Characterization of equine pulmonary endothelin receptors in health and disease

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CHARACTERIZATION OF EQUINE PULMONARY ENDOTHELIN RECEPTORS
IN HEALTH AND DISEASE

A Dissertation

Submitted to the Graduate faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of
Comparative Biomedical Sciences

by
Sumanth Polikepahad
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May 2006
I dedicate this dissertation to my parents, Mr. P. Rameshwar Rao and Mrs. D. Suguna Devi, and all biomedical scientists for their incessant pursuit to make this world a better place to live.
ACKNOWLEDGEMENTS

I would like to thank several people without whose help I couldn’t have finished my dissertation successfully. First of all, I thank my parents and my wife Dr. Menaka Matsyaraja for always being there with me in many spells of stress all along my Ph.D. I express my sincere gratitude to my mentor Dr. Changaram S. Venugopal for his help and guidance towards the completion of my dissertation. I am thankful to Dr. Rustin Moore not only for his help in smooth conductance of my project but also for teaching me the correct ways to write scientific papers. I am also thankful to Dr. Daniel Paulsen for all his input in teaching me the basics of immunohistochemistry.

Also, I am thankful to Dr. Steven Barker for his continuous encouragement and invaluable suggestions all along my Ph.D. I am also grateful to Dr. Michael Welsch and Dr. Joseph Francis for their input towards the successful completion of my defense. I would like to thank many student workers and technicians of Equine Health Studies Program including Mike Keowen, Catherine Koch and Earnistine Holmes for their technical input in smooth conductance of the whole project. Finally, I would like to convey my special thanks to Dr. Masud-ul-haq for playing a pivotal role in my learning of molecular techniques.
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ΔPpl</td>
<td>transpleural pressure</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>BALF</td>
<td>broncho-alveolar lavage fluid</td>
</tr>
<tr>
<td>c-AMP</td>
<td>cyclic adenosine mono phosphate</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CR curves</td>
<td>concentration response curves</td>
</tr>
<tr>
<td>CS</td>
<td>clinical score</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleicacid</td>
</tr>
<tr>
<td>ECE</td>
<td>endothelin converting enzyme</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminotetraacetic acid</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
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<td>endothelin-A</td>
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<tr>
<td>ET-B</td>
<td>endothelin-B</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>HETE</td>
<td>hydroxy eicosatetraenoicacid</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleicacid</td>
</tr>
<tr>
<td>NANC</td>
<td>non-adrenergic non-cholinergic</td>
</tr>
<tr>
<td>NK</td>
<td>neurokinin</td>
</tr>
<tr>
<td>NK-A</td>
<td>neurokinin-A</td>
</tr>
<tr>
<td>NK-B</td>
<td>neurokinin-B</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGDF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PGE-2</td>
<td>prostaglandinE-2</td>
</tr>
<tr>
<td>PKC</td>
<td>phosphokinase C</td>
</tr>
<tr>
<td>RAO</td>
<td>recurrent airway obstruction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleicacid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase- polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>SPAOPD</td>
<td>summer pasture-associated obstructive pulmonary disease</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)methylamine</td>
</tr>
<tr>
<td>TXA-2</td>
<td>thromboxane A-2</td>
</tr>
<tr>
<td>TXB-2</td>
<td>thromboxane B-2</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells</td>
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ABSTRACT

Endothelin-1 (ET-1) has been implicated in allergic type of respiratory inflammatory diseases in various species of animals including horses. This peptide elicits its actions by acting through endothelin-A (ET-A) and endothelin-B (ET-B) receptor sub-types. In this project, we have hypothesized that endothelin receptors (both ET-A and ET-B) are altered in terms of affinity and expression, in the lungs of summer pasture-associated obstructive pulmonary disease (SPAOPD)-affected horses. Objective of this dissertation was to determine the alterations in the affinity and expression of endothelin receptors in the lungs of healthy and SPAOPD-affected horses. To pursue our hypothesis, we have employed pharmacological, immunohistochemical and molecular studies. Totally 33 horses were used in this study. All the horses were examined and grouped in to 16 healthy and 17 SPAOPD-affected, based on clinical evaluation, clinical scoring, pulmonary function testing and broncho-alveolar lavage fluid (BALF) analysis. Horses were then euthanatized, and tissue specimens were immediately collected from all lung lobes. In pharmacological studies, cumulative concentration response curves and pA2 values were determined and compared in both groups of horses. The pA2 value of ET-B receptors was significantly greater in the SPAOPD-affected horses when compared with healthy horses. In immunohistochemical studies, expression of these receptors was determined in the bronchial smooth muscles and epithelium of both groups of horses. The percentage of immunostaining was significantly greater in the smooth muscles of SPAOPD-affected horses when compared with healthy horses. In molecular studies, by employing RT-PCR, western blotting and realtime PCR, expression of these receptors has been determined and compared. The molecular expression of ET-B receptors was significantly greater in the peripheral lungs of SPAOPD-affected horses when compared with healthy horses. Specific antagonists, primers and polyclonal primary
antibodies of ET-A or ET-B receptors were used for these three studies. On the whole, it can be concluded that ET-B receptors show a clear tendency of up-regulation in the lungs of SPAOPD-affected horses. These findings help us in the better understanding of the pathogenesis of this progressive, career-ending disease.
CHAPTER 1. REVIEW OF THE LITERATURE ON SUMMER-PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE AND ENDOTHELIN
1.1. Introduction to Recurrent Airway Obstruction and Summer-Pasture-Associated Obstructive Pulmonary Disease

Recurrent airway obstruction or (RAO), also known as heaves, is a common inflammatory/allergic obstructive airway condition that affects many horses worldwide. This disease has two forms. The first and most widely reported form of RAO is chronic obstructive pulmonary disease (COPD), which is most prevalent in the northern hemisphere where horses are stabled for large portions of their lives and are fed hay. The other form is known as summer-pasture-associated obstructive pulmonary disease (or SPAOPD), which frequently occurs in the southeastern United States, Britain and California in horses that are kept on pasture when the weather is warm and humid. Evidence suggests that the clinical signs and pathology of these two forms of RAO are the same but with different causative factors.

The first description of SPAOPD was reported by Dr. Ralph E. Beadle in 1983. In book chapter, he described this disease as “a syndrome with signs similar to the equine chronic obstructive pulmonary disease or heaves, a condition observed in stabled horses exposed to moldy hay or bedding and dust, but occurring in horses grazing pasture land in the summer in the southeastern region of USA, especially Florida, Georgia, Louisiana and Mississippi” (Beadle, 1983). In 1993, Seahorn and Beadle reported a retrospective study of 21 cases of SPAOPD-affected horses. In this study, they reviewed the medical records of case accessions from 1983 to 1991 to identify the horses with COPD and a history of worsening clinical symptoms after exposure to pasture (i.e. SPAOPD). They concluded that SPAOPD is a disease of adult horses (mean age, 13.6 ± 3.6 years) that are on pasture most of the time in summer. They also reported the existence of significant breed and reproductive status differences in the occurrence of this disease. However, they concluded that those differences were confounded and clinically unimportant (Seahorn and Beadle 1993). Occurrence of this condition in the United Kingdom
was first reported by McGorum in 1990, and later was supported by Dixon and co-workers in 1995 and Mair in 1996 (Dixon et al. 1995, Mair 1996). Nevertheless, the condition in the UK differs from that of US in that exacerbation of the disease occurs during hot dry weather following exposure to dust that originate from adjacent fields when crops are harvested or straw is burned (McGorum and Dixon 1999).

The etiology of SPAOPD is unclear; however, involvement of pollen and fungal spores is suggested. Previous studies have established the importance of fungi in the causation of airway allergic diseases in animals and humans. In 1994, Seahorn and Beadle have conducted a study to identify the predominant fungi present in the nasal passages of horses clinically affected with SPAOPD (Seahorn and Beadle 1994). They demonstrated that the most frequently isolated fungi from the control or unaffected horses in the pasture environment were *Cladosporium* spp., *Acremonium* spp., *Choanephora* spp., *Curvularia* sp., *Fusarium* sp., and *Alternaria* spp. The most frequently isolated fungi from eight SPAOPD-affected horses which did not show any clinical signs and seven SPAOPD-affected horses that did show clinical signs were *Cladosporium* spp., *Curvularia* spp., *Fusarium* spp., and *Alternaria* spp. They concluded that the fungal species *Choanephora* sp., *Curvularia* sp., and *Fusarium* spp. might play a vital role in the etiology of SPAOPD. In addition, they reported that *Micropolyspora feani* and *Aspergillus fumigatus*, which are the most important causes for COPD, were not identified in any of the SPAOPD-affected horses. This suggests that even though COPD and SPAOPD share many pathological features, they differ regarding the causative factors. On the whole, numerous future studies are needed to establish the exact etiology of SPAOPD.
1.2 Clinical Features of SPAOPD

The clinical symptoms of equine SPAOPD are similar to those of equine COPD and human asthma, which include airway hyperresponsiveness and airway obstruction. The susceptible horses manifest the symptoms when they are exposed to pasture in the late summer to early fall.

A detailed description of the clinical symptoms exhibited by SPAOPD affected horses was reported by Seahorn and Beadle in 1993. In this survey, they reported the observations made by horse owners and veterinarians regarding the clinical symptoms of SPAOPD-affected horses (Seahorn and Beadle 1993). In this report they included a total of 21 SPAOPD-affected horses. All affected horses were typically tachypneic and had signs of expiratory dyspnea, but they were not febrile, anorectic or depressed. Auscultation of the thorax often revealed inspiratory and expiratory crackles and wheezes. Vibration from the mucus within the trachea was common. The hemogram was characterized by normal WBC count, but the neutrophil count was slightly increased, which may have been associated with stress of transportation and hospitalization. Aspirates collected by transtracheal wash fluids were characterized by the presence of nondegenerate neutrophils and proteinaceous swirls of mucus (i.e Curschmann’s spirals). Curschmann’s spirals are the casts of inspissated mucus plugs originating from the smaller airways which have been recognized in horses with chronic lung diseases. Bacteria were infrequently found in the transtracheal wash and were rarely grown on culture, suggesting that bacteria were unimportant in the etiopathogenesis of the disease.

Similar findings were reported by Costa and co-workers in 2000. In addition to the aforementioned features, these authors noticed the presence of small strands of yellow to white mucus scattered through the trachea (Costa et al. 2000). The bronchoalveolar lavage fluid
(BALF) of SPAOPD-affected horses contains a significantly greater percentage of nondegenerate neutrophils, compared with healthy horses.

Over-inflation of the lungs was a common feature of SPAOPD horses. However, true emphysema was not frequently observed. Over-inflation of the lungs was due to the air trapped in the post-obstructive areas. Cytologic evaluation of the BALF from SPAOPD-affected horses revealed the predominance of neutrophils, followed by lymphocytes, macrophages and mast cells. The SPAOPD-affected horses had significantly lower percentages of lymphocytes, macrophages, and mast cells in BALF, compared with normal horses. Results of bacteriological culture were negative in all horses. The lungs of SPAOPD-affected horses failed to collapse when the thoracic cavity was opened. Lungs retained the impressions made by the ribs. The lungs were orange-pink and had spongy texture. Many had a white, foamy substance on the visceral and parietal pleural surfaces. In clinically healthy horses, the lungs collapsed after the thoracic cavity was opened. In these horses, lungs had normal texture, pale-pink appearance and minimal or no foam on the external and cut surfaces.

These authors also reported that histologically the most consistent finding was the presence of extensive amounts of basophilic stained mucus within the small airways. Peribronchiolar inflammation was variable and characterized by the predominance of neutrophils and lymphocytes. Peribronchiolar smooth muscle hypertrophy was not a consistent feature. Presence of peribronchial eosinophils was rarely noticed. However, the authors did not rule out the possibility of the presence of eosinophils in the BALF of SPAOPD-affected horses at some point during the disease course. They also suggested the likelihood of the involvement of multiple pathogenic mechanisms in the development of this disease, which could result in variations in the proportion of the type of inflammatory cells. They also concluded that, as far as
the pathological changes are concerned, there were no qualitative or quantitative differences among the lobes. Finally, they stated that the clinical, cytological and histological features observed in SPAOPD were quite similar to those of equine COPD.

1.3. Innervation of Equine Airways

Similar to most of the other species, equine airways are also innervated by cholinergic, adrenergic and non-adrenergic-non-cholinergic inhibitory (iNANC) and excitatory nervous systems (eNANC) (Matera et al. 2002). The airway innervation plays an important role in the regulation of the airway smooth muscle tone, airway secretion, blood flow and vascular permeability. Furthermore, airway innervation also plays a vital role in the modulation of inflammatory mechanisms involved in pulmonary disease.

1.3.1 Cholinergic Nervous System – The cholinergic nervous system innervates the entire tracheobronchial tree. The preganglionic cholinergic nerves originate in the brain stem, pass via the vagus nerve and relay in the ganglia located in the alveolar walls. The postganglionic nerves originating from these ganglia innervate the airway smooth muscles and submucosal glands. The ganglionic neurotransmission is mediated by acetylcholine via nicotinic receptors, whereas the airway smooth muscle contraction is mediated by acetylcholine via muscarinic receptors.

The in vivo administration of atropine to RAO-affected horses results in the dilatation of all the levels of the tracheobronchial tree, suggesting that cholinergic innervation is present in the trachea and larger and smaller bronchi (Matera et al. 2002). This also indicates that cholinergic bronchoconstriction in horses is mediated by muscarinic receptors. At least 5 subtypes of muscarinic receptors have been cloned and characterized (i.e. M₁ to M₅), while four subtypes are recognized pharmacologically (i.e. M₁ to M₄). Each of these subtypes has a distinct role in the maintainence of normal airway physiology.
The M₁ and M₂ receptors are localized on parasympathetic ganglia and postganglionic nerves, respectively. M₁ receptors facilitate the ganglionic neurotransmission mediated by the nicotinic receptors. They also play an important role in the reflex pathways in various species. In humans and guinea pigs, M₂ receptors are located in the prejunctional regions of the postganglionic nerves and play a vital role in the auto-regulation of acetylcholine secretion in the ganglia. When acetylcholine acts on these receptors, its release is inhibited in the ganglia. However, recently it has been suggested that the auto-receptor subtype in guinea pigs are of the M₄ subtype rather than M₂ (Matera et al. 2002). In equine airways, muscarinic auto-receptors also exist; however, it is still unknown which sub-type is involved in the auto-regulation of acetylcholine secretion. Wang and co-workers demonstrated that the muscarinic auto-receptors in equine airways do not belong to M₁, M₂ or M₃, but may belong to a novel sub-type (Wang et al. 1995). Zhang and co-workers have demonstrated that equine RAO is not associated with dysfunction of muscarinic auto-receptors as observed in human asthma (Zhang et al. 1999). This is because human asthma is characterized by an increase in the number of eosinophils in the airways and these eosinophils synthesize inflammatory proteins such as major basic protein in the lungs. These proteins induce the release of excessive amounts of acetylcholine when they act upon muscarinic auto-receptors. In equine RAO, there is no increase in the levels of eosinophils in airways. Instead, a significant increase in the levels of neutrophils is noticed in this disease and therefore no dysfunction of muscarinic auto-receptors is observed. However, these authors have not ruled out the possibility of the influence of various inflammatory mediators in the regulation of acetylcholine release from post-ganglionic nerves.

In many species, the main function of the M₃ receptor sub-type in airways is smooth muscle contraction. In addition, these receptors are also located in the airway epithelium,
submucosal glands and pulmonary vascular endothelium where they yield cholinergic-mediated vasodilatation. In vitro studies have demonstrated that the cholinergic-mediated airway smooth muscle contraction in equine species is mediated by the M₃ receptor sub-type (Wang et al. 1995). In horses, these receptor sub-types are located in the trachea and small airways.

In airway allergic diseases of horses as well as in the other species, cholinergic-mediated mechanisms play a vital role in the etiology of the hyperresponsiveness. This is the reason why anti-cholinergic drugs are as effective as beta agonists in the treatment of these diseases (Robinson et al. 1993).

1.3.2 Adrenergic Nervous System – Little information is available regarding the sympathetic innervation in equine airways. Sonea and co-workers have demonstrated that the sympathetic nerves are found more in the larger than in smaller airways (Sonea et al. 1993). They also reported the presence of numerous immunoreactive adrenergic nerves in pulmonary and bronchial vessels.

Adrenergic-mediated actions are elicited by two receptor subtypes i.e. α and β. At least three subtypes of β receptors exist: β₁, β₂ and β₃. Among these three subtypes, β₂ receptors are widely distributed in the lungs and are located in various types of cells in the lungs. The β₂ agonists decrease airway smooth muscular tone by causing relaxation of the smooth muscles; this is mediated by the accumulation of intracellular c-AMP which in turn reduces the intracellular concentration of calcium by activating protein kinase-A. In equine species, it has been shown that even though both β₁ and β₂ adrenergic receptors are present and activated in RAO, airway dilatation is primarily mediated by β₂ receptor sub-types (Scott et al 1991). The α receptors are further classified into α₁ and α₂. In canine and guinea pig airways, α₁ receptors have been demonstrated in the airway smooth muscles and they mediate contraction.
However, in ponies affected with RAO, the role of these receptors in causing airway smooth muscle contraction is negligible (Scott et al. 1988). The $\alpha_2$ receptors are inhibitory receptors unlike that of $\alpha_1$ receptors. In addition, these receptors have been shown to be located on the cholinergic nerves, which when activated, could be responsible for the decrease of acetylcholine release. In other words, they act as auto-regulatory receptors (Zhang et al. 1999).

Besides its well known direct effects, the sympathetic nervous system also elicits indirect effects on the airways by interacting with the cholinergic nervous system. For example, exogenously applied adrenaline causes the inhibition of ganglionic neurotransmission in cat and ferret isolated airways by acting through $\alpha$ and $\beta$ adrenergic receptors (Johnson 1998). In human airways, $\beta_2$ agonists decrease the contractions elicited by cholinergic nerves and tachykinins (Johnson 1998). In equine airways, the presence of $\alpha_2$ and $\beta_2$ receptors on the cholinergic nerves has been demonstrated with the former being predominant (Zhang et al. 1995). These receptors have been shown to have a modulatory effect on the cholinergic neurotransmission in the equine tracheobronchial tree. Particularly, $\alpha_2$ receptor subtypes have an inhibitory effect on the release of acetylcholine from the cholinergic nerves in equine large and small airways. The $\beta_2$ receptors are distributed in the cholinergic nerves throughout the equine airways. In contrast to other species, activation of $\beta_2$ receptors present on the cholinergic nerves of equine airways leads to an increased release of acetylcholine from the postganglionic cholinergic nerves. Similar behavior of $\beta_2$ receptors has been observed in guinea pig trachea (Belvisi et al. 1996). Zhang and co-workers have suggested that this idiosyncratic effect of $\beta_2$ receptors in equine airways could be due to the paradoxical effect of c-AMP, which might cause augmented release of acetylcholine from the cholinergic nerves (Zhang et al. 1996).
1.3.3 Non-Adrenergic-Non-Cholinergic (NANC) Nervous System – The non-adrenergic-non-cholinergic (NANC) nervous system is comprised of the neurally mediated responses which are not blocked by the antagonists of either the adrenergic or cholinergic nervous system or both. The NANC has two components: excitatory NANC (e-NANC) and inhibitory NANC (i-NANC). In the airways, it has been shown pharmacologically and histologically that e-NANC nerve fibers form diffuse networks in the smooth muscles, submucosal glands and blood vessels (Barnes et al., 1990). The neuropeptides such as tachykinins act as the transmitters of the NANC system. These peptides are synthesized in the ganglionic neural cell bodies and are transported to the peripheral nerve endings and are released into the peripheral tissues. These neuropeptides are contained mainly in the afferent chemosensitive C-fibers. Tachykinins mediate a multitude of functions in the body such as cholinergic neurotransmission, mucus secretion, smooth muscle contraction, inflammatory cell activation and plasma protein leakage from the tracheobronchial microcirculation etc.

It is well established that substance P (SP) is the neurotransmitter of the e-NANC. Recently other tachykinins such as neurokinin A (NKA) and neurokinin B (NKB) have also been recognized as the neurotransmitters of e-NANC. Substance P acts via neuropeptide receptors known as neurokinin 1 (NK-1) receptors, whereas NKA and NKB acts through NK-2 and NK-3 receptors, respectively. By employing autoradiography techniques, Sonea and co-workers have demonstrated that the SP binding sites in the equine lungs were very dense over small bronchial vessels, tracheobronchial glands and airway epithelium in large and small airways (Sonea et al., 1999). However, the density of SP binding sites over airway smooth muscle was much lower than in the aforementioned tissues. These authors have also suggested that in equine lungs NK-1 receptor density is greater compared with that of NK-2. In addition, they stated that the receptor-
mediated effects of SP in the equine lung most likely involve regulation of vascular tone and airway secretions based upon the density of SP binding sites in these tissues. Furthermore, they concluded that the activation of intrapulmonary afferent nerves containing SP by noxious stimuli such as inhaled allergens or irritants may lead to increased mucus secretion and decreased airway diameter due to vascular congestion. The same authors have also demonstrated the distribution of pulmonary nerves immunoreactive for SP by using immunohistochemical methods on healthy lungs from adult equids (Sonea et al. 1994). The nerve fibers immunoreactive for SP were more frequently observed near the hilus of the lung than in the caudal lobes or in the periphery of the lung. In addition, these nerve fibers immunoreactive to SP were most abundant in the lamina propria of the trachea and larger airways, particularly within and directly below the airway epithelium; they were also frequently associated with bronchial and pulmonary vessels. Presence of nerve fibers immunoreactive for SP in peribronchial neural ganglia indicated that these sensory nerves may modulate parasympathetic regulation of pulmonary function. The authors have concluded that the nerve fibers immunoreactive for SP were well placed to detect inhaled agents and to contribute to the pulmonary response to irritants and pathogens.

In equine airways, i-NANC nerves supply the trachea and central bronchi, but are absent in the third generation of bronchi in the horses affected with RAO. By employing electrical field stimulation studies, Yu and co-workers have studied the distribution of inhibitory nerves and the mediator of the i-NANC system in smooth muscles of equine airways (Yu et al. 1994). They concluded that in equine airways, the i-NANC nerves supply the trachea and central bronchi and, nitric oxide (NO) mediates the i-NANC function. The functions of NO include smooth muscle relaxation, inhibition of smooth muscle proliferation, inhibition of platelet aggregation and adhesion, and act as the neurotransmitter of bronchodilator nerves. In horses acutely affected by
COPD, there is a consistent lack of i-NANC function in the larger bronchi (Yu et al. 1994). However, it is still unclear whether the reduction of i-NANC control is a result of the inflammatory response during acute COPD or is a basic characteristic of COPD-susceptible horses.

1.4 Role of Inflammatory Mediators in the Pathogenesis of SPAOPD

Even though it is widely believed that inflammatory mediators play a vital role in the pathogenesis of RAO, there is currently a paucity of the literature regarding their exact role. Gray and co-workers have conducted a study to investigate the involvement of the cyclooxygenase products in the pathogenesis of RAO. In that study, they measured plasma and BALF concentrations of metabolites of thromboxane TXA-2 and prostaglandins PGI-2 and PGD-2 in five affected ponies and their age and gender-matched controls prior to and during acute airway obstruction precipitated by housing the ponies in a barn and exposing them to hay dust. They reported that plasma TXB-2 was the only metabolite that increased significantly during the acute disease state. When the ponies were treated with a cyclooxygenase inhibitor flunixin meglumine, TXB-2 production was inhibited but the degree of airway obstruction or airway hyperreactivity was not altered when measured at pasture and in the barn. They concluded that cyclooxygenase products of arachidonic acid metabolism are altered but do not play a role in the airway obstruction and hyperreactivity observed in ponies with heaves (Gray et al. 1989). The same investigators have measured plasma immunoreactive 15-hydroxyeicosatetraenoic acid (i15-HETE) concentrations in carotid artery and right ventricle blood samples in five affected ponies and their age- and gender-matched control ponies. They found that the plasma i15-HETE concentrations were greater in carotid artery samples compared with right ventricle samples in affected ponies, suggesting that the lung was a source of i15-
HETE. Carotid artery i15-HETE concentrations were significantly greater in affected ponies than in control ponies. They concluded that affected ponies produce greater quantities of i15-HETE than control ponies and that exposing affected ponies to a barn environment produces acute airway obstruction and increased plasma concentrations of i15-HETE (Gray et al. 1992a).

In another study, the same authors have measured PGE-2 and 15-HETE production in vitro in tracheal epithelium obtained from six affected horses at the time of acute airway obstruction as compared with six matched control horses. Strips of epithelium and subepithelial tissue were prepared and stimulated with histamine and bradykinin. The PGE-2 and 15-HETE levels were quantitated by radioimmunoassay. They found that 15-HETE above the limits of accurate detection was found in epithelial strips of only two principal animals and in none of the control horses, and the amount of 15-HETE was not increased when strips were stimulated. In addition, they found that the epithelial strips from affected horses tended to produce less PGE-2 than those of control horses, and there was a significant correlation between epithelial PGE-2 production and the time taken for affected animals to develop airway obstruction. They concluded that the equine tracheal epithelium is not a significant source of 15-HETE and airway mucosal PGE-2 production is reduced in horses with heaves, which suggests that a relative decrease in this bronchorelaxant substance may be a factor in the pathogenesis of RAO (Gray et al 1992b). Similarly, Costa and co-workers have demonstrated that the expression of inducible nitric oxide synthase (i-NOS) was greater in bronchial epithelial cells of horses with SPAOPD, compared with non-affected horses, suggesting that NO may play a role in amplifying the inflammatory process in the airways of horses with this disease (Costa et al. 2001). However, in a study conducted by Watson and co-workers, it was found that the concentrations of PGE-2 and PGF were significantly greater in BALF from horses with COPD than in BALF from normal
horses, but no differences were detected in TXB-2, 6-keto-PGF-1 alpha, PGD-2, LTB-4 or LTC-4 (Watson et al. 1992). Based on the aforementioned studies, it can be inferred that while the excitatory prostanoids might not play a major role, inhibitory prostanoids may play an important role in the pathogenesis of equine RAO (Robinson et al 1996). This conclusion is supported by the findings of a study conducted by Yu and co-workers who demonstrated that in the bronchi of horses with heaves the inhibitory function of prostanoids is reduced (Yu et al. 1994).

The role of histamine appears to be limited in the pathogenesis of equine COPD. Even though the levels of histamine in the BALF of horses challenged with antigens increase when compared to control horses, clinical observations suggest that COPD-affected horses do not respond considerably to anti-histamines (McGorum et al 1993, Beech 1991). Similar findings were reported by Venugopal and co-workers, who demonstrated that the response of bronchial rings from horses with SPAOPD to 5-hydroxytryptamine (5-HT) was significantly greater than those from control horses, whereas the response to histamine was significantly lower (Venugopal et al. 2001). Benamou and co-workers conducted a study to determine the role elicited by endothelin-1 (ET-1), a potent smooth muscle constrictor and inflammatory mediator, in the pathogenesis of RAO (Benamou et al. 1998). In this study, they investigated the levels of ET-1 in systemic blood as well as in BALF from horses with RAO. They also studied how these values might correlate with those of lung function tests and pulmonary artery pressure. The RAO horses had significantly greater systemic ET-1 levels than control horses. They also had a negative arteriovenous ET-1 difference that may correspond to a net uptake of ET-1 in the lung. The RAO horses in crisis had increased amounts of immunoreactive ET-1 in BALF compared to normal control subjects. Additionally, the reduction in lung function observed in RAO horses in
crisis was significantly correlated with lower epithelial lining fluid ET-1 levels. Finally, they suggested that ET-1 may play an important role in the pathogenesis of RAO.

In a study conducted by Olszewski and co-workers, it was concluded that inflammatory mediators can increase endogenous cholinergic responses of equine airways via both prejunctional and postjunctional mechanisms (Olszewski et al. 1999). They found that LTD-4 acts solely on smooth muscle, whereas 5-HT and histamine additionally act on neuronal receptors to facilitate release of ACh. In addition they also concluded that the excitatory effects of histamine, including the direct contractile effect and augmentation of the endogenous cholinergic response, are both mediated via H-1 receptors, whereas the inhibitory H-3 receptors partially oppose the direct contractile effect of this mediator. Therefore, inflammatory mediators play an important role in the alteration of neural control of airways in RAO. In another study performed by the same authors, it was demonstrated that in equine small airways; 1) the endogenous cholinergic responses are subject to strong facilitation by inflammatory mediators, 2) activated neutrophils do not affect cholinergic responses; and 3) in acute episodes of equine COPD, histamine, LTD-4 and serotonin (mediators primarily associated with type I allergic reactions) rather than mediators derived from neutrophils most likely contribute to increased cholinergic airway tone.

On the whole, it can be inferred that various inflammatory mediators might play major roles in the pathogenesis of equine RAO. Therefore, any significant finding in this discipline could form strong foundation in the development of effective therapeutic regimens for this disease.
1.5 Introduction to Endothelin-1

In 1985, Hickey et al demonstrated that the culture media obtained from bovine aortic endothelial cells cause progressive dose-dependent increments in the isometric tension of porcine, bovine and canine coronary arteries (Hickey et al. 1985). Discovery of this novel peptidergic endothelium-derived contraction factor (EDCF), a vasoactive peptide, initiated a new area in the field of biomedical sciences, which promised a clear and better understanding of pathogenesis of several important diseases. This peptide was subsequently isolated from porcine aortic endothelial cells, sequenced, cloned and named endothelin-1 (ET-1) by Yanagisawa in 1988. There are three distinct human endothelin-related genes encoding for the three related, but distinct isomers of endothelins: a) the predominant endothelin (ET-1), b) endothelin-2 or ET-2 (with Trp and Leu at 6th and 7th positions, respectively), and c) endothelin-3 or ET-3 (with Thr, Phe, Thr, Tyr, Lys and Tyr at positions 2,4,5,6,7 and 14, respectively) (Inoue et al. 1989) (figure 1.1). Another peptide belonging to the endothelin family was cloned in mice and was named vasoactive intestinal contractor (VIC or ET-4) (with Asn, Trp and Leu at positions 4, 6 and 7 respectively) (Saida et al. 1989, Luscher and Barton 2000). In addition, a 31 amino acid residue ET-1 has also been identified (Nakano et al. 1997, Kishi et al. 1998, Goldie et al. 2000). All the four isoforms of endothelin possess 21 amino acids and an identical hydrophobic C-terminal hexapeptide (Ohlstein and Ruffolo Jr. 1995).

Among all ET isoforms, ET-1, which has a molecular weight of 2,492 daltons, plays a pivotal role in various physiological as well as pathological processes (Ohlstein and Ruffolo Jr. 1995). It is the only isoform synthesized in the vascular endothelium and is now considered to be the most potent vascular smooth muscle constrictor in mammals (White et al. 1991). Upon systemic administration, ET-1 causes more prolonged elevation in blood pressure than any other
The ET-1 has an influence on DNA synthesis, the expression of cancer genes, cell proliferation and cellular hypertrophy. Mitogenesis caused by ET-1 involves activation of complex and multiple transduction pathways, such as the production of second messengers, the release of intracellular pools of calcium, and influx of extracellular calcium. ET-1 acts synergistically with various regulatory and growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF), etc., to regulate cellular transformation and replication (Dube et al. 2000). Several of these factors in turn stimulate the synthesis and release of endothelins. The synthesis and release of endothelins are also increased in acute and chronic pathological conditions such as atherosclerosis, postangioplastic restenosis, hypertension, and carcinogenesis (Battistini, et al. 1993).

1.6 ET-1 Biosynthesis

Similar to all the other functional peptides in the body, ET-1 is synthesized from a large inactive precursor protein. This precursor (preproendothelin-1 or PPET-1) is cleaved enzymatically by different proteolytic enzymes, finally leading to the synthesis of ET-1 (Itoh et al. 1988). Human PPET-1 contains 212 amino acids and porcine PPET-1 contains 203 amino acids (Yanagisawa et al. 1988). In most processing steps, cleavage of the inactive PPET-1 occurs on the carboxyl side pairs of basic amino acids, usually Lys-Arg or Arg-Arg or Arg-X-X-
Arg, followed by the removal of basic amino acids by carboxypeptidases (Denault et al. 1995). A family of calcium-dependent serine proteases called subtilin-like proprotein convertases (SPCs) is involved in cleaving these basic amino acids (Blais et al. 2002). ET-1 is derived from PPET-1 residues 53-73, which are preceded by paired basic amino acids (Bloch et al. 1989). First, a signal peptide is removed from the PPET-1 by a signal peptidase in the lumen of rough
endoplasmic reticulum, yielding a precursor of 195 amino acids, proendothelin-1 (pro-ET-1) (Denault et al. 1995). Pro ET-1 contains six potential recognition sequences (Arg-X-X-Arg cleavage sites named as R1a, R1b, R2, R3, R4 and R5) for members of SPCs. This peptide is converted to 38 (human) or 39 (porcine) amino acid big ET-1-Lys-Srg, by the furin convertase and PC7, which are the members of SPCs (figure 1.2). Furin convertase has better efficiency than PC7. Pro-ET-1 is the only member of the ET family that contains six potential convertase cleavage sites, three being located in the C-terminal fragment. Pro-ET-2 contains one cleavage site in its COOH-terminal, whereas the ET-3 precursor does not have a carboxy terminal.

Conversion of big ET-1 in to the biologically active ET-1 is believed to occur via specific metalloproteases called endothelin converting enzymes (ECE). The ECE cleaves the C-terminal halves of big ET-1 between Trp^{21} and Val/Ile^{22}, yielding the N-terminal, 21-residue active endothelins (Emoto and Yanagisawa 1995). The ET-1 has 140-fold more powerful vasoconstrictive properties than big ET-1 and pro-ET-1 has no vasomotor action (Kimura et al. 1989).

### 1.7 Endothelin Receptors

#### 1.7.1 Introduction –

Maggi and co-workers performed the first pharmacological studies to characterize endothelin receptors. They confirmed the existence of two distinct receptors and named them ET-A and ET-B. They also reported that in ET-B receptors, the free acid of tryptophan in position 21 plays a key role in the activity of endothelins (Maggi et al. 1989).

Subsequently, existence of multiple receptor subtypes was shown in various human and porcine tissues by ligand and affinity binding studies (Takayanagi et al. 1991). Many groups have cloned these two receptors in various species, including humans (Arai et al. 1990, Sakurai et al. 1990, Baynash et al. 1994, Nakamuta et al. 1991, Lin et al. 1991, Adachi et al. 1991).
To date, four types of ET receptors have been cloned and characterized. Mammals possess ET-A and ET-B receptors (Arai et al. 1990, Lin et al. 1991, Sakurai et al. 1990, Sakamoto et al. 1991). The ET-C receptor, which is sensitive to ET-3, is found in frogs (Karne et al. 1993). This receptor has 47 and 53% primary amino acid sequence identity to the mammalian ET-A and ET-B receptors, respectively. The fourth type of receptor was discovered in rats and named ‘dual angiotensin II/ET-1 receptor’ (Ruiz-Opazo et al. 1998). Discovery of this dual receptor provided evidence of a molecular link between the ET-1 and angiotensin II hormonal systems. In addition to the above mentioned types of ET receptors, some other subtypes were also discovered in various species. For example, a novel subtype of ET-B receptor was discovered in birds (Lecoin et al. 1998). Another receptor called as ET-AX has been cloned and characterized in *Xenopus leavis* heart (Kumar et al. 1994). The other receptor that was found in *Xenopus leavis* liver was
named ET-BX. These receptors resemble mammalian ET-B receptors in their affinities for ET-1 and ET-3 (Nambi et al. 1994). All the aforementioned types and subtypes of ET receptors are comparatively similar in size, ranging from 415 to 444 amino acid residues. The regions of greatest similarity are found in the transmembrane region and the regions of greatest diversity are found in extramembranous amino and carboxy termini (Derk et al. 1995). The affinity of ET-A receptors for ET-1 and ET-2 is over 100-fold greater than for ET-3, whereas ET-B receptors bind ET isopeptides with similar affinity (Masaki 1995). This explains why ET-B is referred as a non-isopeptide selective receptor. Cross-talk between ET-A and ET-B receptors has been reported; however, whether or not this cross-talk affects receptor function is unknown (Simonson and Herman 1993, Ozaki et al. 1997, Zahradka et al. 1998). Many pharmacological studies on blood vessels have indicated the existence of a subtype of ET-A receptors (Bax et al. 1993, Salom et al. 1993, Clark and Pierre 1995 and Maguire et al 1996). Similar results were obtained from pharmacological studies in isolated human bronchus and isolated sheep bronchus (Hay et al. 1998, Henry and King 1999). However, radio ligand binding studies on blood vessels and isolated sheep bronchus have shown contradictory results (Maguire et al. 1996, Henry and King 1999). For the ET-B receptor, a further division into ET-B_1 and ET-B_2 receptors has been suggested in human bronchus and blood vessels, and rat brain and atrium (Sokolovsky et al. 1992, Hay et al. 1998). The ET-B_1 receptor is principally found on endothelial cells and causes vasodilatation because of the formation of NO and /or prostacyclin. The ET-B_2 receptor is present on the smooth muscle cells where it mediates vasoconstriction and bronchoconstriction.

1.7.2 Structure of ET Receptors – The structure of mature ET receptors has been deduced from the nucleotide sequences of the DNAs. Both ET-A and ET-B receptors contain seven transmembrane stretches of 20 to 27 hydrophobic amino acid residues and belong to the seven-
transmembrane (7 TM) domain, heterotrimeric G-protein coupled rhodopsin type receptor super-family. Human ET receptors have several amino acid residues and sequence motifs shared by other members of the G-protein receptor superfamily, including cysteines at position 158 and 239 (ET-A) and 174 and 255 (ET-B), which are postulated to cross link extracellular loops 1 and 2 by a sulfide bond. Both ET-A and ET-B receptors are heptahelical receptors having an N-terminal signal sequence with relatively long extra-cellular N-terminal portion preceding the first transmembrane domain. There are two separate ligand interaction subdomains in each of the ET receptors. The extracellular loops, particularly between TM 4 to 6, determine selectivity (Davenport 2002). In general, ET receptors have one hydrophobic extracellular amino terminal sequence, three putative extracellular loops, three intracellular loops and a carboxy terminal. Four cysteine residues are present in the amino terminals and also three cysteines in each of the three extracellular loops (Derk et al. 1995).

The activation of ET-A receptors induces long-lasting vasoconstriction and cell proliferation in different tissues (Pollock et al. 1995, Ohlstein et al. 1992). In contrast, the activation of endothelial ET-B receptors stimulates the release of NO and prostacyclin, prevents apoptosis, and inhibits ECE-1 expression in endothelial cells (Warner et al. 1989, Hirata et al. 1993, Shichiri et al. 1997, Luscher and Barton 2000). The ET-B receptors also mediate the pulmonary clearance of circulating ET-1 and the re-uptake of ET-1 by endothelial cells (Fukuroda et al. 1994, Ozaki et al. 1995). The human ET-A receptor is 427 amino acids long with a MW of 48,516 daltons. The human ET-B receptor has 442 amino acids and has a molecular weight of 46,901 daltons. There is 58.9% homology between these two receptors in humans (Table 1.1) (Elshourbagy et al. 1993). The degree of homology between human ET-A and ET-B receptors occurs more in the transmembrane hydrophobic domains (86-96%)
compared with the extracellular domains (65-83% in extra cellular loops and 50% in carboxy termini) (Ohlstein et al. 1995, Elshourbagy et al. 1993). The human ET-A receptor has 94% and 91% primary amino acid sequence identity with bovine and rat ET-A receptors, respectively (Elshourbagy et al. 1993, Cyr et al. 1991). The ET-B receptor shares 90%, 89% and 88% identity with porcine, bovine and rat ET-B receptors, respectively (Elshourbagy et al. 1993, Ohlstein et al. 1995). Equine ET-B receptors are 443 amino acids long and shares 89, 91 and 85% homology with human, bovine and mouse ET-B receptors, but it is only 55% identical with human, bovine and rat ET-A receptors (Yang et al. 1998). Bovine lung ET-B receptor is 441 amino acids long; 26 constitute an NH2-terminal signal peptide and 415 constituting the mature receptor (Saito et al. 1991). In general, ET receptors have a large extracellular amino terminal region which plays an important role in tight binding to ET (Kemp and Pearson 1990). But this is not the case with the bovine ET-B receptor, which has no sequence homology at the NH2 terminal either with bovine ET-A receptors or any other subtypes of ET-B receptors (Saito et al. 1991). It has 63 and 85% homology with bovine ET-B and rat ET-B receptors, respectively (Saito et al. 1991). ET-AX receptor has 415 amino acids and has 61, 74, 60, 51, 72, and 74% homology with porcine ET-B, human ET-A, ET-B, *Xenopus* ET-C, bovine and rat ET-A receptors, respectively (Elshourbagy et al. 1992, Elshourbagy et al. 1993, Arai et al. 1990). Like other ET receptors, it also has a leader sequence, a large extracellular domain (70 amino acids long) and a short third cytoplasmic loop (29 amino acids) (Kumar et al. 1994). Transmembrane domains IV-VI and adjacent loops are important for ET-B selective pharmacology, where as transmembrane domains I, II, III and VII plus intervening loops are essential for ET-A selective pharmacology (Sakamoto et al. 1993). It is suggested that tight association of ET with its receptor is due to a strong interaction of its hydrophobic domain with the membrane lipids and/or its internalization.
within cells (Hirata et al. 1989a). Endothelin receptors as with other cell surface receptors are subject to ligand-induced down-regulation. Exposure of cultured smooth muscle cells and cardioocytes to ET-1 for 18-24 hours caused a marked decrease in ET-1 binding sites and no significant change in affinity (Hirata et al. 1989a, Hirata et al. 1989b, Ohlstein et al. 1995).

**Table 1.1** – Homology of the amino acid sequences of ET-A and ET-B receptors among various mammalian species.

<table>
<thead>
<tr>
<th>Species</th>
<th>ET receptor type</th>
<th>Amino acids</th>
<th>Percentage of homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>ET-A</td>
<td>427</td>
<td>58.9 ET-B (Hu) 91 ET-A (Rat)</td>
</tr>
<tr>
<td></td>
<td>ET-B</td>
<td>442</td>
<td>89 ET-B (Bo) 89 ET-B (Eq)</td>
</tr>
<tr>
<td>Porcine</td>
<td>ET-A</td>
<td>427</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ET-B</td>
<td>443</td>
<td>90 ET-B (Hu)</td>
</tr>
<tr>
<td>Bovine</td>
<td>ET-A</td>
<td>427</td>
<td>55 ET-B (Eq) 94 ET-A (Hu)</td>
</tr>
<tr>
<td></td>
<td>ET-B</td>
<td>441</td>
<td>91 ET-B (Eq)</td>
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<tr>
<td>Rat</td>
<td>ET-A</td>
<td>426</td>
<td>55 ET-B (Eq)</td>
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<tr>
<td></td>
<td>ET-B</td>
<td>442</td>
<td>88 ET-B (Hu)</td>
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<tr>
<td>Equine</td>
<td>ET-B</td>
<td>443</td>
<td>55 ET-A (Hu)</td>
</tr>
<tr>
<td>Mouse</td>
<td>ET-B</td>
<td>442</td>
<td>81 ET-B (Eq)</td>
</tr>
</tbody>
</table>

Once the endothelin receptor complex has been formed, the complex undergoes rapid internalization into lysosomal compartments where the acidic environment promotes ligand disassociation (Hirata et al. 1989b, Marsault et al. 1991a, Marsault et al. 1991b). Thus, the receptors are recycled. It is also proved that endothelin receptors exist intracellularly, mostly in the Golgi complex and nuclei. Even though the density of these receptors in Golgi is much less (<10%), it can be suggested that a recycling pathway for these receptors exists inside the cell (Hocher et al. 1992). The recycled receptors are migrated to the plasma membrane through a cycloheximide-insensitive pathway. Endocytotic recycling and externalization of ET receptors comes from the observation that both dansylcadavarine (a transglutamate inhibitor) and
phenylarsine oxide (a recycling inhibitor) inhibit the recovery of endothelin-binding sites in the plasma membrane following exposure to ET-1 (Marsault et al. 1993, Resink et al. 1990).

1.7.3 ET Receptor Genes – The genes for human ET-A and ET-B receptors are located on chromosomes 4 and 13, respectively (Masaki 1995). The gene of ET-A receptors spans more than 40 Kb of DNA and that of ET-B receptor spans approximately 24 Kb of DNA. Having such large genes is typical of proteins belonging to the G-protein coupled receptor family. The ET-A receptor gene is expressed as a single mRNA transcript of 4.3 Kb (Hosoda et al. 1991). The ET-B receptor gene is predominantly expressed as two mRNA molecules that are 4.3 and 1.7 Kb in length. A third ET-B receptor mRNA transcript, 2.7 Kb in length is also present in placental tissue (Ogawa et al. 1991). These three different transcripts may arise from alternative polyadenylation of the ET-B mRNA, which would not effect the final translation of the ET-B receptor. Alternatively, the multiple ET-B mRNA species may reflect alternative splicing of the precursor mRNA, which could potentially give rise to structural variants (subtypes) of the ET-B receptor protein (Shyamala 1994, Cheng et al. 1993). The human ET-A receptor gene contains eight exons and seven introns and the ET-B receptor gene comprises 7 exons and 6 inrons. The structural organization of the genes of both receptors is essentially the same. The only major difference is that the human ET-B receptor gene lacks an intron corresponding to intron 1 of the human ET-A receptor gene, which is present on the 5’ non-coding region. Furthermore, these two genes share an intron at the same position immediately after the third transmembrane domain. Thus, the intron phase and the nucleotide sequence around the exon-intron splice site are highly conserved. The 5’-flanking regions of both genes lack typical TATA and CCAAT boxes but contain Sp1 transcription factor binding sites, GATA binding motifs and APPRE sequences that induce mRNA expression under acute stress in vivo. All of these results suggest that both
receptors evolved from a common ancestral gene (Arai et al. 1993). Despite these similarities, the tissue specific patterns of expression of these two genes are quite different (Rubanyi and Polokoff 1994).

1.8 Mechanism of Action of ET-1

All endothelin isoforms elicit various biological actions by binding to specific receptors located on the external surface of the cell membrane. This binding activates complex but highly coordinated signal transduction pathways involving various secondary intracellular messengers which activate enzymes such as kinases and phostases and in turn phosphorylate or dephosphorylate key cellular proteins, leading ultimately to biological responses. The coupling of endothelin receptors to intracytoplasmic and plasma membrane effector molecules occurs through a family of GTP (guanosine triphosphate) binding proteins known as guanine nucleotide regulatory proteins or G-proteins. G proteins are heterodimers and have three subunits, namely \( \alpha, \beta \) and \( \gamma \). The \( \alpha \) subunits bind to guanine nucleotides and the \( \beta \gamma \) complex functions as a single unit. As many as twenty \( \alpha \) subunits, at least four \( \beta \) subunits, and six \( \gamma \) subunits and their respective genes have been isolated and characterized. Endothelin and receptor complex generate an interaction between the receptors and G-proteins on the intracellular surface of the membrane. This interaction promotes disassociation of GDP from the \( \alpha \) subunit and stimulates association of GTP to it, resulting in the disassociation of \( \alpha \) subunit from the \( \beta \gamma \) complex. The released \( \alpha \)-GTP subunit regulates the activity of secondary messengers. The \( \alpha \) subunit also shows endogenous GTP-ase activity resulting in the inactivation of the cycle. The \( \beta \gamma \) complex is also involved in direct modulation of secondary mediator molecules such as adenylate cyclase, phospholipase C, phospholipase A2 and K+ channels. A single G-protein can activate multiple effectors, and a single receptor can couple to multiple G proteins to activate single or multiple effector systems.
Differential expression of ET receptor subtypes and their coupling to single or multiple G proteins can result in the activation of selective pathways that are appropriate for the biological responses in different tissues.

1.8.1 Secondary Mediators Involved in Endothelin-Mediated Signal Transduction – Calcium: It is believed that ET-1 stimulates Ca+2 influxes into vascular smooth muscle cells via non-dihydropyridine-sensitive Ca+2 channels. Many of the antagonist studies have shown that Ca+2 channel blockers such as verapramil, nifedipine and nicardipine markedly attenuate ET-1 induced vasoconstriction (Egashira et al. 1990, Encabo et al. 1992, Luscher et al. 1990, Kasuya et al. 1989, Hardebo et al. 1989, Lougee et al. 1990, Takenaka et al. 1992). However, in some other studies no such attenuation was observed (Wallnofer et al. 1989, Blackburn and Highsmith 1990, Chabrier et al. 1989, Bodelsson et al. 1992). Dosages of ET-1 and Ca+2 channel antagonists and the sources of the blood vessels and smooth muscle cells used in these experiments could have lead to this contradiction. It is believed that ET-1 induces smooth muscle contraction by increasing intracellular Ca+2 levels (Figure 1.3). There are two possible mechanisms for this increase. First, a rapid and transient initial phase in which calcium is mobilized from intracellular stores. ET-1 stimulates an increase in inositol 1-4-5 trisphosphate (IP3) which causes increased mobilization of intracellular calcium (Little et al. 1992). The second sustained phase is dependent upon extracellular calcium influx into the cell. ET-1 is not a ligand to L type Ca+2 channels. It causes Ca+2 influx through indirect actions. One of the possible mechanisms is membrane depolarization. The most important cause attributed for this depolarization is activation of chloride channels. Increase in intracellular sodium levels either by inhibition of Na+/K+ ATP-ase or by stimulation of the Na+/H+ antiporter, is also another mechanism of depolarization. In addition to membrane depolarization, platelet activating factor
(PAF) and TXA-2 also mediate Ca\(^{2+}\) uptake (Takayasu et al. 1989). Influx of extracellular calcium also triggers calcium release from intracellular stores (Gardner et al 1992). It is also reported that ET-1 can cause smooth muscle cell contractions by altering the sensitivity of the myofilaments to calcium. That means, even if the intracellular calcium levels are not increased either by influx or mobilization from intracellular stores, ET-1 can cause smooth muscle cell contraction. This is believed to occur through a G-protein dependent pathway probably involving PKC (Nishimura et al. 1992).

**Inositol-3-Phosphate (IP3) and Diacylglycerol (DAG):** Numerous reports indicate that ET-1 stimulates phospholipase-C (PLC) mediated hydrolysis of PI (phosphatidylinositol 4, 5-biphosphate) resulting in the rapid transient formation of IP3 and more sustained DAG generation in smooth muscles. In addition to IP3, a variety of other inositol phosphates such as inositol tetrakisphosphate may be formed by ET-1 (Simonson et al. 1989). DAG is formed mainly by hydrolysis of IP3. However, it can be formed by the hydrolysis of phosphatidylcholine by PLC and degradation of phasphatidic acid by phospholipase D also. IP3 is responsible for the stimulation of release of calcium from intracytoplasmic sarcoplasmic reticulum and calcisome (Bialecki et al. 1989, Hirata et al. 1989a). Neutral DAG on the other hand, mediates ET-1 induced activation of phosphokinase-C. Inositol tetrakisphosphate acts in synergy with IP3 to stimulate an increase in calcium in ET-1 stimulated cells.

**Phospholipase-D (PLD):** Application of endothelins to rat mesangial cells, VSMCs and fibroblasts result in activation of PLD, which hydrolyzes phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, to produce phosphatidic acid and either choline or ethanolamine, depending on the phospholipids involved. ET-1 activates PLD in both PKC - dependent and - independent pathways (MacNulty et al. 1990).
Figure 1.3 – Mechanism of action of ET-1
**Protein kinase-C (PKC):** Several studies using activators (phorbol esters) and inhibitors of PKC (staurosporin) provided evidence that activation of PKC plays a role in ET-1 mediated vascular contraction. PKC, a Ca+2 and phospholipid dependent enzyme, can be activated by DAG and tumor promoting phorbol esters (Nishizuka 1989).

**Adenylate Cyclase:** In contrast to the activation of PLC and generation of inositol phosphates, cyclic nucleotide levels are generally unaffected by ET-1 (Sugiura et al. 1989). However, in the iris sphincter of various species, such as cat, dog, cow, monkey and human, ET-1 has been shown to increase cAMP formation (Abdel-Latif and Zhang 1991). This increase is not by the direct action but by indirect actions of ET-1, either by the activation of PLC or PGE-2 dependent pathway (Tomita et al. 1990, Simonson et al. 1990).

**Guanylate Cyclase:** Reiser and coworkers have demonstrated that in mouse neuroblastoma/rat glioma hybrid cells, the addition of ET-1 caused an increase in cGMP levels that was dependent on calcium presence (Reiser et al. 1990). This increase in rat glomeruli was mediated by ET-B receptors and was blocked by the addition of L-arginine, a NO synthase inhibitor, suggesting that cGMP stimulation by ET-1 was mediated through NOS (Edwards et al. 1992).

**Phospholipase A2 (PLA2) and Arachidonic Acid Metabolites:** ET-1 stimulates arachidonic acid metabolism via activation of phospholipase A2 (De Nucci et al. 1988a). It causes release of PGI-2 and TXA-2 from isolated perfused rat and guinea pig lung preparations. ET-1 also mediates release of prostaglandins in kidney, intestine and ion secretion in liver, eye, airways and, gestational tissues (Brown and Smith 1991, Gandhi et al. 1992, Granstam et al. 1991, Ninomiya et al. 1992, Mitchell et al. 1990).
**Na+-H+ Exchange:** ET-1 promotes intracellular alkalinization by promoting Na+-H+ exchange. A wide variety of agonists that are linked to PLC activation and to Ca+2 mobilization increase intracellular pH via PKC - mediated activation of Na+-H+ exchange by increasing the intracellular H affinity for the antiporter (Grinstein and Rothstein 1986). This is also true with ET-1 (Lonchampt et al. 1991).

1.8.2 **Effects on Gene Expression** – ET-1 regulates gene expression to evoke long-lasting cellular responses. It stimulates the expression of the immediate early response genes c-fos and c-myc in vascular smooth muscle cells (Komuro et al. 1989). In rat-1 fibroblasts, ET-1 rapidly increased mRNA levels of five members of the fos / jun gene family (c-fos, fos-B, fos-1, c-jun and jun-B) (Pribnow et al. 1992). For c-fos induction, calcium influx is necessary. And one of the important mechanisms by which calcium promotes c-fos induction is via CaMK (calmodulin dependent protein kinase) (Wang and Simonson, 1996). ET-1 is reported to have activated the enzyme mitogen activated protein kinase (MAPK) in cultured ventricular cardiomyocytes and in cultured vascular smooth muscle cells (Bogoyevitch et al. 1993, Koide et al. 1992). ET-1 also stimulates DNA synthesis, protein synthesis and /or proliferation in pulmonary artery smooth muscle cells and in rat 6 fibroblasts (Janakidevi et al. 1992). ETs can participate in long term adaptive changes in various tissues, including vascular remodeling, cardiac hypertrophy, and bone remodeling, and also in the pathogenesis of proliferative disorders, such as atherosclerosis, renal diseases and bronchial asthma. ET-1 evokes nuclear signaling cascades leading to differential expression of genes that control cell growth and/or differentiation. The c-Src or Src family of protein-tyrosine kinases contributes to a nuclear signaling cascade, linking ET-1 receptors to the c-fos promoter. Activation of the c-fos promoter by ET-1 requires both the CArG box of the serum response element (SRE) and the Ca/CRE

1.9 ET-1 and Lungs

Among various organ systems in the body, the lung has the greatest levels of ET-1 secreted by pulmonary vascular endothelium, smooth muscle, airway epithelium and a variety of other cells. Immunoreactivity for ET-1 can be detected in pulmonary endocrine cells of developing human lung, in the airway epithelium, pulmonary vascular endothelial cells, clara cells (alveolar type II pneumocytes), goblet cells, neuroendocrine cells, smooth muscle cells, platelets, mucus cells, serous cells, parasympathetic ganglia within the airways, and alveolar macrophages (McKay et al., 1991a, Rozengurt et al. 1990, Giaid et al. 1991). Most of the non-ciliated cells synthesize endothelins rather than basal and ciliated cells (Giaid et al. 1991). Finding of ET-1 and ET-2 on parasympathetic ganglia suggested a neuromodulatory role for these peptides in the lungs (McKay et al. 1991a). ET-3 also was found to have a neuromodulatory role on cholinergic parasympathetic transmission in rabbit airways exerted at a prejunctional site (McKay et al. 1993). The ECE and endothelin degrading enzymes also coexist in the airway tissue and both enzymes are sensitive to phosphoramidon. Levels of ECE are elevated in patients with chronic rhinitis and idiopathic pulmonary fibrosis (Furukawa et al. 1996, Noguchi et al. 1991). The presence of immunoreactive ET-1 and its abundant binding sites in airways and the finding that ET-1 is secreted toward both the luminal side and the serosal or abluminal side of the airways suggested that this peptide is an important autocrine and/or paracrine modulator of airway functions (Blouquit et al. 2003). Elevated levels of ET-1 in BAL and plasma of patients with various airway diseases and reduction in the symptoms when endothelins, endothelin forming enzymes and their receptors are antagonized provides evidence
that these peptides are not only important in normal physiology but also in pathogenesis of lung diseases. It is already established that endothelins cause sustained and powerful contraction of the airway smooth muscle in vivo and in vitro directly and indirectly. ET-1 also acts on airway epithelium. It increases ciliary beat frequency, activates mucus secretion by submucosal glands, and stimulates proliferation of epithelial cells. In humans, ET-1 stimulates lactoferrin and mucus glycoprotein release from serous and mucus cells in cultured nasal mucosal explants, and affects expression of genes such as that of fibroectin in bronchial epithelial cells. In feline trachea, ET-1 induces mucus glycoprotein secretion via ET-A receptors, promoting Ca\(^{2+}\) influx into the cells; however, ET-2 and ET-3 have no such effect (Shimura et al. 1992). Intranasal administration of ET-1 in allergic and non-allergic subjects induces symptoms of rhinoerrhea and increases the amount of secretions. ET-1 induces secretion of chlorides in airways of dogs, sheep and humans. In humans, this effect is caused by increasing the intracellular levels of cAMP and not by increasing calcium levels, whereas in dogs this effect is the result of increased intracellular levels of cAMP, calcium and cyclooxygenase products such as PGE-2. Receptor antagonism studies have proved that ET-1 causes all of these effects on chloride secretion by acting via ET-B receptors (especially those located in the apical membrane of the epithelial cells) and not through ET-A receptors (Blouquit et al. 2003).

1.10 ET Receptor Localization, Distribution and Functions in the Lungs

All of the effects of ET-1 in the airways are mediated via stimulation of ET-A and ET-B receptors that are widely but differentially distributed throughout the lungs. They are mainly located in the airway epithelium, airway smooth muscles, inflammatory cells, alveolar septa, vascular endothelium of pulmonary blood vessels, mucosal glands and lung parenchyma. Both receptors have different but important functions in different regions of the lungs in different
species. Takimoto and coworkers demonstrated that ET-1 induces DNA synthesis in human lung cell lines predominantly through ET-A receptors, via pertussis toxin-insensitive G protein (Takimoto et al. 1996). However, intracellular cAMP (also formed by ET-A receptors) inhibits this DNA synthesis. Stimulation of ET-B coupling to G protein modulates ET-A mediated DNA synthesis by inhibiting cAMP formation. This suggested that ET-B receptors do not play a dominant role in ET-1 induced smooth muscle mitogenesis. In contrast to ET-1, ET-3 did not reveal any stimulation of DNA synthesis (Takimoto et al. 1996).

In rabbit airways, ET-1, by activating ET-B receptors, triggers the influx of extracellular calcium through voltage-dependent channels, and induces a contractile response that is, in part, dependent upon stimulation of PKC. The same mechanism is triggered in dog bronchus; however, the receptors involved in this species are of ET-A type. In human airways, the contractile response to ET-1, while independent of extracellular calcium influx, is dependent upon PKC activation after binding of the peptide to ET-B receptors (McKay et al. 1996). This suggests that considerable species variation in receptor functions exists in lungs. In general, ET-A receptors are found more in bronchi and pulmonary vascular smooth muscles, whereas ET-B receptors are more in the lung parenchyma and vascular endothelium. In dogs, Dupius and coworkers have shown that ET-B receptors play an important role in the pulmonary elimination of ET-1 (Dupuis et al. 1996a). They also found similar results in rat lungs. The endothelin-induced potentiation of parasympathetic neural responses in the rabbit bronchus is mediated via ET-B receptor activation. Confirmation of ET-B receptor involvement in the neuropotentiation was obtained by demonstration of a significant amelioration of the potentiation in the presence of the ET-B receptor selective antagonist BQ-788 (McKay et al. 2000). In guinea pig tracheal epithelial cells, the ET-B receptors are exclusively located on the apical membranes of the
ciliated columnar cells. There exists a marked species difference in the relative proportions of ET-A and ET-B receptors present in the airway smooth muscles, ranging from almost pure populations of ET-A receptors present in the ovine tracheal smooth muscles through to predominance of ET-B receptors in guinea pig bronchus (Goldie et al. 1994, Hay et al. 1993b). Human bronchial smooth muscle from non-diseased lungs contains a greater proportion of ET-B receptors than ET-A, where as tracheal smooth muscles from rats and mice contain approximately equal proportions of the two receptor subtypes (Henry 1999). Significant regional variation in receptor distribution is also present. For example the ratio of A to B receptors on airway smooth muscles changes from 30:70 to 70:30 upon moving from the trachea to the bronchus in the porcine airway (Goldie et al. 1996). The ratios are 30:70 and 90:10 in peripheral lungs of non-asthmatic and asthmatic lungs, and in human pulmonary artery, respectively (Hay 1999). On the alveolar wall tissue of human lung, ET-A and ET-B receptors are in the ratio of 30:70 (Knott et al. 1995). ET-1 induced contractions in both large and small sized isolated human bronchi are ET-B mediated (Goldie et al. 1995). These receptors are primarily linked to intracellular release of calcium rather than influx of extracellular calcium (McKay et al. 1991b). In human airway smooth muscle cell cultures, the proliferative responses to ET-1 are mediated via ET-A receptors, even though it is only to a small extent (Panettieri et al. 1996). However, ET-1 is a powerful co-mitogen, enhancing the mitogenic response to EGF several fold (Panettieri et al. 1996). Interestingly, ET-1 interacts with EGF-induced mitogenic axis via a pertussis toxin sensitive G_i protein-dependent pathway, which appears to be distinct from its direct mitogenic pathway (Fujitani et al. 1997). In rat, rabbit and murine airways, the neuro-modulatory action of ET-1 is mediated via the stimulation of ET-A and ET-B receptors located prejunctionally and linked to the enhanced release of Ach during nerve stimulation (Knott et al. 1996). Although
only ET-B receptors have hitherto been ascribed with such a role in human airways (Fernandes et al. 1996), recent studies suggest that both ET-A and ET-B receptors facilitate parasympathetic neuro-transmission in airways of rat, rabbit and mouse trachea (Goldie et al. 1996). ET-B receptors have also been detected immunocytochemically in the parasympathetic neurons of guineapig airways (Fernandes et al. 1998). Airway epithelial cells are the major cellular sources of ET-1 in the airway wall. ET-1 depresses tracheal mucus velocity in sheep (used as an index of mucociliary clearance) via an action on ET-A receptors that was independent of prostanoids and peptidoleukotrienes (Sabater et al. 1996). The ET-A receptor mediated up-regulation in gene expression and release of fibronectin, a potent chemotactic factor for fibroblasts, has also been reported in human bronchial epithelial cells (Marini et al. 1996). Sen and co-workers have demonstrated that ET-1 acts on alveolar type II cells and releases surfactant phosphatidylcholine (Sen et al. 1994). Markeqitz et al. (1995) have demonstrated that ET-1 acts on alveolar epithelial cells and releases PGE-2 and cAMP via ET-A receptors. Therefore, the distribution and numerous physiological functions of endothelin receptors are species and region specific.

1.11 Role of ET-1 in the Pathogenesis of Allergic Airway Diseases

1.11.1 ET-1 and the Pathogenesis of Human Chronic Obstructive Pulmonary Disease – In humans, chronic obstructive pulmonary disease (COPD) is a pulmonary disorder that is characterized by reduced maximal expiratory flow and slow forced emptying of the lungs; features that do not change markedly over several months. This limitation in airflow is only minimally reversible with bronchodilators. Some of the most common types of COPD are emphysema, chronic bronchitis, asthma, cystic fibrosis, and bronchiectasis. Typically, the symptoms of COPD include cough, sputum (mucus) production, shortness of breath, especially with exercise, wheezing and chest tightness. There is also epidemiological evidence that there is
an increased cardiovascular risk in patients with COPD. ET-1 plays an important role in causing these symptoms and effects of COPD (Roland et al. 2001). Other effects of ET-1 related to COPD include recruitment and activation of neutrophils and alveolar macrophages (Roland et al. 2001). Corticosteroids are generally used to reduce the exacerbations of the COPD. One of the mechanisms by which they act is by reducing the production of ET-1 from airway epithelial cells (Radington et al. 1997). Sputum and plasma levels of ET-1 are increased during the exacerbations of COPD. COPD exacerbations have been strongly related to the actions of IL-6. Plasma ET-1 levels are increased in chronic smoking that is a frequent cause of COPD. In patients with COPD, the plasma ET-1 level is not affected by acute progression of pulmonary hypertension and hypoxemia during exercise, and persistent hypoxemia may be associated with an increase in the plasma ET-1 level. In addition, the findings suggest that atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) may modulate pulmonary vascular tone by interacting with ET-1 in these patients (Fujii et al. 1999). One of the important and potentially serious complications of COPD is pulmonary vascular tension that also includes pulmonary hypertension. There are several reports describing an increase in plasma ET-1 levels in patients with pulmonary hypertension, with a correlation between levels and disease severity evident in some studies (Allen et al. 1993). Abnormalities in ET-1 pulmonary metabolism have been reported in patients with pulmonary hypertension, asthma and chronic obstructive pulmonary disease (COPD). BAL ET-1 levels are not elevated in COPD patients. This is the major difference between human COPD and asthma. Also, arterial ET-1 levels in stable COPD patients were found to be significantly greater than in patients with asthma (Nikolaou et al. 2003). Smoking is not associated with an induction of ET release in BAL (Reichenberger et al. 2001). Plasma ET-1 levels in COPD patients were similar during exacerbation and recovery and were
not significantly different from those in healthy subjects. Measurement of 24-hr urinary excretion of ET-1 showed that it is increased in COPD patients during acute exacerbation; it decreased during recovery, but remained elevated when compared to normal subjects. A negative correlation was found between arterial oxygen pressure and ET-1 excretion; no correlation was found between plasma and urinary ET-1 values. Hence, COPD patients excrete higher amounts of ET-1 compared with healthy subjects. Urinary ET-1 values are further increased during acute exacerbation of the disease (Sofia et al. 1994). Smokers have impaired ET-1 mediated vasodilatation that correlates with bronchial hyperresponsiveness and may contribute to pulmonary hypertension. It was shown that the responsiveness of pulmonary arterial smooth muscle to dilator and constrictor agents is increased in patients showing reversibility of airway constriction. Thus, hyperresponsiveness of airway smooth muscle may be associated with a similar phenomenon in the surrounding vascular smooth muscle (Cases et al. 1996).

1.11.2 Role of ET-1 in the Pathogenesis of Asthma – Asthma is a chronic inflammatory airway disease characterized by airway obstruction, airway inflammation and bronchial hyperresponsiveness or bronchoconstriction. These symptoms are a result of airway smooth muscle contraction, mucus hypersecretion, and epithelial damage (contributing to mucus plug formation), microvascular leakage, the influx of inflammatory cells, edema, and airway thickening. Chronic inflammation in asthmatic airways leads to pathophysiologic changes in the structure of tissues, a process often referred to as airway remodeling. Such changes include thickening of the smooth muscles in the airway wall, which may influence airway responsiveness. Other structural changes that may affect airway function include disruption of the bronchial epithelium, subepithelial fibrosis, and goblet cell hyperplasia together with increased mucus accumulation in the airways (Salmon et al. 2000). ET-1 has important roles in
all of these symptoms. Increased levels of ET-1 in BALF, bronchial biopsies, and peripheral blood and alveolar macrophages in asthmatic patients confirm this finding (Radington et al. 1995, Springall et al. 1991). This increase is due to increased local production of ET-1 from epithelial cells in the airways in asthmatic individuals. ET-1 causes immediate bronchoconstriction in asthma (Radington et al. 1995, Chalmers et al., 1997). ET-1 in asthmatic subjects also stimulates mucus secretion, airway edema through microvascular leakage, smooth muscle mitogenesis, and also bronchial hyperresponsiveness. The expression of ET-1 is increased in the vascular endothelial cell of asthmatic patients. In allergic sheep, it has been demonstrated that aerosolized ET-1 causes bronchoconstriction, in part, by stimulation of ET-A receptors. ET-1 is released in the airways after antigen challenge, and this peptide contributes to the severity of the allergic responses, by increasing airway smooth muscle responsiveness (Noguchi et al. 1995). ET-1 inhalation is not associated with a late bronchoconstrictor response. Inhaled ET-1 does not appear to stimulate an acute inflammatory response in asthma as assessed by differential cell count, TNF-α, IL-1β and albumin concentrations in induced sputum (Chalmers 1999). In asthma, ET-1 enhances vascular permeability by mediating and/or modulating the secondary release of TXA-2 (Sirois et al. 1992). The bronchial epithelial cells of all the asthmatic patients expresses preproendothelin-1 mRNA, as assessed by in situ hybridization, and released high amounts of mature and biologically active ET-1. Addition of corticosteroids results in decreased release of ET-1 from cultured asthmatic bronchial epithelial cells (Vittori et al. 1992). Inhibition of ET-A or combined ET-A and ET-B receptors additionally leads to decreased airway inflammation in antigen challenged animals, suggesting that the proinflammatory effects of ET-1 in the airway are mediated mainly by ET-A receptors (Fujitani et al. 1997). However, most of the bronchoconstriction elicited by ET-1 in asthmatic individuals
is through ET-B receptors (Reichenberger et al. 2001). It was shown that the plasma concentrations and production from cultured mononuclear sites from asthmatic children are significantly greater compared with healthy children (Chen et al. 1995). Adult asthmatics have normal levels between attacks, but during acute attacks serum ET-1 levels are increased and correlate inversely with airflow measurements and decrease with treatment (with steroids, beta-adrenergic agonists or phosphodiesterase inhibitors)(Aoki et al. 1994). ET-1 in asthmatics is similarly increased to concentrations that cause bronchoconstriction and inversely correlates with forced expiratory volume (Sofia et al. 1993). Patients with asthma also have been shown to have increased amounts of immunoreactive endothelin in BALF compared with normal control subjects or subjects with chronic bronchitis in absence of any significant alteration in the levels of circulating ET-1. Treatment of asthmatic patients with oral corticosteroids and inhaled beta-agonists for 15 days resulted in improvement of airflow obstruction and in more than 3-fold reduction in the contents of ET-1 in lavage fluid. All these findings indicate that ET-1 contributes significantly to the pathogenesis of airflow obstruction in asthma (Mattoli et al. 1991). ET-1 and ET-3 are found in equal amounts in BAL fluid as well as in cultured epithelial cells from asthmatics (Fagan et al. 2001). ET-1 plays a role in the overnight worsening of asthma and perhaps is more tightly tissue bound, resulting in lower levels of BAL fluid (Kraft et al. 1994).

1.11.3 Role of ET-1 in the Pathogenesis of Equine Recurrent Airway Obstruction (RAO) –

To date very few studies have been conducted regarding the role of ET-1 in the pathogenesis of equine RAO. Benamou and co-workers have performed a study to investigate the effect of ET-1 on smooth muscle from isolated equine pulmonary artery and bronchus (Benamou et al. 2003). In this study, they also investigated the roles of ET-A and ET-B receptors in ET-1 mediated
contraction of these tissues. For this purpose, the force generation of ring segments from pulmonary arteries or third-generation airways was studied in an organ bath at 37°C in response to exogenous ET-1 and selective ET-A or ET-B receptor antagonists. They found that ET-1 produces concentration-dependent contractions of the equine pulmonary artery and bronchus. They also reported that ET-1 potency was 25 times greater in equine pulmonary artery than in equine bronchus. They also found that in pulmonary artery, ET-1 induced contractions were significantly inhibited by the ET-A receptor antagonist, but not by the ETB antagonist. In bronchus, dose-response curves to ET-1 were shifted to the right by an ET-A antagonist, but not by an ET-B antagonist. In the presence of both antagonists, the dose-response curve to ET-1 was shifted to the right by 4.5-fold. They concluded that ET-1 is a potent spasmogen of equine third generation pulmonary artery and bronchus, and that contractions are mediated via ET-A receptors in the former and both ET-A and ET-B receptors in the latter.

In another study performed by the same authors, the levels of ET-1 in systemic blood, as well as in BALF from horses with RAO were investigated (Benamou et al. 1998). They also studied how these values might correlate with those of lung function tests and pulmonary artery pressure. Five horses with RAO were evaluated both in remission and in crisis and compared to five control horses. They demonstrated that the RAO horses had significantly greater systemic ET-1 levels than control horses. They also found that the RAO-affected horses had a negative arteriovenous ET-1 difference that may correspond to a net uptake of ET-1 in the lung. Another important finding in this study was that the RAO horses in crisis had increased amounts of immunoreactive ET-1 in BALF when compared to normal horses. Additionally, they also showed that the reduction in lung function seen in RAO horses in crisis was significantly correlated with lower epithelial lining fluid ET-1 levels. The same authors have examined for the
possible variations in ET-1 concentrations occurring during exercise in COPD-affected horses (Benamou et al. 1999). They compared the effects of intense treadmill exercise on the recovery of ET-1 in the BALF as well as in arterial and venous blood, in a group of 5 healthy horses and a group of 5 COPD-affected horses studied alternately in remission and while symptomatic. They also investigated the possible correlations between ET-1 levels and pulmonary function tests during the study. They found that while exercise did not affect the ET-1 levels recovered in BALF among controls, it caused a significant increase among symptomatic COPD-affected horses. During remission, wide variations of ET-1 levels among horses at rest and during exercise made the interpretation difficult. Furthermore, no correlation could be found between exercise-induced changes in ET-1 concentrations and pulmonary function tests or changes in arterial oxygen tension with exercise. They concluded that exercise appears to affect the release of ET-1 by the airways in COPD-affected horses, in contrast to healthy horses. The findings of all three studies conducted by Benamou et al, suggest that ET-1 might play a pivotal role in the pathogenesis of equine RAO.

1.12 Epithelium and Airway Allergic Diseases

1.12.1 Functions of Epithelium – The epithelium has many important physiological functions in the lungs. It basically acts as a barrier to airborne irritants. The cilia present on the luminal side of the epithelial cells beat rhythmically to push the irritants and the mucus present in the airways. Epithelium mediates a vital role in the modulation of airway tone. It releases many airway smooth muscle relaxing factors such as epithelium-derived relaxing factor (EpDRF) and NO. Similarly, in horses it has been shown that epithelium is a source of inhibitory prostanoids such as PGE-2, which inhibits smooth muscle contraction and prevents bronchospasm (Gray et al. 1992). In addition, airway epithelium is metabolically active, since there are several enzymes
such as acetylcholinesterase, histaminase and peptidases, which can inactivate contractile agonists. Furthermore, the presence of receptors for inflammatory mediators, neurotransmitters and cytokines and the significant influence exerted on isolated airway smooth muscles highlights that the epithelium is more than a simple barrier. The loss of such a barrier, allowing an increased access of noxious chemicals and high molecular weight allergens, together with the exposure and subsequent up-regulated activation of sensory nerves, may contribute to the state of airway hyperresponsiveness which usually accompanies chronic obstructive lung diseases.

1.12.2 Role of Epithelium in Airway Allergic Diseases – Epithelial disruption is a feature of the inflammation associated with asthma (Laitinen et al. 1993, Laitinen et al. 1985). In a study done by Candenas and co-workers to investigate the effect of epithelial removal and enkephalinase inhibition on bronchoconstrictor responses induced by ET-1, it was demonstrated that the removal of the epithelium significantly enhanced the two stages of the contractile responses to the three endothelins (Candenas et al. 1992). The first stage was observed with the low concentrations of agonists and corresponded to low intrinsic activity and the second phase was with high concentration which corresponded with high intrinsic activity. Epithelium removal also abolished the differences in potency efficacy that were observed between ET-1, ET-2 and ET-3 when the epithelium was present. Phosphoramidon, an enkephalinase inhibitor, was as potent as epithelium removal in enhancing the contractile responses to these agonists at low concentrations. However, with high concentrations of endothelins, phosphoramidon was less potent than epithelium removal in enhancing the contractile responses. In epithelium-denuded strips, preincubation with phosphoramidon did not further increase the maximal contractions induced by/or the potencies of ET-1, ET-2 or ET-3. After epithelium removal, responses to low doses of endothelins were attenuated by nicardipine, a calcium channel blocker, whereas
responses to high doses of the endothelins were not affected, as was also observed when the epithelium was present (Barnes PJ. 1994, Candenas et al. 1992). Thus epithelial damage contributes significantly to the enhanced contractile response of the bronchi to endothelins. Intact epithelium in normal subjects forms a physical barrier to ET-1 coming into contact with submucosal target cells or increases in clearance and metabolism of ET-1, either by binding and internalization of ET-1 by ET-B receptors (Fukuroda et al. 1994) or by enzymatic degradation. In humans, it has been shown that ET-1 decreases bronchial epithelial cell migration, causes inhibition of repair of epithelium and it also enhances remodeling (Dosanjh and Zuraw 2003)

There is also evidence in human non-asthmatic tissue that ET-1 can act on ET-A receptors in bronchial epithelium causing release of NO and other relaxant factors, which could attenuate bronchoconstrictor effects of ET-1 in normal airways (Chalmers et al. 1997). It has also been demonstrated in vitro that ET-1 may interact with other mediators implicated in asthma, with potentiation of its activity, including prostaglandins PGD-2 and PGF-2α, LTD-4, and angiotensin II.

In vitro studies conducted on equine airway epithelium derived from healthy and COPD-affected horses have shown that epithelial strips from COPD-affected horses tended to produce smaller amounts of PGE-2, an inhibitory prostanoid, than did the strips from control horses (Gray et al. 1992). This finding suggests that the defective production of PGE-2 is one of the factors responsible for the pathogenesis of equine COPD. Moreover, in isolated bronchi from horses with heaves the inhibitory function of the prostanoids is reduced (Yu et al. 1994). All these findings suggest that epithelial alteration could play an important role in the pathogenesis of equine RAO.
1.13 Goals of this Dissertation

In our preliminary *in vitro* pharmacological studies, we found that when equi-molar concentrations of ET-1 are applied to the bronchial tissues of healthy and SPAOPD-affected horses, ET-1 caused a concentration-dependent contraction of these tissues in both groups of horses; however, the response was significantly greater in the diseased horses than the healthy horses. Owing to these findings and extremely important roles played by ET-1 and its receptors in the pathogenesis of many airway allergic diseases in various species, we wanted to investigate if there are any alterations in the affinity, expression, density and distribution of ET receptors in the lungs of SPAOPD-affected horses. In addition, we also studied the effect of epithelial denudation combined with ET-receptor antagonism, on ET-1 mediated airway smooth muscle constriction.

As exaggerated bronchoconstriction is an important feature of obstructive pulmonary diseases, we wanted to investigate if there is any alteration in the affinity of ET-receptors in the bronchial smooth muscles of SPAOPD-affected horses. For that purpose we employed pharmacological studies to determine and compare the pharmacological affinities of ET-A and ET-B receptors by using specific antagonists in both healthy and SPAOPD-affected horses. In these studies we determined the pA2 values of each ET-receptor antagonist in healthy and SPAOPD-affected horses. The pA2 value is a statistical constant which is calculated to assess the pharmacological affinity of the antagonist for its receptor. We also conducted immunohistochemical studies on bronchial smooth muscles and bronchial epithelial cells to examine for any changes in the expression of ET-A and/or ET-B receptors.

Most important histopathological findings in equine RAO can be observed in peripheral lungs which include bronchioles, capillaries, alveolar septa and inflammatory cells in the alveoli.
In fact, the principal lesion in COPD and SPAOPD-affected horses is bronchiolitis (Ronbinson et al. 1996 and Costa et al. 2000). Hence, we wanted to investigate for the presence of alteration in ET receptor expression in peripheral lungs. Previous studies have revealed that immunohistochemical, molecular biological and western blotting techniques are some of the most reliable and accurate techniques to characterize and compare the expression of ET receptors in lungs. For example, studies which employed immunohistochemical techniques to study the changes in ET receptor expression in developing human perinatal lung, rat pulmonary veins subjected to hypoxia, rat and guinea pig airway nerves, human scleroderma associated fibrotic lung disease, and rat lungs affected with pulmonary hypertension, have revealed that immunochemistry is a very reliable and dependable technique to characterize ET receptors in lungs of various species (Yorikane et al. 1993, Abraham et al. 1997, Fernandes et al. 1998, Takahashi et al. 2001 and Levy et al. 2005).

Similarly, studies that employed western blotting to characterize the presence of ET receptors in human lungs affected with pulmonary edema, in rat lungs affected with post-obstructive pulmonary vasculopathy and in the bronchial smooth muscles of rats have revealed that western blotting is a very useful technique to detect the presence of ET-receptors in the lungs of various mammalian species (Chiba et al. 2005, Kim et al. 2002 and Carpenter et al. 2003). Also, studies employing RT-PCR to detect the presence of ET receptor expression in the airway smooth muscles of allergic lungs, in the bronchial epithelium and lung cancer of humans, in the bronchial biopsies of patients affected with asthma and COPD have shown that this technique is highly accurate to determine and compare the expression of ET receptors (Moller et al. 1999, Ahmed et al. 2000 and Granstrom et al. 2004). Hence, to investigate the presence of alteration in the ET receptor expression in the peripheral lungs of SPAOPD affected horses, we
employed immunohistochemical, molecular biological and western blotting studies to determine and compare the expression of ET-A and ET-B receptors in the peripheral lungs of healthy and SPAOPD-affected horses.

1.14 References


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CHAPTER 2. *IN VITRO* EFFECT OF RECEPTOR ANTAGONISM AND EPITHELIAL DENUDATION ON THE ET-1 INDUCED CONCENTRATION-DEPENDENT RESPONSES OF THE BRONCHI OF CLINICALLY HEALTHY AND SPAOPD-AFFECTED HORSES
2.1 Introduction

Summer pasture-associated obstructive pulmonary disease (SPAOPD) of horses is a form of recurrent airway obstruction (RAO) that commonly occurs in subtropical regions of the US from late summer to early fall. The susceptible horses develop clinical exacerbations when they are allowed to graze lush pastures during these seasons (Beadle 1983, Seahorn and Beadle 1994, Robinson 2001). The RAO is induced by allergic reactions and characterized by respiratory distress, airway inflammation, airway hyperreactivity, and airway remodeling during the progression of the disease (Robinson 2001, Naylor et al. 1992). Airway hyperreactivity is a condition characterized by hypersensitivity and hyperresponsiveness to various stimuli. During periods of acute exacerbations of RAO, horses develop airway hyperreactivity in response to the inflammatory mediators released into the airways, leading to severe bronchoconstriction (Yu et al. 1994, Benamou et al. 1998). These mediators can also induce increased vascular permeability, mucus hypersecretion and airway epithelial disruption (Robinson 2001).

Endothelin-1 (ET-1) is a 21 amino acid peptide and has been implicated in airway hyperreactive diseases such as human asthma, human COPD and equine RAO (Benamou et al. 1998, Mattoli et al. 1991). In the lungs, ET-1 is synthesized by various types of cells, including pulmonary vascular endothelium, airway smooth muscle and airway epithelium (Rubanyi and Polokoff 1994). ET-1 exerts its effects by acting through two types of receptors, namely ET-A and ET-B receptors (Rubanyi and Polokoff 1994).

Benamou et al., have shown that ET-1 induces potent contraction of equine third generation bronchial smooth muscle by acting through both ET-A and ET-B receptors (Benamou et al. 2003). The same investigators have reported that ET-1 levels in the systemic circulation and in the bronchoalveolar lavage fluid (BALF) of RAO horses are significantly greater than
those of clinically normal horses (Benamou et al. 1998). The findings of these studies suggest that ET-1 might play an important inflammatory mediator role in equine airway hyperreactive diseases.

Our pilot *in vitro* pharmacological studies showed that when bronchial rings from SPAOPD-affected and unaffected horses were applied with equi-molar concentrations of ET-1, the contractile responsiveness of the rings from the affected horses was significantly greater than that of unaffected horses. This prompted us to investigate the possibility of alterations in the affinity of ET receptors to ET-1 in the airways of SPAOPD-affected horses.

Epithelial disruption is a feature of the inflammation associated with airway allergic diseases such as asthma (Laitinen et al. 1993, Laitinen et al. 1985). It has been demonstrated that the removal of the epithelium significantly enhances the contractile responses of bronchi to endothelins (Candenas et al. 1992). This is because the intact epithelium in normal subjects forms a physical barrier to ET-1 coming into contact with submucosal target cells or increases in clearance and metabolism of ET-1, either by binding and internalization of ET-1 by ET-B receptors or by enzymatic degradation (Fukuroda et al. 1994). There is also evidence in human non-asthmatic tissue that ET-1 can act on ET-A receptors in bronchial epithelium causing release of NO and other relaxant factors which could attenuate bronchoconstrictor effects of ET-1 in normal airways (Chalmers et al. 1997). In vitro studies carried out on equine airway epithelium derived from healthy and COPD-affected horses have shown that epithelial strips from COPD-affected horses tended to release smaller amounts of PGE₂, an inhibitory prostanoid, than did strips from control horses (Gray et al. 1992). This finding suggests that the defective production of PGE₂ is one of the factors responsible for the pathogenesis of equine COPD. Moreover, in isolated bronchi from horses with heaves the inhibitory function of the prostanoids is reduced.
Yu et al. 1994). All these findings suggest that epithelial alteration could play an important role in the pathogenesis of equine RAO.

In this study, we hypothesized that 1) the bronchi of SPAOPD-affected horses would show a significantly greater contractile response to ET-1 when compared to unaffected horses, and this exaggerated responsiveness could be due to alteration in the pharmacological affinity of ET receptors to ET-1, and 2) the bronchi in both groups of horses would respond greater when epithelium is removed than with intact epithelium. Therefore, the objectives of this study were 1) to determine and compare the pharmacological responses of bronchi from SPAOPD-affected and unaffected horses to graded concentrations of ET-1, 2) to detect and compare the alterations in the bronchial smooth muscle ET-A and ET-B receptor affinities, by using receptor antagonists and their pA2 values, a statistical constant that indicates receptor affinity, (Schild 1947) and 3) to determine and compare the pharmacological responses of bronchi with intact epithelium to those with epithelium removed, in both groups of horses.

2.2 Materials and Methods

2.2.1 Horses – This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. All horses were procured by donation. All SPAOPD-affected horses had a history of signs of obstructive pulmonary disease after they were exposed to pasture in the summer (Seahorn and Beadle 1993). Eight clinically healthy (control or unaffected) horses, 10 to 20 years old (mean ± SD, 15.5 ± 4.9 years) and eight SPAOPD-affected horses, 10 to 20 years old (mean ± SD, 14.3 ± 3.9 years) were included.

2.2.2 Clinical Evaluation – On the day of clinical evaluation, the general demeanor and respiratory behavior of the horse were observed. Rectal temperature (°F), heart rate (beats/min), respiratory rate (breaths/min), nostril flare and abdominal lift were recorded. In addition, the
respiratory sounds were assessed by auscultation. Finally, the clinical score (CS) and change in transpleural pressures (ΔPpl) were determined.

2.2.3 Clinical Scoring (CS) – Clinical scores were determined by use of the following algorithm (Seahorn and Beadle 1997).

\[
CS = \frac{(\text{Medial nostril flare} + \text{Lateral nostril flare})}{2} + \text{Abdominal lift}
\]

Each of the variables in the above algorithm was scored from 0 to 4. A score of “0” indicates that the nostril had little movement and the ventral flank showed little or no movement. A score of “4” indicates that the nostril remained maximally flared throughout the respiratory cycle and the abdominal lift resulted in a “heave line” that extended cranially to the 5th intercostal space. Thus the maximum clinical score is “8”.

2.2.4 Change in Transpleural Pressure (ΔPpl) – The change in transpleural pressure (ΔPpl) was measured indirectly by using an esophageal balloon\(^a\) secured over the end of a catheter connected to a pressure transducer interfaced with a polygraph\(^b\). A 10-cm long, 3.5 cm circumference balloon was placed over the end of a 2-m long, 2-mm diameter canula. The balloon was inserted through a lubricated nasogastric tube that was passed into the rostral 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 one minute. Changes in the esophageal pressure (peak inspiratory minus peak expiratory pressures) during tidal breathing reflect the change in transpleural pressure.

2.2.5 Grouping – For inclusion in the study, all horses must have had normal reference values of rectal temperature (~38.6°C), heart rate (~30 to 40 per minute), respiratory rate (~10 to 20 per minute) and thoracic auscultation. In addition, none of the horses included in this study had received any medication at least 7 days prior to clinical evaluation. To be included in the
SPAOPD-affected group, in addition to the history of SPAOPD, the horses must have had a CS ≥ 5.0 and a $\Delta$Ppl ≥ 15.0 cm of H2O. Similarly, to be included in the clinically healthy group, the horses must have had a CS ≤ 4.0 and $\Delta$Ppl ≤ 10.0 cm of H2O.

2.2.6 In vitro Concentration-Response Studies – The next day after evaluation and grouping, horses were humanely euthanatized using an intravenous overdose (90 mg/kg) of sodium pentobarbital. Gross post-mortem evaluation of the lungs was conducted before removing them from the thoracic cavity. Ten bronchial rings, each measuring approximately 3-4 mm in diameter, were prepared from right diaphragmatic lung lobe in the region of fourth to seventh generation bronchi, a region that has high airflow resistance under normal conditions (Venugopal et al. 2001). This region is also preferred for its ease to identify and maintain the consistency of location of airways in different animals (Venugopal et al. 2001). Immediately after preparing the bronchial rings, each ring was mounted separately in an organ bath containing 95% oxygenated Tyrode’s solution at 37°C. Each ring was fixed on one side to the floor of the bath and the other side was attached by a silk thread to a force transducer interfaced with the polygraph. An initial tension of 2 gm was applied to each ring to mimic the airway tone observed under in vivo conditions (Venugopal et al. 2001). Out of the ten bronchial rings, four were used for the ET-A receptor antagonist (BQ-123) study, another four were used for the ET-B receptor antagonist (IRL-1038) study and the remaining two rings were used as controls i.e. one ring with intact epithelium and the other ring with denuded epithelium, both receiving no antagonist. One of the 4 rings in each receptor antagonist study was manually denuded of epithelium and incubated with $10^{-5}$ M concentration of the antagonist; the other three rings were incubated with one of three concentrations of the antagonists ($10^{-9}$ M, $10^{-7}$ M and $10^{-5}$ M) after the first wash. The bronchial epithelium was mechanically removed by very gentle abrasion of its lumen with small
forceps. Effectiveness of epithelium removal was evaluated by subjecting extra rings (i.e. these rings were not used in the experiment) to the same procedure and then hematoxylin and eosin staining. The rings were allowed to equilibrate for 45 minutes and the bath fluid was gently replaced with fresh, warm Tyrode’s solution at 15-minute intervals. After each solution change, the tension was reapplied to maintain 2 gm except following the last solution change. Antagonists were also added after each wash including the final wash.

After completion of the equilibration period, cumulative concentration-response (CR) relationships were determined by adding graded concentrations of ET-1 (ranging from $10^{-8.5}$ to $10^{-6}$ M) to each bath, at 3-minute-intervals (Van Rossum 1963, Venugopal et al. 2001). The maximum concentration of ET-1 was limited to $10^{-6}$ M due to the high-cost involved. The maximal contraction of the rings was determined by adding $10^{-3}$ M of carbachol. Thus, the response induced by the carbachol was considered as 100% and was used to calculate the percentage response induced by each concentration of ET-1. The responses of the rings on a weight basis (mg of tension/mg of dry weight) were also calculated to compare with the method used in percentage response of maximal carbachol response. Since there was no significant difference in these two methods, EC50 values (the effective concentration of ET-1 to produce 50% of the maximal contraction) were determined from the cumulative CR curves. These EC50 values were used for calculation of the pA$_2$ values.

2.2.7 Determination of the pA$_2$ Values – A slightly modified linear regression analysis method known as Schild plot was used to determine pA$_2$ values (Schild 1947). The pA$_2$ value is a statistical measure of the pharmacological affinity of a competitive antagonist for its receptor. It is defined as the negative logarithm of the molar concentration of an antagonist that will reduce the effect of twice the dose of an agonist to that of a single dose (Schild 1947, Arunlakshana and
Schild 1950). In general, the greater the pA₂ value, the greater is the affinity of the antagonist for the receptor.

The pA₂ value was determined by using the CR curves of ET-1 in the absence (control ring) and presence of the antagonist. The CR curve of ET-1 is shifted in the presence of the antagonist and this shift is reflected in the EC50 value of ET-1. After determining the EC50 values for ET-1 in each of these curves (control rings as well as those treated with antagonists), the next step was to determine concentration ratios for each antagonist. The concentration ratio is the ratio of the EC50 value of the control curve (without the antagonist) to the EC50 value of the ET-1 in the presence of the antagonists. Three concentrations of the antagonist were used in the experimental design. Therefore, three concentration ratios for each antagonist were obtained in each receptor study. Finally, pA₂ values were determined by plotting the log (concentration ratio – 1) on the y-axis against the log molar concentration of the antagonist on the x-axis. The slope was extended to intersect the point on the x-axis that corresponds to the molar concentration of the antagonist. This point is zero on the y-axis. Because the y-axis is plotted as a value of log (concentration ratio – 1), the value of zero on the y-axis represents an actual concentration ratio of two. The negative logarithm of the molar concentration at which the line intersects the x-axis is the pA₂ value (Schild 1947, Venugopal et al. 2001).

2.2.8 Statistical Analyses – Graph pad Prism ver.4.0f was used for performing all statistical analyses. The CS and ΔPpl data was analyzed and comparison between clinically healthy and SPAOPD-affected horses using the Mann-Whitney test. Cumulative CR relationships in SPAOPD-affected and unaffected horses were analyzed and compared using two-way ANOVA. Wherever significance was indicated, post-hoc comparisons were made using Bonferroni’s test. The pA₂ values for ET-A and ET-B receptor antagonists in SPAOPD-affected and unaffected
horses were analyzed for significance by comparing their 95% confidence intervals, which were determined using a “t” distribution with n-2 degrees of freedom. The statistical significance was set at a \( p \leq 0.05 \) for all tests.

2.3. Results

2.3.1 Clinical Evaluation – The mean values of heart rate, respiratory rate and rectal temperature for SPAOPD-affected and unaffected horses are given in Table 2.1. All horses were in relatively good body condition and had a normal appetite and demeanor. All SPAOPD-affected horses had respiratory wheezes on auscultation, nostril flaring, increased abdominal lift and cough. Few SPAOPD-affected horses showed a marked ‘heave’ line extending along the abdominal wall. The CS (median 5.5; range, 5.0 to 7.0) of SPAOPD-affected horses was significantly greater than that of clinically healthy horses (median 2.0; range, 1.0 to 3.5; Table 2.1). Similarly, the \( \Delta P_{pl} \) of SPAOPD-affected horses (median 25.0 cm of H\(_2\)O; range 20 to 32) was significantly greater than that of clinically healthy horses (median 7.0 cm of H\(_2\)O; range, 4.0 to 9.0).

Table 2.1 – Median and range values for signalment, clinical signs, clinical score and \( \Delta P_{pl} \) for clinically healthy and SPAOPD-affected horses. * Indicates significant \(( p \leq 0.05 \) difference between clinically healthy and SPAOPD-affected horses. \(^{a,b}\) Values with different superscript letters differ significantly \(( p \leq 0.05 \). HR = heart rate, RR= respiratory rate, CS = clinical score, \( \Delta P_{pl} \) = transpleural pressure, y = years, F = Fahrenheit, bpm = beats per minute, rpm = respirations per minute.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clinically healthy</th>
<th></th>
<th>SPAOPD-affected</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Age (y)</td>
<td>15.5</td>
<td>10-20</td>
<td>14.3</td>
<td>10-20</td>
</tr>
<tr>
<td>Temp (F)</td>
<td>100.4</td>
<td>100.0-102.0</td>
<td>100.2</td>
<td>98.6-102.0</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>39</td>
<td>34-63</td>
<td>43</td>
<td>31-68</td>
</tr>
<tr>
<td>RR (rpm)</td>
<td>22</td>
<td>17-24</td>
<td>23</td>
<td>18-37</td>
</tr>
<tr>
<td>CS*</td>
<td>2.0</td>
<td>1.0-3.5</td>
<td>5.50</td>
<td>5.0-7.0</td>
</tr>
<tr>
<td>( \Delta P_{pl} ) (cm H(_2)O)*</td>
<td>7.0 (^{a})</td>
<td>4.0-9.0</td>
<td>25 (^{b})</td>
<td>20-32</td>
</tr>
</tbody>
</table>
2.3.2 Gross Post-Mortem Evaluation of Lungs – The lungs of all SPAOPD-affected horses were over-inflated and orange-pink in color. In these horses, after the thoracic cavity was opened, the lungs did not collapse. A tough texture was observed in some regions of the lungs. Non-uniformly distributed, pale, fibrotic patches were observed on the surface of the lungs of many affected horses. In addition, the surface of the lungs of some affected horses had indentions or impressions caused by pressure from the ribs. Mucus plugs were frequently observed in the bronchi; however, this observation was not uniform throughout the lung fields.

Healthy horses had lungs which were of normal size, texture and pale pink in color. Neither fibrotic patches nor rib impressions were observed on the lung surface. The lungs collapsed immediately and completely after opening the thoracic cavity. Bronchial mucus plugs were noticed on only a few occasions.

2.3.3 Responses of Control Rings with Intact Epithelium – Graded concentrations of ET-1 caused a concentration-dependent contraction of control bronchial rings in both groups of horses. The contraction caused by ET-1 on control rings obtained from SPAOPD-affected horses was significantly greater than that of unaffected horses (Fig 2.1). The significance was evident at 10^{-7} M to 10^{-6} M concentrations of ET-1.

2.3.4 Responses of Rings of Healthy Horses in the Presence of ET-A Antagonist – The EC50 values of the concentration-dependent contractions elicited by ET-1 on bronchial rings collected from unaffected horses in the presence of ET-A receptor antagonist bq-123 at 10^{-9} M, 10^{-7} M and 10^{-5} M concentrations are illustrated in Figure 2.2. In general, the potency of a drug is indirectly proportional to the EC50 value; thus, the lower the EC50 value, the greater is the potency of the drug or greater the sensitivity of the target tissues to the drug. In these horses, blockade of ET-A receptors (i.e. ET-B receptors were open) resulted in an increase in the response to ET-1 in
2.3.5 Responses of Rings of Healthy Horses in the Presence of ET-B Antagonist – In healthy horses, when ET-B receptors were blocked (i.e. ET-A receptors were open) with ET-B receptor antagonist IRL-1038, the response to ET-1 was decreased in antagonist concentration-dependent manner, when compared to control rings. In other words, EC50 values were increased when the
Figure 2.2 – Effect of ET-A receptor antagonism (BQ-123) on ET-1 induced responses of the bronchial rings from healthy horses. * indicates a statistically significant difference between the responses of bronchial rings supplied with $10^{-5}$ M concentration of ET-A receptor antagonist and control rings.

ET-B receptors were blocked (Figure 2.3). Therefore, classical competitive antagonism took place in this case. However, statistical significance was evident only at $10^{-5}$ M concentration of the antagonist.

2.3.6 Responses of Rings of SPAOPD Horses in the Presence of ET-A Antagonist – In SPAOPD-affected horses, when ET-A receptors were blocked (i.e. ET-B receptors open), the response of the rings to ET-1 was increased similar to that of healthy horses. In other words, the EC50 values decreased when the ET-A receptors were blocked (figure 2.4). This increase was statistically significant at $10^{-7}$ M and $10^{-5}$ M concentrations of the antagonist.
**Figure 2.3** – Effect of ET-B receptor antagonism on the ET-1 induced responses of the bronchial rings from healthy horses. * indicates a statistically significant difference between the responses elicited by the bronchial rings supplied with $10^{-5}$ M concentration of ET-B receptor antagonist and rest of the rings.
Figure 2.4 – Effect of ET-A receptor antagonism on the responses induced by ET-1 on the bronchial rings from SPAOPD-affected horses. * indicates a statistically significant difference between the responses of bronchial rings supplied with $10^{-5}$ M concentration of ET-A receptor antagonist and control rings.
Figure 2.5 – Effect of ET-B receptor antagonism on the ET-1 induced responses of bronchial rings of SPAOPD-affected horses. * indicates a statistically significant difference between the responses of bronchial rings supplied with $10^{-7}$ and $10^{-5}$ M concentration of ET-B receptor antagonist, and control rings.

2.3.7 Responses of Rings of SPAOPD Horses in the Presence of ET-B Antagonist – Similar to the healthy horses, when ET-B receptors were blocked (i.e. ET-A receptors were open) in bronchial rings of SPAOPD-affected horses, the response to ET-1 decreased in antagonist concentration-dependent manner when compared to the control rings. In other words the EC50 values increased when the ET-B receptors were blocked (Figure 2.5). Therefore, classical competitive antagonism also took place in this case. Statistical significance was evident at $10^{-7}$ M and $10^{-5}$ M concentrations of the antagonist.

2.3.8 pA2 Values – In both groups of horses, the pA2 values for the ET-A antagonist could not be determined because of shifting of the curve towards the left when the ET-A receptors were
blocked. Because of this leftward shift, the dose ratio could not be calculated since the logarithm of a negative number could not be determined. However, the pA₂ values for the ET-B antagonist could be determined in both groups of horses. This is because the curve shifted toward the right when the ET-B receptors were blocked. The pA₂ values for ET-B antagonist were 3.08 ± 2.91 and 9.98 ± 3.36, for unaffected and SPAOPD-affected horses, respectively (Figures 2.6 and 2.7).

![Schild plot to determine the pA₂ value of bronchial ET-B receptors in healthy horses](image)

**Figure 2.6** – Schild plot to determine the pA₂ value of bronchial ET-B receptors in healthy horses

The ET-B receptors showed less affinity to ET-1 to induce a contractile response in unaffected horses (3.08 ± 2.91), whereas in SPAOPD-affected horses the affinity was increased significantly (9.98 ± 3.36). The pA₂ value for the ET-B receptor antagonist in SPAOPD-affected horses was significantly greater (P=0.01) than unaffected horses.
2.3.9 Effect of Epithelium Removal on ET-1 Induced Bronchoconstriction – Removal of epithelium significantly increased the ET-1 induced responses of bronchial rings in both groups of horses. However, this significance was greater in healthy horses than in SPAOPD-affected horses.

![Figure 2.7](image)

**Figure 2.7** – Schild plot to determine the pA₂ value of bronchial ET-B receptors in SPAOPD-affected horses.

2.4. Discussion

This study revealed important findings regarding the functional role of ET-1 and its receptors in equine airways in health and disease. Firstly, ET-1 causes a potent, concentration-dependent contraction of bronchial rings of SPAOPD-affected and unaffected horses by acting through both ET-A and ET-B receptors. Secondly, the bronchial rings from SPAOPD-affected horses show significantly greater contraction to ET-1 when compared to unaffected horses.
Thirdly, blockade of ET-A receptors caused an increase in the ET-1 induced responses of the bronchial rings in both groups of horses, suggesting a possibility of the presence of an interaction between these receptors.

![Graph showing effect of epithelium removal on ET-1 induced responses of bronchial rings.](image)

**Figure 2.8** – Effect of epithelium removal on the ET-1 induced responses of the bronchial rings of healthy and SPAOPD-affected horses. * indicates statistically significant difference between the responses elicited by epithelium removed control bronchial rings and epithelium intact control bronchial rings in healthy horses. † indicates statistically significant difference between the responses elicited by epithelium removed control bronchial rings and epithelium intact control bronchial rings in SPAOPD-affected horses.

Fourthly, the affinity of ET-B receptors for ET-1 increases significantly in the SPAOPD-affected horses when compared to unaffected horses. Finally, removal of epithelium causes a significant increase in the responses elicited by ET-1 in both groups of horses; however, this increase is significantly greater in healthy horses when compared to SPAOPD-affected horses, suggesting that either the epithelium by itself or its properties are altered in SPAOPD-affected horses.
Benamou and co-workers have demonstrated that ET-1 induces in vivo bronchoconstriction in equine airways by acting through both ET-A and ET-B receptors. One of the findings of the current study supplements those reports. In addition, we demonstrated that control rings from the SPAOPD-affected horses contract with significantly greater magnitude than those of healthy horses. This suggests that ET-1 could be an important factor in the generation of airway hyperresponsiveness associated with this disease. Such a role of ET-1 has already been demonstrated in the hyperresponsiveness associated with human asthma, human COPD and experimental allergic inflammation. This study is the first to demonstrate such a role for ET-1 in equine SPAOPD.

In this study, we wanted to determine and compare the pA2 values of both ET-A and ET-B receptors in both groups of horses to evaluate the pharmacological affinities associated with these receptors. However, we were unable to calculate the pA2 values for ET-A receptors in both groups of horses. The reason is that, in order to estimate the pA2 values, we need to calculate the logarithm of (dose ratio-1). The dose ratio is the ratio of the EC50 of the bronchial ring to which an antagonist is applied to the EC50 of the control ring. The (dose ratio-1) must always be a positive value to determine the logarithm of this parameter. That means the EC50 of the control rings must always be lower than that of the rings to which antagonists are added. When competitive antagonism takes place, the EC50 of the control ring is always less compared with the rings to which antagonist is applied. In other words, the concentration-response curve associated with the agonist will shift toward the right when the antagonist is applied. This is because we need to add more agonist to displace the antagonist from the receptor on these tissues for them to reach the same maximal contraction. However, in the current study, when we blocked the ET-A receptors, ET-B receptors were activated and appeared to take over the
function of contraction. Furthermore, this ET-B mediated contraction (i.e. ET-A receptors are blocked) appeared to be more powerful than that of the control rings whereby the ET-A receptors were not blocked. In other words, the concentration-response curves shifted to the left when the ET-A blocker was added to the tissues. For this reason, the (dose ratio-1) value happened to be a negative number for these rings. Because we cannot calculate the logarithm of a negative value, the pA₂ values for ET-A receptors could not be determined in either group of horses. One of the possible reasons for this observation could be because of the presence of an interaction between ET-A and ET-B receptors. Mickley and co-workers have demonstrated the presence of such interaction in rat isolated mesenteric arteries (Mickley et al. 1997). Similar observations have been obtained in rabbit pulmonary artery and human bronchi (Fukuroda et al. 1994, Fukuroda et al. 1996). Even though the exact mechanism of this receptor crosstalk has not been demonstrated, interaction at second messenger level has been suggested as a possible cause. Existence of allosteric interaction between these two receptors has also been demonstrated in rat heart (Sokolovsky 1993).

Nevertheless, the curves shifted toward the right in both groups of horses when ET-B receptors were blocked. Hence we were able to determine the pA₂ values for ET-B antagonists. These values were 3.08 ± 2.91 and 9.98 ± 3.36 in normal and SPAOPD-affected horses, respectively. In addition, the pA₂ value of the ET-B antagonist in SPAOPD-affected horses was significantly greater than that of healthy horses. Therefore, the pharmacological affinity of bronchial smooth muscle ET-B receptors is significantly increased in SPAOPD-affected horses. As described previously in this discussion, when the ET-A receptors were blocked, the curve shifted toward the left in both groups of horses. However, this shift was greater in SPAOPD-affected horses compared with that of healthy horses. In healthy horses, the significance was
evident only at $10^{-5}$M concentration of the antagonist. But in SPAOPD-affected horses, the significance was observed at $10^{-7}$M and $10^{-5}$M concentration of the antagonist. One of the possible reasons for the greater shift of the curve to the left in SPAOPD-affected horses could be due to an increased affinity of the ET-B receptors in these horses. As the affinity of ET-B receptors increases, a little blockade of ET-A receptors may be sufficient for the ET-B receptors to assume the function of bronchoconstriction. Similarly, the rightward shift of the curve when the ET-B receptors were blocked was significantly greater in SPAOPD-affected horses than that of healthy horses. The significance was evident only at $10^{-5}$M concentration of the antagonist in healthy horses. However, the significance was evident at $10^{-7}$M and $10^{-5}$M concentrations of the antagonist in SPAOPD-affected horses. That means, while a $10^{-7}$ M concentration of the antagonist was able to block all of the ET-B receptors in healthy horses it was unable to block the ET-B receptors completely in SPAOPD-affected horses because of the up-regulation of ET-B receptors in these horses. All of these observations suggest that ET-B receptors play an important role in the ET-1 induced hyperresponsiveness associated with equine RAO.

Another important finding of the current study was that the epithelium plays an important role in the regulation of bronchial tone of both healthy and SPAOPD-affected horses. Epithelium produces many smooth muscle relaxing agents such as NO and prostanoids. These agents antagonize the smooth muscle constriction elicited by agents such as ET-1. When the epithelium is removed, these agents will not be released and the contraction is exaggerated. This was observed in both groups of horses. However, this increase in the contraction of epithelium-denuded bronchial rings over the epithelium intact rings was much greater in healthy horses compared with SPAOPD-affected horses. This might either be because of the damage to the epithelium that occurs in the acute exacerbation of this disease or decreased production of the
smooth muscle relaxants by the epithelium in diseased horses. Future studies are warranted to
determine the exact cause of this observation.

2.5 Summary

In summary, the present study demonstrated that the SPAOPD-affected horses show
hyperresponsiveness to ET-1. We also showed that this ET-1 induced hyperreactivity in
SPAOPD-affected horses is associated with an increase in the affinity of bronchial smooth
muscle ET-B receptors. This study also suggests that an interaction or receptor cross-talk could
exist between ET-A and ET-B receptors in equine bronchi. Further studies are needed to
establish the exact nature of this receptor interaction. Furthermore, we reported the importance of
epithelium in the regulation of ET-1 induced bronchial tone in horses. We believe that the
findings of the current study could form a basis for the future clinical studies to develop a
therapeutic regimen using ET-B receptor antagonists.

2.6 Footnotes

a Esophageal balloon, AE Medical, Farmingdale, NJ.

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

c Sodium pentobarbital (Beuthanasia solution), Scherring-Plough Animal Health Corp,
Kenilworth, NJ.

d Grass model FT.03, Grass Medical Instrument Division of Astro-Med, Inc, West Warwick, RI

e ET-A receptor antagonist, BQ-123 and ET-B receptor antagonist, IRL-1038, American Peptide,
Inc, Sunnyvale, CA

f Graphpad Prism Verion 4.0, GraphPad Software, Inc., San Diego, CA.

2.7 References

1949; 14: 48-58.


CHAPTER 3. EXPRESSION OF ENDOTHELIN RECEPTORS IN THE BRONCHIAL SMOOTH MUSCLE AND EPITHELIUM OF CLINICALLY HEALTHY AND SUMMER PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE (SPAOPD)-AFFECTED HORSES: AN IMMUNOHISTOCHEMICAL STUDY
3.1 Introduction

Recurrent airway obstruction (RAO) is a common pulmonary condition affecting numerous horses worldwide (Bowles et al. 2002). There are basically two forms of RAO. One is chronic obstructive pulmonary disease (COPD) which occurs in stabled horses in winter months. The other form which is known as summer pasture-associated obstructive pulmonary disease (SPAOPD) mainly occurs in grazing horses in summer months in the Southeastern United States (Bowles et al. 2002). These are severely debilitating diseases characterized by airway inflammation, bronchoalveolar lavage fluid (BALF) neutrophilia, bronchial spasm, mucus hypersecretion, chronic cough and dyspnea. Equine SPAOPD mainly affects mature horses, which work at sub-maximal intensities most of the time. Affected horses show reluctance to work and exercise intolerance (Art et al. 1998). In inflammatory airway diseases such as SPAOPD and asthma, many inflammatory mediators such as histamine, bradykinin, and endothelin-1 are released in the lungs during the periods of disease exacerbation (Mattoli et al. 1991, Robinson et al. 1996, Benamou et al. 1998). Some of these mediators cause potent bronchial spasm which decreases airway caliber, resulting in hypoxia, hypoxemia and subsequent ventilation/perfusion mismatching (Mattoli et al. 1991, Robinson et al. 1996).

Endothelin-1 (ET-1) is a multi-functional 21 amino acid peptide, which was first isolated from porcine aortic endothelial cells (Yanagisawa et al. 1988). It has an important role in the maintenance of vascular and airway tone (Benamou et al. 1998). It elicits its effects, including smooth muscle contraction, by acting through two types of receptors, namely endothelin A (ET-A) and endothelin B (ET-B). It can also cause vasodilatation and bronchodilatation by releasing nitric oxide and prostaglandin E₂ (PGE₂) from vascular endothelium and airway epithelium, respectively. Additionally, by acting through ET-B receptors, ET-1 can potentiate cholinergic
nerve-mediated contractions of isolated mouse and human tracheae (Henry and Goldie 1995, Fernandes et al. 1996). It has also been demonstrated that ET-1 can induce the release of acetylcholine from nerve endings by acting through ET-A and ET-B receptors in isolated rat and rabbit tracheae (Yoneyama et al. 1995, Knott et al. 1996). ET-1 can also cause bronchoconstriction by releasing secondary mediators such as thromboxanes and platelet activating factor (PAF) (Filep et al. 1990, Battistini et al. 1990). ET-1 stimulates DNA synthesis and cell proliferation in various cells, including smooth muscles (Bagnato and Natali 2004). ET-1 can act as a co-mitogen by acting synergistically with other growth factors including epidermal growth factor (EGF), PAF and platelet-derived growth factor (PDGF) (Bagnato and Natali 2004). ET-1 can also elicit proinflammatory effects on airways, and in pulmonary allergic inflammation it upregulates cytokines such as interleukin (IL)-1, IL-8, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and IL-4 (Finsnes et al. 2001, Millul et al. 1991).

Most of the vital organs in the body synthesize ET-1; however, lung is the primary organ of synthesis and degradation of ET-1 (Henry PJ 1999). Important cells that synthesize 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 (Henry PJ 1999, Sirvio et al. 1990, Giaid et al. 1991, McKay et al. 1991a). In lungs, the greatest density of ET receptors is present in smooth muscles and the alveolar septae (Henry PJ 1999).

Pulmonary ET-1 levels are shown to be increased in human COPD, experimental allergic inflammation in rats, bronchiectasis, bronchiolitis obliterans, bleomycin-induced pulmonary fibrosis in rats and in cultured rat pulmonary endothelial cells subjected to oxidant stress (Roland et al. 2001, Salmon et al. 2000, Zheng et al. 2000, Schersten et al. 1996, Mutsaers et al. 1998,
Michael et al. 1997). Similarly, Benamou et al., reported that the ET-1 levels in the systemic circulation and bronchoalveolar lavage fluid (BALF) are greater in horses affected with RAO than those of normal horses (Benamou et al. 1998). These researchers also demonstrated the spasmogenic action of ET-1 in the isolated third generation pulmonary arteries and bronchi in horses. In these studies, they showed that ET-1 elicits bronchial constriction in horses by acting through both ET-A and ET-B receptors (Benamou et al. 2001, Benamou et al. 2003). This close relationship between ET-1 and lungs has prompted us to conduct preliminary in vitro pharmacological studies, which revealed that ET-1 can induce significantly exaggerated bronchoconstriction in SPAOPD horses, when compared with healthy horses. This result had prompted us to hypothesize that ET receptors are altered in the bronchi of SPAOPD-affected horses. In this study, we particularly wanted to investigate ET receptor alterations in bronchial epithelium and smooth muscles because these are not only the most affected tissues in obstructive pulmonary diseases but also are rich sources of ET receptors (Henry PJ 1999, McKay et al. 1991). Therefore, any findings regarding alterations in the levels of ET receptors in these tissues could help us to better understand the role of ET-1 and its receptors in the pathogenesis of SPAOPD. Additionally, it is reported that equine as well as human COPD does not uniformly affect the entire lungs (Benamou et al. 2003, Kaup et al. 1990, Saito and Thurlbeck 1995). In the current study, the gross pathological changes that we noticed in the post-mortem evaluation of the lungs of SPAOPD-affected horses were not uniformly distributed throughout the lungs. Therefore, we wanted to investigate if there is any interlobar difference in the receptor alteration in SPAOPD-affected horses. Hence, the purpose of the study reported here was to determine and compare the expression of ET receptors immunohistochemically in bronchial smooth muscles and epithelium among five lung lobes of clinically healthy and SPAOPD-affected horses.
3.2 Materials and Methods

3.2.1 Horses – This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. All horses were procured by donation. All SPAOPD-affected horses had a history of signs of obstructive pulmonary disease after they were exposed to pasture in the summer. Eight clinically healthy (or control) horses (2 females, 5 geldings, 1 stallion; 6 Quarter horses, 1 Paint, 1 Arabian), 10 to 20 years old (mean ± SD, 15.6 ± 3.3 years) and eight SPAOPD-affected horses (3 geldings, 1 stallion, 4 females; 3 Quarter horses, 1 Appaloosa, 1 Thoroughbred, 3 Paint), 10 to 20 years old (mean ± SD, 14.8 ± 2.4 years) were included.

3.2.2 Clinical Evaluation – On the day of clinical evaluation, we observed the general demeanor and respiratory behavior of the horse. Rectal temperature (°F), heart rate (beats/min), respiratory rate (breaths/min), nostril flare and abdominal lift were recorded. In addition, we assessed respiratory sounds by auscultation. Finally, clinical score (CS) and change in transpleural pressures (∆Ppl) were determined.

3.2.3 Clinical Scoring (CS) – Clinical scores were determined by use of the following algorithm (Seahorn et al. 1997).

\[
CS = \frac{(\text{Medial nostril flare} + \text{Lateral nostril flare})}{2} + \text{Abdominal lift}
\]

Each of the variables in the above algorithm was scored from 0 to 4. A score of “0” indicates that the nostril had little movement and the ventral flank showed little or no movement. A score of “4” indicates that the nostril remained maximally flared throughout the respiratory cycle and the abdominal lift resulted in a “heave line” that extended cranially to the 5th intercostal space. Thus the maximum clinical score is “8”.

3.2.4 Change in Transpleural Pressure (∆Ppl) – The change in transpleural pressure (∆Ppl) was measured indirectly by using an esophageal balloon secured over the end of a catheter.
connected to a pressure transducer\textsuperscript{f} interfaced with a polygraph\textsuperscript{g}. A 10-cm long, 3.5 cm circumference balloon was placed over the end of a 2-m long, 2-mm diameter canula. The balloon was inserted through a lubricated nasogastric tube that was passed into the rostral 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 one minute. Changes in the esophageal pressure (peak inspiratory minus peak expiratory pressures) during tidal breathing reflect the change in transpleural pressure.

3.2.5 Grouping – For inclusion in the study, all horses must have had normal reference values of rectal temperature (~38.6 °C), heart rate (~30 to 40 per minute), respiratory rate (~10 to 20 per minute) and thoracic auscultation. In addition, none of the horses included in this study had received any medication at least 7 days prior to clinical evaluation. To be included in the SPAOPD-affected group, in addition to the history of SPAOPD, the horses must have had a CS \geq 5.0 and a change in $\Delta$Ppl > 15.0 cm of H$_2$O.

3.2.6 Tissue Collection and Processing – The day after grouping, horses were humanely euthanatized using an intravenous overdose (90 mg/kg) of sodium pentobarbital\textsuperscript{h}. Gross post-mortem evaluation of the lungs was made before removing them from the thoracic cavity. Three samples each measuring approximately 3 x 3 cm were prepared from each of the five lobes (Right diaphragmatic, Left diaphragmatic, Right apical, Left apical and Accessory) of the lungs. It was ensured that each sample that was collected contained at least one bronchus (~ 4 - 8 mm wide). The samples were immediately immersed in zinc-formalin and were fixed for 12 hours. Fixed samples were dehydrated with graded concentrations of ethyl alcohol (70%, 90% and 100%) and subsequently cleared with xylene. Finally, the samples were embedded in paraffin. These paraffin blocks containing tissue sections were stored at room temperature until used for
immunohistochemistry studies. Rat lung samples, which were used as positive controls, were also collected in a similar manner (Wendel et al. 2004). For Western blotting, equine lung samples (containing ~ 4 to 8 mm wide bronchi) and rat lung samples (used as positive controls) were collected, snap frozen in liquid nitrogen and stored at -80°C.

3.2.7 Antibodies and Controls – The anti ET-A receptor polyclonal primary antibodies that were used in the present study were raised in sheep against the immunogen sequence (Q410-E-Q-N-H-N-T-E-R-S-S-H-K422 amino acid residues), which is part of the C-terminal region of rat ET-A receptor. Similarly, anti ET-B receptor polyclonal primary antibodies were raised in sheep against the immunogen sequence (K424-A-N-D-H-G-Y-D-N-F-R-S-S-N-N438 amino acid residues), which is a part of the C-terminal region of rat ET-B receptors. The ET-B receptors of horse and rat share 85% homology (Yang et al. 1998). Moreover, the C-terminal peptide of rat ET-B receptor that was used to generate antibodies bears 100% homology to the C-terminal region of equine ET-B receptor at the 424-438 amino acid locations. This observation is based on the sequence similarity search on Swiss Institute of Bioinformatics (SIB) Basic Local Alignment Search Tool (BLAST) network service. However, ET-A receptors are not yet sequenced in horses. Owing to the extreme conservation of ET receptor sequences in mammalian species, we assumed that the antibodies raised against rat ET-A receptors would cross react with equine ET-A receptors (Yang et al. 1998). To confirm the antibody cross-reactivity, we performed Western blot analysis. In addition, we included the necessary positive and negative controls in immunohistochemistry studies.

3.2.8 Western Blotting to Validate Primary Antibodies – Lung protein extracts were prepared by homogenizing the thawed equine and rat lung samples in Camiolo buffer [0.075M Potassium acetate, 0.3M NaCl, 0.1M L-arginine basic salt, 0.01M EDTA-HCl and 0.25% Triton X-100]
containing a protease inhibitor cocktail. Extracts were clarified by centrifugation (10,000g for 15 min at 4°C). Supernatant fractions were assayed for protein concentration, using Bradford reagent and used for Western blot analysis. Western blot analysis was performed as previously described (McMullan et al. 2001, Black et al. 1997). Briefly, protein extracts (150 µg) were separated on 10% denaturing polyacrylamide gels for ET-A and ET-B receptors. Pre-stained color protein markers were also run in the gel. All gels were electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween. After blocking, the membranes were incubated for 1 hour at room temperature with 1:500 dilution of either ET-A or ET-B antisera, washed with Tris-buffered saline containing 0.1% Tween, and then incubated with donkey anti-sheep IgG-horseradish peroxidase conjugate. After washing, protein bands were visualized by enhanced chemiluminescence according to manufacturer’s instruction using Bio-Max MR film. Protein band sizes were determined by using Quantity One Image analysis software.

3.2.9 Preparation of Slides for Immunohistochemistry – Among the three paraffin blocks from each lobe of the lung, the block with a bronchus closest to the desired diameter (4-8 mm) was selected. From the selected paraffin block, tissue sections measuring 4 µm in thicknesses were cut with a microtome and mounted on clean sialinized slides. From each paraffin block, three slides were prepared. Two of three slides were used for detecting the ET receptors (ET-A and ET-B). The other slide was used as a negative control. These tissue sections were deparaffinized with xylene and subsequently rehydrated with graded concentrations of ethyl alcohol (100%, 90% and 70%), respectively. Then all of the slides were subjected to immunohistochemistry staining (Furuya et al. 2005, De Falco et al. 2002).
3.2.10 Immunohistochemistry – The automated Dako Autostainer was used to perform the immunohistochemistry studies. Optimization of the antibody showed that the staining was best when no antigen retrieval method was used. Except when noted, all the rinsing steps were conducted with TRIS-Buffered Saline (TBS) at pH 7.6 containing 0.05% Tween-20. After rinsing the slides with TBS, the endogenous peroxidase activity of the tissues was blocked by applying 3% H₂O₂ for 10 min. Sections were then rinsed and endogenous avidin and biotin were blocked for 10 minutes each by using avidin/biotin blocking agent. After rinsing all of the slides, endogenous protein was blocked for 30 minutes at room temperature by adding normal rabbit serum. Manufacturer’s guidelines were followed to prepare the blocking serum. After blowing off the excess blocking serum, the tissue sections on the slides were incubated with either sheep anti ET-A receptor polyclonal primary antibodies or sheep anti ET-B polyclonal primary antibodies at 1:200 dilutions each, for 30 minutes at room temperature. In the negative controls, the primary antibody was replaced by sheep gamma globulin at 1:1,000 dilutions. Antibodies and sheep gamma globulins were diluted in DAKO antibody diluent. After rinsing all of the slides, biotinylated rabbit anti-sheep IgG secondary antibodies were applied to the sections for 30 minutes. After rinsing the slides, Vectastain elite ABC immunoperoxidase system was applied to the tissue sections. Then, all of the tissue sections were washed with TBS. Sites of immunostaining were visualized by developing sections in Nova red (substrate for peroxidase), which was applied to the sections for 8 minutes. After washing with TBS followed by deionized water, the slides were counterstained with Mayer’s hematoxylin for 5 minutes. Then, the slides were thoroughly washed with TBS and deionized water, respectively. The sections were dehydrated through graded concentrations of ethyl alcohol and cleared with xylene. Finally, the sections were mounted with Permount and glass cover slips and allowed to dry.
3.3 Image Analysis

Photographs of the stained slides were obtained with a Spot RT Color camera on a Nikon E400 microscope at 20X magnification. Corresponding software was used to calibrate the image set up parameters before capturing the images. Light intensity and exposure settings were kept constant for all photographs. A total of five photographs were captured per slide. Photographs were exported to Image-Pro Plus 4.1.1.2 software for analysis. Using the software, colored markers were applied on the images to differentiate the color of immunostained tissue with the other colors (Francisco et al. 2004). The red color marker was applied to the immunostained sections of the tissues, whereas blue, light blue and yellow color markers were applied to the stained colors of nuclei, non-specific tissue, and white background of the slide, respectively (Figure 3.1). These colored markers represent the intensity of staining. For example, the greater the intensity of immunostaining, the greater is the pixel value for the red color marker and hence the greater is the percentage total per area for red color marker.

A template image was created by using a positive control slide in which the aforementioned colored markers were applied. This template was used to attribute the color markers to all of the slides under analysis. Total area of the tissue of interest (either bronchial epithelium or bronchial smooth muscle) was manually selected. It was ensured that the white background did not exceed more than 10% of the total area selected. Percentage of immunostained area (total stained area/total selected area multiplied by 100) of the tissue was determined for each of the five photographs taken. This procedure was applied to all three types of slides, i.e., the slide to which the ET-A antibody was applied, the slide to which the ET-B antibody was applied and the slide which was used as a negative control. Percentage of immunostained area on the negative control slide was subtracted from the percentage of total
immunostained area of either of the other slides. The data for five images were averaged and exported to MICROSOFT EXCEL.

Figure 3.1. Colored markers were applied by using image analysis software to determine the percentage of immunostained area in a selected tissue of interest. The arrows in the figure indicate the immunostaining color.

3.4 Statistical Analyses

The CS and ΔPpl data were reported as median and range values. These variables were compared between clinically healthy and SPAOPD-affected horses, using the Mann-Whitney test. The immunohistochemical data was analyzed by using two-way ANOVA and pair-wise
comparisons were made by employing post-hoc Bonferroni tests. For epithelium and smooth muscle, the following three comparisons were made. First, in all five lung lobes, comparisons were made between clinically healthy and SPAOPD-affected horses to determine the differences in receptor expression between the two groups. Second, comparisons were made in both groups within the five lobes to determine the interlobar differences in receptor expression. Third, in both groups of horses, comparisons were made to determine the differences of expression between ET-A and ET-B receptor sub-types in all of the lung lobes. In addition, in both groups of horses, comparisons were made to determine the differences in the receptor expression between epithelium and smooth muscle of all the lung lobes. Finally, data from all of the lung lobes was pooled and all the comparisons just mentioned were made to determine the receptor expression differences in the whole lung. A P ≤ 0.05 was considered statistically significant for all the tests.

All the tests were analyzed using the SAS 9.1.2z version of the software.

Table 3.1– Signalment, clinical variables, clinical score, and ∆Ppl for SPAOPD-affected horses and healthy horses. * Clinical score assigned on a scale of 0 to 8. a,b Values with different superscript letters differ significantly (P ≤ 0.05). y = years, bpm = beats per minute, rpm = respirations per minute, CS = clinical score and ∆Ppl = transpleural pressure

<table>
<thead>
<tr>
<th>Variable</th>
<th>SPAOPD-affected horses</th>
<th>Healthy horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>14.8</td>
<td>10-20</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>37.8</td>
<td>37-38.9</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>46</td>
<td>35-66</td>
</tr>
<tr>
<td>RR (rpm)</td>
<td>26</td>
<td>18-40</td>
</tr>
<tr>
<td>CS*</td>
<td>5.5</td>
<td>5.0-6.0</td>
</tr>
<tr>
<td>∆Ppl (cm H₂O)*</td>
<td>25⁺</td>
<td>20-32</td>
</tr>
</tbody>
</table>

3.5 Results

3.5.1 Clinical Evaluation and Grouping – In all horses, the mean values of heart rate, respiratory rate and rectal temperature were close to the reported normal values (Table 3.1).
All horses were in good body condition and had a normal appetite and demeanor. All SPAOPD-affected horses had respiratory wheezes on auscultation, nostril flaring, increased abdominal lift and cough. Few SPAOPD-affected horses showed a marked ‘heave’ line extending along the abdominal wall. The SPAOPD-affected horses had a CS (median 5.50; range, 5.0 to 7.0) significantly greater than clinically healthy horses (median 1.5; range, 1.0 to 3.5). Similarly, the \( \Delta P \text{pl} \) of SPAOPD-affected horses (median 25.0 cm of H\(_2\)O; range 20 to 32) were significantly greater than clinically healthy horses (median 7.0 cm of H\(_2\)O; range, 4.0 to 9.0; Table 3.1).

3.5.2 Post Mortem Assessment of Lungs – All SPAOPD-affected horses had hyperinflated orange-pink colored lungs. The lungs did not collapse after the thoracic cavity was opened. Some regions of the lungs had a tough texture and a marked difficulty was noted during excision of samples from those regions. Lungs of many affected horses showed distinct pale fibrotic patches distributed non-uniformly on the surface. The surface of the lungs of some affected horses had indentions or impressions caused by the pressure from the ribs. Mucus plugs were observed in the airways; however, this observation was also not uniform throughout the lung fields. Lungs of healthy horses were of normal size, normal texture and were pale pink in color. None of them had noticeable fibrotic patches or rib impressions on the surface. Samples were easy to excise and the lungs collapsed immediately and completely after opening the thoracic cavity. On only a few occasions were mucus plugs noticed inside the bronchi.

3.5.3 Antibody Specificity – Western blotting analysis revealed that the band sizes of either ET-A or ET-B receptors were identical in rat and horse lung samples (Fig 3.2). In immunohistochemistry, rat lung sections showed intense immunostaining for both receptors and no staining was observed in negative controls (Figures 3.3, 3.4 and 3.5). Similarly, the horse
lung sections showed immunostaining for both receptors and no staining was detected in the negative controls (Figures 3.6 and 3.7).

Figure 3.2 – Western blotting of ET receptors was used to confirm the specificity of the primary antibodies used in the immunohistochemistry studies. Rat lung tissues were used as positive controls. Lane 1 and 3 demonstrate the bands for rat ET-A and ET-B receptors, respectively. Lane 2 and 4 illustrate the bands for equine ET-A and ET-B receptors. For all of the bands, the molecular weight was ~ 55 Kd.
Figure 3.3 – Photomicrograph of the immunostaining of rat bronchial tissue which was used as negative control (20X magnification). In this control, the primary antibody was replaced with sheep gamma globulin.
**Figure 3.4** – Photomicrograph of the immunostaining of rat bronchial tissue using anti ET-A receptor primary antibody, which was used as a positive control (20X magnification). The same tissue was used as that of the negative control. The arrows in the figure indicate the color of ET-A receptor immunostaining.
Figure 3.5 – Photomicrograph of the immunostaining of rat bronchial tissue using anti ET-B receptor primary antibody, which was used as positive control (20X magnification). The same tissue was used as that of the negative control. The arrows in the figure indicate the color of ET-B receptor immunostaining.
Figure 3.6. Photomicrograph of the immunostaining of equine bronchial tissue, which was used as a negative control (20X magnification). Tissue from an SPAOPD-affected horse is shown. The primary antibody is replaced with sheep gamma globulin.
**Figure 3.7.** Photomicrograph of the immunostaining of equine bronchial tissue to which anti ET-B antibody is applied (20X magnification). The same tissue as that of the negative control is shown here. Staining of bronchial smooth muscle and bronchial epithelium can be clearly observed. The arrows indicate the color of ET-B receptor immunostaining.
Figure 3.8 – Mean ± SEM percentages of immunostained area for ET-A 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 3.9 – Mean ± SEM for percentage of ET-B receptor immunostained area of bronchial epithelium and smooth muscle of healthy and SPAOPD-affected horses. The * indicates a statistically significant (P < 0.05) difference in the percentage of immunostained areas between epithelium and smooth muscle of SPAOPD-affected horses. The † indicates a statistically significant (P < 0.05) difference in the percentage of immunostained areas between smooth muscle of healthy and SPAOPD-affected horses.
3.5.4 Immunostaining – Changes in the Whole Lungs: Pooled data analysis revealed that in SPAOPD-affected horses, the percentage of immunostained area of bronchial smooth muscle ET-A receptors was significantly greater than that of epithelium; however, this percentage was not significantly greater than the smooth muscle ET-A receptors of healthy horses (Figure 3.8). In addition, there was no statistical difference in the percentage of immunostaining of ET-A receptors between epithelium and smooth muscles of healthy horses. Similarly, there was no statistically significant difference in the percentage of immunostaining of epithelial ET-A receptors between healthy and SPAOPD-affected horses. Regarding ET-B receptors, pooled data analysis revealed that in SPAOPD-affected horses bronchial smooth muscles showed
significantly greater percentage of immunostaining than that of epithelium (Figure 3.9). However, there was no difference in the immunostaining between epithelium and smooth muscles of healthy horses. Bronchial smooth muscle ET-B receptors of SPAOPD-affected horses showed a significantly greater percentage of immunostaining than that of healthy horses. However, there was no difference in the immunostaining of epithelial ET-B receptors between healthy and SPAOPD-affected horses.

On the whole, there was no difference in the percentage of immunostaining between ET-A and ET-B receptors of either epithelium or smooth muscles of normal and SPAOPD-affected horses.

**Interlobar Differences:** Specimens from the five lung lobes of SPAOPD-affected horses showed a greater percentage of ET-A receptor immunostained area in the bronchial smooth muscle when compared to that of normal horses (Figure 3.10). In SPAOPD-affected horses, the percentage of immunostained area was ~45% in the bronchial smooth muscle of four out of five lobes, whereas the percentage was < 30% in the smooth muscles of all five lobes in the control horses. However, the difference was statistically significant only in the case of the left diaphragmatic lobe. Additionally, in both groups of horses, no difference in the percentage of smooth muscle ET-A receptor immunostaining was observed among the lobes. Similarly, no difference was observed in the percentage of immunostained area of bronchial epithelial ET-A receptors between control and SPAOPD-affected horses or among the five lobes of both groups of animals. In control horses, no difference was observed in the percentage of immunostained area of ET-A receptors between epithelium and smooth muscles. However, in SPAOPD-affected horses, all lung lobes showed a tendency for greater percentage of immunostained area of bronchial ET-A receptors in smooth muscle compared with the
epithelium. This difference was statistically significant in all lung lobes except the right
diaphragmatic and accessory lobes.

Regarding ET-B receptors, specimens from the five lung lobes showed a greater
percentage of immunostained area in the bronchial smooth muscles of SPAOPD-affected horses
when compared with those of normal horses (Figure 3.11).

![Figure 3.11](image)

**Figure 3.11** – Mean ± SEM for percentage of ET-A receptor immunostained area in different
lung lobe sections: RD= right diaphragmatic, LD= left diaphragmatic, RA= right apical, LA= left
apical and AC= accessory. The * indicates a statistically significant (P < 0.01) difference in the
percentage of immunostained areas (in RD, LD, RA and LA) between epithelium and smooth
muscle of SPAOPD-affected horses. The † indicates a statistically significant (P < 0.05)
difference in the percentage of immunostained areas (in LD, RA, LA and AC) between smooth
muscle of healthy and SPAOPD-affected horses.

In SPAOPD-affected horses, bronchial smooth muscles of all the lobes showed a
percentage of immunostained area in the range of 35 to 50%, whereas, the percentage of
immunostained area was < 25% in all of the lobes in control horses. The difference was
statistically significant in all of the lobes except in the right diaphragmatic lobe (P = 0.08). No lung lobe showed a tendency of greater expression of smooth muscle ET-B receptors than the others in either group of animals. No difference was observed in the percentage of immunostained area of bronchial epithelial ET-B receptors between control and SPAOPD-affected horses and among the five lung lobes of both groups of animals. In control horses, no difference was observed in the percentage of immunostained areas of ET-B receptors between epithelium and smooth muscle. As in the case of ET-A receptors in SPAOPD-affected horses, all of the lobes showed a tendency for a greater percentage of immunostained area of bronchial ET-B receptors in smooth muscle compared with epithelium. This difference was statistically significant in all lobes except the accessory lobe.

Finally, there was no difference in the percentage of immunostaining between ET-A and ET-B receptors of either epithelium or smooth muscle in all of the lung lobes of normal and SPAOPD-affected horses.

3.6 Discussion

This study revealed several important findings regarding the potential role played by ET-1 and its receptors in the pathogenesis of equine SPAOPD. First, both ET-A and ET-B receptors are present in bronchial epithelium as well as bronchial smooth muscle of both healthy and SPAOPD-affected horses. Second, both ET-A and ET-B receptors are equally distributed in bronchial epithelium of either control or SPAOPD-affected horses. Similarly, both of these receptors are equally distributed in the bronchial smooth muscle of either control or SPAOPD-affected horses. Third, in the bronchial epithelium, distribution of the ET-A or ET-B receptors in SPAOPD-affected horses is not different from control horses. In other words, there is no alteration in receptor density in the epithelium of diseased horses. Finally, in the bronchial
smooth muscle, there is an up-regulation of ET-A receptors in the left diaphragmatic lung lobe and of ET-B receptors in all of the lung lobes except the right diaphragmatic in SPAOPD-affected horses when compared to control horses.

An important feature of airway hyperreactive diseases is obstruction of airways, particularly at the level of the bronchi (Parker 2004). The hyper-inflation of the lungs that we noticed during post-mortem analysis of SPAOPD-affected horses was because of air entrapping in post-obstructive areas of the lung. Hence, it is not a true emphysematous condition as is often observed in human COPD. This hyper-inflation 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 can be caused mainly by three factors: constriction of airway smooth muscle, thickening of airway walls due to smooth muscle hyperplasia and/or hypertrophy, and accumulation of mucus or other secretions in the airway lumen. Among these three causes, constriction of airway smooth muscle, especially those of the bronchi (i.e. bronchoconstriction), is the most important (O'Byrne PM and Inman MD 2003). Bronchoconstriction in obstructive pulmonary diseases can be caused by several inflammatory mediators released into the airways. Some of these mediators include histamine, PAF, prostaglandins, bradykinin and ET-1 (Robinson et al. 1996). ET-1 induced bronchoconstriction is well established in human obstructive pulmonary diseases (Mattoli et al. 1991). Additionally, Benamou et al. demonstrated that ET-1 is a potent constrictor of bronchial as well as vascular smooth muscles of equine lungs. Besides being one of the important agents causing bronchoconstriction, ET-1 is also an important factor causing airway remodeling and mucus hypersecretion in obstructive pulmonary diseases (Goldie and Fernandes 2000). Bronchoconstriction elicited by ET-1 is extremely potent in most mammalian species, both
*in vivo* and *in vitro*. ET-1 can induce bronchoconstriction by three mechanisms: first by acting directly on the smooth muscle ET-A and/or ET-B receptors, second by augmenting the cholinergic mediated smooth muscle constriction, and finally by releasing secondary mediators such as thromboxanes (Filep et al. 1990). This ET-1 induced bronchoconstriction is significantly exaggerated in most of the obstructive pulmonary diseases such as COPD, when compared with healthy subjects (Goldie and Fernandes 2000). Even though increased ET-1 levels could serve as the main explanation for this exaggerated ET-1 induced bronchoconstriction, another possible reason could be the up-regulation of ET-A and/or ET-B receptors in the bronchial smooth muscles. Benamou et al. have already established that pulmonary and BAL fluid ET-1 levels are elevated in RAO-affected horses (Benamou et al. 1998). In the present study, we demonstrated that in four out of five lung lobes there was greater expression of ET-B receptors in the bronchial smooth muscle of SPAOPD-affected horses when compared with normal horses. As far as ET-A receptors are concerned, all of the lung lobes showed a tendency of greater expression in the smooth muscle of SPAOPD-affected horses. However, the difference was statistically significant only in the case of left diaphragmatic lobe. We believe that the non-significance of the over expression of ET-A receptors in all the other lobes of SPAOPD affected horses was because of the small sample size (n=8 in each group of horses) in this study and also the inherent variability in the intensities of this naturally acquiring disease.

Airway remodeling constitutes an important pathological change in many airway hyperreactive diseases including equine COPD (Kaup et al. 1990). It is demonstrated that ET-1 can cause potent airway smooth muscle hyperplasia in the isolated rabbit and human airway smooth muscle cells (Glassberg et al. 1994, Novereal et al. 1992). ET-1 has also been implicated in allergen induced airway smooth muscle proliferation in the rat (Martin and Ramos-Barbon ...
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, EGF and PDGF (Salmon et al. 2000, Carratu et al. 1997). Our findings of ET receptor alteration in diseased horses could form a basis for future studies investigating the role of ET-1 in causing smooth muscle hyperplasia, which has been reported in equine COPD (Robinson et al. 1996).

In healthy subjects, bronchial tone is constantly maintained by a balance between smooth muscle constrictor and relaxant agents (Matera et al. 2002). Certain agents such as ET-1 possess both smooth muscle contraction and relaxation properties. ET-1 elicits smooth muscle relaxing effects by acting through both ET-A and ET-B receptors present on the epithelial lining of the airways. By acting through these receptors, ET-1 causes the release of bronchodilator agents like nitric oxide and PGE$_2$ (Emanuelli et al. 1998, Hay et al. 1993b). In obstructive pulmonary diseases such as SPAOPD, the balance between bronchodilation and bronchoconstriction is disturbed either due to alterations in the concentrations of airway tone modulators or alterations in their receptors. For example, the levels of bronchomodulators such as histamine, adenosine and their receptors are altered in the lungs of patients affected with asthma (O’Byrne and Inman 2003). Similarly, pulmonary and peripheral ET-1 levels are shown to be up-regulated in equine COPD (Benamou et al. 1999). In the present study, we showed that in healthy horses, there is no difference in either the ET-A or ET-B receptor population between epithelium and smooth muscles. In other words, the quantity and distribution of ET-A and ET-B receptors were similar between epithelium and smooth muscle. However, in SPAOPD-affected horses, the relative levels of these receptors are shifted toward smooth muscles. In other words, smooth muscles tended to show greater expression of receptors compared with the epithelium. This could be an important cause in the disturbance of the physiological equilibrium between bronchodilating and
bronchoconstricting actions of ET-1 leading to an enhanced bronchoconstriction in diseased horses.

The relative proportion of ET receptors in the airway smooth muscle is species-specific. Human bronchial smooth muscle from non-diseased lungs contains a greater proportion of ET-B receptors compared with ET-A, whereas tracheal smooth muscle from rats and mice contain approximately equal proportions of the two receptors subtypes (Henry 1999). In the bronchial smooth muscle of pigs, the ratio of ET-A to ET-B receptors is 70:30 (Henry 1999). Such species-specific differences in the relative proportions of these two receptor populations within airway smooth muscles could be correlated to the species specific differences in the intensity of complications caused by ET-1 in obstructive pulmonary diseases. Such differences in relative proportions of ET receptors can also be correlated with the type of predominant physiological role exerted by ET-1 in healthy subjects. In the present study, we observed that in both groups of horses, the relative proportion of ET-A and ET-B receptors is equivalent in smooth muscle and epithelium.

In conclusion, for the first time we have demonstrated that ET-B receptor expression is enhanced in the bronchial smooth muscle of SPAOPD-affected horses when compared to those of control horses. In this study, we also showed that ET-A receptors tend to be over-expressed in the bronchial smooth muscle of SPAOPD-affected horses. Both of these receptors, known to have potential roles in the pathogenesis of many obstructive pulmonary diseases such as asthma, were shown that they also could play an important role in the pathogenesis of SPAOPD. Further studies are warranted regarding the exact role of each receptor in the pathogenesis of this disease. Clinical trials using ET receptor antagonists could reveal certain findings which might have

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clinical relevance in developing a therapeutic strategy to treat this progressive career-ending disease.

3.7 Footnotes


b Polikepahad S, Moore RM, Venugopal C et al. Effects of epithelial denudation on endothelin-1 induced bronchoconstriction in clinically healthy and summer pasture associated obstructive pulmonary disease (SPAOPD) affected horses. Abstract # 172.1; FASEB Journal 2004 18 (4); A234.


d Esophageal balloon, AE Medical, Farmingdale, NJ.

e Catheter, PE 350 tubing, VWR Scientific Products, Willard, OH.


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

h Sodium pentobarbital (Beuthanasia solution), Scherring-Plough Animal Health Corp, Kenilworth, NJ.

i Sheep Antibody to Endothelin A Receptor, BIODESIGN International, Saco, Maine.

j Sheep Antibody to Endothelin B Receptor, BIODESIGN International, Saco, Maine.

k Protease Inhibitor Cocktail Tablets, Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN.

l Quick Start Bradford Protein Assay Kit 1, Bio-Rad Laboratories, Hercules, CA

m Donkey anti-sheep IgG-HRP, Santa-Cruz Biotechnology, Santa Cruz, CA.

n Western lightening chemiluminescence reagent, PerkinElmer Life Sciences, Boston, MA.

o BioMax MR film, Eastman Kodak Company, Rochester, NY.
3.8 References


O'Byrne PM, Inman MD. Airway hyperresponsiveness. *Chest*. 2003; 123(3 Suppl):411S-6S.


CHAPTER 4. CHARACTERIZATION OF ENDOTHELIN RECEPTORS IN THE PERIPHERAL LUNGS OF CLINICALLY HEALTHY AND SUMMER PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE (SPAOPD)-AFFECTED HORSES
4.1 Introduction

Recurrent airway obstruction (RAO) is a common pulmonary condition observed in horses worldwide, and is essentially a delayed type of hypersensitivity reaction characterized by neutrophilic infiltration into the airway lumen. Characteristic symptoms of this disease include chronic cough, exercise intolerance, labored expiratory effort and nasal discharges (Robinson et al. 1996). The inflammatory response in this disease is characterized by airway inflammation, bronchoalveolar lavage fluid (BALF) neutrophilia, and increased levels of inflammatory mediators such as histamine, bradykinin, leukotrienes, platelet activating factor (PAF), endothelin-1 and 15-HETE (Robinson et al. 1996). This disease has two forms. The most widely reported form is chronic obstructive pulmonary disease (COPD) which is common in more temperate regions of the world. The other form, which is known as summer pasture-associated obstructive pulmonary disease (SPAOPD), is a seasonal disease afflicting adult horses in the late summer and early fall. This form is common in the Southeastern United States where a warm and humid climate enhances the growth of certain types of molds in the pasture, which are believed to act as potential aeroallergens in susceptible horses (Seahorn et al. 1994).

Endothelin-1 (ET-1) is a potent smooth muscle constrictor and an inflammatory mediator. It elicits its actions by acting through two receptors, namely endothelin-A (ET-A) and endothelin-B (ET-B), both of which are G-protein-coupled heptameric transmembrane receptors. Physiologically, ET-1 plays an important role in the maintenance of vascular and airway tone (Rubanyi and Polokoff 1994). By directly acting through its receptors, ET-1 can cause potent contraction of vascular and non-vascular smooth muscles in vivo and in vitro (Rubanyi and Polokoff 1994). It can also cause vasodilatation and bronchodilatation by releasing nitric oxide and prostaglandin E2 (PGE2) from vascular endothelium and airway epithelium, respectively (de

Additionally, ET-1 can potentiate cholinergic nerve-mediated contractions of isolated mouse and human tracheae by acting through ET-B receptors (Henry and Goldie 1995, Fernandes et al. 1996). The ET-1 can also induce the release of acetylcholine from nerve endings by acting through both ET-A and ET-B receptors in isolated rat and rabbit tracheae (Yoneyama et al. 1995 and Knott et al. 1996). The ET-1 stimulates DNA synthesis and cell proliferation in various cells by synergistically acting as a co-mitogen with other growth factors including epidermal growth factor (EGF), platelet activating factor (PAF) and platelet-derived growth factor (PDGF) (Bagnato and Natali 2004). The ET-1 can elicit proinflammatory effects on airways. It is demonstrated that in experimental pulmonary allergic inflammation, ET-1 can up-regulate cytokines such as interleukin (IL)-1, IL-8, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and IL-4 (Finsnes et al. 2001).

It has been demonstrated that ET-1 plays an important role in the pathogenesis of allergic airway diseases of humans and other species. For example, pulmonary ET-1 levels have been shown to be increased in human asthma, human COPD and experimental allergic inflammation in rats (Finsnes et al. 2001, Roland et al. 2001 and, Hay and Goldie 1998). Similarly, Benamou et al. reported that the ET-1 levels in the systemic circulation and BALF are greater in horses affected with RAO than those of normal horses (Benamou et al. 1998). These researchers also demonstrated the spasmogenic action of ET-1 in the isolated third generation pulmonary arteries and bronchi in horses. In these studies, they showed that ET-1 elicits in vitro bronchial constriction in horses by acting through both ET-A and ET-B receptors (Benamou et al. 2003). In addition, our preliminary pharmacological studies have revealed that when equimolar concentrations of ET-1 are applied to the bronchial tissues of healthy and SPAOPD-horses, the
ET-1 induced concentration-dependent contraction was significantly greater in SPAOPD-horses than that of healthy horses, suggesting that receptor alterations occur in airway hyperreactivity. In the current study, we hypothesized that the expression of ET receptors would be altered in the lungs of SPAOPD-affected horses. The objective of the current study was to determine and compare the expression of ET receptors in the lungs of clinically healthy and SPAOPD-affected horses by employing reverse transcriptase-polymerase chain reaction (RT-PCR), real time PCR, Western blotting and immunohistochemical techniques.

4.2 Materials and Methods

4.2.1 Horses – This study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. All horses were procured by donation. All SPAOPD-affected horses had a history of signs of obstructive pulmonary disease after they were exposed to pasture in the summer (Seahorn and Beadle 1993). Seven clinically healthy (or control) horses, 10 to 20 years old (mean ± SD, 15.8 ± 4.1 years) and seven SPAOPD-affected horses, 10 to 20 years old (mean ± SD, 16.5 ± 3.2 years) were included.

4.2.2 Clinical Evaluation – On the day of clinical evaluation, we observed the general demeanor and respiratory behavior of the horse. Rectal temperature (38.6°C) heart rate (~30 to 40 beats/min), respiratory rate (~10 to 20 breaths/min), nostril flare and abdominal lift were recorded. In addition, we assessed respiratory sounds by auscultation. Finally, the clinical score (CS) and transpleural pressures (∆Ppl) were determined.

4.2.3 Clinical Scoring (CS) – Clinical scores were determined by use of the following algorithm (Seahorn et al. 1997).

\[
CS = \frac{(\text{Medial nostril flare} + \text{Lateral nostril flare})}{2} + \text{Abdominal lift}
\]
Each of the variables in the above algorithm was scored from 0 to 4. A score of “0” indicates that the nostril had little movement and the ventral flank showed little or no movement. A score of “4” indicates that the nostril remained maximally flared throughout the respiratory cycle and the abdominal lift resulted in a “heave line” that extended cranially to the 5th intercostal space. Thus the maximum clinical score is “8”.

4.2.4 Transpleural Pressure (ΔPpl) – The transpleural pressure (ΔPpl) was measured indirectly by using an esophageal balloon\(^b\) secured over the end of a catheter connected to a pressure transducer interfaced with a polygraph\(^c\). A 10-cm long, 3.5 cm circumference balloon was placed over the end of a 2-m long, 2-mm diameter canula. The balloon was inserted through a lubricated nasogastric tube that was passed into the rostral 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 one minute. Changes in the esophageal pressure (peak inspiratory minus peak expiratory pressures) during tidal breathing reflect the transpleural pressure.

4.2.5 Grouping – For inclusion in the study, all horses must have had normal reference values of rectal temperature (38.6°C), heart rate (~ 30 to 40 beats/min), respiratory rate (~ 10 to 20 breaths/min) and thoracic auscultation. In addition, none of the horses included in this study had received any medication for at least 7 days prior to clinical evaluation. To be included in the SPAOPD-affected group, in addition to the history of SPAOPD, the horses must have had a CS > 5.0 and a ΔPpl > 15.0 cm of H\(_2\)O. To be included in the healthy group, the horses must have had a CS < 4.0 and a ΔPpl < 10 cm of H\(_2\)O.

4.2.6 Tissue Collection and Processing – The day after grouping, horses were humanely euthanatized using an intravenous overdose (90 mg/kg) of sodium pentobarbital\(^d\). Gross post-
mortem evaluation of the lungs was conducted while removing them from the thoracic cavity. Samples were randomly collected from the same region of each lung lobe in each horse. For the immunohistochemistry, five samples, one from each lung lobe, measuring approximately 3 x 3 cm were collected, placed in zinc-formalin and fixed for 12 hours. Fixed samples were dehydrated with graded concentrations of ethyl alcohol (70%, 90% and 100%) and subsequently cleared with xylene. Finally, the samples were embedded in paraffin. These paraffin blocks containing tissue sections were stored at room temperature until used for immunohistochemistry studies. For the Western blotting, real time PCR and RT-PCR studies, five samples, collected from the same region of each lung lobe as the samples for immunohistochemistry, were collected from each horse, snap frozen in liquid nitrogen and stored at -80°C. Rat lung samples, which were used as positive controls, were also collected in a similar manner for all three studies.

4.2.7 Antibodies, Primers and Controls – The anti ET-A receptor polyclonal primary antibodies that were used in the present study were raised in sheep against the immunogen sequence (Q^{410}E-Q-N-H-N-T-E-R-S-S-H-K^{422} amino acid residues), which is part of the C-terminal region of rat ET-A receptor. Similarly, anti ET-B receptor polyclonal primary antibodies were raised in sheep against the immunogen sequence (K^{424}A-N-D-H-G-Y-D-N-F-R-S-S-N-N^{438} amino acid residues), which is a part of C-terminal region of rat ET-B receptor. For PCR, the following primers were used: ET-A (sense – 5’ CTTGGAGACCTTATCTACGTGGTC 3’ and antisense – 3’TACACCTGTCCATGTCTCGTCA 5’), ET-B (sense – 5’ AGTCGAGATGTGTAAGCTGGTGC 3’ and antisense – 3’TCAATAGACGCTTAGACGGACGACGAAGT 5’), and β-actin (sense – 5’ AAGGACCTGTACGCCAACAC 3’ and antisense – 3’TAAAGCGGAGTAACAGGTG 5’).
The ET-B receptors of horse and rat share 85% homology (Yang et al. 1998). Moreover, the C-terminal peptide of rat ET-B receptor that was used to generate antibodies bears 100% homology to the C-terminal region of equine ET-B receptor at the 424-438 amino acid locations. Similarly, the ET-B receptor primer that we used in this study has 95% homology with the sequence of ET-B receptor of rats and humans at the corresponding location on the gene (Yang et al. 1998). These observations are based on the sequence similarity search on Swiss Institute of Bioinformatics (SIB) Basic Local Alignment Search Tool (BLAST) network service. However, ET-A receptors are not yet sequenced in horses. Owing to the extreme conservation of ET receptor sequences in mammalian species, we assumed that the antibodies and primers designed for rat ET-A receptors would cross react with equine ET-A receptors (Yang et al. 1998). To confirm the cross-reactivity of antibodies and primers, we performed preliminary Western blot and PCR analyses on rat and equine lung samples. In the immunohistochemistry studies, we also used rat lung samples as positive controls. In addition, negative controls were used in all three studies.

4.2.8 RNA Extraction – Five independent replications per horse (n=14) were used for the RT-PCR studies. By applying RNase Zap, it was made sure that all the microtubes, gloves and equipment were RNAse free. Lung samples were thawed on ice and homogenized in 0.5 mL of TRI reagent with Ultra Turrax T8. After homogenization, all samples were allowed to settle for 5 minutes. To each homogenized sample, 200 µL of chloroform was added immediately and the microtubes were vigorously vortexed for 15 seconds. The samples were allowed to settle for 15 minutes until two phases of liquid were noticed. Then all microtubes were centrifuged at 16,000X g at 4°C for 15 minutes. The supernatants (~200 to 300 µL) were collected in separate microtubes. Approximately 200 to 300 µL of 2-propanol were added to each microtube, mixed
and allowed to settle for 10 minutes at room temperature. All microtubes were then centrifuged at 16,000×g at 4°C for 10 minutes. Proponol was decanted thoroughly from all the microtubes. Subsequently, 300 to 500 µL of 70% ethanol was added to each tube and the microtubes were centrifuged at 16,000 X g at 4°C for 6 minutes. Ethanol was thoroughly decanted and the total RNA was dissolved in nuclease free DEPC-treated water. Total RNA was quantified by using Nano drop ND-1000 spectrophotometer.

4.2.9 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) – The RT-PCR was performed as described previously (McMullan et al. 2001). For reverse transcription, 1.5 µg of total RNA was used. To each sample, oligo dT (0.002µM) and dNTPs (0.5µM) were added. The mixture was incubated at 65°C for 5 minutes. The microtubes were quickly cooled on ice. RT buffer, dithiothreitol (DTT)(0.002M), RT-MLV (10 U) and RNase inhibitor (2 U) were added to the mixture according to manufacturer’s instructions. This final mixture was incubated at 37°C for 55 minutes and at 70°C for 15 minutes, successively. The PCR mixture containing double distilled water, 1.5 µL cDNA, PCR buffer, 2.5 mM MgSO4, 0.5 mM dNTP, 0.25 U Taq polymerase and, sense and antisense primers of ET-A or ET-B or β-actin was prepared according to manufacturer’s instructions. PCR reactions were performed in master cycler gradient PCR machine at 40 cycles. The following conditions were used for PCR: denaturation at 94°C for 15 seconds, annealing at 55°C for 15 seconds for ET-A and 60°C for 15 seconds for ET-B and β-actin, respectively, and extension at 72°C for 30 seconds. In each experimental run, negative controls (without reverse transcriptase) were used. To each microtube containing PCR product, 2 µL of loading buffer were added. The products and the standard (2 µL) were loaded into 1.4% PCR gel placed in an electrophoresis tank containing Tris-Acetate-EDTA(TAE) buffer. Then
electrophoresis was conducted at 80 volts for 40 minutes. Bands were detected by using Bio Rad’s Versa Doc Imaging system.

4.2.10 Real-Time Polymerase Chain Reaction (PCR) – For reverse transcription, 1.5 µg of total RNA was used. To each sample, oligo dT (0.002µM) and dNTPs (0.5µM) were added. The mixture was incubated at 65°C for 5 minutes. The microtubes were quickly cooled on ice. RT buffer, dithiothreitol (DTT) (0.002M), RT-MLV (10 U) and RNase inhibitor (2 U) were added to the mixture according to manufacturer’s instructions. This final mixture was incubated at 37°C for 55 minutes and at 70°C for 15 minutes successively. The PCR mixture containing double distilled water, 1.5 µL cDNA, PCR buffer, 2.5 mM MgSO₄, 0.5 mM dNTP, 0.25 U Taq polymerase and, sense and antisense primers of ET-A or ET-B was prepared according to manufacturer’s instructions. Real-time PCR was conducted by using Biorad’s iTaq SYBR Green Supermix with Rox. Horse Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) was used as house-keeping gene. Following nucleic acid sequences of primers of equine GAPDH were used: Forward primer: 5’-ccagaacatcatccctgctt-3’ and reverse primer: 3’-cgtatttggcagctttctcc-5’. All the real-time experiments were performed in Applied Bioscience 7900 Sequence Detection System. The comparative Cₜ or ΔΔCₜ method was used for data analysis.

4.2.11 Western Blotting – Five independent replications per each horse (n=8) were performed for Western blot studies. We conducted Western blot studies as supportive studies for both RT-PCR and immunohistochemistry studies. Hence we used small sample size i.e. four horses in each group. For the Western blotting, lung protein extracts were prepared by homogenizing the thawed equine and rat lung samples in Camiolo buffer [0.075M Potassium acetate, 0.3M NaCl, 0.1M L-arginine basic salt, 0.01M EDTA-HCl and 0.25% Triton X-100] containing a protease inhibitor cocktail. Extracts were centrifuged at 10,000 g for 15 min at 4°C. Supernatant fractions
were assayed for protein concentration by using Bradford reagent\(^1\) and were used for Western blot analysis. Western blot analysis was performed as previously described (McMullan et al. 2001 and Black et al. 1997). Briefly, protein extracts (40 µg) were separated on 10% denaturing polyacrylamide gels for ET-A and ET-B receptors. Pre-stained protein markers were also run in the gel. All gels were electrophoretically transferred to polyvinylidene fluoride membranes. Efficiency of protein transfer was evaluated by using Ponceau S staining. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween. After blocking, the membranes were incubated for one hour at room temperature with 1:500 dilution of either ET-A or ET-B antisera, washed 3 times (for 10 minutes each wash) with Tris-buffered saline containing 0.1% Tween, and then incubated with donkey anti-sheep IgG-horseradish peroxidase conjugate.\(^m\) The membranes were then washed 3 times (for 10 minutes each wash) with Tris-buffered saline containing 0.1% Tween and protein bands were visualized by enhanced chemiluminescence\(^n\) according to manufacturer’s instruction using Bio-Max MR film.\(^o\) Protein band sizes were determined by using Quantity One image analysis software.\(^l\) The Western blot membranes were stripped in the stripping buffer (62.5 mM Tris at pH of 6.7, 2% SDS and 100 mM \(\beta\) mercaptoethanol) for 30 minutes at 60\(^\circ\)C with occasional agitation. Then the membranes were washed twice in the washing buffer for 10 minutes each. These membranes were used for detecting \(\beta\) actin bands by using goat-polyclonal antibodies. Bands were detected using Bio Rad’s Versa Doc Imaging system.\(^l\)

4.2.12 Detection and Quantification of Bands – Western blot and RT-PCR product bands were analyzed and the average intensity of the bands was determined by using Bio Rad’s Quantity One Quantitation software.\(^l\) The data were collected in terms of average intensity of bands of
ET-A or ET-B receptors per average intensity of bands of β–actin, and imported to MICROSOFT EXCEL.

4.2.13 Preparation of Slides for Immunohistochemistry – Five independent replications were performed (n=14) for immunohistochemical studies. Tissue sections measuring 4 µm in thickness were cut from each paraffin block and mounted on clean sialinized slides. From each paraffin block, three slides were prepared. Two of three slides were used for detecting the ET receptors (ET-A and ET-B) and the other slide was used as a negative control (without primary antibody). These tissue sections were deparaffinized with xylene and subsequently rehydrated with graded concentrations of ethyl alcohol (100%, 90% and 70%), respectively. Then, all slides were subjected to immunohistochemistry (Furuya et al. 2005 and Naylor et al. 1992).

4.2.14 Immunohistochemistry – The automated Dako Autostainer was used to perform the immunohistochemistry studies. Optimization of the antibody showed that the staining was best when no antigen retrieval method was used. Except when noted, all rinsing steps were conducted with TRIS-Buffered Saline (TBS) at pH 7.6 containing 0.05% Tween-20. After rinsing the slides with TBS, the endogenous peroxidase activity of the tissues was blocked by applying 3% H2O2 for 10 min. Sections were then rinsed and endogenous avidin and biotin were blocked for 10 minutes each by using avidin/biotin blocking agent. After rinsing all of the slides, endogenous protein was blocked for 30 minutes at room temperature by adding normal rabbit serum. Manufacturer’s guidelines were followed to prepare the blocking serum. After blowing off the excess blocking serum, the tissue sections on the slides were incubated with either sheep anti ET-A receptor polyclonal primary antibodies or sheep anti ET-B polyclonal primary antibodies at 1:200 dilutions each, for 30 minutes at room temperature. In the negative controls, the primary antibody was replaced by sheep gamma globulin at 1:1,000 dilutions. Antibodies and sheep
gamma globulins were diluted in DAKO antibody diluent. After rinsing all of the slides, biotinylated rabbit anti-sheep IgG secondary antibodies were applied to the sections for 30 minutes. After rinsing the slides, Vectastain elite ABC immunoperoxidase system was applied to the tissue sections. Then, all of the tissue sections were washed with TBS. Sites of immunostaining were visualized by developing sections in Nova red (substrate for peroxidase), which was applied to the sections for 8 minutes. After washing with TBS followed by deionized water, the slides were counterstained with Mayer’s hematoxylin for 5 minutes. The slides were then thoroughly washed with TBS and deionized water, respectively. The sections were dehydrated through graded concentrations of ethyl alcohol and cleared with xylene. Finally, the sections were mounted with Permount and glass cover slips and allowed to dry.

4.2.15 Evaluation of Immunohistochemistry Slides – The immunohistochemistry slides were independently evaluated by two authors (Polikepahad S and Venugopal CS) for staining intensity. The staining intensities of ET-A and ET-B receptors were scored after comparing them to that of negative control, the score of which was considered zero. The results were interpreted as absence of staining (0), weak staining (1+), moderate staining (2+) and strong staining (3+).

4.2.16 Evaluation of Slides for Pathological Changes – Five slides, one from each of the 6 horses in each group were evaluated for the presence of pathological changes in the bronchioles, blood vessels and alveoli. Each slide was evaluated independently by two authors (Polikepahad S and Venugopal CS) and scores were assigned based upon the intensity of the pathological change (Naylor et al. 1992). The pathological changes evaluated included Goblet cell metaplasia, epithelial hyperplasia, mucus plugs in the bronchioles, neutrophils in the bronchiolar lumen, peribronchial eosinophils, alveolar macrophages, peribronchiolar inflammation, perivascular inflammation and alveolar inflammation. The results were interpreted as 0 (absence of the
pathological change), 1+ (weak presence), 2+ (moderate presence) and 3+ (strong presence). The results were reported as median and range values.

4.2.17 Statistical Analysis – Graph pad Prism ver.4.0	extsuperscript{a} was used for performing all statistical analyses. The CS and ∆Ppl data was analyzed and comparison between clinically healthy and SPAOPD-affected horses using the Mann-Whitney test. The immunohistochemistry staining scores and histopathology scores were evaluated and compared between clinically healthy and SPAOPD-affected horses using the Kruskal-Wallis test. Selected pair-wise comparisons were made using Dunn's multiple comparison tests. Data pertaining to band intensities for the Western blotting and RT-PCR, and real-time PCR were statistically analyzed using two-way ANOVA. Pair-wise comparisons were made by employing post-hoc Bonferroni tests. All statistical analyses were performed at the level of significance of p < 0.05.

4.3 Results

4.3.1 Clinical Evaluation – The mean values of heart rate, respiratory rate and rectal temperature for clinically healthy and SPAOPD-affected horses are given in Table 4.1.

Table 4.1– Signalment, clinical variables, clinical score, and ∆Ppl for SPAOPD-affected horses and healthy horses. * Clinical score assigned on a scale of 0 to 8. \textsuperscript{a,b} Values with different superscript letters differ significantly (P \leq 0.05). y = years, bpm = beats per minute, rpm = respirations per minute, CS = clinical score and ∆Ppl = transpleural pressure.

<table>
<thead>
<tr>
<th>Variable</th>
<th>SPAOPD-affected horses</th>
<th>Healthy horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>Median: 16.5</td>
<td>Range: 10-20</td>
</tr>
<tr>
<td>Temp (°F)</td>
<td>100.2</td>
<td>98.6-102.0</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>43</td>
<td>31-68</td>
</tr>
<tr>
<td>RR (rpm)</td>
<td>23</td>
<td>18-37</td>
</tr>
<tr>
<td>CS*</td>
<td>5.50</td>
<td>5.0-7.0</td>
</tr>
<tr>
<td>∆Ppl (cm H_2O)*</td>
<td>25\textsuperscript{a}</td>
<td>20-32</td>
</tr>
</tbody>
</table>
All horses were in relatively good body condition and had a normal appetite and demeanor. All SPAOPD-affected horses had respiratory wheezes on auscultation, nostril flaring, increased abdominal lift and cough. Few SPAOPD-affected horses showed a marked ‘heave’ line extending along the abdominal wall. The CS (median 5.5; range, 5.0 to 7.0) of SPAOPD-affected horses was significantly greater than that of clinically healthy horses (median 1.5; range, 1.0 to 3.5; Table 1). Similarly, the ΔPpl of SPAOPD-affected horses (median 25.0 cm of H2O; range 20 to 32) was significantly greater than that of clinically healthy horses (median 7.0 cm of H2O; range, 4.0 to 9.0).

4.3.2 Gross Post-Mortem Evaluation of Lungs – The lungs of all SPAOPD-affected horses were over-inflated and orange-pink in color. In these horses, after the thoracic cavity was opened, the lungs did not collapse. A tough texture was observed in some regions of the lungs. Non-uniformly distributed pale fibrotic patches were observed on the surface of the lungs of many affected horses. In addition, the surface of the lungs of some affected horses had indentions or impressions caused by the pressure from the ribs. Mucus plugs were frequently observed in the bronchi; however, this observation was not uniform throughout the lung fields.

Healthy horses had lungs which were of normal size, texture and pale pink in color. Neither fibrotic patches nor rib impressions were noticed on the lung surface. The lungs collapsed immediately and completely after opening the thoracic cavity. Bronchial mucus plugs were noticed on only a few occasions.

4.3.3 Microscopic Evaluation for Histological Changes – The microscopic findings are given in the Table 4.2.
Table 4.2 – Different pathological changes were evaluated and scored from 0 to 3, based on the degree of the change. The evaluated scores were statistically compared between clinically healthy and SPAOPD-affected horses.

<table>
<thead>
<tr>
<th>Pathological Change</th>
<th>Healthy (n=6; mean ± SEM)</th>
<th>SPAOPD (n=6; mean ± SEM)</th>
<th>Significant difference (P ≤ 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet cell metaplasia</td>
<td>0.00 ± 0.00</td>
<td>1.33 ± 0.33</td>
<td>Yes</td>
</tr>
<tr>
<td>Epithelial hyperplasia</td>
<td>0.20 ± 0.20</td>
<td>1.87 ± 0.27</td>
<td>Yes</td>
</tr>
<tr>
<td>Mucus plugs in the bronchioles</td>
<td>0.00 ± 0.00</td>
<td>1.94 ± 0.30</td>
<td>Yes</td>
</tr>
<tr>
<td>Neutrophils in the bronchiolar lumen</td>
<td>0.06 ± 0.06</td>
<td>1.94 ± 0.31</td>
<td>Yes</td>
</tr>
<tr>
<td>Peribronchial eosinophils</td>
<td>0.13 ± 0.09</td>
<td>0.80 ± 0.26</td>
<td>No</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>0.06 ± 0.06</td>
<td>0.60 ± 0.17</td>
<td>No</td>
</tr>
<tr>
<td>Peribronchiolar inflammation</td>
<td>0.13 ± 0.09</td>
<td>2.47 ± 0.13</td>
<td>Yes</td>
</tr>
<tr>
<td>Perivascular inflammation</td>
<td>0.13 ± 0.13</td>
<td>0.80 ± 0.14</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 4.1 – Section of SPAOPD-affected lung. Note the presence of mucus plugs in the lumen of bronchioles and peribronchial inflammation.
**Figure 4.2** – Section of SPAOPD affected lung. Note the presence of excessive neutrophils in the lumen of bronchioles in the SPAOPD-affected lung.
The most consistent finding in the lungs of SPAOPD-affected horses was the presence of mucus plugs in the bronchioles (Figure 4.1). Other consistent and significant findings in the SPAOPD-lungs were the presence of goblet cell metaplasia, bronchiolar epithelial hyperplasia, infiltration of neutrophils into the bronchiolar lumen and peribronchial inflammation (Figure 4.2 and Figure 4.3).

In addition, weak to moderate infiltration of eosinophils was noticed in 3 of the 6 SPAOPD-affected horses. Although not statistically different from clinically healthy horses, few alveolar macrophages and mild perivascular and alveolar inflammation were noticed in all SPAOPD-affected horses. None of the SPAOPD-affected lungs had true pulmonary emphysema.
In healthy horses, none of the aforementioned changes were observed except for very mild epithelial hyperplasia and peribronchial and alveolar inflammation in 2 of the 6 horses.

4.3.4 Antibody and Primer Specificity – The protein band sizes and RT-PCR product sizes of ET-A and ET-B receptors of rat lungs matched with those of equine lung samples. For the immunohistochemistry, rat lung sections showed intense immunostaining for both receptors and no staining was observed in negative controls. Similarly, the horse lung sections showed immunostaining for both receptors and no staining was detected in the negative control sections.

4.3.5 Findings of RT-PCR Studies – There was no difference in the mean intensity of ET-A receptor bands between healthy and SPAOPD-affected horses, whereas the mean intensity of ET-B receptor bands was significantly greater (~ 4-fold) in SPAOPD-affected horses compared with healthy horses (Figure 4.4 and Figure 4.5). In healthy horses, there was no difference between the mean intensities of ET-A and ET-B receptors; however, in SPAOPD-affected horses, the mean intensity of ET-B receptors was significantly greater (~ 4-fold) than that of ET-A receptors.

4.3.6 Findings of Western Blotting Studies – The mean band intensities of ET-A and ET-B receptors were significantly greater (~ 1.6-fold and ~ 2-fold, respectively) in lungs of SPAOPD-affected horses compared with values from healthy horses, respectively (Figure 4.6 and Figure 4.7). In healthy horses, there was no difference between the mean intensities of ET-A and ET-B receptors; however, in SPAOPD-affected horses, the mean intensity of ET-B receptors was significantly greater (~ 2-fold) than that of ET-A receptors.
Figure 4.4 – RT-PCR bands of ET-A and ET-B receptors and β-actin in healthy and SPAOPD-affected horses. The mean sizes of the RT-PCR bands for β-actin, and ET-A and ET-B receptors were 160, 190 and 220 base pairs, respectively.

Figure 4.5 – Mean ± SEM for the mean intensity of the ET-A and ET-B receptor bands per mean intensity of β-actin bands in healthy (n=7) and SPAOPD-affected (n=7) horses. The * indicates a statistically significant difference (P < 0.05) in the mean intensities of ET-B receptors between healthy and SPAOPD-affected horses. The † indicates a statistically significant difference (P < 0.05) between the mean intensities of ET-A and ET-B receptors in SPAOPD-affected horses.
Figure 4.6 – Western blot bands of ET-A and ET-B receptors and β-actin in healthy and SPAOPD-affected horses. The mean sizes of western blot bands for β-actin and ET-receptors were 40 and 60 Kd, respectively.

Figure 4.7 – Mean ± SEM for the intensity of the ET-A or ET-B receptor bands per mean intensity of β-actin bands in clinically healthy (n=4) and SPAOPD-affected (n=4) horses. The * indicates statistically significant difference (P < 0.05) in the intensities of ET-B receptors between healthy and SPAOPD-affected horses. The † indicate a statistically significant difference (P < 0.05) between the intensities of ET-A and ET-B receptors in SPAOPD-affected horses. The ‡ indicates statistically significant difference (P < 0.05) in the intensities of ET-B receptors between healthy and SPAOPD-affected horses.
Figure 4.8 – Immunostaining of equine alveolar tissue from an SPAOPD-affected horse (20X magnification) where the primary antibody was replaced with sheep gamma globulin as a negative control.
Figure 4.9 – Immunostaining of equine alveolar tissue from the same tissue sample as used for the negative control, but in which anti ET-B receptor antibody was applied.
Figure 4.10 – Immunostaining of ET-B receptors can be observed in the luminal side of bronchial epithelium of the lung from an SPAOPD-affected horse and epithelial hyperplasia can also be clearly noticed.
Figure 4.11 – Mean (± SEM) of ET-A and ET-B receptor immunostaining in the lungs of healthy (n=7) and SPAOPD-affected horses (n=7). The * indicates statistically significant difference (P < 0.05) in the intensities of ET-B receptors between healthy and SPAOPD-affected horses. The † indicate a statistically significant difference (P < 0.05) between the intensities of ET-A and ET-B receptors in SPAOPD-affected horses. The ‡ indicates statistically significant difference (P < 0.05) in the intensities of ET-A receptors between healthy and SPAOPD-affected horses.
**Figure 4.12** – Mean (± SEM) of relative ET-A and ET-B receptor gene expression in the lungs of healthy (n=7) and SPAOPD-affected horses (n=7). The * indicates statistically significant difference (P < 0.05) in the relative gene expression of ET-B receptors between healthy and SPAOPD-affected horses. The † indicates a statistically significant difference (P < 0.05) in the relative gene expression of ET-A receptors between healthy and SPAOPD-affected horses.
4.3.7 Findings of Immunohistochemistry Studies – The findings of immunohistochemistry studies were similar to those of Western blotting. The mean score for ET-A and ET-B receptor immunostaining was significantly greater (~ 1.7-fold and ~ 2.1-fold, respectively) in lungs of SPAOPD-affected horses, compared with values in healthy horses (Figures 4.8, 4.9, 4.10 and 4.11). In healthy horses, there was no difference between the mean scores for ET-A and ET-B receptor immunostaining; however, in SPAOPD-affected horses, the mean score of ET-B receptor immunostaining was significantly greater (~ 1.6-fold) than that of ET-A receptors.

4.3.8 Findings of Real-Time PCR Studies – Findings of real-time PCR studies have agreed with the other studies. The relative gene expression of ET-B receptors was ~ 50 times greater in SPAOPD-affected horses when compared to clinically healthy horses (Figure 4.12). The relative gene expression of ET-A receptors was ~ 10 times greater in SPAOPD-affected horses when compared to clinically healthy horses.

4.4 Discussion

The findings of this study are interesting and thought provoking. First, by using RT-PCR, we demonstrated that the mRNA of ET-A and ET-B receptors is expressed in the lungs of healthy and SPAOPD-affected horses, and the expression of ET-B receptor mRNA is significantly greater in SPAOPD-affected horses when compared to healthy horses. However, no change was observed in the ET-A receptor mRNA expression between the two groups of horses. Second, by employing Western blotting and immunohistochemistry, we showed that ET-A and ET-B receptors are expressed in equine lungs and the expression of both of these receptors is significantly greater in the lungs of SSAOPD-affected horses than that of clinically healthy horses. Third, all three techniques revealed that there is no difference between the expression of ET-A and ET-B receptors in the lungs of healthy horses. Finally, all three techniques revealed
that ET-B receptors are significantly over-expressed in the lungs of SPAOPD-affected horses, compared with healthy horses and compared with ET-A receptors in the same horses.

Since the present study demonstrated significant differences in the expression of ET-receptors between the two groups of horses, we wanted to confirm that the grouping of horses was properly performed. All SPAOPD-affected horses that were used in this study were kept in the pasture until they began showing clinical exacerbations. Clinical evaluation was conducted immediately after the horses started showing the symptoms of SPAOPD. During the clinical evaluation, all affected horses showed the characteristic signs of obstructive pulmonary diseases such as cough, exaggerated expiratory effort, respiratory wheezes on auscultation, abdominal lift and flared nostrils (Seahorn and Beadle 1993). The findings in our gross post-mortem evaluation and histopathologic evaluation in SPAOPD-affected horses are in agreement with the previous reports (Costa et al. 2000). The hyper-inflation of the lungs that was noticed during post-mortem evaluation of SPAOPD-affected horses is believed to be due to the trapping of air in post-obstructive areas of the lung. This hyper-inflation is the cause of the rib impressions that were observed on the surface of the lungs of some SPAOPD-affected horses. Because of the entrapped air, SPAOPD-affected lungs did not collapse when the thoracic cavity was opened. Histologically, the consistent findings in SPAOPD-affected horses were the presence of neutrophil-filled mucus plugs and goblet cell metaplasia in the bronchioles, and epithelial hyperplasia of bronchi (Costa et al. 2000). All of these changes contribute substantially to obstruction of the airways in this disease.

Previously, Benamou et al suggested involvement of ET-1 in the pathogenesis of equine RAO by demonstrating the presence of elevated levels of ET-1 in the pulmonary circulation and BALF of RAO-affected horses (Benamou et al. 1998). This concept of ET-1 involvement in
equine allergic pulmonary disease is complemented by the findings of the present study, which
showed that the expression of ET-receptors, especially that of ET-B receptors, is significantly
increased in the lungs of SPAOPD-affected horses. Compelling evidence exists for the
involvement of ET-1 in the pathogenesis of human allergic airway diseases (Michael and
Markewitz 1996). For example, in human asthmatic airways, ET-1 has been shown to stimulate
mucus secretion, airway edema through microvascular leakage, smooth muscle mitogenesis and
bronchial hyperresponsiveness (Michael and Markewitz 1996). Similarly, in human COPD, ET-1
has been implicated in the activation of neutrophils, and alveolar macrophages and pulmonary
hypertension secondary to COPD (Ronald et al. 2001). As far as the ET-receptors are concerned,
there is paucity of literature regarding alteration of their expression in airway allergic diseases.
Moller et al. demonstrated that the ratio of ET-A and ET-B receptor mRNA is altered in the
bronchial biopsies from human patients with asthma and chronic airway obstruction. By
employing RT-PCR techniques, they showed that the bronchial biopsies of these patients express
significantly greater ET-B receptor mRNA expression compared with ET-A receptor mRNA
(Moller et al. 1999). In contrast, by employing quantitative autoradiography studies, Knott et al.
showed that asthma is not associated with any significant alteration in the densities of ET-A and
ET-B receptors in the peripheral human lung (Knott et al. 1995). In these studies, they also
demonstrated that in both asthmatic and non-asthmatic individuals, approximately 30% of the
ET-1 binding was to ET-A receptors whereas approximately 70% was to ET-B receptors (Knott
et al. 1995). In the current study, all three techniques showed that ET-B receptor expression is
significantly greater in the lungs of SPAOPD-affected horses compared to healthy horses;
however, although Western blotting and immunohistochemical studies demonstrated the over-
expression of ET-A receptors in SPAOPD-affected horses, RT-PCR studies showed no
difference in the ET-A mRNA expression between the two groups. The reason for this discrepancy is not clear at this point. Perhaps the post-transcriptional regulatory mechanisms might have significant influence during the synthesis of ET-A receptors. Further studies are required to establish the exact cause of this finding.

In the current study, we also demonstrated that the significant over-expression of ET-B receptors in the lungs of SPAOPD-affected horses is much greater than the over-expression of ET-A receptors. These findings suggest that ET-B receptors might play a greater role than ET-A receptors in the pathogenesis of SPAOPD. However, considering the fact that the role of ET-1 is not yet confirmed in the pathogenesis of SPAOPD, it is premature to draw this conclusion. Nevertheless, our findings in this study could form a strong basis for future functional studies regarding the involvement of ET-1 in the pathogenesis of SPAOPD.

In conclusion, the present study provides molecular evidence for the presence of ET-receptors in the lungs of healthy and SPAOPD-affected horses. In addition, we also demonstrated for the first time that both types of ET-receptors, particularly ET-B receptors, are over-expressed in the lungs of SPAOPD-affected horses. We believe that these findings could form a basis for future clinical studies involving use of ET-receptor antagonists in alleviating the symptoms of this disease.

4.5 Footnotes


b Esophageal balloon, AE Medical, Farmingdale, NJ.

c Model 7D polygraph, Grass Medical Instrument Division of Astro-Med, Inc. WestWarwick, RI.

d Sodium pentobarbital (Beuthanasia), Scherring-Plough Animal Health Corp, Kenilworth, NJ.
Sheep Antibody to Endothelin A Receptor and Sheep Antibody to Endothelin B Receptor, BIODESIGN International, Saco, Maine.

RNase Zap® RNase decontamination solution, Ambion®, Inc., Austin, TX.

TRI Reagent® Molecular Research Center, Inc., Cincinnati, OH.

Ultra-Turrax T8®, IKA® Works, Inc., Wilmington, NC

NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE

M-MLV Reverse Transcriptase and Platinum® Pfx DNA Polymerase Invitrogen Corporation, Carlsbad, CA.

Eppendorf® Mastercycler® gradient, Eppendorf, Westbury, NY.

VersaDoc imaging system Model 1000, Quick Start Bradford Protein Assay Kit 1 and Quantity one 1-D image analysis software, Bio-Rad Laboratories, Hercules, CA.

Donkey anti-sheep IgG-HRP, Santa-Cruz Biotechnology, Santa Cruz, CA.

Western lightening chemiluminscence reagent, PerkinElmer Life Sciences, Boston, MA.

BioMax MR film, Eastman Kodak Company, Rochester, NY.

Microsoft Corporation, Redmond, WA.

Dako Autostainer Universal Staining system, DakoCytomation California Inc, Carpinteria, CA.

Avidin/Biotin Blocking Kit, Vector laboratories, Inc., Burlingame, CA.

Vectastain Elite ABC Kit (Sheep IgG) and Vector NovaRED Substrate Kit for Peroxidase, Vector laboratories, Inc., Burlingame, CA.

Jackson ImmunoResearch laboratories, Inc., West Grove, PA.

Graphpad Prism Verion 4.0, GraphPad Software, Inc., San Diego, CA.

4.6 References


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O’Byrne PM, Inman MD. Airway hyperresponsiveness. *Chest.* 2003; 123(3 Suppl):411S-6S.


VITA

Sumanth Polikepahad was born in Hyderabad, Andhra Pradesh, India. He received his Bachelor in Veterinary Science and Animal Husbandry (B.V.Sc & A.H, 2001) degree from the college of veterinary sciences, Acharya N.G.Ranga Agricultural University, Rajendranagar, Hyderabad, India. In January 2002, he joined in the Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University to pursue his doctoral degree in veterinary medical sciences with a specialization in respiratory pharmacology and physiology. In his dissertation research, he has worked to investigate the role played by the endothelin receptors in the pathogenesis of summer pasture-associated obstructive pulmonary disease of horses. He will receive his Doctor of Philosophy degree in the spring 2006 commencement.