In vitro responses of distal airways in horses with recurrent airway obstruction

P. H. LeBlanc, DVM, MS; R. V. Broadstone, DVM; F. J. Derksen, DVM, PhD; N. E. Robinson, PhD, BVetMed

SUMMARY

Distal airway segments (ID, 3 to 4 mm; length, 5 mm) from 2 groups of horses were isolated and suspended in tissue baths filled with Krebs solution, aerated with 5% CO₂ in oxygen and maintained at 37 C. Responses to exogenous acetylcholine, isoproterenol, or electrical field stimulation were compared. Control horses (n = 30) had no history of recurrent airway obstruction, whereas principal horses (n = 15) had recurrent airway obstruction and were studied during an acute episode of airway obstruction. Although the distal airways contracted in response to the cumulative half-logarithmic addition of acetylcholine (10⁻¹⁰M to 10⁻⁸M) in both groups, bronchi obtained from principals were less sensitive to acetylcholine than were bronchi obtained from controls. Tetrodotoxin-sensitive electrical field stimulation-induced contractions were observed in both groups of airways, but the tension achieved in principal bronchi was less than in controls. All electrical field stimulation-induced contractions were abolished by atropine, indicating that the only excitatory innervation of equine distal airways is through the parasympathetic system. To examine the effect of isoproterenol and determine inhibitory innervation, bronchi were precontracted with histamine. Electrical field stimulation did not cause relaxation of precontracted bronchi in either group, thus indicating that distal airways lack inhibitory innervation. Isoproterenol caused similar, dose-dependent relaxation in both groups.

Recurrent airway obstruction (heaves) is a respiratory tract syndrome of horses characterized functionally by spontaneous, yet reversible, airway obstruction and, pathologically, by bronchiolitis. Clinical manifestations vary, but affected horses may have signs of disease that range from mild dyspnea to severe respiratory distress. The disease is precipitated by exposing affected horses to a barn environment and hay. During acute manifestations of disease, horses have increased pulmonary resistance, decreased dynamic compliance, arterial hypoxemia, prolongation of N₂ washout, and airway hyperresponsiveness. A large proportion of airway obstruction associated with this condition is reversible by administration of the nonspecific muscarinic antagonist atropine, but β-adrenergic blockade causes further airway obstruction. These findings implicate the autonomic nervous system in pathogenesis of the disease. The purpose of the study reported here was to characterize the major innervation of the distal airways and their responses to muscarinic and β-adrenergic agonists in clinically normal and affected horses.

Materials and Methods

Horse preparation—Fifteen horses with a history of recurrent airway obstruction (principals) were studied during an acute attack of airway obstruction precipitated by barn housing. Airway obstruction was indicated by expiratory dyspnea, as well as a change in esophageal pressure (> 15 cm of H₂O) during tidal breathing. Esophageal pressure was determined, using an esophageal balloon located in the midthoracic portion of the esophagus and attached to a pressure transducer. Thirty horses that had no history of recurrent airway obstruction or respiratory problems (controls) also were studied. Other tissues from the control horses were used in various experiments.

Preparation of bronchial segments—Horses were euthanized by overdose of sodium pentobarbital (90 mg/kg of body weight). The lungs were immediately harvested, and distal airway segments (ID, 3 to 4 mm) were dissected free of excess tissue and suspended in 10-ml organ baths. The 5-mm-long rings were hung in tissue baths between an immovable and a movable stainless steel stirrup. The movable stirrup was attached via 3-0 silk thread to a force transducer, and the force exerted by the bronchial ring was recorded on a multichannel polygraph. Tissues were equilibrated for 120 minutes at the predetermined optimal tension of 2 g. Baths contained a physiologic saline solution (Krebs solution: NaCl, 118 mM KCl, 4.7 mM; MgCl₂, 0.5 mM; CaCl₂, 2.5 mM, NaH₂PO₄, 1 mM; NaHCO₃, 25 mM; and dextrose, 5.5 mM), which was changed at 15-minute intervals during the equilibration period. The solution was continuously aerated with a mixture of oxygen (95%) and carbon dioxide (5%) to maintain pH of 7.40, P/O₂ of 38 mm of Hg, and P/O₂ of 999.

Received for publication Apr 18, 1990.
From the Pulmonary Laboratory, Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, MI 48824. Supported in part by USPHS grants HL 27619 and HL 01742. The authors thank Cherie Benson, Cathy Bernoy, Susan Eberhart, and Mary Ellen Shea for technical assistance.
Address reprint request to Dr. Patrick H. LeBlanc.
approximately 500 mm of Hg; temperature was kept constant at 37.5 °C.

Two rectangular platinum electrodes (10 × 15 mm) were placed parallel to the tissue strips for electrical stimulation of the tissues. Electrical impulses were produced by a voltage stimulator and passed through a stimulus power booster. The electrical impulses consisted of square waves. Voltage and duration were monitored on an oscilloscope.

All drugs were prepared in deionized water and expressed as final bath molar concentration. Ascorbic acid (0.1%) was added to the isoproterenol to retard oxidation.

Response of the airways to acetylcholine—Response of the bronchial rings to the cumulative addition of acetylcholine (ACH) was examined in airways from 8 controls and 8 principals. The ACH concentrations in the tissue baths ranged from 10^{-9} M to 10^{-3} M and were increased in half-logarithmic increments once the response to the preceding concentration reached a stable plateau. After response to the highest concentration of ACH (10^{-3} M) stabilized, the absolute maximal contractile response was elicited, using barium chloride (BaCl; 3 × 10^{-3} M). The contractile response at each ACH concentration was expressed as percentage of the BaCl-induced response.

Response of the airways to isoproterenol—The cumulative dose response to isoproterenol was investigated in airways from 7 control and 5 principals. Immediately after the 120-minute equilibration period, response to (10^{-9} M) ACH maximal contraction was determined. Tissues were then rinsed (45 to 60 minutes) with buffer until active tension was eliminated and resting tension on the tissues returned to baseline value of 2 g. Histamine was added cumulatively in half-logarithmic increments from 10^{-9} M to the bath until the tissues contracted to 50% of the ACH-induced response. In preliminary studies, small bronchi had histamine-induced phasic contractions; therefore, to prevent such phasic contractions, all were incubated for 30 minutes with indoethacin (3 × 10^{-6} M) prior to the histamine addition. Once the histamine-induced contraction was stable, isoproterenol was added cumulatively in half-logarithmic increments from 10^{-9} to 10^{-3} M. Relaxation in response to isoproterenol was expressed as percentage of precontracted tension.

Response of the airways to electrical field stimulation—Tissues from 12 controls and 6 principals were stimulated at 20 V and at frequencies of 0.25, 0.5, 1, 3, 5, 10, 15, 20, 25, and 32 Hz. Stimuli were applied for a duration of 0.5 ms. Frequency was increased after the contractile response from the preceding stimulation reached a stable plateau. Thirty minutes after electrical stimulation, either atropine (10^{-6} M) or tetrodotoxin (3 × 10^{-6} M) was added to some of the baths for 15 minutes and the frequency response was again tested. After the highest frequency was applied to the tissues (32 Hz), the absolute maximal contractile response was elicited, using BaCl (3 × 10^{-3} M). The contractile response at each electrical frequency was expressed as percentage of the BaCl-induced response.

In a separate set of experiments (n = 3 controls and n = 3 principals), tissues were precontracted with histamine as previously described. Atropine (10^{-6} M), phentolamine (10^{-6} M), and indoethacin (3 × 10^{-6} M) were added to the baths prior to incubation for 30 minutes. While precontracted, tissues were stimulated: 20 V, 0.25 to 32 Hz, and 0.5-ms duration. Results of preliminary studies indicated stability of histamine-induced contraction in the absence of electrical field stimulation.

Statistical analysis of data—Only 1 protocol (dose or frequency response) was performed/segment. Each n value represents the average data for 4 bronchial segments from 1 horse. Results are expressed as mean ± SEM. Differences between means for the 2 groups of horses at specific concentration of drug agonist or specific stimulation frequency were compared by use of the unpaired Student t test. When P < 0.05, means were considered to be significantly different.

Results

Characteristics of the bronchial segments—Segments from control and principal bronchi were easily dissected free of surrounding lung parenchyma. Gross examination indicated that the walls of control airways were thicker than those of control airways and often contained mucus. Histologic examination revealed intact epithelium and cartilage in control and principal bronchi. Resting baseline tension was stable in both groups, and spontaneous phasic contractions were rarely observed.

Response of the bronchi to exogenous acetylcholine—The cumulative addition of acetylcholine induced a concentration-dependent contraction in all bronchi. In both groups, maximal response to the highest concentration of ACH studied (10^{-3} M) was not different from maximal response to the nonspecific agonist BaCl. Additionally, this maximal contractile response (grams) as not different between the 2 groups (Table 1). As indicated by the higher negative logarithmic value of the molar concentration required to produce a contraction that is 50% of the maximal contraction (pD2 value), control bronchi were more sensitive to acetylcholine than were principal bronchi. The increased sensitivity of the control group was further indicated by the significantly greater tension developed by the control group at ACH concentrations ranging from 10^{-7} to 10^{-3} M (Fig 1). Concentration > 10^{-4} M induced contractions that were not significantly different between

| Table 1—Average maximal active force (grams) induced by BaCl and average pD2 values to isoproterenol and acetylcholine (ACH) for control and principal distal bronchi |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | ACH             | Isoproterenol   | Max contractile | pD2 values      |
| Control         | 5.22 (0.13)     | 6.83 (0.29)     | 21.4            | 6.33 (0.29)     | NS              |
| Principal       | 6.82 (0.08)     | 6.89 (0.1)      | 20.7            | 7.1 (0.08)      | NS              |
| P value         | < 0.01          | NS              | NS              | NS              |
| Values represent mean ± SEM. pD2 = 50% of maximal contraction; Max = maximal; NS = no significant difference. |
controls and principals. Acetylcholine-induced contractions were blocked by atropine.

Response of the bronchi to isoproterenol—Histamine contracted principal bronchi to 56.6% of the ACH maximum and to 60.9% in controls; values were not statistically different. The concentration of histamine required to induce this equal tension in principals and controls was $2.1 \times 10^{-6} \text{M}$ and $2.4 \times 10^{-6} \text{M}$, respectively. Although isoproterenol caused a concentration-dependent relaxation of bronchi, in neither group did tension return to baseline (Fig 2). The sensitivity of principals and control tissues to isoproterenol was similar; the $pD_2$ values were not significantly different (Table 1), and the dose-response curves of the 2 groups were not significantly different.

Response of bronchi to electrical field stimulation—Electrical field stimulation (EFS) of distal equine airways induced frequency-dependent contraction. The maximal contraction that was blocked by tetrodotoxin was elicited at 20 V, 0.5 ms, and 32 Hz. This contractile response was also abolished by atropine ($10^{-6} \text{M}$). Response to EFS was expressed as a percentage of the response to BaCl (Fig 3). At every frequency, significantly more contraction was apparent in the control than in the principal bronchi. In controls, the maximal EFS-induced contraction was 65.4 ($\pm 4$)% of the contraction induced by BaCl and was 39.4 ($\pm 4$)% in the principals. These 2 values were significantly different.

Electrical field stimulation of histamine-precontracted bronchi had no effect on airways from either control or principal horses.

Discussion

The bronchial smooth muscle from control horses contracted to ACH in a concentration-dependent manner. All contractions were mediated via muscarinic receptors, as indicated by results of blockade with atropine. The $pD_2$ value of control airways (5.32) is equivalent to an EC$_{50}$ value of approximately $4.5 \times 10^{-6} \text{M}$. This concentration of ACH was similar to that reported in equine third-generation bronchi and trachea, indicating that the sensitivity of airway smooth muscle to ACH does not vary along the tracheobronchial tree.

Because the equine airway has no inherent tone, it was necessary to precontract the airways with histamine to detect relaxation. Indomethacin was added to the tissue baths prior to histamine to prevent phasic contractions. These phasic contractions have also been observed in canine and bovine airway smooth muscle. The observed concentration-dependent relaxation of distal equine airways to isoproterenol confirms the existence of $\beta$-adrenergic receptors in these tissues. Results of other in vitro studies have indicated that $\beta$-adrenergic agents induced dilatation in the trachea of horses and a $pD_2$ value similar to that obtained by us was reported. Epithelium is essential for maximal relaxation in response to isoproterenol and, in the absence of epithelium relaxation, is considerably less. In the trachea and small bronchi (from the horses of our study) with epithelium intact, the magnitude of relaxation induced by isoproterenol appeared identical.

The contractile response of the small bronchi to EFS was
dependent on frequency of stimulation. The stimulus variables (20 V, 0.5 ms) were recommended for equine airways by Mason et al. In small bronchi, as in more central airways of horses, contractile responses were abolished by tetrodotoxin or atropine and, therefore, were mediated through cholinergic nerves. Thus, it appears that the cholinergic system is the predominant excitatory innervation of central and peripheral airways in horses.

The maximal contractile response of control bronchi to EFS was 64.5% of the maximal response to BaCl or ACH. This compares with 89% reported for the trachea and 68% for third-generation bronchi. The diminishing response to EFS, in comparison to ACH, from trachea to more peripheral airways has also been reported in other species and is explained by a less-abundant cholinergic innervation in the peripheral airways or by differences in cell-to-cell contact in airway smooth muscles.

In general, mammalian airway smooth muscle receives 2 types of inhibitory innervation, a sympathetic system and a nonadrenergic inhibitory system. The trachea of horses has both of these systems, whereas third-generation bronchi have only a nonadrenergic system. The nonadrenergic system is responsible for relaxing the trachea by 40%, but the third-generation bronchi by only 20%, in response to EFS. The observed lack of EFS-induced relaxation in precontracted small bronchi indicates lack of either inhibitory nervous system and confirms a pattern of decreasing inhibitory innervation from the central to the peripheral airways. A pattern of central-to-distal reduction in adrenergic innervation of airways has previously been reported in guinea pigs.

During an acute attack of recurrent airway obstruction in horses, airways are hyperresponsive to the inhaled cholinergic agonist methacholine. We examined the in vitro response of distal bronchi obtained from affected horses during acute exacerbation of airway obstruction. As indicated by the lower pD_{2} value and the right shift of the ACH dose-response curve, principal airways were less, rather than more sensitive to ACH than were control airways. In another study, we observed that the trachea and third-generation bronchi of principal horses also were hyporesponsive to ACH. The airway hyperresponsiveness of principal horses to inhaled methacholine is not, therefore, attributable to an exaggerated response of airway smooth muscle to cholinergic stimulation. This agrees with results obtained from human beings and dogs.

Although airway morphometry was not an objective of this study, it was observed that airways from principals were markedly thicker than airways from control horses. Future quantitative studies of airway wall thickness are warranted, because the mathematical findings of Maren indicate that for a given amount of smooth muscle shortening, airways with thick walls may have exaggerated increase in lung resistance.

In horses with recurrent airway obstruction and in asthmatic human beings, β-adrenergic blockade augments airway obstruction. These observations could be explained by upregulation of β receptors in the diseased airways. The dose responses to isoproterenol observed in our study of horses did not support upregulation of β receptors in the airways of principals. The dose-response curves indicated similar β-adrenergic receptor function in the 2 groups.

Szentivanyi proposed that abnormalities in the β-adrenergic system is a feature of bronchial asthma in human beings. Recurrent airway obstruction is used as model of asthma and bronchial hyperreactivity and, therefore, our study provided an opportunity to examine Szentivanyi's hypothesis. This hypothesis has been difficult to prove in human beings, because lung specimens are often obtained from asthmatics administered β-adrenergic agents on a long-term basis. The principal horses used in this study were not given β-adrenergic drugs, and clearly, the β-adrenergic response of the airways was not abnormal.

At all frequencies studied, the contractile response of small bronchi from principal horses was less than that observed in controls. In airways from controls, the maximal response to EFS was 64.5% of the maximal response to ACH. Examination of the ACH dose-response curve (Fig 1) revealed that 64.5% maximal response could be induced by ACH concentration of approximately 1.3 × 10^{-5} M. In the principal group, this concentration induced only 41% of the maximal contraction to ACH, which was identical to the maximal response to EFS (32 Hz; Fig 3). If, at this frequency, the same amount of ACH is released in each group of horses, the lower maximal response of principal airways to EFS can be explained by hyporesponsiveness to ACH.

At submaximal stimulus frequencies, the response of principal airways to EFS was less than could be explained by hyporesponsiveness to ACH. It is, therefore, possible that other factors inhibited the contractile response to EFS. Differences in inhibitory innervation between groups cannot explain the observed results because evidence of an inhibitory nervous system in these small bronchi was lacking. In a previous study, it was determined that airway inflammation develops during an acute attack of recurrent airway obstruction. Because prostaglandins, such as prostaglandin E2, can inhibit release of and response to ACH, it is possible that these inflammatory mediators reduced the EFS-induced response in the principal group.

References


