Immunohistochemical determination of the expression of endothelin receptors in bronchial smooth muscle and epithelium of healthy horses and horses affected by summer pasture-associated obstructive pulmonary disease

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**Objective**—To immunohistochemically determine the expression of endothelin (ET) receptors in bronchial smooth muscle and epithelium of healthy horses and horses affected by summer pasture-associated obstructive pulmonary disease (SPAOPD).

**Sample Population**—Tissue specimens obtained from 8 healthy and 8 SPAOPD-affected horses.

**Procedure**—Horses were examined and assigned to healthy and SPAOPD groups. Horses were then euthanatized, and tissue specimens containing bronchi of approximately 4 to 8 mm in diameter were immediately collected from all lung lobes, fixed in zinc-formalin solution for 12 hours, and embedded in paraffin. Polyclonal primary antibodies against ET-A or ET-B receptors at a dilution of 1:200 and biotinylated IgG secondary antibodies were applied to tissue sections, followed by the addition of an avidin-biotin immunoperoxidase complex. Photographs of the stained slides were digitally recorded and analyzed by use of image analysis software to determine the intensity of staining. Two-way ANOVA was used for statistical analysis.

**Results**—The left diaphragmatic lung lobe of SPAOPD-affected horses had a significantly greater area of bronchial smooth muscle that immunostained for ET-A, compared with that for healthy horses. All lung lobes of SPAOPD-affected horses, except for the right diaphragmatic lobe, had significantly greater staining for ET-B receptors in bronchial smooth muscle, compared with results for healthy horses.

**Conclusions and Clinical Relevance**—This study revealed overexpression of ET-A and, in particular, ET-B receptors in the bronchial smooth muscle of SPAOPD-affected horses, which suggested upregulation of these receptors. These findings improve our understanding of the role of ET-1 in the pathogenesis of SPAOPD. (Am J Vet Res 2006;67:348–357)
humans. It has been reported that ET-1 can induce the release of acetylcholine from nerve endings by acting through ET-A and ET-B receptors in isolated tracheal tissues obtained from rats and rabbits. Furthermore, ET-1 can also cause bronchoconstriction by releasing secondary mediators, such as thromboxanes and platelet-activating factor. Endothelin-1 stimulates DNA synthesis and cell proliferation in various cells, including smooth muscle cells, and ET-1 can act as a comitogen by acting synergistically with other growth factors, including epidermal growth factor, platelet-activating factor, and platelet-derived growth factor. Endothelin-1 can also elicit proinflammatory effects on airways, and in patients with pulmonary allergic inflammation, ET-1 upregulates cytokines, such as IL-1, IL-4, IL-8, tumor necrosis factor-α, and interferon-γ.

Most of the vital organs in the body synthesize ET-1; however, the lungs are the primary site of synthesis and degradation of ET-1. Cells important for the synthesis of ET-1 in the lungs include vascular endothelial cells, epithelial cells, Clara cells, goblet cells, neuroendocrine cells, smooth muscle cells, platelets, mucus cells, serous cells, parasympathetic ganglia within the airways, and alveolar macrophages. The greatest density of ET receptors in the lungs is found in smooth muscles and the alveolar septae.

Pulmonary ET-1 concentrations are increased in humans with COPD; rats with experimentally induced allergic inflammation, bronchiectasis, bronchiolitis obliterans, and bleomycin-induced pulmonary fibrosis; and cultured rat pulmonary endothelial cells subjected to oxidant stress. Similarly, ET-1 concentrations in the systemic circulation and bronchoalveolar lavage fluid are greater in horses affected with RAO, compared with concentrations in clinically normal horses. Researchers in that study detected a spasmodic action of ET-1 in isolated specimens of third-generation pulmonary arteries and bronchi in horses. It has also been reported that ET-1 elicits bronchial constriction in horses by acting through ET-A and ET-B receptors. This close relationship between ET-1 and the lungs has prompted our laboratory group to conduct preliminary in vitro pharmacologic studies; those studies revealed that ET-1 can induce exaggerated bronchoconstriction in SPAOPD-affected horses, compared with responses in healthy horses. This led us to hypothesize that ET receptors are altered in the bronchi of SPAOPD-affected horses.

In the study reported here, we especially wanted to investigate alterations in ET receptors in bronchial epithelium and smooth muscles because these are the tissues most affected in animals with obstructive pulmonary diseases and also are rich sources of ET receptors. Therefore, any findings regarding alterations in the amounts of ET receptors in these tissues would help us to better understand the role of ET-1 and its receptors in the pathogenesis of SPAOPD. Additionally, it has been reported that COPD in horses and humans does not have a uniform effect throughout the lungs. In the study reported here, gross pathologic changes that were noticed in the postmortem evaluation of the lungs of SPAOPD-affected horses were not uniformly distributed throughout the lungs. Therefore, we also wanted to investigate whether there were any interlobar differences in the alteration of receptors in SPAOPD-affected horses. Hence, the purpose of the study reported here was to immunohistochemically compare the expression of ET receptors in bronchial smooth muscles and epithelium for all 5 lung lobes of healthy and SPAOPD-affected horses.

Materials and Methods

Horses—Specimens were obtained from 16 horses for use in the study. All horses were procured by donation. For inclusion in the study, all horses had to have values within the respective reference ranges for rectal temperature, heart rate, respiratory rate, and results of thoracic auscultation. In addition, none of the horses included in this study had received any medication for at least 7 days before clinical evaluation. All SPAOPD-affected horses had a history of signs of obstructive pulmonary disease after they were exposed to pasture during the summer. In addition to the medical history, SPAOPD-affected horses had to have a clinical score ≥ 5.0 and ΔPpl > 15.0 cm H2O.

The horses comprised 8 healthy (control) horses (2 females, 5 geldings, and 1 stallion; 6 Quarter horses, 1 Paint, and 1 Arabian) that were 10 to 20 years old (mean ± SD, 15.6 ± 3.3 years) and 8 SPAOPD-affected horses (4 females, 3 geldings, and 1 stallion; 3 Quarter horses, 3 Paint, 1 Appaloosa, and 1 Thoroughbred) that were 10 to 20 years old (mean, 14.8 ± 2.4 years). The study was approved by the Institutional Animal Care and Use Committee of Louisiana State University.

Clinical evaluation—The general demeanor and respiratory behavior of each horse were observed. Rectal temperature, heart rate, respiratory rate, nostril flare, and abdominal lift (ie, the upward movement of the abdomen from its natural position when the horse tries to exhale during breathing) were recorded. In addition, auscultation was used to assess respiratory tract sounds. Finally, clinical score and ΔPpl were determined.

Clinical scores were determined by use of the following equation:

$$CS = ([Flare \ of \ medial \ nostril + \ flare \ of \ lateral \ nostril] / 2) + \ abdominal \ lift$$

Each of the variables was scored on a scale from 0 to 4. For nostril flare, a score of 0 indicated that the nostril had little movement, whereas a score of 4 indicated that the nostril remained maximally flared throughout the respiratory cycle. For abdominal lift, a score of 0 indicated that the ventral portion of the flank had little or no movement, whereas a score of 4 indicated that a heave line (ie, a line formed by the enlargement of the abdominal muscles along the edges of the ribs attributable to the extra usage of these muscles to force the air out of the lungs) extended cranially to the fifth intercostal space. Thus, 8 was the maximum clinical score for any horse.

The ΔPpl was measured indirectly by use of an esophageal balloon secured over the end of a catheter, which was connected to a pressure transducer interfaced with a polygraph. A balloon (10 cm in length and 3.5 cm in circumference) was placed over the end of a 2-m-long, 2-mm-diameter cannula. The balloon was inserted through a lubricated nasogastric tube that was passed into the cranial portion of the esophagus. Once the balloon was located between the heart and diaphragm, the nasogastric tube was removed. The balloon was inflated with 1.5 mL of water, and measurements were recorded for 1 minute. Changes in esophageal pressure (peak inspiratory pressure minus peak expiratory pressure) during tidal breathing reflect ΔPpl.
Tissue collection and processing—The day after clinical evaluation, horses were euthanatized by administration of an overdose of sodium pentobarbital5 (90 mg/kg, IV). Gross postmortem evaluation of the lungs was performed, and the lungs were then removed from the thoracic cavity. Three specimens, each of which was approximately 3 X 3 cm, were obtained from each of the 5 lung lobes (right diaphragmatic, left diaphragmatic, right apical, left apical, and accessory) of each horse. We ensured that each specimen collected contained at least 1 bronchus (bronchus diameter of approx 4 to 8 mm). Specimens were immediately immersed in zinclin fixative solution and allowed to soak for at least 12 hours. Specimens then were dehydrated by use of graded concentrations of ethyl alcohol (70%, 90%, and 100%) and subsequently cleared by use of xylene. Finally, the specimens were embedded in paraffin and stored until used for immunohistochemical analysis. Lung specimens obtained from rats were used as positive control samples31; they were immunohistochemically stained and allowed to incubate for at least 12 hours. Positive and negative control samples were collected, snap-frozen in liquid nitrogen, and stored at –80°C.

Preparation of antibodies—A polyclonal primary antibody (anti-ET-A receptor) used in the study was raised in rabbits against the equine ET-A receptor.32 To confirm cross-reactivity of the antibody, western blot analysis was performed. The antibodies raised against the rat ET-A receptor were expected to cross-react with the equine ET-A receptor used in the study was raised in rabbits against the equine ET-A receptor.32 To confirm cross-reactivity of the antibody, western blot analysis was performed. The antibodies raised against the rat ET-A receptor were expected to cross-react with the equine ET-A receptor.

Preparation of slides for immunohistochemical analysis—Of the 3 paraffin blocks with tissue from each lung lobe, the block with a bronchus closest to the desired diameter (4 to 8 mm) was selected for use. Tissue sections (4 µm thick) were cut by use of a microtome and mounted on clean slides coated with silane. Three slides were prepared from each paraffin block. Two of the 3 slides were used for detecting ET receptors (ET-A and ET-B on 1 slide each), and the other slide was used as a negative control slide. Paraffin was then removed from these tissue sections by use of xylene, and tissue sections were subsequently rehydrated by use of graded concentrations of ethyl alcohol (100%, 90%, and 70%, respectively). All slides were subjected to immunohistochemical analysis.33,34

Immunohistochemical analysis—Immunohistochemical analyses were performed by use of an automated autostainer.59 Optimization of the antibody revealed that staining was best when an antigen retrieval method was not used. All rinsing steps were conducted by use of TBBS (pH 7.6) containing 0.05% Tween 20, except as indicated. Slides were rinsed with TBBS, and endogenous peroxidase activity of the tissues was blocked by applying 3% H2O2 for 10 minutes. Sections were then rinsed, and endogenous avidin and biotin were blocked by use of avidin-biotin blocking agent and incubation for 10 minutes. Slides were again rinsed, and endogenous protein was blocked by the addition of normal rabbit serum and incubation for 30 minutes at 25°C. Blocking serum was prepared in accordance with the manufacturer’s guidelines.

Excess blocking serum was removed, and tissue sections on the slides were incubated with sheep anti–ET-A receptor polyclonal primary antibody or sheep anti–ET-B polyclonal primary antibody (dilution of 1:200) for 30 minutes at 25°C. For the negative control samples, primary antibody was replaced by sheep γ globulin (dilution of 1:1,000). Primary antibodies and sheep γ globulin were diluted in antibody diluent.58 After incubation, slides were rinsed and biotinylated rabbit anti-sheep IgG secondary antibodies were applied and allowed to incubate for 30 minutes. Slides were then rinsed, and immunoperoxidase system* was applied to the tissue sections. Then, all tissue sections were washed with TBBS. Sites of immunostaining were developed by use of a red peroxidase substrate that was applied to the sections for 8 minutes. Slides were then washed with TBBS followed by deionized water, counterstained with hematoxylin, and allowed to incubate for 5 minutes. The slides were again thoroughly washed with TBBS followed by deionized water. Sections were dehydrated through graded concentrations of ethyl alcohol and cleared with xylene. Finally, the sections were covered by glass coverslips and allowed to dry.

Image analysis—Photographs of the stained slides were obtained at 20X magnification by use of a color camera mounted on a microscope.54 Corresponding software was used to calibrate image variables before each image was photographed. Light intensity and exposure settings were kept constant for all photographs. Five photographs were made of each slide. Photographs were exported to image analysis software.54 The software was used to apply color markers on the images to differentiate immunostained tissue from other tissues. A red marker was applied to immunostained sections of the tissues, whereas blue, light blue, and yellow markers were applied to stained sections of nuclei, non-specific tissues, and the background of each slide, respectively. These

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color markers represented the intensity of staining. For example, the greater the intensity of immunostaining, the greater the pixel value for the red marker and hence the greater the red marker’s percentage of the total area.

A template image was created by use of a positive control slide in which the aforementioned color markers were applied. This template was used to attribute the color markers to all of the slides analyzed. Total area of the tissue of interest (ie, bronchial epithelium or bronchial smooth muscle) was manually selected. It was ensured that the background did not exceed > 10% of the total area selected. Percentage of immunostained area ([total stained area/total selected area] × 100) of the tissue was determined for each of the 5 photographs. This procedure was applied to all 3 types of slides (ie, the slide to which ET-A antibody was applied, the slide to which ET-B antibody was applied, and the slide used as a negative control sample). Percentage of immunostained area on the negative control slide was subtracted from the percentage of total immunostained area for each of the other slides. Mean values for the 5 photographs were calculated and exported to a computer spreadsheet program.*a

Statistical analysis—Clinical scores and ∆Ppl data were reported as median and range values. These variables were compared between healthy and SPAOPD-affected horses by use of the Mann-Whitney test. Immunohistochemical data were analyzed by use of a 2-way ANOVA, and pairwise comparisons were made by use of post hoc Bonferroni tests. For antibody specificity to all of the slides analyzed. Total area of the tissue of interest (ie, bronchial epithelium or bronchial smooth muscle) was manually selected. It was ensured that the background did not exceed > 10% of the total area selected. Percentage of immunostained area ([total stained area/total selected area] × 100) of the tissue was determined for each of the 5 photographs. This procedure was applied to all 3 types of slides (ie, the slide to which ET-A antibody was applied, the slide to which ET-B antibody was applied, and the slide used as a negative control sample). Percentage of immunostained area on the negative control slide was subtracted from the percentage of total immunostained area for each of the other slides. Mean values for the 5 photographs were calculated and exported to a computer spreadsheet program.*a

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Results
Clinical evaluation—Mean values of heart rate, respiratory rate, and rectal temperature for all horses were close to the respective reference ranges (Table 1).

Table 1—Signalment, clinical variables, clinical score, and ∆Ppl for SPAOPD-affected horses and healthy horses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SPAOPD-affected horses</th>
<th>Healthy horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>14.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37.8</td>
<td>38.0</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>46</td>
<td>39</td>
</tr>
<tr>
<td>Respiratory rate (breaths/min)</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>Clinical score*</td>
<td>5.5*</td>
<td>1.5*</td>
</tr>
<tr>
<td>∆Ppl (cm H2O)</td>
<td>25</td>
<td>20–32</td>
</tr>
</tbody>
</table>

*Clinical score assigned on a scale of 0 to 8.

All horses were in good body condition and had a typical appetite and demeanor. All SPAOPD-affected horses had respiratory wheezes detected during auscultation, nostril flaring, an increase in abdominal lift, and coughing. Few SPAOPD-affected horses had a marked heave line extending along the abdominal wall. Clinical score for the SPAOPD-affected horses (median, 5.50; range, 5.0 to 7.0) was significantly greater than the clinical score for healthy horses (median, 1.5; range, 1.0 to 3.5). Similarly, ∆Ppl of SPAOPD-affected horses (median, 25.0 cm H2O; range, 20 to 32 cm H2O) was significantly greater than the value for healthy horses (median, 7.0 cm H2O; range, 4.0 to 9.0 cm H2O).

Postmortem assessment of lungs—All SPAOPD-affected horses had hyperinflated orange-pink lungs that did not collapse after the thoracic cavity was opened. Some regions of the lungs had fibrous texture, and it was difficult to excise specimens from those regions. Lungs of many SPAOPD-affected horses had distinct pale fibrotic patches distributed nonuniformly on the surface of the lungs, and the surface of the lungs of some SPAOPD-affected horses had indentions or impressions caused by pressure from the ribs. Mucus plugs were observed in the airways; however, this observation was not uniform throughout the lung fields.

Lungs of healthy horses were pale pink and of the expected size. The lungs collapsed immediately and completely after the thoracic cavity was opened. The lungs had a typical texture, and it was easy to excise specimens. None of the lungs of healthy horses had noticeable fibrotic patches or rib impressions on the lung surface. Only a few mucus plugs were noticed inside the bronchi.

Antibody specificity—Western blot analysis revealed that the band sizes of ET-A or ET-B receptors were identi-
cal in lung specimens obtained from rats and horses (Figure 1). Immunohistochemical analysis revealed that lung sections from rats had intense immunostaining for both receptors and no staining in negative control samples (Figure 2). Similarly, lung sections from horses had immunostaining for both receptors and no staining in the negative control samples (Figure 3).

**Results of immunostaining**—Analysis of pooled data revealed that the percentage of immunostained area for ET-A receptors in SPAOPD-affected horses was significantly greater in bronchial smooth muscle than in the epithelium; however, this percentage was not significantly greater than that for the ET-A receptors in bronchial smooth muscle of healthy horses (Figure 4). In addition, there was no statistical difference in the percentage of immunostaining for ET-A receptors between epithelium and bronchial smooth muscle of healthy horses. Similarly, there was no significant difference in the percentage of immunostaining for ET-A receptors in the epithelium between healthy and SPAOPD-affected horses.

Analysis of pooled data for ET-B receptors revealed that SPAOPD-affected horses had a significantly greater percentage of immunostaining in the bronchial smooth muscle than in the epithelium (Figure 5). However, there was no difference in immunostaining between epithelium and bronchial smooth muscles of healthy horses. The ET-B receptors in bronchial smooth muscle of SPAOPD-affected horses had a significantly greater percentage of immunostaining, compared with results for bronchial smooth muscle of healthy horses. However, there was no difference in immunostaining for ET-B receptors in the epithelium between healthy and SPAOPD-affected horses. Overall, there was no difference in the percentage of immunostaining between ET-A and ET-B receptors for epithelium or bronchial smooth muscles of healthy and SPAOPD-affected horses.

Specimens obtained from the 5 lung lobes of SPAOPD-affected horses had a greater percentage of immunostained area for the ET-A receptor in bronchial smooth muscle, compared with the percentage in bronchial smooth muscle of healthy horses (Figure 6). The percentage of immunostained area was approximately 45% in the bronchial smooth muscle of 4 of 5 lobes in SPAOPD-affected horses, whereas the percentage was < 30% in the bronchial smooth muscle of all 5 lobes in healthy horses. However, there was a significant difference only for the left diaphragmatic lobe. Additionally, no difference in the percentage of immunostaining for the ET-A receptor in bronchial smooth muscle was observed among the lobes in both groups of horses. Similarly, no difference was observed in the percentage of immunostained area for ET-A receptors in the bronchial epithelium between healthy
and SPAOPD-affected horses or among the 5 lung lobes in both groups of horses. In healthy horses, no difference was observed in the percentage of immunostained area for ET-A receptors between epithelium and bronchial smooth muscle. However, in SPAOPD-affected horses, there was a significantly greater percentage of immunostained area for ET-A receptors in the bronchial smooth muscle, compared with the percentage for the bronchial epithelium, in all lung lobes, except for the right diaphragmatic and accessory lobes.

Specimens obtained from the 5 lung lobes had a greater percentage of immunostained area for ET-B receptors in the bronchial smooth muscle of SPAOPD-affected horses, compared with percentages for bronchial smooth muscle of healthy horses (Figure 7). In SPAOPD-affected horses, bronchial smooth muscle of all lobes had a percentage of immunostained area that ranged from 35% to 50%, whereas the percentage of immunostained area was < 25% in all lobes in healthy horses. There were significant differences between healthy and SPAOPD-affected horses in percentages for all lobes, except the right diaphragmatic lobe ($P = 0.08$). No lung lobe had greater expression of ET-B receptors in bronchial smooth muscle, compared with expression in the other lung lobes, in either group of horses. No difference was observed in the percentage of immunostained area for ET-B receptors in bronchial epithelium between healthy and SPAOPD-affected horses or among the 5 lung lobes of both groups of horses. In healthy horses, no difference was observed in the percentage of immunostained area for ET-B receptors between bronchial epithelium and bronchial smooth muscle. Similar to results for ET-A receptors in SPAOPD-affected horses, all lobes, except for the accessory lobe, had a significantly greater percentage of immunostained area for ET-B receptors in bronchial smooth muscle, compared with the percentage for bronchial epithelium. Finally, there was no difference in the percentage of immunostaining between ET-A and ET-B receptors for bronchial epithelium or bronchial smooth muscle in all lung lobes of healthy and SPAOPD-affected horses.

**Figure 3**—Photomicrographs of immunostained bronchial tissues obtained from an SPAOPD-affected horse in which the primary antibody was replaced with sheep $\gamma$ globulin (ie, negative control sample; A) and tissue was immunostained by use of anti–ET-B receptor primary antibody (ie, positive control sample for ETA receptors; B). Adjacent tissues from the same tissue section were used for both slides. Notice that staining of bronchial smooth muscle and bronchial epithelium is clearly evident. Bar = 500 $\mu$m.

**Figure 4**—Mean ± SEM percentages of immunostained area for ETA receptor in bronchial epithelium of healthy horses (white bars), bronchial smooth muscle of healthy horses (horizontal-striped bars), bronchial epithelium of SPAOPD-affected horses (crosshatched bars), and bronchial smooth muscle of SPAOPD-affected horses (black bars). *Within SPAOPD-affected horses, value differs significantly ($P < 0.05$) from the value for bronchial epithelium.

**Figure 5**—Mean ± SEM percentages of immunostained area for ETB receptors in bronchial epithelium and bronchial smooth muscle of healthy and SPAOPD-affected horses. †Value differs significantly ($P < 0.05$) from the value for bronchial smooth muscle of healthy horses. See Figure 4 for remainder of key.
Discussion

Analysis of results for the study reported here revealed several important findings regarding the potential role played by ET-1 and its receptors in the pathogenesis of SPAOPD in horses. First, both ET-A and ET-B receptors were found in bronchial epithelium as well as bronchial smooth muscle of healthy and SPAOPD-affected horses. Second, both ET-A and ET-B receptors were equally distributed in bronchial epithelium of healthy and SPAOPD-affected horses. Similarly, both of these receptors were equally distributed in the bronchial smooth muscle of healthy and SPAOPD-affected horses. Third, distribution of ET-A or ET-B receptors in the bronchial epithelium of SPAOPD-affected horses did not differ significantly from distribution in healthy horses. In other words, there was no alteration in receptor density in the epithelium of horses with SPAOPD. Finally, there was upregulation of ET-A receptors in the bronchial smooth muscle of the left diaphragmatic lung lobe and of ET-B receptors in all lung lobes, except for the right diaphragmatic lobe, in SPAOPD-affected horses, compared with results for healthy horses.

An important feature of airway hyperreactive diseases is obstruction of airways, particularly the bronchi. Hyperinflation of the lungs that was detected during postmortem analysis of SPAOPD-affected horses was attributed to entrapment of air in obstructed areas of the lungs. Hence, it was not a true emphysematous condition as is often observed in humans with COPD. This hyperinflation was the cause of the rib impressions that were observed on the surface of the lungs. Because of the entrapped air, SPAOPD-affected lungs did not collapse when the thoracic cavity was opened. Airway obstruction is primarily caused by 3 factors (constriction of airway smooth muscle; thickening of airway walls as a result of smooth muscle hyperplasia, hypertrophy, or both; and accumulation of mucus or other secretions in the airway lumen).

Among these 3 factors, constriction of airway smooth muscle, especially that of the bronchi (ie, bronchoconstriction), is the most important. Bronchoconstriction in obstructive pulmonary diseases can be caused by several inflammatory mediators released into the airways. Some of these mediators include histamine, platelet-activating factor, prostaglandins, bradykinin, and ET-1. Bronchoconstriction induced by ET-1 has been established as a factor in obstructive pulmonary diseases of humans. It has also been determined that ET-1 is a potent constrictor of bronchial and vascular smooth muscle of equine lungs. In addition to being an important agent for causing bronchoconstriction, ET-1 is also an important factor causing airway remodeling and mucus hypersecretion in patients with obstructive pulmonary diseases.

Bronchoconstriction elicited by ET-1 is extremely potent in most mammalian species in vivo and in vitro. Endothelin-1 can induce bronchoconstriction by 3 mechanisms. First, it can act directly on ET-A or ET-B receptors (or both) of bronchial smooth muscle. Second, it can augment cholinergic-mediated smooth muscle constriction. Third, it can release secondary mediators, such as thromboxanes. This ET-1–induced bronchoconstriction is substantially exaggerated in most patients with obstructive pulmonary diseases, such as COPD, compared with responses in healthy subjects. Although increased ET-1 concentrations could serve as the main explanation for this exaggerated ET-1–induced bronchoconstriction, another possible reason could be upregulation of ET-A or ET-B receptors (or both) in bronchial smooth muscle. It has been established that concentrations of ET-1 in pulmonary and bronchoalveolar lavage fluid are increased in RAO-affected horses. In the study reported here, we determined that there was greater expression of ET-B receptors in the bronchial smooth muscle in 4 of 5 lung lobes of SPAOPD-affected horses, compared with expression for healthy horses. With regard to ET-A receptors, all lung lobes had slightly greater expression in the smooth muscle of SPAOPD-affected horses, compared with expression for healthy horses; however, there was a significant difference only for the left diaphragmatic lobe. We believe that the nonsignificant differences for expression of ET-A receptors in all other lobes of SPAOPD-affected horses were a result of the small sample size (8 horses in each group) in this study.
Airway remodeling constitutes an important pathologic change in patients with many airway hyperreactive diseases, including horses with COPD. 

Endothelin-1 can cause potent hyperplasia of airway smooth muscle in isolated airway smooth muscle cells obtained from humans 

and rabbits. 

Endothelin-1 has also been implicated in allergen-induced proliferation of airway smooth muscle in rats. 

Moreover, by acting through ET-A and ET-B receptors, ET-1 can augment the potent mitogenic properties of classical growth promoters, such as thromboxanes, epidermal growth factor, and platelet-derived growth factor. 

Our finding of alterations in ET receptors in SPAOPD-affected horses could form a basis for future studies investigating the role ET-1 plays in causing smooth muscle hyperplasia, which has been reported in horses with COPD.

In healthy subjects, bronchial tone is constantly maintained by a balance between constrictor and relaxant agents of smooth muscle. 

Certain agents, such as ET-1, possess contraction and relaxation properties for smooth muscle. Endothelin-1 elicits smooth muscle relaxation by acting through ET-A and ET-B receptors in the epithelial lining of the airways. By acting through these receptors, ET-1 causes the release of bronchodilator agents, such as nitric oxide and prostaglandin E 

.In patients with obstructive pulmonary diseases, such as SPAOPD, the balance between bronchodilating and bronchoconstricting events is disturbed because of alterations in the concentrations of modulators of airway muscle tone or alterations in their receptors. For example, the amounts of bronchomodulators, such as histamine, adenosine, and their receptors, are altered in the lungs of patients affected with asthma. 

Similarly, pulmonary and peripheral ET-1 concentrations are upregulated in horses with COPD. 

In the study reported here, we determined that there is no difference in populations of ET-A or ET-B receptors between bronchial epithelium and bronchial smooth muscle in healthy horses (i.e., the quantity and distribution of ET-A and ET-B receptors were similar between bronchial epithelium and bronchial smooth muscle). However, the relative amounts of these receptors were shifted toward bronchial smooth muscle in SPAOPD-affected horses. 

In other words, bronchial smooth muscle tended to have greater expression of receptors, compared with expression in bronchial epithelium. This could be an important cause for the disturbance of the physiologic equilibrium between bronchodilating and bronchoconstricting actions of ET-1 that leads to enhanced bronchoconstriction in affected horses.

The relative proportion of ET receptors in airway smooth muscle is a species-specific event. Bronchial smooth muscle from nondiseased lungs of humans contains a greater proportion of ET-B receptors, compared with the proportion of ET-A receptors, whereas tracheal smooth muscle from rats and mice contains approximately equal proportions of the 2 receptor subtypes. 

In bronchial smooth muscle of pigs, the ratio of ET-A receptors to ET-B receptors is 70 to 30. 

Such species-specific differences in the relative proportions of these 2 receptor populations within airway smooth muscles could be correlated to species-specific differences in the intensity of complications caused by ET-1 in patients with obstructive pulmonary diseases. Such differences in relative proportions of ET receptors can also be correlated with the type of predominant physiologic role exerted by ET-1 in healthy subjects. In the study reported here, we observed that the relative proportion of ET-A and ET-B receptors was equivalent in bronchial smooth muscle and bronchial epithelium in both groups of horses.

We believe that we have reported for the first time that expression of ET-B receptors is enhanced in bronchial smooth muscle of SPAOPD-affected horses, compared with expression for healthy horses. In the study reported here, we also determined that ET-A receptors tend to be overexpressed in bronchial smooth muscle of SPAOPD-affected horses. It was revealed that both of these receptors, which have potential roles in the pathogenesis of many obstructive pulmonary diseases, such as asthma, also could play an important role in the pathogenesis of SPAOPD in horses. Additional studies are warranted to determine the exact role of each receptor in the pathogenesis of this disease. Clinical trials that use ET-receptor antagonists could reveal findings that may have clinical relevance for the development of therapeutic strategies to treat horses with this progressive career-ending disease.


d. Esophageal balloon, AE Medical, Farmingdale, NJ.


g. Model 7D polygraph, Grass Medical Instrument Division of Astro-Med Inc, West Warwick, RI.

h. Beuthanasia solution, Schering-Plough Animal Health Corp, Kenilworth, NJ.

i. Sheep antibody to endothelin A receptor, BIODISEIGN International, Saco, Me.

j. Sheep antibody to endothelin B receptor, BIODISEIGN International, Saco, Me.

k. Protease inhibitor cocktail tablets, Roche Diagnostics Corp, Roche Applied Science, Indianapolis, Ind.

l. Quick Start Bradford protein assay kit 1, Bio-Rad Laboratories, Berkeley, Calif.

m. Donkey anti-sheep 1G-HRP, Santa Cruz Biotechnology, Santa Cruz, Calif.

n. Western lightening chemiluminescence reagent, Perkin-Elmer Life Sciences, Boston, Mass.


p. Quantity one 1-D image analysis software, Bio-Rad Laboratories, Hercules, Calif.

q. Dako autostainer universal staining system, DakoCytomation California Inc, Carpinteria, Calif.

r. Avidin/biotin blocking kit, Vector Laboratories Inc, Burlingame, Calif.
References

40. Golldie RG, Fernandes LB. A possible mediator role for

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