Mediators of anaphylaxis but not activated neutrophils augment cholinergic responses of equine small airways

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Olszewski, Michal A., N. Edward Robinson, Feng-Xia Zhu, Xiang-Yang Zhang and Patricia K. Tithof. Mediators of anaphylaxis but not activated neutrophils augment cholinergic responses of equine small airways. Am. J. Physiol. Lung Cell. Mol. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L522–L529, 1999.—Neutrophilic inflammation in small airways (SA) and bronchospasm mediated via muscarinic receptors are features of chronic obstructive pulmonary disease in horses (COPD). Histamine, serotonin, and leukotrienes (LTs) are reported to be involved in the exacerbation of COPD, and currently, histamine has been shown to increase tension response to electrical field stimulation (EFS) in equine SA. We tested the effects of these mediators and the effects of activated neutrophils on the cholinergic responses in SA. Histamine, serotonin, and LTD4 had a synergistic effect on EFS responses and only an additive effect on the tension response to exogenous ACh or methacholine. Atropine and TTX entirely eliminated the EFS-induced tension response in the presence of all three inflammatory mediators, indicating that augmentation of the EFS response applies only to the endogenous cholinergic response. Neutrophils isolated from control and COPD-affected horses were activated by zymosan, producing 18.1 ± 2.3 and 25.0 ± 2.3 nmol superoxide·106 cells·1·30 min−1, respectively. However, in contrast to the profound effect of mediators, incubation of SA for over 1 h in a suspension of up to 30 × 106 zymosan-treated neutrophils/ml did not significantly affect EFS responses of SA isolated from either control or COPD-affected horses. We conclude that in equine SA 1) the endogenous cholinergic responses are subject to strong facilitation by inflammatory mediators; 2) activated neutrophils do not affect cholinergic responses in SA; and 3) in acute bouts of equine COPD, histamine, LTD4, and serotonin (mediators primarily associated with type I allergic reaction) rather than mediators derived from neutrophils most likely contribute to increased cholinergic airway tone.

airway smooth muscle; chronic obstructive pulmonary disease; inflammatory mediators; neutrophil activation; zymosan

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) in horses or “heaves” is a naturally occurring syndrome sharing multiple features with human asthma and COPD (25). Exposure of COPD-susceptible animals to natural (hay and straw) antigens precipitates an inflammatory response in the airways, with airway hyperresponsiveness and bronchospasm leading to severe airway obstruction (25). A cholinergic mechanism of airway obstruction in horses with COPD has been clearly demonstrated in several studies (6, 24), but the origin of increased cholinergic tone in the airways remains largely unknown. In vivo, horses with COPD demonstrate airway hyperresponsiveness to a variety of spasmosgens, and airway obstruction can be resolved by the use of antimuscarinic drugs (1, 6, 20). In contrast, the isolated airway responses to ACh and electrical field stimulation (EFS) are decreased (18, 36), and ACh release from airway parasympathetic nerves, measured in vitro, is not elevated in horses with COPD (31).

Inhalation of allergens by heavy horses leads to several inflammatory events in the airways, such as neutrophil recruitment and activation (12, 21), changes in lymphocyte populations, release of histamine from airway mast cells (19), and activation of the arachidonic acid cascade in the airway mucosa, with a significant shift in the lipid mediator profile (14). The latter results in a decrease in mucosal PGE2 production and an increase in proinflammatory lipid mediators such as thromboxane, 15-hydroxyeicosatetraenoic acid (HETE), cysteiny1 leukotrienes (LTs), and platelet-activating factor (10, 13, 14). We propose that inflammatory mediators released in response to antigen challenge are responsible for the altered cholinergic responses of the airways in COPD. In this context, the discrepancy between in vitro tissue behavior and in vivo airway responses could be caused by washout of these mediators from the tissues in vitro before measurement of tension or ACh release. Additionally, earlier studies were conducted on trachea and bronchi (18, 36), whereas in COPD, the most predominant inflammatory response (retention of mucopurulent secretion and airway wall infiltration) occurs in peripheral airways. Therefore, if inflammation is the source of altered cholinergic tone in the airways, detectable changes in cholinergic responses may be limited to small airways (SAs).

Some of these issues were addressed in a previous study by Olszewski et al. (22) that confirmed that peripheral airways from horses, in vitro, produce entirely cholinergic contractions in response to nerve stimulation by EFS. These contractions were increased by cyclooxygenase blockade and application of histamine, indicating that inflammatory mediators can exert a profound effect on the responses to nerve stimulation in equine SAs (22). In the present study, we extended our research to further investigate the effects of inflammation on cholinergic mechanisms in equine SAs. Several approaches were used to reach our goal. We used SAs from both control and acutely heavy animals to compare their responses to EFS. Because histamine had quite dramatic effects on the EFS response and currently, the discrepancy between in vitro tissue behavior and in vivo airway responses could be caused by washout of these mediators from the tissues in vitro before measurement of tension or ACh release. Additionally, earlier studies were conducted on trachea and bronchi (18, 36), whereas in COPD, the most predominant inflammatory response (retention of mucopurulent secretion and airway wall infiltration) occurs in peripheral airways. Therefore, if inflammation is the source of altered cholinergic tone in the airways, detectable changes in cholinergic responses may be limited to small airways (SAs).

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SA responses. Finally, we were particularly interested in the effects of neutrophil-derived mediators on cholinergic airway responses for many reasons. First, in contrast to other inflammatory cells, the number of neutrophils in the airways consistently increases during an exacerbation of COPD (8, 12, 29). Neutrophil recruitment takes place within a few hours of natural antigen challenge and, in most cases, parallels early changes in lung function (12); neutrophils washed from airways of heavy horses are strongly activated (21); and, as shown in multiple studies (32, 33), the number of neutrophils in bronchoalveolar fluid generally increases with the severity of airway obstruction. Additionally, it has been shown in dog airways that neutrophils are critical to the development of both ozone- and intravenous platelet-activating factor-induced hyperresponsiveness (5). Thus, in our final approach, we used isolated and activated neutrophils to test whether mediators released acutely from these cells activated in proximity of SAs are capable of altering their cholinergic responses.

MATERIALS AND METHODS

Animals

Control horses and horses with COPD were tissue donors for this in vitro study, which was approved by the All-University Committee on Animal Use and Care at Michigan State University (East Lansing). The control group consisted of 17 geldings and mares of various breeds, 7.3 ± 0.8 yr old, weighing 901.4 ± 20.2 kg, and free of signs of respiratory disease. At 48 h before euthanasia, animals were brought from the pasture into the barn and kept on hay and straw. To induce airway obstruction in horses with a history of COPD, 10 horses (geldings and mares of various breeds, 13.3 ± 3.5 yr old, weighing 1,034.8 ± 37.2 kg) were brought from the pasture into the barn and kept on hay and straw until significant clinical signs of airway obstruction had developed (on average 1 wk). We assessed the severity of airway obstruction on a daily basis by means of a clinical score system that is tightly correlated with changes in pulmonary functions (26). As blood donors, we used two horses with heaves that consistently developed severe airway obstruction within 1–2 days of stabling and four control horses stabled in the barn.

Tissue Collection

The animals were killed by an intravenous injection of pentobarbital sodium; the rib cage was opened; and the heart, lung, and trachea were excised. Immediately after death, the cardiac region of the right lung was collected and suspended in Krebs-Henseleit (KH) solution (composition in mM: 118.4 NaCl, 25.0 NaHCO3, 11.7 dextrose, 4.7 KCl, 2.6 CaCl2·2H2O, 1.19 MgSO4·7H2O, 7H2O, and 1.16 KH2PO4) saturated with 95% O2-5% CO2. During dissection and experimental protocols, the tissues were kept in the KH solution that was continuously gassed with 95% O2-5% CO2.

Tissue Preparation

SA preparations were isolated from the peripheral part of the cardiac region following procedures described previously (22). In this anatomic location, 1- to 2-mm OD horse airways represent generations 12–16 and are the smallest airways that still contain some cartilaginous elements. Dissected SA preparations were placed in a 2-ml tissue bath (Radnoti Glass Technology) filled with KH solution (38°C), which was replaced every 15 min and bubbled with 95% O2-5% CO2 during the entire experiment. The lower end of the SA preparation was fixed with surgical silk ties to the glass tissue holder, which secured the tissue at the bottom of the tissue bath. The upper tie was hooked to a force transducer (model FT03, Grass Instruments, Quincy, MA) installed on a tension manipulator. Isometric force of the tissue preparations was recorded on a polygraph (model 7D or 7E, Grass Instruments). In this setup, tissues were suspended in the middle of the tissue bath between two platinum wire electrodes built vertically in the wall of tissue bath. During a 2-h equilibration period, optimal passive tension was determined by gently stretching the tissue and using 127 mM KCl to induce contraction, followed by two applications of EF5 (1 Hz, 20 V, 0.5 ms) at 30-min intervals. Optimal tension was in the range of 2–2.2 g. Square-wave EF5 impulses were generated by a stimulator (model 588, Grass Instruments) and delivered to the electrodes via a stimulus power booster (Stimu-Splitter II, Med Lab Instrument, Loveland, CO). After equilibration, we treated the tissues with KCl for a second time to determine the maximal response (100% KCl). The experimental protocols were conducted in eight muscle baths.

Blood Collection and Neutrophil Isolation

For protocols that required neutrophils, peripheral blood (60 ml from each horse) was collected into EDTA-containing Vacutainer tubes via jugular venipuncture from an acutely heavey and a control horse. A two-step isolation method was used. Buffy coat was collected from blood tubes after centrifugation (T) -6R, Beckman Instruments, Palo Alto, CA) at 1,500 rpm for 15 min and gently layered on the surface of a density gradient of 59 and 75% isometric Percoll solutions. After 45 min of centrifugation at 3,000 rpm and 14°C, the neutrophils accumulated in the form of a cloudy band at the gradient interface. After aspiration, the neutrophils were suspended in Ca-, Mg-, and phenol-free Hanks’ balanced salt solution (HBSS) and spun and washed two times. Cell count, purity, and viability were assessed, and activity of the neutrophils stimulated with serum-treated zymosan (STZ) was determined by measurement of superoxide (O2·−) production. For that purpose, we used the superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction assay according to method developed by Babor et al. (2) and adapted by Tithof et al. (28), who provided its detailed description in their papers.

Protocols

We designed two major groups of protocols to test the effects of neutrophils on SA responses.

Inflammatory mediators. In the first subset of experiments, we tested the effects of inflammatory mediators on SAs from either control or COPD horses. In all protocols, one of the tissues was not treated with any inflammatory mediator and served as a time control. Depending on the protocol, other tissues were each treated with one concentration of mediator: histamine (3 µM), LTD4 (0.3, 1, 3, or 10 nM), or 5-HT (0.01, 0.1, 1, or 10 µM). After 15 min of incubation, EF5 frequency-response curves were created by the application of increasing EF5 frequencies (0.05–32 Hz). Frequency was increased when the response to the lower frequency reached a plateau. To confirm that mediators affected exclusively the endogenous cholinergic response to EF5 (neurally released Ach), two additional tissues were treated: one with the sodium-channel blocker tetrodotoxin (TTX; 3 µM) and the other with the muscarinic-receptor antagonist atropine (Atr; 3 µM).
latter tissues were treated before EFS with a concentration of inflammatory mediator that, in a pilot study, had the greatest effect on the EFS response.

After the EFS-response curves were obtained, the tissues were washed thoroughly with fresh buffer (with and without inhibitor) and rested for 30 min. The same concentrations of inflammatory mediator were then added to each tissue bath, and after a 15-min incubation period, the concentration-response curves to ACh or MCh (depending on the protocol) were created.

Effects of neutrophils. In a second subset of experiments, we compared responses to EFS in control and heavey horse SAs and tested the effects of activated neutrophils on these responses. Because neutrophils of horses with COPD may have different mediator profiles or, alternatively, the sensitivity of SAs to neutrophil-derived mediators in these horses could be different, we applied a crossover design with four combinations of neutrophils and tissues isolated from both COPD-affected and control horses. In this protocol, untreated control tissue and tissue treated solely with STZ were included in addition to six tissue baths in which SAs were incubated with 3, 10, or 30 × 10⁶ neutrophils/ml. During experiments with neutrophils, the first EFS frequency-response curve was created before incubation with the neutrophils; the second and third EFS curves were created at 30 and 60 min of incubation, respectively, in the presence of STZ-activated neutrophils.

Before the series of experiments with SAs, we performed pilot studies in which we measured O₂⁻ production in response to chemotactic ligands (formyl-methionyl-leucyl-phenylalanine, human recombinant C5a, and LTB₄) and STZ in the neutrophils isolated from peripheral blood of both control and heavey horses. The purpose of these studies was to select the optimal neutrophil activator and to compare the responses of neutrophils isolated from control and heavey horses. Additionally, to confirm that neutrophils maintained their activity at the time of incubation, activity of the neutrophils was tested. We used a fraction of the neutrophils isolated for our tissue experiments, treated them with STZ, and measured O₂⁻ production by means of the cytochrome c reduction assay.

Agents

On the day of the experiments, acetylcholine hydrochloride, atropine sulfate, histamine hydrochloride, 5-hydroxytryptamine hydrochloride, MCh, and TTX (all from Sigma, St. Louis, MO) were dissolved in deionized water to obtain stock solutions (10 or 100 mM) as needed. LTD₄ (Calbiochem) was diluted in KH solution to 10 µM and frozen in portions that were diluted for use shortly before addition to the tissue baths. Stock solutions of Atr and TTX were directly mixed into the KH solution; other compounds were serially diluted into the KH solution, and each concentration was added to the muscle baths in a volume of 1%. The concentrations of all substances are expressed as their final bath concentration. Cytochrome c, formyl-methionyl-leucyl-phenylalanine, HBSS, Percoll, and STZ were all from Sigma. Sterile Percoll, after adjustment of osmolality and pH by addition of 10× HBSS and 1 N HCl, was diluted to 59 and 75% solutions in sterile Ca-, Mg-, and phenol red-free HBSS and carefully layered in 50-ml tubes as a discontinuous gradient. SOD and cytochrome c were dissolved in sterile HBSS without phenol red. All solutions were prepared directly before use. Zymosan (Sigma) was prepared according to the manufacturer’s guidelines and opsonized in equine serum. Small portions of STZ were frozen and stored at −20°C, and each portion was brought to room temperature directly before use.

Statistics

Tension study data (means ± SE) are expressed as a percentage of the response to 127 mM KCl-substituted KH solution, and n represents the number of horses used in each protocol. To determine drug effects, we applied between-bath comparisons of treated and control tissues. This excluded any effects of time or tachyphylaxis. Data were calculated and analyzed (Excel 7.0, Microsoft, and SSPS for Windows 7.0, SSPS, on a Gateway 2000 PS-133 computer) by means of one-way ANOVA or mixed-design factorial ANOVA as appropriate. A post hoc Dunnett’s test was used to compare means between treatment and control values. Means were accepted to be significantly different at P ≤ 0.05.

RESULTS

SA Responses to EFS

Just as in larger airways, SAs isolated from horses with COPD produced weaker responses to EFS than those from control animals (Fig. 1), although the responses to KCl were identical in both groups of tissues.

Inflammatory Mediators

Effect of LTD₄. LTD₄ contracted the airway in a concentration-dependent manner (Fig. 2); however, there was a great deal of variability among individual tissue responses to LTD₄. The response to EFS was augmented by 0.3–3 nM LTD₄ (Fig. 3A). The increase in the response to EFS was greatest when the tissues were slightly contracted by LTD₄; however, elevation of baseline tension was not absolutely necessary for this augmentation to occur. Both TTX and Atr completely blocked the responses to EFS in the presence of LTD₄ (Fig. 3B). The response to MCh was additive with the LTD₄-induced contraction but was not synergistic (Fig. 3C). Similar to control tissues, in SAs from COPD horses, LTD₄ augmented the response to EFS (Fig. 4A).

![Fig. 1. Comparison of electrical field stimulation (EFS)-induced tension responses in small airways isolated from horses with chronic obstructive pulmonary disease (COPD; heavey) and control horses (n = 5/group). Responses are expressed as a percentage of tissue contraction evoked by 127 mM KCl (%KCl). *Significantly different from control value.](http://ajplung.physiology.org/Downloadedfrom)
whereas the MCh concentration-response curve was only slightly affected (data not shown).

Effect of 5-HT. 5-HT (0.01–10 µM) contracted only some of the SA preparations, and the magnitude of the 5-HT-induced contraction was generally small (Fig. 2). Much greater than the effect on the baseline tension was the dose-dependent increase in SA responses to EFS in the presence of 5-HT (Fig. 5A). Maximal augmentation was observed at 1 µM 5-HT. In the presence of Atr and TTX, tissues treated with 1 µM 5-HT did not respond to EFS (Fig. 5B). 5-HT had no effect on the response to exogenous ACh (Fig. 5C). In SAs from COPD horses, 5-HT produced an effect similar to that in normal tissue (Fig. 4B).

Effect of histamine. Consistent with previous data (22), histamine (3 µM) induced a small contraction of SAs and dramatically augmented the responses to EFS (Fig. 6). In the presence of Atr, responses to EFS in the presence of histamine were abolished (Fig. 6), indicating that augmentation of the EFS response by histamine was due to an increased cholinergic response and not by activation of other mechanisms. Similar augmentation in response to histamine was also observed in SAs from the COPD group (Fig. 4C) but not in their response to MCh (data not shown).

Neutrophils

For each experiment, we were able to isolate a sufficient number of neutrophils (1–4 × 10⁶), with both purity and viability > 98%, to perform the tissue bath experiments with neutrophils from both control and heavy horses. We selected STZ as the best neutrophil activator because it does not affect SA responses to EFS, and in contrast to chemotactic ligands that activate neutrophils only for a 5-min period, the STZ-induced respiratory burst lasted over the period of 1 h (data not shown). Neutrophils isolated from heavy horses stimulated with STZ produced significantly more O₂⁻ than those from control horses (Fig. 7).

Even though a sufficient number of neutrophils were isolated and these cells were strongly activated by STZ during each experiment, we did not observe any effect of neutrophils on the EFS response in SAs (Fig. 8). Coincubation of SAs with neutrophils over the period of 30 (data not shown) and 60 min (Fig. 8) neither increased baseline tension nor significantly affected the response to EFS.

DISCUSSION

In a variety of airway diseases and animal models of airway obstruction, the inflammatory response has been shown to be the crucial event leading to bronchospasm and airway hyperresponsiveness (27). In equine COPD, airway inflammation and cholinergically mediated bronchospasm are also associated, but the role of inflammatory response and the mechanisms by which it might affect airway tone remain obscure. Additionally, as previously shown in larger airways (36) and presently in peripheral ones (Fig. 1), tissues isolated from...
horses with COPD are not hyperresponsive. Paradoxically, they produce weaker responses to cholinergic stimulation. This apparent discrepancy between the in vitro and in vivo airway responses provides important information about the mechanism of cholinergic bronchospasm in COPD. Rather than being caused by some chronic changes in nerve terminals or smooth muscle itself, e.g., by upregulation of M₃ muscarinic receptors on airway smooth muscle (ASM) or decreased acetylcholinesterase activity, the increase in cholinergic airway tone is most likely caused by factors that, when present in the airways, facilitate either local ACh release or the response of smooth muscle to ACh released by nerves.

Because COPD is an inflammatory disease in which both airway cytology and autacoid profile change rapidly in response to antigen challenge, we reasoned that inflammatory mediators may be responsible for altered cholinergic responses of horses with COPD. Several inflammatory mediators are known to cause bronchospasm not only via a direct contractile effect on ASM but also by more complex interactions with mechanisms of airway control. Previously, Olszewski et al. (22) showed that histamine has synergistic effects with SA responses to EFS in vitro. To further investigate the...
effects of mediators on cholinergic airway response, we applied two experimental models. In the first, we tested the effect of several inflammatory mediators implicated in the pathogenesis of COPD. In the second approach, we used activated inflammatory cells.

In response to natural antigen challenge, all three inflammatory mediators, histamine, LTD₄, and 5-HT, are reported to increase in respiratory secretion, urine, and plasma of COPD horses (10, 11, 19). In our experiments, treatment of SAs from either control or COPD horses with any of these mediators caused a quite dramatic leftward shift of the frequency-response curve to EFS. Considering the possibility of interactions of these mediators with the airway responses in vivo, particularly interesting is the large (often manyfold) augmentation of SA contraction at the lower range of EFS frequencies. These are the physiological frequencies at which postganglionic parasympathetic nerves are thought to periodically fire in the airways. With regard to the mechanism, we further confirmed that all mediator effects on the EFS responses were exclusively due to modulation of the cholinergic activity because Atr and TTX (blockade of either muscarinic receptors or neuronal fast sodium channels) abolished the responses to EFS in the presence of all three inflammatory mediators. This is in contrast to dog airways where histamine unmasks otherwise absent α-adrenergic contractions in atropine-treated tissues (3).

Even though the maximal synergistic effect of the mediators was observed when tissues were contracted by the mediators up to the level of 10–20% of their maximal response, the effects of the mediators on the EFS response are not just related to the contractile status of the tissue. As we observed, particularly with the lower concentrations of mediators, baseline elevation was not necessary to produce a quite impressive increase in tissue response to EFS. We also observed that the effect of inflammatory mediators on the EFS response was more long lasting than their direct effect on tissue tension. Although responses to smaller concentrations of mediators started to decrease or even waned after several minutes (compare the maximal responses of LTD₄ in Fig. 2 with the baseline representing remaining tension response after 15 min of incubation in Fig. 3), the increased response to EFS was present for a long period of time and, in the case of histamine, persisted for up to 30 min after the washout (Olszewski, personal observations). The response to exogenous cholinergic stimulation with either ACh or MCh was not subject to similar synergism and was simply additive. This observation indicates that the effects of histamine, LTD₄, and 5-HT are not mediated by an alteration at the level of muscarinic receptors on ASM or by a change in the mechanical properties of the tissue due to elevation of the baseline tension by these mediators. The large effect of the mediator on the EFS response and lack of a similar effect on the exogenous ACh-response curve may provide evidence of prejunctional modulation of ACh release from parasympathetic nerve terminals. To make a firm conclusion, measurements of ACh release from SA cholinergic nerves in the presence of inflammatory mediators is required. We attempted to measure ACh release from SAs in the presence and absence of histamine utilizing HPLC coupled with electrochemical detection. Even though this method is very well established in our laboratory for measurement of ACh release in both bronchi and the trachea (31, 37), we could not determine the release of ACh from equine SAs because the amount of ACh released was very small and below the level of detection. Regardless of the mechanism by which inflammatory mediators exerted their effect on the EFS response, we have shown that inflammatory autacoids may greatly influence the magnitude of the endogenous, cholinergic response in equine terminal airways, and in this respect, several mediators may exert a similar effect. This strong synergism between mediator-induced airway contraction in response to nerve stimulation at a physiological range of frequencies is consistent with the hypothesis that inflammatory mediators released in response to antigen challenge are responsible for the increased cholinergic tone of the airways in horses with COPD. Noteworthy, all of these mediators could produce effects of similar magnitude. In the clinical course of COPD, where many inflammatory mediators act in concert, this may be responsible for the relatively low therapeutic...
tic efficacy of compounds that block the effects of singular mediators (e.g., antihistamines) in contrast to very efficient glucocorticosteroids that blunt all of the inflammatory process (4, 17).

In our second approach, we re-created the milieu of neutrophilic inflammation through direct contact of SA preparations with activated neutrophils. Neutrophils are implicated in the pathogenesis of COPD because 1) they are consistently recruited into the airways and their increase in bronchoalveolar lavage is one of the classic clinical findings in COPD (25); 2) neutrophils isolated from peripheral blood of heavy horses produced more \( \text{O}_2^\bullet^- \) in response to activation with chemoattractant ligands and STZ (Fig. 7) and neutrophils in the airways of horses with COPD are strongly activated (21); and 3) in other species, neutrophils or neutrophil-derived inflammatory mediators such as reactive oxygen species or thromboxane \( \text{A}_2 \) have been shown to contract smooth muscle or affect their responses (7, 15, 35). Even though indirect evidence supports it, the exact role of the neutrophil in the pathogenesis of COPD is not very clear. On one hand, antigen challenge-induced recruitment of neutrophils into the airways of COPD-susceptible horses is generally concurrent with the first changes in lung function, which argues for a strong association between these two events. On the other hand, in some exceptional individuals, changes in lung function precede neutrophil recruitment or the recruitment appears earlier than the alterations in pulmonary function (12). These last few pieces of evidence indicate that even though the association between neutrophil recruitment and changes in lung function may exist, neutrophils are neither sufficient nor necessary for airway obstruction to occur. Our data clarify this even further. Coincubation of SAs with a large number of strongly activated neutrophils over a period of \( \leq 1 \) h did not significantly alter the SA responses. In fact, there was a tendency to decrease the EFS response rather than to cause augmentation. Also, smaller numbers of neutrophils, unactivated neutrophils, or neutrophils activated by stimuli other than STZ failed to alter in vitro responses of SAs or equine tracheae (personal observations). Lack of a synergistic effect of neutrophils was not entirely surprising and parallels some other observations. For example, hydrogen peroxide, one of the reactive oxygen species produced by activated neutrophils, decreases cholinergic responses in equine tracheae (23), and the neutrophil-derived enzyme elastase, in concentrations present in respiratory secretion, decreases tension responses of rabbit trachealis (7).

Our observation changes our view of the pathogenesis of COPD by suggesting that neutrophils may not be as important in the pathogenesis of COPD as previously postulated and that the cholinergic component of airway obstruction in horses is most likely "neutrophil independent." However, the role of the neutrophil cannot be entirely excluded based on our data. Neutrophil products may exert some long-term effects on airways, e.g., promote inflammation, edema formation, and mucous secretion, and in this way contribute to airway obstruction by mechanisms not directly related to neuromuscular regulation of airway tone. In contrast to neutrophil-derived mediators, effects of histamine, \( \text{LTD}_4 \), or \( 5\text{-HT} \) may explain the mechanisms of increased cholinergic airway tone in COPD. These mediators are traditionally associated with a type I allergic reaction (mast cell derived), and in this respect, our data support some current reports (9, 16, 19, 30, 34) favoring a type I reaction as an important mechanism in the development of airway obstruction in COPD.
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