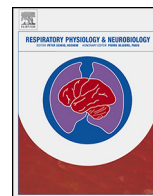




Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiolVagotomy reverses established allergen-induced airway hyperreactivity to methacholine in the mouse[☆]M. Allen McAlexander^{a,1}, Stephen H. Gavett^b, Marian Kollarik^c, Bradley J. Undem^{c,*}^a Integrated Toxicology Program, Duke University, Durham, NC, United States^b EPHD, NHEERL, United States Environmental Protection Agency, Research Triangle Park, NC, United States^c Johns Hopkins School of Medicine, Baltimore, MD, United States

ARTICLE INFO

Article history:

Accepted 26 March 2015

Available online xxx

Keywords:

Allergic inflammation

Vagus nerve

Reflex

Vagotomy

Particulate matter

Airway hyperreactivity

ABSTRACT

We evaluated the role of vagal reflexes in a mouse model of allergen-induced airway hyperreactivity. Mice were actively sensitized to ovalbumin then exposed to the allergen via inhalation. Prior to ovalbumin inhalation, mice also received intratracheally-instilled particulate matter in order to boost the allergic response. In control mice, methacholine (i.v.) caused a dose-dependent increase in respiratory tract resistance (RT) that only modestly decreased if the vagi were severed bilaterally just prior to the methacholine challenge. Sensitized and challenged mice, however, manifested an airway reactivity increase that was abolished by severing the vagi prior to methacholine challenge. In an innervated ex vivo mouse lung model, methacholine selectively evoked action potential discharge in a subset of distension-sensitive A-fibers. These data support the hypothesis that the major component of the increased airway reactivity in inflamed mice is due to a vagal reflex initiated by activation of afferent fibers, even in response to a direct (i.e., smooth muscle)-acting muscarinic agonist.

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1. Introduction

In recent years the mouse has become the most commonly used animal model in the study of asthma and allergic airway inflammation. In these investigations, the mouse is usually actively or passively sensitized to an antigen, then the airways are exposed to the sensitizing antigen, resulting in a “T_H2 type” allergic inflammatory response. Although the strains of mice differ among studies, and there are subtle differences in the sensitization-and-challenge schemes, invariably an eosinophil-rich inflammation is evoked that is associated with “airways hyperreactivity” to methacholine. The hyperreactivity is typified by an increase in the slope of the

methacholine dose–response curve, with relatively modest changes in methacholine sensitivity compared to those seen in asthmatics (Takeda et al., 1997; Park et al., 2004; Leigh et al., 2004; Williams and Galli, 2000). Despite a large and growing literature describing the essential immunologic features associated with this type of mouse airway hyperreactivity, there is comparatively little information regarding its physiological mechanisms.

Exposing mammalian airways to contractile agonists activates vagal sensory mechanosensors. Depending on the mechanosensory phenotype, this can lead to increases or decreases in parasympathetic contraction of bronchial smooth muscle (Canning, 2006). As a consequence, the bronchoconstriction evoked by contractile agonists, including methacholine, is likely to be at least partially mediated by central reflexes that result in the release of acetylcholine from postganglionic parasympathetic nerve terminals (Wagner and Jacoby, 1999; Canning, 2006). Inflammation can stimulate a subset of afferent C-fibers collectively referred to as nociceptors. One consequence of afferent C-fiber activation in airways is an amplification parasympathetic and cough reflexes. Within the context of airway inflammation, this feed-forward bronchoconstrictor reflex may be amplified through multiple mechanisms occurring at each of the sites in the sensory-CNS-autonomic reflex arc as reviewed in (Undem et al., 2000). A recent study has revealed that selective ablation of vagal C-fiber neurons in the mouse prevented entirely allergen-induced airway

Abbreviations: ROFA, residual oil fly ash; Mch, methacholine/acetyl-beta-methylcholine; OVA, ovalbumin; JNC, jugular/nodose ganglia complex; BAL, bronchoalveolar lavage.

[☆] The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views or the policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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<http://dx.doi.org/10.1016/j.res.2015.03.007>
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hyperreactivity to acetylcholine, without apparently inhibiting the inflammatory response (Trankner et al., 2014). These data are consistent with the idea that the role inflammation plays in causing airways hyperreactivity in the mouse is dependent entirely on the effect it has in stimulating vagal C-fibers. A C-fiber mediated hyper-reflexive state in the airways would, in theory, result in greater reflex responses for any given mechanical perturbation, resulting in methacholine dose–response curves with steeper slopes.

In this study, we specifically addressed the hypothesis that the airway hyperreactivity associated with acute (1 day) allergic inflammation in mouse lungs is due, at least in part, to an exaggeration specifically in the reflex component of the methacholine response. We used a previously characterized (Gavett et al., 1999) model in which tracheal application of particulate matter (residual oil fly ash, ROFA) serves as an adjuvant that augments the allergic response, resulting in a more vigorous and consistent inflammation and airway hyperreactivity.

2. Methods

2.1. Experimental animals

Female Balb/cJ mice ($n = 7$ – 12 per group), 7-week old on arrival and weighing 18–22 g, were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were provided with Prolab RMH-3000 meal (PMI Feeds, St. Louis, MO) and tap water ad libitum. Animals were maintained on a 12 h light/dark cycle at $22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity in an AAALAC-approved facility, and held for a minimum of 4 days before treatment. All experiments were approved by the Institutional Care and Use Committee of the organization where they were performed and met ethical standards outlined in the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Allergen sensitization and challenge and exposure to residual oil fly ash

Exposure to ROFA, as well as allergen sensitization and challenge, were performed as previously described (Gavett et al., 1999). Mice were sensitized with 20 mg ovalbumin (OVA; Grade V, Sigma Chemical, St. Louis, MO) in 0.2 mL aluminum hydroxide gel adjuvant (Alhydrogel; Accurate, Westbury, NY) delivered via an intraperitoneal (i.p.) injection. Control mice were injected with Alhydrogel only. Two weeks later, all mice were placed in a wire rack inside a 135 L chamber and challenged for 1 h with an aerosol of 1% ovalbumin in sterile saline. The solution was nebulized using three jets of a 6-jet atomizer (Model 9306; TSI, Minneapolis, MN) at 8 L/min/jet, and house air was added to bring the total flow rate to 40 L/min (18 exchanges/h).

Residual oil fly ash (ROFA) was collected from the flue gas of a power plant burning low sulfur (1%) residual oil downstream from a 2.5-mm cutoff cyclone. One hour after ovalbumin challenge, mice were anesthetized with methoxyflurane (Mallinckrodt, Mundelein, IL) vapor in a 2.7 L Plexiglas chamber. Following anesthesia, mice were intratracheally instilled with sterile saline vehicle or ROFA in saline using a 100 μL syringe and round-tip needle (dose = 3 mg/kg, $\sim 50 \mu\text{L}$ volume; $\sim 60 \mu\text{g}$ ROFA instilled per mouse. Saline was used as a vehicle since the saline-soluble portion (94% by weight) has been shown to mediate ROFA's toxic effects (Gavett et al., 1997). After 1-day of recovery, airway responsiveness to intravenously administered methacholine (Mch) was assessed in each mouse. After these measurements, bronchoalveolar lavage (BAL) was performed to collect and analyze cells. Statistical differences were determined using analysis of variance (ANOVA).

2.3. Airway responsiveness measurements

Mice were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg) and tracheostomized using an 18 g cannula and kept warm on a 37°C -heated circulating water pad. Animals were ventilated with constant inspiratory flow (flexiVent, Scireq, Montreal, Quebec) using hospital grade oxygen, and spontaneous breathing was eliminated with pancuronium bromide (0.8 mg/kg i.p.). Breathing frequency ($50 \text{ kg}^{-0.25}/\text{min}$) and tidal volume (7.5 mL/kg) were determined by animal body weight. Heart rate and waveform were monitored (SRA-200, MicroMed, Louisville, KY). Animals that did not maintain a baseline heart rate of ≥ 400 bpm were excluded from the study.

Mice were cannulated via the jugular vein using a 27 g needle inserted into PE20 tubing which was attached to an automated syringe pump loaded with 0.2 mg/mL Mch in saline. Baseline measurements of heart rate and total respiratory resistance (RT) were taken prior to drug infusion as described in more detail here (Gavett et al., 1999). Bolus doses of Mch were delivered over two seconds every 2 min in half log doses ranging from 10.0 to 316.2 $\mu\text{g}/\text{kg}$. Following drug infusion, RT was measured and recorded at 6-s intervals for a total of one minute. The peak response was recorded and that generally was observed 12–30 s after drug infusion. To standardize lung volumes 30 s before each dose, the expiratory port was occluded until airway pressure reached 20–30 cm H_2O . Resistance of the tracheal cannula was subtracted from measured values of RT. Responses from each dose were summed to determine area under the curve for one minute. To standardize lung volumes prior to the first dose and 1 min after each dose, the expiratory port was briefly occluded until airway pressure reached ~ 30 cm H_2O . This maneuver eliminated artifactual differences in baseline resistance caused by focal atelectases, which could affect responses to subsequent doses of Mch. Following a series of doses, the vagi were severed bilaterally and the Mch challenge protocol was repeated. No tachyphylaxis was observed when sham vagotomy was performed between methacholine dose–response curves ($95 \pm 2.1\%$ of maximum original response, $n = 3$).

2.4. BAL fluid differential cell count

Mice were lavaged with two aliquots of Ca^{2+} and Mg^{2+} -free Hanks' balanced salt solution (35 mL/kg; HBSS). Approximately 90% of the delivered volume was consistently recovered. The lavage fluid was placed on ice and centrifuged at $360 \times g$ for 12 min at 4°C . Cells were then resuspended in 1.0 mL HBSS and counted (Coulter, Hialeah, FL). Slides of BAL fluid cells were made (Cytospin 3; Shandon, Pittsburgh, PA) and stained with Leuko-Stat (Fisher Scientific, Fair Lawn, NJ) and at least 500 cells per sample were differentiated.

2.5. Sensory nerve recordings

Mice were killed by CO_2 inhalation and exsanguination. The blood from the pulmonary circulation was washed out by in situ perfusion with indomethacin (3 μM) containing Krebs bicarbonate buffer (KBS, composed of: NaCl, 118 mM; KCl, 5.4 mM; NaH_2PO_4 , 1.0 mM; MgSO_4 , 1.2 mM; CaCl_2 , 1.9 mM; NaHCO_3 , 25.0 mM; dextrose, 11.1 mM, and gassed with 95% O_2 –5% CO_2 , pH 7.4) through the right ventricle. The airways and lungs with intact left- and right-side extrinsic vagal innervation (including left and right jugular/nodose ganglia complex, JNC) were dissected and the tissue was pinned in a small Sylgard-lined Perspex chamber. The right and left JNC, along with the rostralmost vagi were pulled through a small hole into an adjacent chamber for extracellular recording as described in detail elsewhere (Kollarik et al., 2003).

Briefly, extracellular recordings were performed using an aluminosilicate glass microelectrode (pulled with Flaming-Brown

micropipette puller, Sutter Instrument Company, Novato, CA, USA) and filled with 3 M sodium chloride (electrode resistance $\sim 2\text{ M}\Omega$). The recording microelectrode was manipulated into the right vagal sensory ganglion. The receptive field of a single vagal sensory nerve fiber was identified in the lung tissue using a concentric stimulation electrode (100 V, 0.5 ms, 1 Hz). The electrode was sequentially positioned at different places on the surface of the lung lobes and the activity recorded from JNC was observed. When electrical stimulation of the tissue evoked action potentials, the tissue was probed with a mechanical probe (Von Frey hair, 60–1800 mN). The mechanosensitive receptive field was identified when the mechanical stimulus evoked a burst of action potentials. Conduction velocity was calculated by dividing the distance along the nerve pathway by the time between the shock artifact and the action potential evoked by electrical stimulation of the mechanosensitive receptive field. The response to a 1 mL intratracheal infusion of methacholine was monitored and quantified as the total number of action potentials evoked and the peak frequency taken as the maximum number of action potentials occurring in any 1 s bin.

Statistical significance of comparisons was made using Student's *t*-test. For the cellular data a non-paired analysis of the number of a given cell type in the BAL of control vs. ROFA/OVA challenged mice was carried out. For the methacholine results an ANOVA was carried out at each dose, followed where significant, by a Student's *t*-test of unpaired data.

3. Results

3.1. Inflammatory response to ROFA/OVA

The BAL of control (saline treated) animals contained on average $13.5 \pm 3.5 \times 10^4$ cells, which were primarily mononuclear. One day after vehicle challenge there were essentially no eosinophils in the BAL of saline treated animals. One day after intra-tracheal infusion of (ROFA) particulate there was a minimal increase in BAL eosinophils ($\sim 2\%$ of the total cells). One day following OVA exposure in the ROFA treated animals caused a marked increase in BAL eosinophils, lymphocytes and neutrophils (Fig. 1). In these allergically-inflamed animals the total cell count increased >5 -fold to $74.9 \pm 8.2 \times 10^4$ with $12.4 \pm 2.1 \times 10^4$ eosinophils. The OVA response in the ROFA treated animals, with respect to eosinophil accumulation, was substantially larger than ROFA alone ($8 \pm 1 \times 10^3$) or OVA alone ($11 \pm 2 \times 10^3$). This augmentation of

allergic inflammation by ROFA treatment is consistent with a previous more detailed report (Gavett et al., 1999)

3.2. Airway response to methacholine

We quantified the airway response to methacholine (i.v.) in control animals and in animals 1 day following ROFA/OVA-induced airway inflammation. In saline treated animals, methacholine caused a dose-related increase in RT. At a dose of $316\text{ }\mu\text{g/mL}$, the largest dose studied, the resistance increased $11 \pm 4\text{ cm H}_2\text{O s mL}^{-1}$ above baseline. The methacholine response was significantly increased in the ROFA/allergen exposed animals (Fig. 2). The hyper-reactivity to methacholine was manifested as an increase in the slope of the dose–response curve. At the largest dose tested, there was >3 -fold increase in methacholine efficacy.

3.3. Effect of vagotomy on airway response to methacholine

Severing the vagus nerves did not significantly influence baseline RT ($P > 0.1$; see Fig. 2 legend). In control animals the response to methacholine was modestly attenuated by vagotomy (slope was decreased roughly 2-fold, $P > 0.05$) as compared to those animals in which the vagus nerves were intact. Cutting the vagi in the ROFA–OVA animals between two consecutive methacholine dose–response challenges, however, completely normalized the inflammation-induced hyperreactivity such that the response to methacholine was the same as that observed in control vagotomized group (Fig. 2). Following vagotomy, the response of the OVA/ROFA treated animals to methacholine was not different than that observed in control animals ($P > 0.1$).

3.4. Vagal afferent nerve recordings

The data are consistent with the hypothesis that in addition to directly contracting bronchial smooth muscle, methacholine initiates a reflex response that is strongly enhanced in the allergically inflamed lungs. Only possible initiator of a parasympathetic reflex are the bronchopulmonary C-fibers or subtypes of

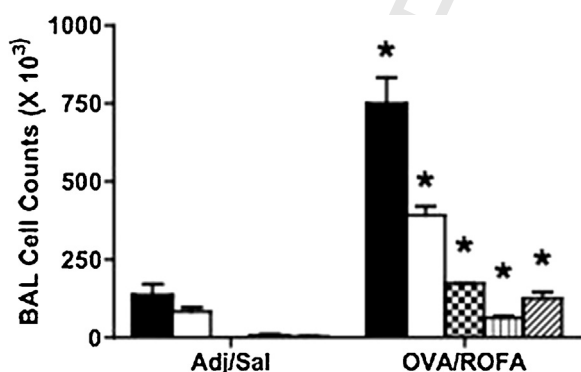


Fig. 1. Allergen and particulate matter exposure causes robust airway inflammation. Cell counts in the bronchoalveolar lavage fluid obtained from mice that were treated with vehicle control (adjuvant, adj/saline, sal) or residual oil fly ash (ROFA) and allergen (ovalbumin, OVA). Solid bars = total cells, open bars = mononuclear cells; checked bars = eosinophils; vertical hashed bars = lymphocytes, and diagonally hashed bars = neutrophils. Each bar represents the mean $\pm$ SEM of 7–12 experiments. Asterisk (*) denotes a statistically significant ($P < 0.05$, ANOVA) difference between cell counts in OVA/ROFA vs. adj/sal groups.

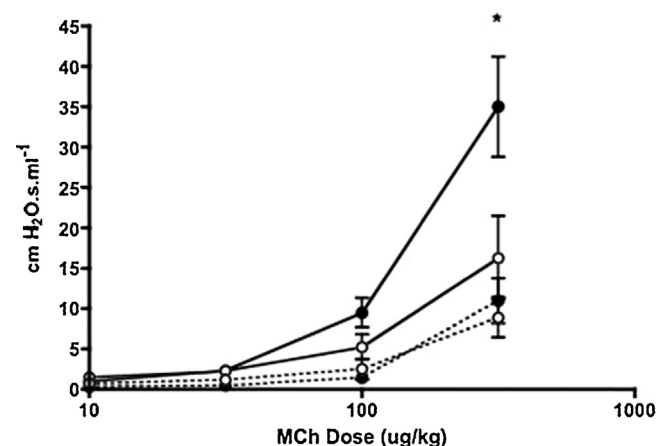


Fig. 2. Vagotomy reverses established airway hyperreactivity in inflamed mice. Change in RT evoked by intravenous administration of methacholine in saline treated animals (O) and allergically inflamed animals treated 1 day earlier with residual fly ash and ovalbumin as described in methods (●). Dose–response curves were administered to mice with vagus nerves left intact (sham, solid lines), and following bilateral vagotomy just prior to methacholine administration (dashed lines). The baseline resistance was not different between the four groups of animals. In control and OVA/ROFA animals with the vagus nerve intact the baseline resistance ($\text{cm H}_2\text{O s mL}^{-1}$) averaged 1.8 ± 0.1 ($n = 5$) and 1.9 ± 0.1 ($n = 8$), respectively. In control and OVA/ROFA animals with the vagus nerves cut the baseline resistance averaged 1.6 ± 0.2 ($n = 5$) and 1.9 ± 0.1 ($n = 7$). Data reflect the mean $\pm$ SEM.



Fig. 3. Methacholine activates mouse airway mechanosensitive A-fibers ex vivo. The upper trace is a single unit recording of a vagal A-fiber (conduction velocity = 9.5 m s^{-1}) responding to methacholine ($1 \mu\text{M}$) with action potential discharge (peak frequency of discharge was 11 Hz). The lower trace is the perfusion pressure (vertical line = $10 \text{ cm H}_2\text{O}$). More details are presented in the text.

bronchopulmonary A-fibers. In the isolated vagally innervated mouse lung preparation, at least two distinct sensory nerve phenotypes can be observed, C-fibers and distension-sensitive A-fibers. Previously, we have noted that less than 10% of the fibers studied using this design were A-fibers (only 9 of 92 recorded fibers) (Kollarik et al., 2003). This may reflect either a large preponderance of C-fibers in murine lungs or an unintended selection bias due to the experimental design. We reported that C-fibers terminating in the mouse lungs are not activated by methacholine (Kollarik et al., 2003). Here we evaluated the response of distention-sensitive A-fibers to methacholine. Methacholine evoked marked action potential discharge in four of the six intrapulmonary vagal afferent fibers that conducted action potentials in the A range (e.g., Fig. 3). In the four experiments on methacholine-sensitive fibers, the peak frequencies of action-potential discharge were 11, 9, 13, and 8 impulses per second. As expected, lung distension also activated the four A-fibers. In one of the two experiments in which the A-fiber did not respond to methacholine, it also failed to respond to lung distension. These studies were carried out ex vivo where the lungs were being perfused with buffer, obscuring a determination of adaption index that would indicate whether the A fibers were of the rapidly or slowly adapting type as defined in in vivo recordings.

4. Discussion

Our findings support the hypothesis that, under certain conditions, intravenous administration of methacholine can increase airway resistance in the mouse not only by directly contracting airway smooth muscle, but also by evoking a vagal reflex. In antigen and particulate matter-exposed mice, the inflammation-induced airway hyperreactivity to methacholine appears to be due to a selective increase in the vagal reflex component of the response.

In guinea pigs, histamine, like methacholine, can increase airflow resistance, both by directly contracting bronchial smooth muscle and by evoking a cholinergic parasympathetic reflex bronchial contraction (Mazzone and Canning, 2002). Consistent with our results, Costello et al. (1999) demonstrated that exposing guinea pigs to allergen caused a marked increase in the reactivity to histamine by enhancing the cholinergic reflex component of the response. Histamine does not evoke a strong bronchoconstriction in the mouse. In fact the few known stimuli that elicit bronchoconstriction in the mouse act primarily through anticholinergic-sensitive mechanisms (Tilley et al., 2003; Weigand et al., 2009). Given these limitations of the mouse airway physiology, we elected to use acute vagotomy as a mechanism to discriminate between the direct and indirect effects of methacholine, our model bronchoconstrictor.

We chose to evaluate a model of allergen-induced airway inflammation in which the airways were “primed” by instillation of particulate matter. In previous work this caused neutrophil influx,

an amplification of T_H2 type inflammation and a robust and consistent airway hyperreactivity to methacholine (Gavett et al., 1999). In agreement with the previous study, the particulate matter alone did not evoke airway hyperreactivity, but caused an exaggerated inflammation and airway response to methacholine when administered in combination with antigen challenge.

The observation that, in control animals, removing the airway reflex arc by vagotomy modestly decreased the response to methacholine is consistent with the conclusion that in the absence of inflammation, only a small component of the methacholine response is likely due to the additive effect of increased parasympathetic cholinergic activity. That is to say that in naïve mice, the direct constrictive action of methacholine on the airway smooth muscle is the sole or at least primary determinant of airflow obstruction. A methacholine-induced reflex increase in parasympathetic cholinergic airway tone has been observed in other animal models (Wagner and Jacoby, 1999). As expected from studies on other mammals, methacholine was ineffective at stimulating vagal C-fibers in the mouse, but effectively evoked action potential discharge in vagal mechanosensitive A-fibers. Stimulation of some types of mechanosensitive A fiber, such as the rapidly adapting fibers, may be responsible for initiating the reflex component of the methacholine response (Canning, 2006). In addition, sensory fibers outside the lung could have been activated by these high, i.v.-administered doses of methacholine and these may have contributed to the net reflex. Regardless of the nature of the initiating stimulus, it is sufficient to induce a reflex airflow obstruction only in the inflamed mice.

Airway inflammation markedly increased the central reflex (vagus nerve-dependent) component of the methacholine response in our model, but the location(s) within the reflex arc where the amplification occurred is not known. The effect we observed with vagotomy, namely an interruption of airway hyperreactivity, is mimicked by knocking out or inhibiting the Transient Receptor Potential Ankyrin 1 (TRPA1) ion channel that is, at least in naïve mice, thought to be predominantly expressed in C-fibers (Caceres et al., 2009) or by selectively deleting vagal C-fiber neurons using Cre diptheria toxin receptor methods, leaving intact afferent A-fibers (e.g., RAR and SARs) as well as the parasympathetic nerves (Trankner et al., 2014). Further study will be required to determine the relative contributions of individual nerve subtypes and neuronal effectors in this type of model. Of note, our findings not only compliment but extend this work, as many genetic manipulation studies in mice such as the elegant ones performed by Trankner et al. (8) must be done in an unpaired manner. Thus, unpaired experiments are capable of demonstrating protection from – rather than reversal of – a phenomenon. In our mouse model, we have demonstrated that vagotomy acutely reverses established airway hyperreactivity, proving that, under these conditions, vagal reflex arcs are obligatory for maintaining airway hyperreactivity and also that the hyperreactivity is acutely reversible.

The vagal C-fibers innervating the mouse respiratory tract comprise two distinct subtypes. One type has their cell bodies situated in the neural crest derived jugular ganglia, the other in the placodal derived nodose ganglia (Nassenstein et al., 2010). We have previously found that in sensitized mice, OVA strongly activates only the subset of C-fibers arising from the jugular ganglion, and not those arising from the nodose ganglia (Potenzieri et al., 2012). Considered together, we may hypothesize that, in inflamed mice, action potentials arising from bronchopulmonary jugular C-fibers are likely to lead to an amplification of mechano-sensory-mediated parasympathetic reflex activity in the airways by augmenting synaptic transmission in the brain stem by a phenomenon referred to as “central sensitization” (Mazzone et al., 2005). Allergen challenge may also increase afferent input into the brainstem by increasing the sensitivity of vagal mechano-sensors (including

normally high-threshold C fibers) in the airways (Zhang et al., 2008; Riccio et al., 1996) and/or via induction of neurokinin synthesis in vagal A-fiber mechanosensors (Myers et al., 2002; Chuaychoo et al., 2005). If these neurokinins are released centrally at the level of the brainstem, this would likely lead to an amplification of central excitatory synaptic transmission. We chose to evaluate the response to systemic administration of methacholine rather than aerosol administration as this provides for a more consistent dose response. Although we favor the hypothesis that methacholine triggers reflex action by activating mechanosensitive A-fibers in the pulmonary system we cannot exclude the possibility that the vagal reflex was initiated by activation of nerves outside of the respiratory tract.

Although the studies by Trankner et al. (8) indicate that parasympathetic nerves per se, in the absence of vagal C-fiber afferent nerves, are insufficient for the development of airway hyperreactivity in the allergic mouse model, effects of allergic inflammation on the parasympathetic nerves may enhance the overall response. For example, the filtering of preganglionic input at the synaptic level in bronchial parasympathetic ganglia may be decreased by allergic inflammation (Myers, 2001). We have recently found that antigenic activation of airway mast cells leads to membrane depolarization of principal neurons in the mouse airway parasympathetic ganglia (Weigand et al., 2009). The acetylcholine released from the postganglionic terminals per action potential discharge may also be augmented in allergically inflamed airways via muscarinic M2 receptor inhibition (Fryer and Wills-Karp, 1991). That a component of the increased reflex activity is due to neuromodulation of the effector limb in mouse models of airway inflammation is supported by studies in which allergic inflammation lead to an increase in the cholinergic contraction of isolated trachea in response to electrical field stimulation, but not to direct application of methacholine (Larsen et al., 1992).

In summary, our results are in general agreement with the conclusion drawn from nerve deletion studies (Trankner et al., 2014) that vagal afferent nerve activation is a sine qua non of allergen-induced airways hyperreactivity to muscarinic agonists. These findings emphasize that, particularly during conditions of injury and inflammation, reflex arcs may serve as factors critical to the amplification of airway obstruction caused by multiple means, including muscarinic agonists that constrict airway smooth muscle via direct action.

Acknowledgments

The authors wish to thank Najwa Haykal-Coates for expert technical assistance. Funding provided by US EPA-Duke University Cooperative agreement # CT826514 and National Institute of Health grant HL038095.

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