured parameters: Ptseg, RL, and Cdyn. Vagotomy causes a lengthening of inspiration, so an increase of respiratory rate after physostigmine is reflected in respiratory pattern overall. Referring to this theory, these dynamic changes are expected to be associated with a decrease in lung resistance and pressure in the tracheal segment, as shown in our study. However, the increase of dynamic compliance was dose dependent and partially supported by our findings. Hence, these results suggest that the respiratory function is dominantly regulated by the cholinergic drive, which underlies to feedback mechanisms of downregulation and adaptation. After vagotomy feedback mechanisms are via peripheral chemoreceptors and act to maintain respiration. Additionally, the lengthened inspiratory phase evokes the phrenic potential to become larger which would impact the RL and Ptseg if the vagi were intact (Haxhiu *et al.*, 1989, 1986).

The effect of physostigmine acts through ACh which is produced and released by neurons. Thus, this effect was diminished after vagotomy, which in turn decreased airway tone and affected the baseline variables (Ptseg, RL) but not significantly. However, ACh might be partially compensated from nonneuronal sources (Buels and Fryer, 2012).

In previous studies Dick *et al.*, showed the role of the vagal neural pathways in the control of respiratory cycle. The authors stated that respiratory function is under control of neuronal and non-neuronal factors. Within these neuronal factors, the key role is attributed to the primary respiratory network which interfaces the neuronal respiratory drive and sensory feedback from airways. Furthermore, the control of respiratory function is a complex circuit, and in a case of central neural or local constraints, this circuit is complementary adjusted through other neuronal and non-neuronal factors. Accordingly, after vagotomy, the function will recover partially due to other factors involved in the control of respiration (Dick *et al.*, 2015, 2009).

Although there have been extensive studies on the subject, inconsistencies linger and questions persist regarding role of the vagal control of respiration. According to Colebatch

and colleagues, after bilateral cervical vagotomy, the inspiratory resistance to airflow decreases, and expiratory resistance increases, resulting in changes in the pattern of breathing, while the lung compliance is not modified (Colebatch and Halmagyi, 1963). Contrary to this, Zimmermann *et al.* report that, after vagotomy, exposing cats to bronchoconstrictor substances such as histamine and acetylcholine does not evoke significant changes in the breathing pattern neither in respiratory rate nor tidal volume. This evidence does not diminish the role of vagal innervation in the respiratory function, rather, it suggests that after vagotomy the role of vagus in central respiratory control might be transferred to other structures of the respiratory circuit (Zimmermann and Ulmer, 1986).

However, the blunting of the effects of physostigmine expressed after vagotomy could be because, in our experimental model, the excitatory innervation of the airways was mainly cholinergic (Haxhiu *et al.*, 2003). The pressure in the tracheal segment before vagotomy increased significantly and dose-dependently related to physostigmine, and after vagotomy, this was diminished. These results indicate that the effects of enhanced cholinergic outflow on airway smooth muscle tone are highly dependent on vagal innervation.

The lung resistance responses to asphyxia tests before vagotomy were correlated with an increase in the dose of physostigmine and achieved the highest difference at the maximal applied dose. After vagotomy, the effects of physostigmine were neither significant nor consistent. These results confirm the role of intact vagal innervations in lung resistance before vagotomy, while after vagotomy, sporadic changes may have resulted from spontaneous release of ACh persisting due to accumulation of physostigmine. We surmise that the postganglionic parasympathetic nerves are mostly inactive in the absence of preganglionic drive, which is consistent with baseline inhibition of cholinesterase having minimal effects on lung mechanics (Schelegle and Walby, 2012).

We observed in this study that with the vagi intact, dynamic compliance decreased progressively from baseline with higher doses of physostigmine. Contrary to this, the lung resistance increased and was in accordance with a previously reported study (Iben *et al.*, 2006). After vagotomy when the airway tone was abolished, there was decrease in baseline dynamic compliance. The inconsistency of this finding, when compared to other, similar studies, suggests the presence of other factors which have an effect on elastic recoil. Plausible explanations is the development of atelectasis and or fluid retention in the lungs which caused edema following vagotomy during the experiment.

We conclude that our results support the theory advanced by McAlexander and co-investigators. They postulated that the importance of an increased local response through the vagal reflex in pathological respiratory function is triggered by the stimulation of afferent fibers and then, vagal preganglionic neurons activating postganglionic neurons and releasing ACh to activate the muscarinic receptors (McAlexander *et al.*, 2015).

Taken together, the results of our study indicate that potentiation of cholinergic outflow induced by blockade of acetylcholinesterase after vagotomy is not associated with an impact on a pressure in the tracheal segment and an overall change in lung resistance. Conceivably, the basal tone of the airways may be maintained by local factors which may result in a

compensatory effect after descending central mechanisms of control are abolished. We theorize that in the absence of central neuronal control, local factors may regulate activities for respiratory function of the lung and airway.

In conclusion, our study confirms the importance of vagal neural pathways and the impact of cholinergic outflow in airway smooth muscle tone and lung mechanics. Nevertheless, the heterogeneity in our results before and after vagotomy reveals complexity of central and peripheral mechanisms as well as neuromodulatory pathways on lung mechanics and respiratory function. Cholinergic mediators can stimulate the central mechanisms which affect preganglionic neurons related to airway control, probably promoting the release of different amino acids, or causing an imbalance between inhibitory non-adrenergic/non-cholinergic components, and central excitatory cholinergic control. An obvious future direction would be to evaluate the role of these specific receptors within the groups of neurons related to airway control.

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Highlights

- Investigation of central mechanism in airway constriction after cholinesterase blockage
- Systemic administration of physostigmine evokes airway constriction from central nervous system
- Vagal nerves have a crucial role in regulating respiration process by contraction or relaxation of airway smooth muscle tone
- Inhibition of cholinesterase activity by systemic administration of physostigmine induces increase in cholinergic outflow to the airways by activation of central mechanisms

Fig. 1. Protocol for experiment

(A) After a steady state had been reached, physostigmine was administered systemically, in increasing doses from 10, 40 and 100 $\mu\text{g/kg bw}$ was administered as a final step before proceeding with the rest of the protocol. A 15 minutes interval was allowed between doses. After each dose of physostigmine, RL, Cdyn, and Ptseg were measured. Physostigmine in the same doses as prior to vagotomy was given and RL, Cdyn, and Ptseg were measured as before. (B) Tracings of the pressure signal on the tracheal segment (Ptseg, cm H₂O) from an anesthetized, paralyzed and an artificially ventilated ferret. Bilateral vagotomy (BLV) significantly diminished the tracheal smooth muscle response to cholinergic outflow stimulation after 100 $\mu\text{g/kg bw}$ of physostigmine.

Fig. 2. The effect of increasing dose of physostigmine in pressure of bypassed tracheal segment (Ptseg) in response to asphyxia test before (BV) and after (AV) vagotomy; N=7; Mean $\pm$ SEM

Fig. 3.

The effect of increasing dose of physostigmine in Lung Resistance (RL) in response to asphyxia test before (BV) and after (AV) vagotomy; N=7; Mean $\pm$ SEM

Fig. 4.

The effect of increasing dose of physostigmine in dynamic compliance (Cdyn) in response to asphyxia test before (BV) and after (AV) vagotomy; N=7; Mean ± SEM