INVESTIGATION OF ARACHIDONIC ACID METABOLISM IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (HEAVES) OF PONIES AND HORSES

By

Peter Royston Gray

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Large Animal Clinical Sciences

1991

ABSTRACT

INVESTIGATION OF ARACHIDONIC ACID METABOLISM IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (HEAVES) OF PONIES AND HORSES

By

Peter Royston Gray

Chronic obstructive pulmonary disease, or "heaves," is a spontaneously occurring pulmonary disease of horses and ponies characterized by repeated episodes of airway obstruction and airway hyperresponsiveness. To determine whether there are alterations in arachidonic acid metabolism during the occurrence of this disease, I first measured plasma and bronchoalveolar fluid concentrations of the arachidonic acid metabolites, 15-hydroxyeicosatetraenoic acid (15-HETE), thromboxane(Tx) A_2 (as TxB_2), prostaglandin(PG) I_2 (as 6-keto-$PGF_{1\alpha}$), and PGD_2 (as 9α,11β-PGF_2). Plasma 15-HETE and TxA_2 concentrations increased significantly in the affected ponies during airway obstruction. To determine the potential importance of the increase in plasma TxB_2 in development of airway obstruction and hyperresponsiveness, I measured pulmonary function and airway responsiveness in affected and control ponies prior to and following cyclooxygenase inhibition with flunixin meglumine. Inhibition of TxB_2 production did not prevent the development of airway obstruction or hyperresponsiveness. I concluded from these data that plasma TxA_2, although increased, was not important in the development of the disease. To determine whether the lung was the source of the 15-HETE measured in jugular blood samples, I simultaneously collected blood samples from the right ventricle and

carotid artery. Fifteen-HETE concentrations in carotid artery blood samples were significantly greater than those in right ventricular blood samples, and carotid artery blood 15-HETE concentrations were significantly greater in affected ponies compared to controls. I concluded that the lung was a source of 15-HETE production and that this production was increased in affected ponies compared to controls. I next attempted to determine the site of the increased production of 15-HETE within the lung. Evidence from experiments with human tissue suggested that tracheal epithelium is a major source of 15-HETE, especially in asthmatics, so I developed an in-vitro technique for determining the profile of arachidonic acid metabolites produced by equine tracheal epithelium. In the final experiment, I utilized this technique to measure 15-HETE and PGE_2 production by tracheal epithelium collected from affected horses during clinical disease and from control horses housed in the same environment. Tracheal epithelium was not a significant source of 15-HETE in either group of horses. Prostaglandin E_2 production by airway epithelium was similar in both groups, but PGE_2 production by subepithelial connective tissue stimulated with 50 μM bradykinin was significantly greater in control animals. I concluded from this that the tracheal epithelium was not the source of the increased concentrations of 15-HETE measured in carotid blood samples from affected ponies. The significance of the decreased PGE_2 production by subepithelial tissues in principal horses remains to be determined.

This dissertation is dedicated to my wife and friend,

Mary Frances McConnell

For her support, both emotional and scientific,

that helped me to reach this goal

ACKNOWLEDGMENTS

I wish to express my appreciation and thanks to my major professor, Dr Frederik J. Derksen, and to the members of my guidance committee, Drs. Thomas G. Bell, N. Edward Robinson, Robert A. Roth, and William L. Smith.

I would like to acknowledge the assistance of Drs. Laurie Carpenter-Deyo, Herbert Johnson, and Neil Olson for their assistance with sample analysis.

Finally, I would also like to acknowledge the advice and assistance of Dr. Marc Peters-Golden from the University of Michigan. The work relating to airway epithelium could not have occurred without his timely and unselfish support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Chronic obstructive pulmonary disease of horses and ponies is not a new disease, having been first described by Aristotle in 333 BC. It is an important disease for the equine industry both financially, due to the cost of treating affected animals, and emotionally, due to its tendency to affect older animals and to shorten the careers of otherwise sound performance animals. Its importance to the equine industry is matched by its potential importance in the area of comparative pulmonary pathophysiology.

Equine and human lungs are anatomically similar, and chronic obstructive pulmonary disease is a naturally occurring disease of domestic animals that has the potential to serve as a model for human asthma. Characteristics shared by both diseases include the natural occurrence, intermittent episodes of airway obstruction, non-specific airway hyper-responsiveness, the presence of pulmonary inflammation and excessive mucus production during disease episodes, and the response of affected individuals to treatment with corticosteroids, anticholinergic drugs such as atropine, and some beta agonists.

While equine COPD is potentially a useful model for asthma in humans, human asthma has, in turn, served as a model for directing and focusing research into COPD of horses. Much of the information contained in the literature review that follows has been derived from studies in human asthmatics, as has the impetus to investigate the potential involvement of arachidonic acid metabolites in the pathogenesis of equine COPD. The expectation of this research was that, if similar alterations in arachidonic acid metabolism occurred in both species, equine COPD could serve as a useful model for determining the potential significance of those changes in human asthmatics.

The possible involvement of inflammatory mediators, and arachidonic acid metabolites in particular, in the pathogenesis of asthma has received a great deal of attention. Lung tissues collected from asthmatics show marked airway inflammation, with disruption of airway epithelium, and increased numbers of inflammatory cells are present in bronchoalveolar lavage fluid samples collected during disease episodes. Similar changes have been reported in lung tissue and bronchoalveolar lavage fluid samples collected from horses and ponies with COPD. Altered arachidonic acid metabolism has also been reported in human asthmatics, with increased production of both lipoxygenase and cyclooxygenase metabolites during disease episodes, but as yet, no equivalent studies have been performed in ponies or horses with COPD. The purpose of my work was to begin this examination of arachidonic acid metabolism in equine COPD, using the results of human asthma research as a

guide. My initial objective was to determine whether any measurable alterations in arachidonic acid metabolism could be detected during the acute disease phase. If changes were detected, the next step was to attempt to block production of the identified metabolite and determine what effect this had on the occurrence of the disease. If no suitable antagonist or inhibitor was available, the alternative was to identify the source of the altered arachidonic acid metabolism to determine whether production of that particular metabolite at that site could account for any of the observed symtoms.

During the course of my research, increasing attention began to focus on the possible role of airway epithelium and its metabolites in the pathogenesis of human asthma. Tracheal epithelium was shown to produce factors that modulated airway smooth muscle responsiveness (as measured by a left shift in the dose response curve), and in some species, these substances appeared to be arachidonic acid metabolites. Since human airway epithelium was a source of one of the metabolites which I had shown to be increased in ponies with COPD, the final part of my research was directed towards developing a method for examining arachidonic acid metabolism in equine tracheal epithelium to see whether this was the source of that particular metabolite, or of other epithelial-derived arachidonic acid metabolites that had been shown to alter airway smooth muscle responsiveness.

CHAPTER 1

LITERATURE REVIEW

In this review I will initially discuss the clinical and histological features of chronic obstructive pulmonary disease in horses and ponies. Since non-specific airway responsiveness is a feature of both equine COPD, and human asthma, I will then discuss potential mechanisms by which this hyperresponsiveness is thought to occur, focusing finally on the possible contribution of inflammatory mediators. In the next section I will briefly describe the inflammatory mediators and cells that have been postulated to play a role in the pathogenesis of asthma, before covering in greater detail arachidonic acid metabolites and their possible role in allergic airway disease. The review will then conclude with a discussion of airway epithelium and its potential role in modulating airway smooth muscle reactivity.

Heaves/Chronic Obstructive Pulmonary Disease

Heaves is a term applied to a complex of clinical signs rather than to a specific disease.[34] It is used to describe a horse or pony with a chronic respiratory disorder exhibiting some or all of the following: exercise intolerance,

intermittent or chronic purulent nasal discharge, cough, and dyspnea characterized by an accentuated abdominal effort at end expiration.[34,284] A variety of synonyms for the condition exist, including chronic obstructive pulmonary disease, recurrent airway obstruction, broken wind, chronic bronchitis, chronic bronchiolitis, equine pulmonary emphysema, and chronic alveolar emphysema.[34,65,284] I have chosen to use the term chronic obstructive pulmonary disease (COPD) in this section since it most accurately describes the disease present in the ponies and horses used in my studies. These animals exhibited repeated episodes of dyspnea and exaggerated respiratory effort when housed in a barn and these signs were accompanied by evidence of airway obstruction and airway hyperresponsiveness.

Epidemiology. Chronic obstructive pulmonary disease is associated with housing or confinement. It is common in countries where horses are fed hay and stabled for long periods of time and rare in warmer areas where animals are able to be kept outside on grass year round.[65] The disease incidence increases with age, but there appears to be no breed, sex, or genetic predisposition to the condition.[238]

Etiology. The causes of COPD are unknown, but proposed factors include exposure to allergens, prior respiratory infection, and pneumotoxin ingestion.[34] Although it is unlikely that any one factor is responsible for the disease, the strongest evidence supports an allergic basis to the disease in many cases, while the evidence for other contributing factors such as pneumotoxin ingestion or viral infection is largely circumstantial.

Allergen exposure. Lowell was the first to show a relationship between the occurrence of acute disease and the feeding of hay, and he suggested that the disease was precipitated by the inhalation of "heaves producing factors" in hay.[215]

Horses with COPD have a significantly greater percentage of positive reactions to aerosol or intradermal challenge with extracts of common environmental thermophilic moulds and fungi than do unaffected animals.[141,239] The two most commonly implicated antigens are *Micropolysporum faeni* and *Aspergillus fumigatus,*[141] and the greatest number of reactions are associated with *M. faeni* challenge. *M. faeni* has been associated with farmers lung in humans, a form of hypersensitivity pneumonitis.[105] Factors that increase environmental contamination with these antigens, such as inadequate ventilation or feeding poor quality (dusty/mouldy) hay, are associated with an increased incidence of COPD.[238] A similar association between COPD and environment was seen with the affected animals used in my studies.[135] Airway obstruction, exhibited as increased pulmonary resistance and decreased dynamic compliance, airway hyperresponsiveness to aerosol histamine, and hypoxemia could be repeatedly induced by placing these animals in the barn and feeding them poor-quality hay. Remission of symptoms occurred when they were returned to pasture and not exposed to hay or dust. The same response has been observed in previous studies utilizing a similar group of affected animals.[6,40,82,296]

However, an allergic response to environmental antigens is unlikely to be the sole cause of the disease. Lawson et al. demonstrated that precipitins to

M. faeni and *A. fumigatus* are not restricted to animals with heaves, while animals without precipitins develop clinical signs of heaves.[203] In addition, experimental sensitization of ponies with *M. faeni* followed by aerosol challenge results in airway inflammation but does not reproduce the characteristic changes in pulmonary function.[83,88]

Infection. Prior infection with influenza virus is associated with an increased incidence of chronic pulmonary disease in Swiss horses,[121] while other authors report increased levels of hemagglutination inhibiting activity against influenza A equine 1 in serum and tracheal mucus samples from horses with COPD.[327] Abnormal airway function following viral infection occurs in humans,[43,209] rats,[50] and dogs.[210] These effects may be due to induction of airway smooth muscle hyperreactivity,[42,210] epithelial hyperplasia with consequent airway narrowing,[245] impaired epithelial function,[173] or altered production of inflammatory mediators.[186] Viral infection is unlikely to be a major factor in the ponies used in my studies, since these animals were all vaccinated against equine influenza and most had been utilized by the laboratory for over four years, during which time their disease status has not changed. In addition, if viral infections were an important factor in the pathogenesis of COPD, control ponies should have also been affected, since they share the same environment and are in constant close contact with the affected ponies.

Pneumotoxin ingestion. Ingestion of 3-methylindole, a metabolite of dietary L-tryptophan, has been suggested as a possible cause of COPD.[35] Oral administration of 3-methylindole (0.1 to 0.2 gm/kg bodyweight) to ponies induces

obstructive lung disease characterized by necrotizing bronchiolitis and alveolar emphysema.[35] Similar lesions were reported by Derksen et al. in ponies treated orally with 0.1 gm/kg.[85] Treated animals become dyspneic, and respiratory rate, minute ventilation, functional residual capacity, and total lung resistance increase significantly. There is a significant decrease in dynamic compliance and $PaCO_2$ but no change in PaO_2. Although these changes reflect the presence of pulmonary dysfunction, they are not typical of those seen with COPD, where the changes in dynamic compliance and pulmonary resistance are more marked, tachypnea is less evident, and there is a significant decrease in PaO_2 but no change in $PaCO_2$.[6,40,82,296] A role for 3-methylindole in causing disease in the animals that I studied appears unlikely, because tryptophan is found on pasture where affected ponies remain in remission, and the experimental design, which pairs heavey and control ponies to ensure the same environmental exposures, would make it unlikely that only affected animals would be exposed to any potential pneumotoxin. However this does not preclude a possible role for this substance in producing COPD in other horses or ponies.

Pathophysiology. Pulmonary function changes in animals with COPD include hypoxemia, decreased dynamic compliance, increased pulmonary resistance, and prolongation of nitrogen washout, which together reflect the presence of diffuse airway obstruction.[82,284,356] Broadstone et al. demonstrated that a major component of the increased resistance is atropine responsive and therefore mediated via muscarinic receptors.[40] Atropine administered intravenously or by aerosol does not affect pulmonary resistance, dynamic

compliance, or airway responsiveness to histamine in control or affected ponies during remission or in control ponies during barn exposure, but in the principal ponies during barn exposure it significantly decreases pulmonary resistance without affecting dynamic compliance or responsiveness to histamine. Factors apart from parasympathetic activity must contribute to the alterations in pulmonary function in principal ponies, because atropine is unable to return pulmonary resistance to baseline values and also does not reverse the decrease in dynamic compliance or reduce the responsiveness to histamine.

During disease episodes, affected ponies exhibit airway hyperresponsiveness to stimuli other than histamine. Aerosol saline, histamine, methacholine and citric acid, and intravenous histamine can all produce exaggerated bronchoconstriction in affected ponies.[6,82,87,135] This hyperresponsiveness is accompanied by a significant increase in the number of neutrophils present in bronchoalveolar lavage fluid in affected ponies, but there is no significant change in circulating neutrophil numbers, which suggests a localized inflammatory response.[86] This response is different to that seen with hypersensitivity pneumonitis in humans, where the acute phase is characterized by a leukocytosis with marked left shift.[105] When ponies are removed from the barn and kept at pasture,[86] neutrophil numbers and airway responsiveness return to control levels within 14 days The involvement of inflammation in the pathogenesis of equine COPD is indirectly supported by the effectiveness of corticosteroid therapy in resolving clinical signs in affected animals.[18]

Histological lesions. The main histological lesions in lungs from affected animals is a bronchiolitis characterized by diffuse epithelial hyperplasia, mucous plugging of airways and neutrophilic, lymphocytic, and plasmacytic infiltrates. Peribronchial eosinophilia may be prominent in some animals and absent in others. A degree of acinar overinflation is usually present, but emphysema with destruction of alveolar septae is uncommon.[34,328] Epithelial damage with loss of ciliated cells, goblet cell metaplasia, and increased numbers of undifferentiated cells with superficial microvilli are also observed in electron micrographs of tissues from affected horses.[93,363]

In summary, COPD is a chronic airway disease of horses and ponies associated with housing and exposure to dusty/mouldy hay. Disease episodes are characterized by an inflammatory response in and around airways, epithelial injury, and airway hyperresponsiveness to a variety of non-specific agonists. Knowledge about the mechanisms involved in this airway hyperresponsiveness is important, not only for establishing appropriate treatment regimens for affected animals, but also because airway hyperresponsiveness to a variety of specific and non-specific agonists is one of the important clinical features of human asthma. Knowledge gained about COPD in horses may help to establish mechanisms of hyperresponsiveness in the human disease.

Airway Hyperresponsiveness

Airway responsiveness is the term used to define the sensitivity with which airways narrow in response to a variety of stimuli. These stimuli can be

classified as non-specific or specific. Non-specific stimuli include drugs such as methacholine, air pollutants such as ozone, and physical stimuli such as cold air or water, dust, and exercise. Specific stimuli include allergens and occupational agents such as toluene diisocyanate and western red cedar dust. As discussed in the previous section, an increase in airways responsiveness to such stimuli is a characteristic feature of asthma and also of equine COPD. The mechanism by which this heightened responsiveness occurs has not been determined, but research has focused on four broad areas. These are:

i) Abnormalities of neurohumoral control of airway caliber;

ii) Altered intrinsic airway smooth muscle responsiveness;

iii) Altered mechanical properties of the airways; and

iv) Cellular dysfuntion and inflammation with altered mediator release.

Abnormalities of Neurohumoral Control. Innervation of the lung is provided by the sympathetic (adrenergic) and parasympathetic (cholinergic) branches of the autonomic nervous system and the nonadrenergic-noncholinergic system.

Parasympathetic nervous system. The parasympathetic system is the major bronchoconstrictor network in the lung. Preganglionic fibers arise in the dorsal motor nucleus and descend in the vagus nerve to ganglia located around airways and blood vessels in the lung. Postganglionic fibers then innervate airway smooth muscle, bronchial glands, and blood vessels. Vagal stimulation causes airway constriction, pulmonary circulatory vasodilation, and increased secretion by bronchial glands.

A role for the parasympathetic nervous system in equine COPD was supported by Broadstone et al., who studied the effect of aerosol or intravenous atropine on pulmonary resistance, dynamic compliance, and airway responsiveness to aerosol histamine challenge in control ponies and ponies with COPD.[40] They found that atropine administered intravenously or by aerosol significantly reduces the increase in pulmonary resistance seen in affected ponies during an episode of COPD but has no consistent effect on dynamic compliance. Derksen et al. reported a similar effect in an investigation of vagal mechanisms in equine COPD. They found that vagal cooling causes a significant decrease in pulmonary resistance, but no change in dynamic compliance.[84] These data suggest that a large portion of the increased pulmonary resistance resistance in affected animals is mediated via muscarinic receptor activity in larger airways. Mechanisms other than parasympathetic activity contribute to the altered pulmonary function in affected ponies, since atropine does not return pulmonary resistance to baseline values,[40] has no effect on dynamic compliance,[40,84] and also fails to alter the dose-response curve to aerosol histamine.[40] Broadstone et al. could not determine whether the increase in pulmonary resistance was due to augmented vagal reflex activity, enhanced reactivity of airway smooth muscle to a normal level of vagal activity, or loss of inhibitory factors that normally oppose vagally mediated bronchoconstriction. Derksen et al. suggested that increased sensitivity of airway smooth muscle to normal vagal tone rather than augmented vagal reflex activity could play a role in ovalbumin-induced airway obstruction in ponies,

since ovalbumin challenge of one lung does not alter pulmonary resistance measured in the other lung.[84]

Parasympathetic mechanisms have been reported to be involved in other experimental and spontaneous lung diseases. Drazen et al. reported that in guinea pigs sensitized to ovalbumin, like in the pony, cholinergic mechanisms are involved in regulating the changes in large and central airway tone following antigen challenge.[92] They found that antigen challenge significantly decreases dynamic compliance and pulmonary conductance, and that atropine prevents the change in conductance but not the change in dynamic compliance. Gold et al. studied pulmonary function in dogs sensitized to *T. canis, A. suum,* and mixed grass pollen. They found that airflow resistance increases significantly after antigen aerosol, and that this change is abolished by complete vagal blockade (vagotomy or vagal cooling to 0°C), efferent blockade with atropine, and afferent blockade (vagal cooling to 7-10°C). Unlike the results of Derksen et al. in ponies, unilateral challenge of one lung in these sensitized dogs causes bilateral bronchoconstriction, which is inhibited by cooling the vagus nerve supplying the challenged lung. These data suggest that vagally mediated reflex bronchoconstriction is a major component of acute antigen-induced canine asthma.[131] In humans, Gross et al. reported that in patients with COPD, anticholinergics given at usual clinical doses are more effective than adrenergic agents at increasing airflow and decreasing lung volumes, and that the effect of anticholinergics is predominantly on central airways.[139]

Sympathetic nervous system. Sympathetic nerves originate from the upper thoracic segments of the spinal cord with postganglionic fibers arising from the cervical and upper thoracic ganglia. These postganglionic fibers enter the lung at the hilum together with the vagus nerves.[252] Compared to the distribution of cholinergic nerves, sympathetic innervation is relatively sparse with considerable species variation. Sympathetic stimulation results from activation of alpha or beta receptors either by sympathetic nerves or by circulating catecholamines. Sympathetic stimulation can cause bronchial relaxation, constriction of pulmonary blood vessels and inhibition of glandular secretions.[140] However, the degree of bronchodilation in response to sympathetic stimulation can depend on the degree of pre-existing vagal tone.[45] Cabezas et al. studied the quantitative relationship between sympathetic and parasympathetic effects on canine airway smooth muscle. They found that sympathetic stimulation causes maximum relaxation when vagal tone is moderate but could never completely inhibit constriction induced by vagal stimulation. Following vagotomy, sympathetic stimulation has no effect on airway dimensions.[45] These data suggest that some degree of pre-existing airway tone must be present for sympathetic stimulation to produce bronchodilation.

Possible abnormalities of sympathetic function that can result in bronchoconstriction include:

i) Decreased circulating catecholamines;

ii) Partial beta adrenoceptor blockade; and

iii) Increased alpha:beta adrenergic activity.

Circulating catecholamine levels have not been measured in ponies with COPD, but in human asthmatics, catecholamine levels are in the normal range even during severe attacks. Ind et al. measured plasma catecholamines in 15 patients with acute severe asthma and found a 2-3 fold increase in noradrenaline concentrations, but no change in plasma adrenaline concentrations.[169] Similar results are reported for asthmatics during exercise and hyperventilation-induced asthma.[10] In this study, control subjects had a 5.5 fold increase in noradrenaline and a 3.2-fold increase in adrenaline plasma concentrations during exercise, while asthmatics had only a 2-fold increase in noradrenaline and no change in plasma adrenaline concentrations. All of the asthmatics developed bronchoconstriction following exercise. The importance of these changes in plasma concentrations of adrenergic agents in modulating bronchoconstriction was questioned since hyperventilation also produces bronchoconstriction in the asthmatics, with no change in circulating catecholamines in either group.[10] These results were interpreted to mean that circulating catecholamines do not have a direct role in exercise-induced bronchospasm.

It has been proposed that asthma may be associated with partial blockade of beta adrenoceptors, making affected individuals insensitive to endogenous and exogenous beta agonists.[323] Such a theory is not supported by the observation that normal subjects treated with beta adrenergic antagonists do not develop asthma, whereas asthmatics are normally very sensitive to beta agonists. In ponies with COPD, the beta adrenergic system appears to be involved in the

control of central airway caliber during disease episodes. Scott et al. showed that treatment with propranolol, a beta antagonist, does not alter pulmonary resistance, dynamic compliance, or airway responsivenes to histamine in control ponies or affected ponies in remission, but it significantly increases pulmonary resistance in affected ponies during disease episodes.[296] This propranolol-induced bronchoconstriction is prevented by atropine, suggesting that it results from unopposed cholinergic activity.[296]

Alpha adrenergic receptors may mediate bronchoconstriction, so alpha receptor stimulation in excess of beta stimulation has been proposed as a cause of asthma and bronchial hyperresponsiveness.[12,323] Barnes et al. studied alpha and beta receptor populations in lung homogenates from guinea pigs sensitized to ovalbumin following daily aerosol challenge with ovalbumin for 4 weeks. There is a significant increase in alpha and a small but significant decrease in beta receptor binding in sensitized versus control guinea pigs, and the ratio of alpha:beta receptors is 1:30 in control versus 1:12 in sensitized guinea pigs.[12] No equivalent binding studies have been performed on lung tissue from ponies with COPD, but Scott et al. studied the response of ponies with COPD to aerosol challenge with alpha-adrenergic agonists. They pretreated ponies with atropine and propranolol to decrease cholinergic and beta-adrenergic effects, then challenged the ponies with phenylephrine, an alpha-adrenergic agonist. Phenylephrine has no effect on control ponies, but decreases dynamic compliance and increases pulmonary resistance in the affected ponies during both remission and disease episodes. This response suggests that ponies with

COPD have increased density and/or activity of alpha receptors relative to control animals, but alpha receptor involvement in bronchoconstriction is thought to be minimal since treatment with prazosin, an alpha 1 antagonist, produces no significant improvement in pulmonary function.[297] This lack of response to treatment with an alpha adrenergic antagonist is similar to that reported by Utting in a study of 39 chronic asthmatics. These patients were treated with indoramin, a long-acting alpha-antagonist, and no difference was found between drug and placebo treatment.[333] These results suggest that altered alpha/beta receptor activity is unlikely to be a primary problem in asthma or COPD.

Non-adrenergic non-cholinergic nervous system. This system incorporates neural mechanisms that are neither cholinergic nor adrenergic. Non-adrenergic inhibitory nerves relax airway smooth muscle, whereas non-cholinergic excitatory nerves cause contraction. Non-adrenergic inhibitory nerves have been demonstrated in vitro in in several species, including humans, guinea pigs, and ponies.[41,282,324] Taylor et al. studied human bronchial strips and guinea pig tracheal chains, and found that electrical field stimulation induces relaxation in tissues pretreated with atropine. This relaxation is slightly diminished by propranolol in guinea pig but not human tissue, suggesting that the relaxation may be due to a neurally mediated factor(s).[324] Electrically mediated relaxation is also reported by Richardson for human airway smooth muscle precontracted with histamine. This relaxation is blocked by treatent with tetrodotoxin but unaffected by adrenergic blockade.[282] Similar results have been found in airway

smooth muscle from ponies by Broadstone et al.[41] Muscle from trachea and third generation airways were treated with atropine and propranolol and precontracted with histamine. Electrical field stimulation induces relaxation in tracheal muscle from affected and control ponies and in third-generation muscle from control ponies. There is no relaxation of third-generation airway muscle from affected ponies, suggesting that non-adrenergic non-cholinergic innervation is absent in these ponies.

The neurotransmitter in the non-adrenergic, non-cholinergic system is thought to be a peptide, with substance P and vasoactive intestinal peptide being the most likely agents. A functional defect in this system may develop as a result of inflammatory processes in the airway wall during disease. Increased degradation of peptide transmitters by inflammatory cell peptidases could disrupt the the modulating action of the non-adrenergic inhibitory system on cholinergic nerves and lead to exaggerated bronchoconstrictor responses.[9] Airway inflammation is reported in ponies with COPD during acute episodes of airway obstruction, and this inflammation could result in degradation of peptide neurotransmitters and thus explain the lack of non-adrenergic non-cholinergic relaxation in tissues from these animals. However, the significance of the absence of a non-adrenergic non-cholinergic inhibitory system from third-generation airways of ponies with COPD awaits further study.

Abnormalities of Airway Smooth Muscle. Alterations in the intrinsic physiological properties of airway smooth muscle has been suggested as a cause of the airway hyperresponsiveness seen with asthmatics.[4] This suggestion is

made on the basis of research performed on airway smooth muscle collected from ovalbumin-sensitized dogs and challenged in vitro with ovalbumin. There were no differences between tissues from control and sensitized dogs in response to electrical field or carbacol stimulation, but muscle from some of the sensitized dogs demonstrated increased velocity and amount of shortening when stimulated.[4] These conclusions are not supported by more recent experiments that examine responsiveness of airway smooth muscle from the same patient in vivo and in vitro. Vincenc et al. compared the responsiveness in vitro of parenchymal and bronchial tissue from surgical specimens with presurgery in vivo responsiveness of patients to aerosol histamine. They found no correlation between the dose of histamine required to decrease the one second forced expiratory volume by 20%, and the histamine concentration that contracts airway smooth muscle to 50% of maximum in vitro.[342] Similar results were reported by Armour et al., but in addition to determining histamine responsiveness, they quantitated airway smooth muscle morphometrically. They found no correlation between the provocative concentration of histamine required to decrease the one second forced expiratory volume by 20%, and reactivity in vitro or percentage of smooth muscle.[5] These results are consistent with those reported for ponies with COPD, in which tracheal smooth muscle from affected ponies is hyporesponsive to acetylcholine stimulation in vitro compared to control horses, yet affected ponies are hyperresponsive to aerosol methacholine challenge.[6,41] These data suggest that the hyperresponsiveness seen in asthmatics and ponies with COPD is unlikely to be due to intrinsic hyperresponsiveness of

airway smooth muscle. To assume that such a defect could comprise the primary abnormality also fails to account for other features of asthma and COPD such as mucosal edema, airway inflammation, and increased airway secretions.

Altered Mechanical Properties of Airways. Changes in mechanical properties of the airways can potentially result in an exaggerated degree of airway narrowing in response to normal degree of smooth muscle contraction. Such theories are based on physical models and mathematical calculations of the changes in pulmonary function that would result from altering physical properties of the airways, rather than on actual experimental data.

Moreno et al discussed a number of such factors, which can be broadly divided into those that decrease preload, permitting increased shortening of airway smooth muscle with consequent exaggerated airway narrowing, and those that decrease airway diameter, resulting in exaggerated airway narrowing in response to a normal degree of muscle shortening. Softening of airway cartilage via the action of enzymes released during inflammatory reactions, or loss of the airway tethering effect of lung parenchyma in conditions such as emphysema can both decrease the preload that airway smooth muscle must overcome during contraction. This will then permit a greater degree of muscle shortening and airway narrowing in response to normal stimuli. Factors that can result in decreased baseline airway caliber include increased airway wall thickness as a result of edema, cellular infiltration or hyperemia, and increased

airway secretions. These factors can result in a greater decrease in airway diameter for the same degree of muscle shortening.[245]

These theories are not supported by experimental results. Cartier et al. examined the relationship between the magnitude of the late asthmatic response and the magnitude and duration of histamine responsiveness in 7 asthmatics. They measured the concentration of histamine required to decrease the one second forced expiratory volume by 20% before and after allergen challenge. Four of the 7 subjects were still hyperresponsive to histamine when their airway caliber, as documented by total lung capacity, residual volume, one second forced expiratory volume, and maximum expiratory flow rate, had returned to within $\pm$ 10% of control levels. These data suggest that the increased responsiveness of these asthmatics to histamine is due to factors apart from a decrease in baseline airway caliber.[48] A similar conclusion was reached by Broadstone et al. in a study of ponies with COPD. They found that atropine treatment can reverse the airway narrowing present in affected ponies, but that this increase in airway caliber does decrease the airway hyperresponsiveness to aerosol histamine challenge.[40]

Cellular Dysfunction and Inflammation with Altered Mediator Release. Several studies have suggested that inflammation may play an important role in the hyperresponsiveness seen in asthma and COPD. Histologic studies of lung tissue collected from patients who have died during asthma attacks show marked airway inflammation with infiltration by inflammatory cells, disruption of airway epithelium, and plugging of the airway lumen with viscous secre-

tions.[94] Similar changes have been described in bronchial biopsy specimens collected from asthmatics[197]. Horses with COPD also exhibit airway inflammation[34,328] and evidence of epithelial injury.[93] Bronchoalveolar lavage samples from both asthmatics and ponies with COPD have increased numbers of inflammatory cells during disease episodes,[86,127,241] and there is evidence in these and other species that the presence of these inflammatory cells or their mediators is important in the pathogenesis of airway hyperresponsiveness.

Ferguson et al. reported that histamine hyperresponsiveness in 22 children with chronic, stable asthma is closely correlated with increases in eosinophils and alveolar macrophages in bronchoalveolar fluid samples. There is no correlation between hyperresponsiveness and neutrophil or lymphocyte numbers, or between the degree of airway obstruction and any cell type.[104] A similar association between eosinophils and human asthma was reported by Demonchy et al. in a study of inflammatory cell populations in bronchoalveolar lavage fluid samples from 19 asthmatics and 5 controls. They found a significant increase in bronchoalveolar fluid eosinophil numbers in samples collected 6-7 hours after inhalation challenge in asthmatics that developed a late response.[81]

Murphy et al. studied the relationship between inflammation and airway reactivity to histamine in rabbits sensitized to ragweed.[251] They found that antigen challenge produces a significant decrease in specific airway conductance and hyperresponsiveness to histamine challenge. This increased responsiveness can be eliminated by neutrophil depletion with nitrogen mustard, and restored

by neutrophil repletion, suggesting that neutrophils play a direct role in the development of hyperresponsiveness to histamine. In another study using rabbits, Larsen et al. reported that antigen challenge results in edema within large airways accompanied by neutrophil and eosinophil infiltration. These changes are accompanied by increased airway reactivity that is also abolished by granulocyte depletion and restored by granulocyte repletion.[202]

Several studies have suggested that it is not the presence of inflammatory cells per se, but mediators released by these cells, that are important in the development of airway hyperresponsiveness. O'Byrne et al. studied hyperresponsiveness in dogs induced by aerosol LTB_4,[259] or ozone exposure,[260,261] and found that both challenges result in airway hyperresponsiveness accompanied by significant increases in neutrophil numbers in bronchoalveolar fluid samples. Neutrophil depletion prevents the hyperresponsiveness,[261] but treatment with indomethacin[260] or OKY 046, a thromboxane synthase inhibitor,[259] prevents the hyperresponsiveness without stopping the neutrophil influx. These data suggest that it is a neutrophil-derived mediator that is the cause of the hyperresponsiveness. A similar result is reported for guinea pigs sensitized to ovalbumin, in which repeated aerosol challenge results in increased numbers of eosinophils in bronchoalveolar fluid samples and in airway hyperresponsiveness to acetylcholine. Pretreatment with SDZ 64-412, a PAF antagonist prevents the hyperresponsiveness without stopping the increase in eosinophils.[171]

While many studies like these have demonstrated a correlation between hyperresponsiveness and the presence of inflammatory cells, others have shown

that the presence of inflammatory cells per se does not necessarily result in airway hyperresponsiveness.[88,109,123,250] Gibson et al. compared sputum cell counts in 7 non-smokers with steroid responsive productive cough and 8 smokers with COPD, with those from asthmatics collected during an asthmatic attack. They found no difference in total cell count and percentage of eosinophils in sputum samples from asthmatics and non-smokers, despite a marked increase in airway responsiveness in the asthmatics.[123] In guinea pigs challenged with endotoxin, there is a significant increase in bronchoalveolar fluid neutrophil numbers and airway hyperresponsiveness, but there is no relationship between neutrophil numbers and changes in pulmonary function.[109] Similar results in guinea pigs exposed to ozone were reported by Murlas et al. Ozone exposure causes neutrophil infiltration of the airway mucosa and hyperresponsiveness to acetylcholine challenge, but the changes in pulmonary function and airway responsiveness precede the neutrophil influx.[250]

An alternate hypothesis that has been proposed is that the involvement of inflammatory mediators in airway responsiveness is not merely a function of the amount of these mediators released into the airways, but also of the ability of mediators once released to gain access to underlying nerves and smooth muscle. Increased epithelial permeability could allow greater access of mediators to airway smooth muscle or irritant receptors located in the epithelium, resulting in increased airway responsiveness. Although such a hypothesis seems logical, there is little experimental evidence showing a relationship between alterated airway epithelial permeability and airway

hyperresponsiveness. Kennedy et al. studied the effect of epithelial permeability and airway reactivity to histamine in 10 smokers and 8 non-smokers.[185] They found increased airway permeability in smokers, with no evidence of altered responsiveness to histamine. Elwood et al. performed a similar study in asthmatics and non-asthmatics. Compared with the non-asthmatics, the asthmatics are hyperresponsive to aerosol methacholine, but there is no demonstrable difference in mucosal permeability between the two groups.[102] An absence of epithelial permeability changes in ponies with COPD is supported by the observation that albumin concentrations in bronchoalveolar lavage fluid samples from control ponies and ponies with COPD are not significantly different and there is no difference in albumin concentrations in BAL fluid samples from affected animals in remission and during disease episodes.[86]

The experiments discussed above provide evidence that in many species, inflammatory cells or the mediators that they release are involved in the pathogenesis of airway hyperresponsiveness. Because my thesis deals with the potential role on inflammatory mediators in equine COPD, I will now concentrate of the inflammatory cells and mediators that have been proposed to play a role in the pathogenesis of reactive airway disease in a variety of species.

Pulmonary Inflammation - Cells and Mediators

Airway inflammation has long been recognized as a prominent feature of asthma in humans,[94,126] and chronic obstructive pulmonary disease in horses.[34,215] A tremendous volume of research has since focused on the involvement of individual cells and specific mediators in the pathogenesis of airway obstruction and airway hyperresponsiveness. Much of the early research on the contribution of airway inflammation to human asthma focused on the role of mast cells and histamine, since antihistamines are effective in treating the early, but not the late asthmatic response. Eosinophils and their metabolites are currently attracting a great deal of attention in asthma research, but there is also evidence to support a role for other inflammatory cells such as neutrophils, lymphocytes, and macrophages in allergic airway disease. While there is an association between the presence of airway inflammation and the occurrence of allergic airway disease, it is likely that no one cell or class of mediator is the sole cause. Inflammation is a dynamic process, and interactions between different inflammatory cells or between inflammatory cells and other cell types present in the lung have been shown to occur in vitro and could potentially occur in vivo. The outcome of such interactions is difficult to predict.

In an attempt to present a concise overview of pulmonary inflammation, I will cover the various cell types and mediators that have been studied with suggestions as to how each may contribute to the pathogenesis of allergic airway disease. Most of this information is obtained from research into the

mechanisms of human asthma, since, apart from observations documenting the presence of inflammatory cells in equine COPD, there is little information concerning inflammatory cell function or mediator release in the disease. Where such information is available, I will refer to it specifically.

The cell types I will discuss are:

i) Mast Cells

ii) Neutrophils

iii) Eosinophils

iv) Macrophages

v) Lymphocytes

vi) Epithelial Cells

vii) Platelets

Mediators of inflammation I will discuss are:

i) Histamine

ii) Kinins

iii) Adenosine

iv) Complement

v) Sensory neuropeptides

vi) Oxygen Radicals

vii) Platelet Activating Factor

viii) Cytokines

ix) Eicosanoids

Cells.

i) Mast Cells. Mast cells are found throughout the lung in the connective tissue beneath the airway basement membrane, near submucosal blood vessels, in interalveolar septa, and in the airway lumen.[352] The term "mast cell" comes from the German "mast," meaning excessively well-fed or fattened,[115] which refers to their prominant cytoplasmic granules. These granules contain a variety of preformed mediators, including histamine, serotonin, and neutrophil/-eosinophil chemotactic factors.[115] Cell activation and release of granule contents to the exterior can be classically induced by binding of antigen to adjacent membrane bound IgE molecules,[115] but a variety of other factors can trigger mast cell degranulation. Opiates such as morphine and codeine can induce degranulation by binding to specific mast cell receptors,[49] whereas human neutrophils activated with zymosan or phorbol myristate acetate release a factor(s) that stimulates histamine release from cutaneous mast cells.[353] The eosinophil-derived mediators, major basic protein and eosinophil cationic protein also stimulate histamine release from rat peritoneal mast cells,[364] whereas human lung macrophages maintained in culture release a soluble, non-dialyzable factor (molecular weigh < 2000 daltons) that stimulates histamine release from human lung mast cells.[294]

In addition to the release of preformed mediators, activated mast cells can generate a range of eicosanoids. Human mast cells incubated with antihuman IgE or A32187 show a 14-fold increase in PGD_2 release and a 7-fold increase in TxB_2 release,[158] whereas Peters et al. showed that human mast cell

incubated with [^{3}H]arachidonic acid and stimulated with anti-human IgE release LTC_4/LTD_4 and 5-HETE in addition to PGD_2 and TxB_2.[274]

Much of the evidence for the involvement of mast cells in asthma comes from studies on the effects of histamine in asthmatics, which will be discussed in the mediator section. However, Wardlaw et al. studied human mast cells isolated from asthmatics and control subjects by bronchoalveolar lavage and reported a significant increase in mast cell numbers in the asthmatics, accompanied by a significant increase in spontaneous histamine release, which is greatest in patients with marked airway reactivity.[344] No similar increase in bronchoalveolar lavage fluid mast cell numbers was reported by Derksen et al. when they examined changes in bronchoalveolar lavage fluid cytology during the occurrence of COPD.[86]

ii) Neutrophils. Neutrophils form part of the initial host defense against injurious stimuli migrating in the vasculature to the focus of an insult in response to the release of various chemotactic factors. Once there, they emigrate from the blood vessels into the extravascular space where they phagocytize and degrade bacteria, immune complexes, and necrotic debris. Neutrophils possess large azurophil (or primary) granules, which contain pro-inflammatory mediators, including cationic proteins, acid hydrolases, and neutral proteases.[68] De novo synthesis and release of superoxide anions, leukotriene B_4, thromboxane A_2, and platelet activating factor also occur in activated neutrophils.[110]

Neutrophil infiltration is associated with impaired pulmonary function and airway hyperresponsiveness in a variety of animal species. Derksen et al. reported significantly increased numbers of neutrophils in bronchoalveolar lavage fluid samples collected from ponies with COPD during episodes of acute airway obstruction and airway hyperresponsiveness.[86] However, conflicting evidence for the role of neutrophils in equine COPD is provided by the same researchers in a subsequent study where they attempted to reproduce the natural disease by challenging affected and control ponies with aerosol *M. faeni*. Both groups of ponies develop a similar increase in bronchoalveolar lavage fluid neutrophil numbers, but only affected ponies exhibit evidence of impaired pulmonary function.[83] In rabbits sensitized to ragweed extract, aerosol challenge causes increased reactivity to histamine, which is accompanied by increased numbers of polymorphonuclear leukocytes (neutrophils/eosinophils) in bronchoalveolar lavage fluid.[230] O'Byrne et al. administered LTB_4 to dogs by aerosol and produced increased airway responsiveness to acetylcholine that is accompanied by increased neutrophil numbers and TxB_2 concentrations in bronchoalveolar lavage fluid.[259] Administration of a thromboxane synthase inhibitor (OKY 046) blocks the increase in airway responsiveness and bronchoalveolar lavage fluid TXB_2 but not the neutrophil influx. A similar effect is seen in dogs challenged with aerosol PAF.[55] There is an increase in airway responsiveness to acetylcholine that persists for 6 hours, a 2.3-fold increase in pulmonary resistance, and an 8-fold increase in bronchoalveolar lavage fluid neutrophil numbers. Treatment with OKY 046 prevents the

increase in pulmonary resistance and acetylcholine responsiveness without affecting neutrophil numbers. Further experiments by the same group studying ozone-induced hyperresponsiveness to acetylcholine in dogs again demonstrate increases in bronchoalveolar lavage fluid neutrophil numbers temporally associated with the occurrence of airway hyperresponsiveness. Treatment with indomethacin, or neutrophil depletion with hydroxyurea, prevents the increase in airway reactivity induced by ozone exposure.[260,261] These data suggest that in the dog, release of a cyclooxygenase metabolite, most probably TxA_2, by neutrophils attracted to the airways is involved in the observed airway hyperresponsiveness. However, in guinea pigs the presence of neutrophils does not correlate with airway hyperresponsiveness.[250] In these studies, guinea pigs exposed to ozone demonstrate an increase in airway reactivity to acetylcholine within 2 hours of exposure, which corresponds to an increase in mucosal mast cells and a decrease in mucosal goblet cells.[250] Neutrophil influx into airways occurrs later than 2 hours and persists beyond the remission of hyperresponsiveness. In human asthmatics, eosinophils rather than neutrophils appear to be the more important inflammatory cell. Wardlaw et al. examined neutrophil and eosinophil numbers in bronchial washings and bronchoalveolar lavage fluid samples from mildly affected asthmatics and control subjects. They found a greater number of eosinophils in samples from asthmatics, but no difference in neutrophil numbers compared to controls. Interestingly, they reported a significant increase in neutrophil numbers in bronchial washings compared to bronchoalveolar lavage in both asthmatics and controls, with

neutrophils comprising up to 50% of the total cell count in bronchial washes from nonatopic controls. They interpreted these data to mean that neutrophils are normal inhabitants of airway mucosal surfaces and that the small volume (20 ml) bronchial wash collects a greater proportion of airway cells than does the large volume (180-240 ml) bronchoalveolar lavage.[344]

These data suggest that the potential importance of neutrophils in allergic airway disease varies from species to species, and that even in ponies and dogs where they appear to play a role in pulmonary disease, their potential state of activation rather than their mere presence may be the important factor.

iii) Eosinophils. Compared with neutrophils, eosinophils are relatively ineffective at phagocytosis and rely primarily on extracellular release of cytotoxic compounds to destroy microbes and parasites.[76] Preformed mediators are contained within prominent cytoplasmic granules, and include eosinophil cationic protein, major basic protein, eosinophil peroxidase and eosinophil protein X. Of these, major basic protein has been studied most extensively. Its concentration in bronchoalveolar lavage fluid increases during allergen-induced late phase reactions,[90] and it is cytotoxic to respiratory epithelium. Studies using guinea pig tracheal epithelium in vitro found that major basic protein damages ciliated cells in a dose-related fashion. Low doses slow, and higher doses stop cilia beating. Histological evidence of cell damage occurrs at concentrations as low as 9×10^{-7} M, and superficial cells are destroyed at concentrations of 3.4×10^{-5} M, leaving underlying basal cells exposed.[125] Concentrations of major basic protein in sputum samples from asthmatics

approximate or exceed these concentrations, suggesting that major basic protein has the potential to cause epithelial injury in vivo.[125]

In addition to these granule proteins, activated eosinophils can generate large quantities of membrane-derived mediators, including 15-HETE, LTC_4, platelet activating factor, and superoxide anions.[205,276,300,332] The state of activation of eosinophils and hence their ability to secrete these inflammatory mediators can change rapidly when exposed to various cytokines. Veith et al. showed that supernatants from cultured monocytes of individuals with activated eosinophils significantly enhance the ability of eosinophils from normal subjects to kill *Schistosoma mansoni* larvae in vitro.[341] Incubating eosinophils with tumor necrosis factor also enhances their ability to kill *S. mansoni* larvae in a dose-dependent manner.[304]

Eosinophils, rather than neutrophils, appear to be the important inflammatory cell associated with airway hyperresponsiveness in humans. DeMonchy et al. examined the late asthmatic reaction to mite dust inhalation in 19 asthmatics and 5 controls.[81] They found no change in bronchoalveolar lavage neutrophil numbers, but an increase in eosinophil numbers and the eosinophil cationic protein/albumin ratio in patients with a late asthmatic response. In another study, circulating eosinophil numbers are significantly elevated 24 hours after allergen exposure in late phase asthmatics, and the magnitude of the changes in eosinophil numbers is correlated with airway reactivity to methacholine.[95] Similar results were reported by Horn et al., who measured blood eosinophil counts in 53 asthmatics and found a significant

eosinophilia in 52 of them during active disease.[163] In this study there are significant correlations between the eosinophil count and specific conductance, forced expiratory volume, and maximum mid-expiratory flow rate. Eosinophils figure prominently in histological sections obtained from asthmatics dying in status asthmaticus[94] and also in some horses with COPD,[328] but changes in blood eosinophil numbers are not seen in ponies with COPD during episodes of airway obstruction.[86]

Despite these reports associating increased eosinophil numbers with airway hyperresponsiveness in humans, Gibson et al.[123] report a series of cases in which patients exhibit increased numbers of eosinophils in their airways without evidence of airway hyperresponsiveness. Therefore, as was the case with neutrophils in ponies, the mere presence of inflammatory cells in the airways is not the only requirement for the occurrence of airway hyperreactivity.

iv) Macrophages. Three types of macrophages are present throughout the respiratory tract. Alveolar macrophages are present in the airspaces, while interstitial macrophages are emeshed in the connective tissue of the lung parenchyma,[207] and intravascular macrophages are located in the lumen of capillaries in alveolar walls of several species.[21,346] Most attention has been directed at the alveolar macrophage and its role in allergic airway disease. Mature alveolar macrophages phagocytize and remove bacteria and other particulate matter from the airways and alveoli and, depending on their state of activation, also secrete degradative enzymes and reactive oxygen metabo-

lites.[122] Activated alveolar macrophages can produce platelet activating factor[3] and eicosanoids, including $PGF_{2\alpha}$, PGD_2, TxA_2, LTB_4, and 5- and 12-HETE.[21,219] Apart from these pro-inflammatory mediators, macrophages can release factors which modulate the function of other cells. These factors include C5a,[254] tumor necrosis factor, and interleukin 1.[329] They are also involved in the processing and presentation of antigen to immunocompetent T lymphocytes, which is necessary for the induction of cell-mediated immunity.[330] Because of their location at the epithelial surface, their ability to be activated by immunological stimuli, and their ability to produce a wide variety of chemoattractants and inflammatory mediators, macrophages are well suited to play a central role in orchestrating the inflammatory response in airways.[280]

v) Lymphocytes. Lymphocytes play an integral part in the body's ability to mount an immune response. The two main types of lymphocytes are B cells, which are involved in antibody production, and T cells, which give rise to cell-mediated immunity. For B cells to respond to antigen requires the coordinated actions of specific macrophages to process antigen and present it to the B cell while fixed to the macrophage surface, and helper T cells, which secrete cytokines such as interleukin 2 to enhance the response of B cells to the presented antigen.[330] Once activated, the B cell begins to divide, forming antibody-secreting plasma cells and memory cells. T cells display greater heterogeneity than do B cells, with some responding to antigen as part of the cell-mediated immune response while others enhance (T helper) or suppress (T suppressor) the response of other T and B cells to antigen. Like B cells, the

response of T cells to antigen is regulated by interleukins released by macrophages and T helper cells. Antigen-sensitive T cells respond to antigen by dividing and generating memory cells and effector cells. The effector cells secrete a wide range of antigen-specific and non-antigen-specific proteins, including chemotactic factors for neutrophils, eosinophils, and macrophages, interleukins, interferon, and a variety of factors that regulate macrophage function.[330] As an example, Parish et al. reported that T lymphocytes isolated from bronchial mucus of patients with extrinsic asthma spontaneously release a factor(s) of molecular weight 30-60,000 that stimulates chemotaxis and chemokinesis and enhances chemotactic response to activated complement in eosinophils from control subjects.[269]

The normal immune response is a complex process that requires interaction between a number of different cells. If these regulatory processes are disrupted, the body could mount an inappropriate response to a normal antigenic challenge. In some asthmatics there are alterations in the type and number of lymphocytes present as well as in their state of activation. Large numbers of lymphocytes of unspecified type are present in the airways of asthmatics,[94] and atypical or activated lymphocytes are reported in bronchial biopsies from mild asthmatics.[175] Corrigan et al. examined T lymphocyte subsets and lymphocyte activation markers in patients with severe asthma. They found increased numbers of activated T helper cells in peripheral blood of patients with acute severe asthma, and these cells decrease in number with treatment and clinical improvement.[67] A similar increase in circulating T helper cells is

reported in early responder asthmatics following inhalation challenge with antigen.[132] These authors also reported a decrease in T helper and an increase in T suppressor cells in bronchoalveolar lavage fluid samples from early, but not late responders. They suggested that mobilization of suppressor cells into the lung after the allergen-induced early response may be important in preventing a subsequent late-phase reaction. There have been no equivalent studies on alterations in lymphocyte population in ponies or horses with COPD.

vi) Epithelial Cells. Epithelial cells will be discussed in a separate section, starting on page 57.

vii) Platelets. Platelets are a major source of TXA_2, which has been proposed to be an important contributor to bronchoconstriction in allergic airway disease.[365] Initial interest in the involvement of platelets in airway disease was prompted by the presence of platelets in bronchoalveolar lavage fluid from asthmatics after allergen challenge,[241] and increased plasma concentrations of platelet factor 4 in atopic asthmatics, suggesting platelet activation.[189] These results have since been questioned.[138] Whereas platelets may have a role in the pathogenesis of the Adult Respiratory Distress Syndrome,[151] their importance in diseases such as asthma is being increasingly questioned.[184]

Mediators.

i) Histamine. Histamine is probably the best characterized of all mediators of asthma. It can induce many of the physiological alterations characteristic of asthma, including bronchoconstriction, edema formation and increased mucus

secretion. Aerosol histamine administration produces bronchoconstriction in ponies, measured as a decrease in dynamic compliance and an increase in pulmonary resistance,[82] and a similar response in humans is reported by Woolcock et al.[361] Propst et al. studied changes in pulmonary alveolar membrane permeability in dogs challenged with intravenous histamine and found that histamine increases alveolar permeability to substances with a molecular weight of 10,000 daltons or less.[278] Exogenous histamine administration stimulates increased release of mucous glycoproteins from human bronchioles in vitro, and this action is blocked by cimetidine, an H2-receptor antagonist.[301]

Further evidence for the role of histamine in allergic airway disease is provided by the observation that asthmatics and ponies with COPD appear more sensitive than control subjects to the bronchoconstrictor action of histamine. In the study by Derksen et al., the dose of histamine required to decrease dynamic compliance to 65% of baseline values is significantly lower in ponies with COPD compared to controls,[82] while Woolcock et al. reported that the provocative dose of histamine required to reduce the one second forced expiratory volume by 20% is significantly lower in asthmatics than in normal subjects.[361] A similar increased sensitivity was reported as long ago as 1946 by Curry, who studied the action of histamine on vital capacity in normal patients, patients with "severe allergic tendencies," and asthmatics/bronchitics. Only the asthmatics show increased sensitivity to histamine, and this sensitivity correlates with the degree of asthma present.[70] Not only are asthmatics more sensitive to

the action of histamine, but there is also evidence that histamine release occurs during asthmatic attacks. Durham et al. measured plasma histamine concentrations in asthmatics and found a significant increase in plasma histamine, which parallels the decrease in the one second forced expiratory volume.[96] Despite this evidence for histamine's potential involvement in COPD, treatment of affected horses or ponies with antihistamines is relatively ineffective, suggesting that other factors are involved in the pathogenesis of the disease.[18]

ii) Kinins. Bradykinin is generated from alpha-2-globulins (kininogens) in plasma by the action of kininogenases such as plasma or tissue kallikrein. Bradykinin produces bronchoconstriction in guinea pigs when administered by aerosol,[156] causes vasodilation and increased airway mucosal thickness in dogs,[198] and stimulates chloride ion transport and PGE_2 production by cultured canine tracheal epithelium.[208]

Dolovich et al. reported the presence of bradykinin-like activity in nasal secretions from asthmatics challenged with local administration of ragweed antigen, and also in sputum specimens from subjects with asthma and bronchiolitis.[91] To investigate a possible role for bradykinin in asthma, Fuller et al. studied the effects of bradykinin aerosol challenge in asthmatics and controls. They found that bradykinin is ten times as potent as histamine or methacholine at inducing bronchoconstriction, and that asthmatics are more sensitive to its effects.[117] Aerosol administration of ipratropium, an anticholinergic drug, significantly attenuates the bronchoconstriction caused by

bradykinin, suggesting that bradykinin is acting in part through a cholinergic mechanism. In the same study these researchers examined the effect of bradykinin on isolated human bronchial smooth muscle and found that bradykinin is only a weak contractile agonist in vitro, suggesting that the bronchoconstriction seen with bradykinin in vivo is not due to a direct effect on airway smooth muscle. A similar study of bradykinin in asthmatics and controls revealed no effect in controls but a decrease of up to 30% in vital capacity in 13 of the 15 asthmatics studied.[156] These results contrast with those of Simonsson et al., who found no difference between normal and asthmatic subjects in the bronchoconstrictor response to aerosol bradykinin. However, within the asthmatic group there were individuals who were extremely sensitive to bradykinin, and in these individuals, atropine reverses the bronchoconstriction.[305] These data are interpreted to mean that bradykinin stimulates reflex vagal bronchoconstriction. No similar studies have been performed in ponies with COPD.

iii) Adenosine. Adenosine is a purine nucleoside released by a number of cells, including rat serosal mast cells stimulated with A23187 or compound 48/80[229] and mixed populations of human leukocytes stimulated with A23187.[224]

Aerosol administration of adenosine induces bronchoconstriction in asthmatics but not normal subjects, with a potency of one-third that of histamine.[72] Mann et al. reported that adenosine, adenosine monophosphate, and adenosine diphosphate all produce concentration-related bronchoconstriction in asthmatics.[223] In the same study, there was a significant increase in

plasma adenosine concentrations in asthmatics following allergen challenge, which occurs within 2 minutes and precedes peak bronchoconstriction. Adenosine's mechanism of action is uncertain, but may involve selective enhancement of histamine release from airway mast cells, since Rafferty et al. report that the time course of adenosine monophosphate-induced bronchoconstriction in asthmatics parallels that for histamine and can be prevented by treatment with terfenadine, an H1 receptor antagonist.[279] It has been suggested that adenosine acts as a secondary mediator in asthma and that release of endogenous adenosine may exacerbate asthma rather than initiate it.[57]

Theophylline is a cyclic nucleotide phosphodiesterase inhibitor that also acts as an adenosine receptor antagonist. It inhibits bronchoconstriction in asthmatics induced by adenosine inhalation,[71] and is a bronchodilator in ponies with COPD.[237] Fredholm et al. demonstrated that theophylline blocks adenosine-potentiated histamine release in rat mast cells at a concentration below that needed to inhibit cyclic nucleotide phosphodiesterase. They attribute this inhibition to adenosine receptor antagonism and propose that this may be the mechanism by which theophylline acts to prevent asthma attacks.[114] However, enprofylline, a xanthine derivative practically devoid of adenosine antagonistic effects, is 3-4 times more potent that theophylline as a bronchodilator in asthmatics,[217] so some other mechanism must be responsible for theophylline's bronchodilator action.

iv) Complement. Complement was first reported in 1895, and acts as the principal effector arm of antibody-mediated allergic reactions.[196] The

complement cascade consists of a complex series of interacting proteins triggered directly by interaction with damaged tissue, viruses, or bacterial products, and indirectly by the interaction of antibody with antigen. It provides a general amplification system for inflammatory reactions following such a "trigger response."[196] The complement pathway consists of nine central plasma protein components produced primarily by macrophages and hepatocytes, together with a number of ancillary factors and control proteins that help to regulate the cascade. Two products of the complement cascade with documented airway effects are the anaphylatoxins C3a and C5a.[20,170,318] These products can cause bronchoconstriction, increased vascular permeability, and inflammatory cell chemotaxis. Irvin et al. reported that aerosolization of C5a des Arg into rabbits causes neutrophil influx, decreased compliance, increased resistance, and increased responsiveness to aerosol histamine.[170] These respiratory effects are prevented by prior neutrophil depletion with nitrogen mustard,[170] or treatment with indomethacin.[20] Vascular permeability changes require C3a or C5a, a vasodilator prostaglandin such as PGE_2 or PGI_2, and neutrophils for a full effect.[27,179,355] C5a and C5a des Arg have neutrophil chemotactic activity equal to or greater than that of LTB_4 [130,249] and can also stimulate lysozomal enzyme release, oxygen-free radical production, and arachidonic acid metabolism in human neutrophils[59] and rabbit alveolar macrophages.[235]

Despite these many pro-inflammatory actions, the role of complement in asthma and airway hyperresponsiveness is unclear. The complement pathway can be activated by cotton dust, extracts of house mite dust, and plicatic acid,

which is responsible for asthma induced by western red cedar wood.[51,266,315] In a study of biopsy samples collected from 50 cases of severe adult onset asthma, Molina et al. reported that there is consistent evidence of association between IgA, IgG, and IgM, and the C3 component of complement localized mainly in the superficial portion of the basement membrane beneath the bronchial mucosa.[244] However, in a series of studies of circulating complement components in asthmatics during allergen-induced asthmatic attacks, no evidence for complement activation is found.[96,168,182,316] There have been no equivalent studies on complement activation in cases of equine COPD.

v) Sensory Neuropeptides. Sensory neuropeptides such as vasoactive intestinal peptide and substance P are localized to afferent C-fibers in the airways. They are synthesized in sensory neurones in the nodose and jugular ganglia and transported peripherally.[216] The precise physiological role of these peptides is not yet known, but in addition to their possible role as neurotransmitters in the non adrenergic-non cholinergoc nervous system, they are proinflammatory and potent secretagogues. Payan et al. showed that intradermal injection of substance P could induce a wheal and flare reaction, which is partially blocked by treatment with an H1 receptor blocker,[272] while Coles et al. demonstrated that canine tracheal explants treated with substance P have a dose-dependent increase in secretion of high molecular weight proteins predominantly from submucosal glands.[64] The wheal and flare reaction induced by intradermal administration of substance P, calcitonin gene-related peptide,

and neurokinin A is reduced by pretreatment with aspirin, suggesting that cyclooxygenase metabolites may be involved in the reaction.[11]

Sensory neuropeptides may be involved in neurogenic inflammation and amplification of the inflammatory response in airways of asthmatics, but direct evidence for such involvement awaits the development of specific antagonists suitable for clinical use.[13]

vi) Oxygen Radicals. Oxygen-derived molecules include superoxide anion (O_2^-), perhydroxyl radical ($HO_2^\cdot$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^\cdot OH$). These molecules can interact with cell proteins, lipids, and sugars, damaging membranes, receptors, and enzymes, and altering cell function.[256] Of these, hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) are produced enzymatically by neutrophils, eosinophils, and macrophages.[7] Production of these metabolites is enhanced during inflammatory reactions in the lungs and can result in further inflammation, epithelial injury, edema, and connective tissue deposition.[37]

There is no direct evidence for involvement of oxygen radicals in asthma or equine COPD, although these metabolites have been shown to affect airway smooth muscle in vitro. Hydrogen peroxide contracts canine lung parenchymal strips and bovine trachealis muscle, and these contractions are blocked by indomethacin and partially reversed by oxygen radical scavengers such as dimethylsulphoxide, reduced glutathione, and ascorbic acid.[317] In guinea pigs, H_2O_2 contracts tracheal strips to 50% of maximum carbachol contraction, and this action is inhibited by indomethacin.[14] Engels et al. studied isoproterenol-

induced relaxation of guinea pig tracheal rings exposed to supernatant from stimulated alveolar macrophages. They found that addition of macrophage supernatant significantly decreases isoproterenol-induced relaxation and that this effect is blocked by adding catalase or a hydroxyl radical scavenger.[103] These data suggest that beta receptors could have been damaged by oxygen radicals released from the alveolar macrophages. Whether such damage could occur with airway inflammation in vivo and result in increased airway responsiveness remains to be shown.

vii) Platelet Activating Factor. Platelet activating factor is an ether-linked phospholipid produced by many cell types including human eosinophils[205] and neutrophils,[214] rat alveolar macrophages,[3] rabbit platelets,[19] vascular endothelial cells,[47] and mouse bone-marrow cells.[240]

Platelet activating factor is able to reproduce many of the pathological changes associated with asthma, including bronchoconstriction, airway hyperresponsiveness, increased vascular permeability and edema formation, and activation of inflammatory cells. Intravenous injection of platelet activating factor in guinea pigs causes bronchoconstriction,[340] while in rabbits, 0.6 μg/kg of platelet activating factor/mg administered intravenously increases respiratory rate and pulmonary resistance and decreases dynamic compliance.[142] Cuss et al. administered platelet activating factor by aerosol to normal humans and reported a dose-dependent bronchoconstriction and increased responsiveness to methacholine challenge that persists for 7-28 days,[73] while Chung et al. reported that aerosolized platelet activating factor administered to dogs increases

pulmonary resistance 2-3 fold and significantly increases airway responsiveness to acetylcholine. Both of these effects are blocked by OKY 046, a platelet activating factor antagonist.[55] In rhesus monkeys, the threshold for platelet activating factor-induced bronchoconstriction is 1/600 and 1/20,000 that for LTD_4 and histamine, respectively,[270] which indicates the potency of this compound. Abraham et al. showed that prior treatment with WEB-2086, a platelet activating factor antagonist, significantly decreases the late response to aerosol *A. suum* antigen in sensitized sheep,[1] suggesting that platelet activating factor is involved in this response. Intradermal injection of as little as 52 picogram of platelet activating factor induces increased vascular permeability in guinea pigs,[166] and this action is potentiated by vasodilators such as PGE_2.[246,348]

Platelet activating factor aggregates and degranulates rabbit platelets and neutrophils,[263] while human eosinophils incubated with 10^{-9} to 10^{-7} platelet activating factor show dose-dependent enhancement of cytotoxicity to *Schistosoma mansoni* larvae.[220] Wardlaw et al. report that platelet activating factor in doses of 10^{-8} to 10^{-5} induces directional locomotion in human eosinophils in a time- and dose-dependent fashion,[345] and that this effect is much greater than that seen with histamine and LTB_4.

Despite its ability to induce airway inflammation, bronchoconstriction, and airway hyperresponsiveness, there is little direct evidence for the involvement of platelet activating factor in human asthma. Developing more effective antagonists, and more accurate methods for quantitating platelet

activating factor concentrations in blood and tissue samples will help to resolve the question of its involvement in both asthma and equine COPD.

viii) Cytokines. These are part of a rapidly increasing number of mediators that includes the interleukins, interferon-gamma, tumor necrosis factor, and stimulating factors, such as granulocyte/monocyte colony stimulating factor. These substances are important for cell-cell communication or interaction and their production and release can have marked effects on the capacity of inflammatory cells to synthesize and release mediators. Pretreatment of neutrophils with granulocyte/monocyte colony stimulating factor is essential if they are to produce LTB_4 when stimulated with C5a,[75] while interleukin-3 pretreatment markedly increases histamine release from human basophils stimulated with allergens.[195] Cytokines are also involved in communication between inflammatory cells and connective tissue cells such as fibroblasts. Here, inflammatory cells regulate fibroblast function, and fibroblasts in turn regulate inflammatory cell function.[100] In this complex network, the effect of an individual cytokine varies with the state of activation of the target cell, the presence of other cytokines in the local microenvironment, and the ability of the target cell to produce bioactive autocoids such as prostaglandins.[100]

Summary. As can be seen from the above brief description, there are a tremendous variety of inflammatory cells and mediators that could potentially play a role in the pathogenesis of airway disease. The mere fact that so many cells and mediators have been considered suggests that no single cell or mediator is likely to be responsible for airway obstruction and

hyperresponsiveness in any situation, but rather that the development of an inflammatory response is the product of extensive communication and interaction between inflammatory cells and other cell types in their immediate environment. If one mediator or class of mediators were to play a central role in such a process, then a strong candidate would be the metabolites of arachidonic acid. They are mediators produced by all of the cell types mentioned above, as well as by most other cells in the body, and appear to occupy a central role in the regulation of cell-cell interaction and normal or pathological processes in the body. Because of this, I have chosen to discuss them in greater detail in the next section.

Arachidonic Acid Metabolites

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is a long chain fatty acid found esterified at the 2 position of cell membrane phospholipids, particularly phosphatidyl inositol, phosphatidyl choline, and phosphatidyl ethanolamine.[62] Arachidonic acid can undergo a series of oxygenation reactions to yield 20-carbon oxygenated metabolites called eicosanoids, which have a wide range of physiological actions.[311] Eicosanoid synthesis requires the free, nonesterified form of arachidonate since the oxygenating enzymes will not act on substrate bound to phospholipid.[201] Release of arachidonic acid from membrane phospholipids can occur via the activation of phospholipase A2 or the combined activation of phospholipase C and a diacylglycerol lipase. This release step is the primary site for regulation of eicosanoid synthesis.

Once released, free arachidonate can undergo three possible fates:

1. Diffusion out of the cell,[314] with subsequent binding to albumin,[312] or incorporation and metabolism by other cells.[98]

2. Reacylation into membrane phospholipids via the actions of an arachidonoyl coenzyme A synthetase and an acyltransferase.[199,357]

3. Metabolism via the cyclooxygenase or lipoxygenase pathways.

Cyclooxygenase Pathway. The cyclooxygenase pathway is substrate limited in that the enzymes are always active but substrate is lacking unless released from membrane lipids. The initial steps in the pathway are catalyzed by prostaglandin endoperoxide synthase, a single protein with cyclooxygenase and peroxidase activity. The cyclooxygenase activity catalyzes the addition of two O_2 molecules to arachidonate, producing PGG_2, an unstable cyclic endoperoxide. The peroxidase activity then reduces PGG_2 to PGH_2, its 15-hydroxy analogue.[255] Generation of oxygen radicals during the peroxidase step results in enzyme inactivation, which may serve to limit in vivo prostaglandin synthesis.[265] The cyclooxygenase enzyme is also inhibited by aspirin-like drugs,[338] and this inhibition is limited to the cyclooxygenase activity.[243] The aspirin effect involves acetylation[286] of a single serine residue.[285] PGH_2 can be metabolized to thromboxane A_2 (TxA_2), and prostaglandins D_2 (PGD_2), E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$), and I_2, or prostacyclin (PGI_2). Both TxA_2 and PGI_2 are extremely labile in aqueous solution and are rapidly hydrolyzed to their inactive forms (TxB_2 and 6-keto-$PGF_{1\alpha}$ respectively).[200]

Lipoxygenase Pathway. Lipoxygenases catalyze incorporation of one oxygen molecule into polyunsaturated fatty acids containing a 1,4-cis,cis-pentadiene system to yield a 1-hydroperoxy-2,4-trans,cis-pentadiene product.[255] The regional specificity of the lipoxygenase is designated by the number of the carbon bearing the hydroperoxy group. The hydroperoxy group thus formed can then be reduced to its hydroxy analogue. A large number of lipoxygenases have been described, but in this review I will concentrate on metabolites of the 5- and 15-lipoxygenases that are thought to have a role in pulmonary disease and in asthma in particular.

i) 5-lipoxygenase. This is a calcium requiring enzyme that catalyzes the initial peroxidation of arachidonic acid to form 5-hydroperoxy-eicosatetraenoic acid (5-HPETE), a key intermediate in the formation of leukotrienes.[291] 5-HPETE is metabolized to leukotriene A_4 (LTA_4), an unstable epoxide that can be metabolized to leukotriene B_4 (LTB_4) by an epoxide hydrolase. An alternate pathway involves the addition of glutathione to the C-6 position of LTA_4 by a glutathione transferase, resulting in the formation of a group of sulfidopeptidal leukotrienes. The first of these is leukotriene C_4 (LTC_4). Removal of glutamic acid by gamma-glutamyl-transpeptidase converts LTC_4 to LTD_4 and removal of glycine by a different dipeptidase converts LTD_4 to LTE_4.[211] These three leukotrienes together constitute slow reacting substance of anaphylaxis (SRS-A).[255]

ii) 15-Lipoxygenase. This enzyme initially catalyzes the formation of a 15-hydroperoxy-eicosatetraenoic acid (15-HPETE), which can then undergo three

possible reactions. The first is a reduction to the 15-hydroxyeicosatetraenoic acid (15-HETE), the second is a dehydration to 14-15 LTA_4, and the third involves the formation of lipoxins A and B, which are trihydroxylated compounds with a conjugated tetraene structure.[255]

Individual Metabolites.

i) PGD_2. Prostaglandin D_2, released from human lung mast cells[293] and alveolar macrophages[219] is a potent constrictor of human airways, in vivo[149] and in vitro.[28] On a molar basis, PGD_2 is about 10 times more potent than histamine as a bronchoconstrictor. [149] PGD_2 also potentiates the bronchoconstrictor response to histamine and methacholine in asthmatics,[118] as well as histamine- and carrageenan-induced skin edema.[107] Mucus secretion is significantly increased in human airway tissue explants exposed to PGD_2.[227] Increased concentrations of PGD_2 are reported in bronchoalveolar lavage fluid during the acute asthmatic response.[253]

ii) $PGF_{2\alpha}$. $PGF_{2\alpha}$ contracts human airway smooth muscle in vitro,[28] and aerosol administration of $PGF_{2\alpha}$ in humans causes bronchoconstriction, which is more marked in asthmatics.[232] Sensitivity to $PGF_{2\alpha}$ in asthmatics challenged with antigen is positively correlated with the degree of bronchial responsiveness present.[233] Increased plasma concentrations of 15-keto-13,14-dihydro-$PGF_{2\alpha}$, a metabolite of $PGF_{2\alpha}$ are reported to occur in asthmatics during bronchoconstriction.[137] $PGF_{2\alpha}$ can also stimulate increased mucus production in vitro[281] and in vivo.[213] In dogs, subthreshold doses of $PGF_{2\alpha}$ increase airway responsiveness to acetylcholine aerosol challenge. This effect is inhibited by treatment with

hexamethonium, a ganglionic blocking agent that blocks sympathetic and parasympathetic nerve pathways,[258] implying that $PGF_{2\alpha}$ is acting by affecting nervous pathways.

iii) TxA_2. Thromboxane A_2 is released from human lung tissue during anaphylaxis[303] and can cause bronchoconstriction in vitro.[321] This contractile action may be the result of potentiation of cholinergic neurotransmission, since a TxA_2 mimetic (U46619) potentiates cholinergic neurotransmission in canine airways.[56] Allergen stimulation of atopic asthmatics results in increased levels of TxA_2 metabolites in urine, but inhibition of TxA_2 synthesis with indomethacin does not prevent the bronchoconstrictor response to allergen.[308] An increase in TxA_2 concentrations in plasma and bronchoalveolar lavage fluid samples from ponies with COPD has also been reported,[135] but here again, cyclooxygenase inhibition blocks TxA_2 production but does not improve pulmonary function. In contrast to this, treating asthmatics with a thromboxane synthetase inhibitor reduces bronchial hyperresponsiveness to acetylcholine without affecting baseline pulmonary function.[116]

iv) PGI_2. PGI_2 is synthesized predominantly by macrophages and pulmonary vascular endothelium,[164,225] and its production is increased after anaphylactic challenge of human and guinea pig lung fragments.[30,303] It causes relaxation of vascular and bronchial smooth muscle from humans, dogs, and guinea pigs in vitro,[119,180,347] and can protect against bronchoconstriction induced by PGD_2 and methacholine,[24,149] although it has little effect on normal airway caliber in monkeys[271] or normal and asthmatic humans.[148]

v) PGE_2. PGE_2 is synthesized by airway epithelium,[15,52,58,89,174,354] alveolar macrophages,[287] eosinophils,[165] and fibroblasts[100] in response to a variety of mediators. It is a bronchodilator in both normal and asthmatic humans,[74,183] although a bronchoconstrictor action has also been reported.[232] PGE_2 administration reverses bronchoconstriction caused by administration of $PGF_{2\alpha}$,[309] and it relaxes isolated human[322] and equine[124] tracheal smooth muscle, possibly by inhibiting acetylcholine release at a prejunctional site.[15] Decreased production of PGE_2 by tracheal epithelium is associated with endotoxin-induced hyperreactivity in guinea-pig isolated trachea.[108] In addition to its actions on smooth muscle, PGE_2 has an anti-inflammatory action, regulating production of tumor necrosis factor and interleukin 1 by macrophages.[193,194] This may form part of a negative feed-back system for regulating the inflammatory response, since both tumor necrosis factor and interleukin 1 stimulate production of PGE_2.[8,100] A similar anti-inflammatory action is reported during endotoxin challenge of sheep lungs, in which PGE_2 infusion attenuates pulmonary dysfunction, possibly by preventing endogenous release of other eicosanoids.[38] Sulfur dioxide-induced pulmonary inflammation and eosinophilic pneumonia are associated with increased bronchoalveolar lavage fluid concentrations of PGE_2,[257,264] whereas enhanced PGE_2 synthesis is reported from fibroblasts stimulated after exposure to mononuclear cell products[191] and from tracheal epithelium exposed to eosinophil-derived major basic protein,[174] PAF, and bradykinin.[354]

vi) 15-HETE. 15-HETE is produced by tracheal epithelial cells,[167] pulmonary endothelial cells,[176] polymorphonuclear leukocytes,[336] eosinophils,[332] lymphocytes,[128] and alveolar and pulmonary intravascular macrophages.[21] It is a major metabolite in homogenates of human lung tissue and is produced in greater quantities in tissues from asthmatics.[77,143,192] There is also a marked increase in bronchoalveolar lavage fluid 15-HETE concentrations collected from asthmatics following aerosol challenge with specific antigen.[253] Results from in vitro and in vivo experiments show that 15-HETE is a potent secretagogue,[178,228] causes neutrophil chemotaxis[178] and can activate[337] or inhibit[128,335] the 5-lipoxygenase enzyme, depending on the cell type. A 15-HETE metabolite, 8S,15S-diHETE is also chemotactic for canine neutrophils in airways.[187]

vii) Leukotrienes. These consist of the sufidopeptide leukotrienes (LTC_4, LTD_4, LTE_4) and the dihydroxy-eicosatetraenoic acid LTB_4. They are produced by a variety of cells, including lung tissue,[290] mast cells,[283] eosinophils,[351] alveolar macrophages,[231] and neutrophils.[289] LTB_4 is a potent neutrophil chemotactic and chemokinetic agent in vitro,[112] which can also induce attachment of leukocytes to vascular endothelial surfaces[162] and thus assist in migration of inflammatory cells from the vasculature to the tissues. LTB_4 has some smooth muscle constricting activity in vitro, but this action appears to be due to the production of thromboxane.[306] The sulfidopeptide leukotrienes are now known to constitute the biological activity previously known as slow-reacting substance of anaphylaxis (SRS-A). Allergen challenge is a potent stimulus for their release from sensitized human and animal lung tissue.[78] They can produce

increased vascular permeability[273,360] and smooth muscle contraction.[79,144] LTC_4 and LTD_4 are at least 1000 times more potent than histamine at causing bronchial smooth muscle contraction in vitro,[79,144] and 600 to 9500 times more potent bronchoconstrictors than histamine when administered by aerosol to humans.[350] LTE_4 is approximately one-tenth as potent as LTD_4 and has a longer duration of action.[80] Both $LTC_4$and LTD_4 are potent stimuli for mucus secretion from human airways in vitro,[63,226] and dog trachea.[177]

A number of observations suggest that the sulfidopeptide leukotrienes have a role in the pathogenesis of asthma. Polymorphonuclear leukocyte 5-lipoxygenase activity is increased in cells from asthmatics,[242] and asthmatics are hyperresponsive to inhaled leukotrienes.[26,80,310] Several studies report increased leukotriene concentrations in plasma[172,295] and urine[325] from asthmatics following an attack. However, experiments performed with currently available leukotriene antagonists in vivo have produced conflicting results. Treatment with a selective LTD_4/LTE_4-receptor antagonist improves pulmonary function in patients with mild, chronic asthma,[60] whereas L-649,923, a LTD_4 antagonist, has no significant protective effect against bronchoconstriction following allergen challenge.[39] The 5-lipoxygenase inhibitor/leukotriene receptor antagonist REV 5901 also fails to antagonize LTD_4-induced bronchoconstriction in asthmatics.[334] It appears that definitive proof of a role for leukotrienes in asthma awaits the development of more selective leukotriene antagonists.

Transcellular Eicosanoid Metabolism. As presented above, the capacity for individual cell types to generate eicosanoids has been well defined.

However, in the body, cells do not usually exist as pure populations isolated from other cell types, and there is growing evidence that when different cell types are mixed, transcellular transfer and metabolism of endogenous arachidonic acid can occur.[25,53,98,221] In addition to this, it appears that communication between cells can result in enhanced synthesis of normally produced eicosanoids.[46,188] These data suggest that cell-to-cell interaction is an important factor in determining the type and quantity of eicosanoids that are produced, and may be potentially important in inflammatory diseases such as asthma and COPD. One potentially important interaction that is receiving increasing attention is that between airway epithelium and inflammatory cells or airway smooth muscle. Airway epithelium is able to synthesize several eicosanoids, and in the final section of this review I will present some of the information currently available that supports the potential role of epithelium and epithelial-derived arachidonic acid metabolites in reactive airway disease.

Airway Epithelium

One of the characteristic features of both bronchial asthma in humans and COPD in horses is loss of, or damage to, airway epithelial cells. Histological examination of airway epithelial biopsies from asthmatics during remission[197] and following death in status asthmaticus[94] shows widespread epithelial damage, affecting principally ciliated epithelial cells. A relationship between epithelial damage and bronchial hyperreactivity is supported by reports that exposure to agents, such as ozone, which are known to damage the airway mucosa results

in increased bronchial reactivity. In guinea pigs, this hyperreactivity is related to signs of airway mucosal injury and preceeds neutrophil infiltration.[250]

Possible mechanisms by which epithelial damage may result in bronchial hyperresponsiveness include the following. Noxious stimuli could more readily interact with superficially located sensory nerve endings, which terminate in the epithelial layer, resulting in exaggerated vagal reflexes. Also, epithelial damage may cause release of chemotactic factors, resulting in an influx of neutrophils into the airways. Inflammatory mediators released by these cells may then result in tracheal smooth muscle hyperresponsiveness. Alternatively, epithelial cells may synthesize and release an epithelium-derived relaxant factor that normally modulates the response of tracheal smooth muscle to contractile stimuli. Epithelial cell destruction could decrease production of such a factor, with a consequent increase in tracheal smooth muscle responsiveness. Finally, epithelial cells may possess metabolic pathways for inactivating inflammatory mediators released into the airway lumen. For example, epithelial cells contain enzymes such as enkephalinase, which break down inflammatory peptides. Reduced levels of these enzymes during influenza infection have been associated with airway hyperreactivity.[173]

The identity or existence of an epithelium-derived relaxant factor is the subject of much debate. In the rabbit, mechanical removal of the epithelium from bronchial segments increases the sensitivity of bronchial smooth muscle to bethanecol.[44] In the guinea-pig, relaxation of tracheal smooth muscle exposed to arachidonate is converted to contraction following epithelium removal,[331]

while in ovalbumin-sensitized animals, epithelium removal causes a greater increase in sensitivity to antigen than to either methacholine or histamine.[150] In humans, contractile responses of bronchial smooth muscle to acetylcholine, histamine, and $PGF_{2\alpha}$ are significantly increased following epithelium removal, while subsequent addition of chopped epithelium to the muscle bath significantly decreases the contractile response to acetylcholine.[2] Contractile responses of dog tracheal smooth muscle to electrical field stimulation are significantly inhibited when the supernatant from bradykinin-stimulated epithelial cell cultures is added to the muscle bath.[15]

Immunoperoxidase staining of lung sections with a monoclonal antibody for cyclooxygenase shows intense staining in the epithelium, indicating that this cell layer possesses the capacity to synthesize prostaglandins.[44] In several preparations, the effects of epithelial removal can be reproduced by treating tissues with indomethacin, a cyclooxygenase inhibitor.[15,44,331] PGE_2 is a major arachidonic acid metabolite produced by intact epithelium and has properties similar to those described for epithelium-derived relaxant factor. Its production can be stimulated by a variety of substances, including eosinophil major basic protein,[174] neutrophils and reduced oxygen molecules,[181] bradykinin, platelet activating factor, and calcium ionophore (A23187).[354] PGE_2 relaxes respiratory airway smooth muscle and inhibits contractile responses evoked in intact and epithelium-denuded tracheal preparations.[32] Endotoxin-induced airway hyperreactivity in guinea-pigs coincides with decreased PGE_2 production by the epithelial layer, which is thought to be due to decreased mobilization of

arachidonic acid from membrane phospholipids rather than reduced activity of the cyclooxygenase enzyme.[108] PGE_2 inhibits the initiation of excitatory junction potentials and contractile responses to electrical field stimulation in tracheal smooth muscle. It also depresses responses to acetylcholine but to a significantly lesser extent, which is consistent with a major presynaptic and lesser postsynaptic inhibitory action.[22,343] It is possible that a decreased production of PGE_2 by airway epithelium from ponies with COPD could remove this modulating action on acetylcholine release and increase the responsiveness of airway smooth muscle to stimuli that act via parasympathetic mechanisms and stimulate acetylcholine release.

Based on my understanding of the literature, there was evidence for altered arachidonic acid metabolism in human asthma as well as other animal models of airway hyperresponsiveness. Metabolites of particular interest for human asthma were TxA_2, PGD_2 and 15-HETE, whereas in the dog, TxA_2 appeared to be an important mediator. My initial aim was to determine whether similar alterations could be detected in ponies and horses with COPD. If such alterations were detected, I intended to try and identify the site of the altered eicosanoid production, and also to study the significance of these changes by using appropriate inhibitors to block eicosanoid production where possible. Experiments designed to help achieve these aims are presented in the next five chapters.

CHAPTER 2

MEASUREMENT OF PLASMA AND BRONCHOALVEOLAR FLUID CONCENTRATIONS OF EICOSANOIDS IN PONIES WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE (HEAVES)

Introduction

Arachidonic acid is a ubiquitous, esterified fatty acid found in cell membranes. It is a precursor for a large group of C20 fatty acids or eicosanoids that have a broad range of biologic actions.[311] Eicosanoids are produced by lung tissue,[365] and their actions include smooth muscle contraction,[17,321] leukocyte chemotaxis,[33,178] stimulation of mucus secretion,[177,178,226,227] and increased vascular permeability.[222] In addition, eicosanoids can produce bronchoconstriction when inhaled by animals or human volunteers.[17,149,309] Because these alterations are all characteristic features of asthma, it has been proposed that eicosanoids may play a role in the pathogenesis of this disease.[111] However, reports documenting the isolation of eicosanoids during episodes of clinical disease are less common, as are reports correlating alterations in eicosanoid concentrations with the onset or severity of asthma.[152] Elevated leukotriene (LT) C_4 concentrations have been reported in plasma from children during acute asthma attacks,[295] and an

increase in 15-hydroxyeicosatetraenoic acid (15-HETE) and prostaglandin (PG)D_2 concentrations occurs after antigen challenge in asthmatics.[253] Asthmatics also have increased concentrations of PGD_2, $PGF_{2\alpha}$, and 9α,11β-PGF_2 in samples obtained by bronchoalveolar lavage when compared with non-asthmatics.[212]

The similarities between human asthma and COPD in ponies have been well documented with reference to the occurrence of airway obstruction and non-specific airway hyperresponsiveness.[6,40,82,87,296] There had been no studies to determine whether the association between altered eicosanoid concentrations and pulmonary dysfunction seen in humans asthma was also present in ponies with COPD.

To test this possibility, I elected to measure plasma and bronchoalveolar lavage fluid concentrations of the lipoxygenase metabolite, 15-HETE, and the cyclooxygenase metabolites, thromboxane (Tx)A_2, PGI_2, and PGD_2.

Studies using lung tissue from asthmatics have shown that TxA_2 and PGI_2 are the major cyclooxygenase products formed, and that PGE_2, PGD_2, and $PGF_{2\alpha}$ occur in lesser concentrations.[78] TxA_2 contracts guinea pig bronchial smooth muscle in vitro and in vivo,[321] and inhibition of TxA_2 synthesis decreases bronchial hyperresponsiveness to inhaled acetylcholine in asthmatics.[116] In a series of experiments studying airway hyperresponsiveness in dogs, O'Byrne et al. showed that neutrophil-derived TxA_2 was responsible for the increased responsiveness that occurred following ozone exposure or aerosol challenge with PAF and LTB_4.[259,260,261] PGI_2 relaxes bronchial smooth muscle in vitro,[119] but its effects in studies performed in vitro are less conclusive. It has

little effect on dynamic compliance or airway resistance when administered by aerosol to rhesus monkeys[271] and no consistent effect on airway caliber when administered as an aerosol to normal and asthmatic subjects.[148] 9α,11β-PGF_2 is a metabolite of PGD_2 in human lung,[298] and a contractile agonist of isolated guinea pig and human airways. It also produces bronchoconstriction in humans when inhaled.[17] 15-HETE, a major metabolite produced in homogenates of human lung tissue,[143] is produced in greater quantities in tissues from asthmatics.[78,143,192] A marked increase in 15-HETE concentrations in bronchoalveolar lavage fluid from asthmatics following aerosol challenge with specific antigen was reported by Murray et al.[253] 15-HETE is a potent secretagogue,[178,228] causes neutrophil chemotaxis,[178] and is involved in regulation of leukotriene synthesis.[335-337]

My hypothesis was that the airway obstruction and hyperresponsiveness measured in ponies with COPD is associated with increased production of 15-HETE or a bronchoconstrictor eicosanoid such as TxA_2 and PGD_2, or decreased production of a bronchodilator prostaglandin such as PGI_2. To test this hypothesis I measured plasma and bronchoalveolar lavage fluid concentrations of these substances or their stable metabolites.

Methods

Five mixed-breed ponies with a history of heaves (principals) were matched for age and sex with five ponies with no history of heaves. Each pony had a chronic tracheostoma created in the midcervical region and a carotid

artery that had been relocated to a subcutaneous site in the neck. Ponies were paired (principal and control) and each pair was fed, housed, and transported together to ensure that they received the same environmental exposures.

Experimental Protocol. Baseline pulmonary function was measured when ponies had been kept at pasture for 2 months with no exposure to hay, straw, or a barn environment (Period A). They were then housed in a barn, bedded on straw, and fed hay. Hay was shaken beside the ponies 3 times daily to increase airway exposure to hay dust. When the principal pony developed clinical signs of airway obstruction, pulmonary function tests were repeated on both ponies (Period B). In addition to pulmonary function measurements, plasma and bronchoalveolar lavage fluid samples were collected at Periods A and B, and concentrations of 15-HETE were measured. Concentrations of the stable metabolites TxB_2, 6-keto-$PGF_{1\alpha}$, and $9\alpha,11\beta$-PGF_2 were also measured as estimates of TxA_2, PGI_2, and PGD_2 concentrations, respectively.

Pulmonary Function Measurements. Each pony was tranquilized with intravenously administered xylazine (Rompun; Haver Lockart, Shawnee Missions, KS) (0.5 mg/kg) and restrained in stocks. A 45-cm-long, cuffed endotracheal tube (20-mm internal diameter) was inserted into the trachea via the tracheostoma. A pneumotachograph (Fleisch No. 4; Dynasciences, Blue Bell, PA) and associated pressure transducer (DP45-22; Validyne, Northridge, CA) were attached to the end of the endotracheal tube. The pneumotachograph transducer system produced a signal proportional to flow that was electronically integrated to give tidal volume. Prior to each experiment, this system was

calibrated by forcing a known volume of air through the pneumotachograph using a 2-L syringe (Super syringe; Hamilton Syringe Co., Warminster, PA). An esophageal balloon (10-cm length, 3.5-cm perimeter, 0.06-cm wall thickness) was sealed over the distal end of a polypropylene catheter (3-mm internal diameter, 4.4-mm outer diameter, 140-cm length) that had several spirally arranged holes in the portion covered by the balloon. The balloon volume was adjusted to 0.5 ml with room air, and the catheter was inserted via the nares until the balloon was positioned in the midthoracic esophagus. The external end of the catheter was then connected to a pressure transducer (DP45-34; Validyne) for measurement of esophageal pressure.

Transpulmonary pressure was defined as the pressure difference between atmospheric and esophageal pressure. Transpulmonary pressure, tidal volume, and airflow were recorded on light-sensitive paper (VR12; Electronics for Medicine, White Plains, NY). Dynamic compliance, pulmonary resistance, minute ventilation, and frequency were calculated by a pulmonary function computer (Model 6; Buxco Electronics, Sharon, CT) and data logger (Model DL12; Buxco). Blood samples were collected from the exteriorized carotid artery and analyzed for arterial oxygen tension and arterial carbon dioxide tension using an automated blood gas analyzer (Model ABL 3; Radiometer, Copenhagen, Denmark).

Bronchoalveolar Lavage. A 1-m-long, 12-mm-diameter, fiberoptic endoscope (American Cytoscope Makers, Pelham Manor, NY) was inserted through the tracheostoma. Three ml of 2.0% lidocaine were used to desensitize

the airways, and the endoscope was wedged in a left or right ventral lobe bronchus. Three 100-ml aliquots of room-temperature, phosphate-buffered saline were infused through the biopsy channel and gently aspirated. An average of 50% of the infused fluid was recovered. The recovered fluid was filtered through a double layer of gauze into a chilled graduated cylinder that contained sufficient indomethacin to provide a final concentration of 10 μM/ml in 300 ml of bronchoalveolar lavage fluid. The lavage fluid was centrifuged (800 g for 15 min) at 4°C, the supernatant was drawn off, and lipid extractions were performed.

Plasma Collection. Sixty mls of blood were collected from the external jugular vein into three 20-ml syringes via a 14-gauge needle. Each syringe contained 200 μL of a 15% EDTA solution, 0.3 mg of indomethacin, and 100 μL of eicosatetraynoic acid solution (48 mg in 2 ml of dimethylsulfoxide). To minimize the problem of iatrogenic eicosanoid formation, we used a standardized collection technique. The external jugular vein was occluded until it became visibly distended, then a 14-gauge, 4-cm-long hypodermic needle was inserted into the center of the distended vein. When blood flowed freely from the end of the needle, a syringe was connected to the needle and gentle suction was applied. Using this technique, 20-ml of blood syringe could be withdrawn in 15 seconds. After blood collection, each syringe was gently inverted to mix the blood with anticoagulant, indomethacin, and eicosatetraynoic acid; the syringe was then placed in an ice-bath. Blood samples were combined and centrifuged (800 g for 10 min). The plasma was drawn off, recentrifuged (1100

g for 10 min) to ensure that no further cells remained, and lipid extractions were performed.

Extraction and Measurement of Eicosanoids. Extraction of TxB_2 and 6-keto-$PGF_{1\alpha}$ was performed using the method of Powell.[277] Plasma (5 ml) and bronchoalveolar lavage fluid (40 ml) samples were acidified to pH 3.0 to 3.5 with 1 N HCl. The acidified sample was centrifuged (800 g for 5 min) and the supernatant placed on a preconditioned Sep-Pak C_{18} column, washed sequentially with distilled water, ethanol:water (15:85), petroleum ether, and then eluted with methyl formate. A similar extraction technique was used for 9α,11β-PGF_2, except that the ethanol:water step was followed with elution in ethyl acetate. Samples for 15-HETE extraction were acidified to pH 4.0-4.5 with 1 N H_3PO_4 before being placed on the Sep-Pak, then washed with 20 ml of distilled water, and eluted with 20 ml of ethyl acetate. Eluates were dried under a stream of nitrogen at room temperature and stored under nitrogen at -70°C until ready to be assayed.

Samples for 15-HETE and 9α,11β-PGF_2 measurement were resuspended in 500 μl of methanol:water (70:30) and duplicate 100 μL aliquots were subjected to radioimmunoassay. Tritiated 15-HETE (New England Nuclear, Boston, MA) (35-40Ci/mM) diluted to 1:200 to give approximately 5000 cpm, and rabbit anti-15-HETE antibody (The Upjohn Company, Kalamazoo, MI) were used for the 15-HETE assay.

Tritiated 9α,11β-PGF_2 was obtained from New England Nuclear and rabbit anti-9α,11β-PGF_2 antibody from The Upjohn Company. Samples

containing TxB_2 were dissolved in 50 μL of methanol and then 950 μL of distilled water was added. The samples were centrifuged and duplicate 100-μL aliquots taken for radioimmunoassay. A similar technique was used for samples containing 6-keto-$PGF_{1\alpha}$, except that ethyl acetate was used to dissolve the samples. Antibodies and standards for the TxB_2 and 6-keto-$PGF_{1\alpha}$ assays were obtained from Amersham (Arlington Heights, IL) and tritiated TxB_2 and 6-keto-$PGF_{1\alpha}$ from New England Nuclear. Incubations were performed at room temperature for 60 minutes, then at 4°C for 20 hours. Separation of bound from unbound trace or sample/standard was achieved by adding 1 ml ice-cold dextran-coated charcoal (5 mg Norit A and 0.5 mg Dextran T-70 per ml of 0.1 M phosphate buffered saline) to each tube, allowing the tubes to stand on ice for 10 minutes, then centrifuging at 0°C (1200 rpm for 12 min). One ml aliquots of supernatant were then added to 15 ml of Safety Solve radioimmunoassay scintillation cocktail and counted in a liquid scintillation counter (Beckman, Palo Alto, CA).

Statistical Analysis. Measured values for individual animals at Periods A and B were compared using Student's t test for paired values, while values for principal and control animals at each time period were compared using Student's t test for unpaired values. Significance was set at $p < 0.05$.

Results

Pulmonary Function Measurements. At Period A, pulmonary function measurements were similar in control and principal ponies, except for

pulmonary resistance, which was significantly greater in the principals (Table 2-1). After barn exposure (Period B), there was no change in lung function in the control group, but in the principal group there was a significant decrease in arterial oxygen tension and dynamic compliance and a significant increase in pulmonary resistance and respiratory frequency.

Eicosanoid Concentrations. Plasma and bronchoalveolar lavage fluid concentrations of 15-HETE, TxB_2, 6-keto-$PGF_{1\alpha}$, and 9α,11β-PGF_2 were similar in control and principal ponies at period A (Table 2-2). At period B, plasma 15-HETE and TxB_2 concentrations increased significantly in the principals, but no other significant changes were found. The increase in plasma 15-HETE was consistent for each of the principal ponies (Fig 2-1).

Table 2-1 Pulmonary function measurements in principal and control ponies at pasture (A) and when principal ponies had acute airway obstruction (B) (n = 5).

	Principal		Control	
	A	B	A	B
PaO_2 (mmHg)	92.7 ± 4.2	68.3 ± 3.8*+	90.9 ± 4.1	99.6 ± 6.6
$PaCO_2$ (mmHg)	40.4 ± 1.1	39.1 ± 1.5	39.1 ± 1.7	37.8 ± 1.4
Cdyn (L/cmH_2O)	0.62 ± 0.13	0.13 ± 0.03*+	1.00 ± 0.20	0.94 ± 0.18
R_L ($cmH_2O/L/sec$)	1.32 ± 0.21+	5.81 ± 1.58*+	0.50 ± 0.05	0.59 ± 0.05
f (breaths/min)	14.5 ± 1.7	23.8 ± 2.7*+	11.6 ± 1.4	12.1 ± 1.3
V_T (L)	2.31 ± 0.35	1.63 ± 0.20	2.20 ± 0.27	2.14 ± 0.24
V_E (L)	31.4 ± 2.8	39.4 ± 7.8	23.5 ± 2.0	24.6 ± 1.8

Cdyn = dynamic compliance, R_L = pulmonary resistance. Values are means ± SEM. * Significant difference from period A ($p < 0.05$). + Significant difference from control group at period B ($p < 0.05$).

Table 2-2 Plasma and bronchoalveolar lavage (BAL) fluid concentrations of TxB_2, 6-keto-$PGF_1\alpha$, and $9\alpha,11\beta$-PGF_2 in principal and control ponies at pasture (A) and when principal ponies had acute airway obstruction (B) (n = 5).

	Principal		Control	
	PLASMA			
	A	B	A	B
TxB_2 (pg/ml)	102.0 ± 47.0	314.8 ± 64.6*	85.0 ± 26.2	172.6 ± 47.3
6-k-$PGF_1\alpha$ (pg/ml)	33.0 ± 2.30	38.3 ± 7.7	21.3 ± 5.6	28.0 ± 7.6
$9\alpha,11\beta$-PGF_2 (ng/ml)	3.4 ± 3.5	2.8 ± 1.8	3.0 ± 1.8	2.8 ± 2.1
15-HETE (ng/ml)	101.0 ± 15.2	463.0 ±163.0*	206.3 ± 51.0	107.2 ± 42.6
	BAL FLUID			
TxB_2 (pg/ml)	16.5 ± 9.7	37.5 ± 14.5	18.1 ± 4.8	25.4 ± 5.9
6-k-$PGF_1\alpha$ (pg/ml)	6.1 ± 3.6	7.5 ± 2.2	2.8 ± 1.2	5.7 ± 1.1
$9\alpha,11\beta$-PFG_2 (ng/ml)	1.2 ± 0.5	0.9 ± 0.3	1.2 ± 0.3	1.4 ± 0.5
15-HETE (ng/ml)	9.3 ± 2.0	8.4 ± 2.7	5.4 ± 1.0	6.4 ± 0.7

Values are means ± SEM. * = Significant difference from period A ($p < 0.05$)

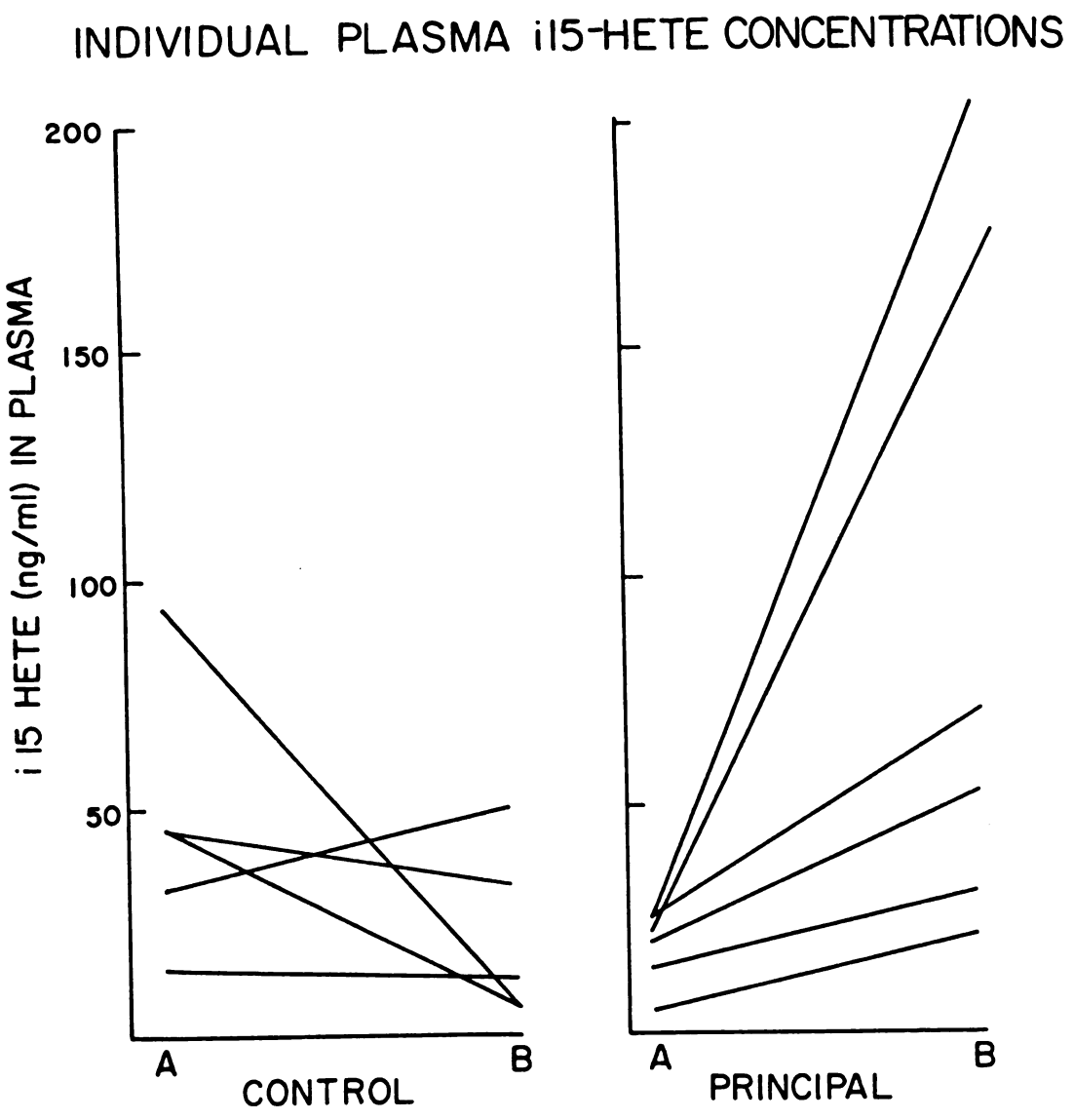


Figure 2-1 Immunoreactive 15-HETE concentrations in plasma samples from individual principal and control ponies collected at pasture (Period A) and when principal ponies had acute airway obstruction (Period B).

Discussion

A significant increase in plasma TxB_2 and 15-HETE occurred in the principal ponies at Period B, which coincided with the development of airway obstruction. No other significant changes in eicosanoid concentration were seen in either group of ponies at either Period A or B.

Platelets are a major source of TxA_2,[311] but other cells capable of synthesizing TxA_2 include macrophages, monocytes, neutrophils, and intrapulmonary endothelial cells.[152,259] Plasma TxB_2 can be an imprecise indicator of endogenous TxA_2 synthesis, particularly because of artifactual formation of thromboxane during sample collection.[133,134] This iatrogenic TxB_2 formation can be reduced by using a cyclooxygenase inhibitor in the collection container, free-flowing collection techniques rather than vacutainer tubes, and adequate amounts of anticoagulant in the collection container to prevent activation of the clotting process.[134] My sampling method was designed to minimize this problem, but despite these precautions some artifactual TxB_2 production could have occurred during sample collection. In this study, I was examining relative changes in plasma eicosanoid concentrations. If alterations in plasma TxB_2 were the result of iatrogenic formation, I would have expected a random distribution of such error between Periods A and B. However, all 5 principal ponies showed an increase in plasma TxB_2 at Period B relative to Period A, suggesting that the alteration in plasma TxB_2 was related to barn exposure.

A similar uniform increase in plasma 15-HETE occurred between Periods A and B in principal ponies. Previous experiments using affected

ponies have shown that airway hyperresponsiveness as well as airway obstruction is present at period B. Airway hyperresponsiveness and airway obstruction are also characteristics of human asthma, and several studies have reported an association between asthma and 15-HETE production in humans.[77,143,192,253] Hamberg et al. identified 15-HETE as a major arachidonic acid metabolite in homogenates of human lung. Samples of lung tissue from asthmatics and non-asthmatics were studied, and it was found that conversion of arachidonic acid to 15-HETE was greatest in lung tissue from asthmatics.[143] Similar results have been reported in vitro by other investigators.[77,192] Murray et al. reported increased 15-HETE production in vivo in asthmatic subjects.[253] 15-HETE can be produced by tracheal epithelial cells,[160,161,167] pulmonary endothelial cells,[176] polymorphonuclear leukocytes,[336] eosinophils,[332] lymphocytes,[128] and alveolar and intravascular macrophages.[21] The changes in 15-HETE production that we measured in this study provide another characteristic shared by both equine COPD and human asthma.

While there was a consistent increase in i15-HETE between periods A and B with each of the principals, the quantities of i15-HETE measured were very high (greater than 200 nanograms per ml of plasma in some ponies). This suggested that there could be some other factor present in the plasma that was cross-reacting with the anti-15-HETE antibody. If this were so, then high performance liquid chromatographic separation of samples would be needed to establish a more accurate estimate of plasma i15-HETE concentrations.

The first step in demonstrating the possible involvement of a mediator in a disease such as equine COPD is to show that measurable increases in the proposed mediator occur during disease episodes. The second is to block the action of the mediator, either by preventing its production, or by blocking its action with a receptor antagonist. O'Byrne et al and Chung et al [55,259-261] showed that airway hyperresponsiveness and airway obstruction in dogs is associated with increases in neutrophils and TxB_2 concentrations in bronchoalveolar lavage fluid. This hyperresponsiveness could be prevented by prior administration of the cyclooxygenase inhibitor indomethacin, or a specific thromboxane synthase inhibitor, OKY 046.

Having shown that TxB_2 production was increased in principal ponies at Period B, I therefore sought to determine whether, as in the dog, TxA_2 contributed to the airway obstruction and hyperresponsiveness by blocking its production and determining whether this altered pulmonary function in any way.

CHAPTER 3

EFFECT OF CYCLOOXYGENASE INHIBITION ON PULMONARY FUNCTION AND AIRWAY REACTIVITY IN PONIES WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Introduction

The first step in demonstrating the possible involvement of a mediator in a disease such as heaves is to show that increases in the proposed mediator occur during disease episodes. In the first experiment I determined that plasma concentrations of TxB_2 and 15-HETE were increased in principal ponies during the occurrence of acute airway obstruction.

The next step is to block the action of the mediator, either by preventing its production, or by using a receptor antagonist, and seeing whether this prevents the disease. No specific 15-lipoxygenase inhibitor was currently available for in vivo use, but TxA_2 production could be blocked with either cyclooxygenase or thromboxane synthase inhibitors. I therefore sought to determine the significance of the increased TxA_2 production measured in the first experiment by blocking its production and determining what effect this had on the development of airway obstruction or airway hyperresponsiveness

during the acute disease phase. In humans, cyclooxygenase inhibitors may exacerbate[339] or lessen[190] the symptoms of asthma, but in the majority of cases they have no effect on either the severity or duration of these symptoms.[320] No equivalent studies have been performed on the effect of cyclooxygenase inhibition on heaves in horses.

The inhibitor that I chose for this second experiment was flunixin meglumine, a non-steroidal cyclooxygenase inhibitor commonly used in horses and ponies,[206] which effectively blocks TxA_2 production without adverse side effects at clinically used dosages.[146,299] The problem with using a broad inhibitor such as flunixin meglumine was that it was not possible to examine the role of any single cyclooxygenase metabolite in isolation. While blocking production of bronchoconstrictor eicosanoids such as TxA_2 it also blocked production of PGE_2 and PGI_2, which may function to counteract the effects of TxA_2. The use of a specific thromboxane synthase inhibitor could have overcome this problem, but a safe, effective inhibitor was not currently available for use in the horse. In the one reported use of a thromboxane synthase inhibitor in horses, the inhibition that resulted lasted less than two hours after intravenous administration, and was markedly less effective than that produced with flunixin meglumine.[146]

The hypothesis that I set out to test in this experiment was that TxA_2 production is important in the pathogenesis of heaves and that inhibition of its production with flunixin meglumine would prevent or ameliorate the airway

obstruction and/or airway hyperresponsiveness to histamine that develops when affected ponies are housed in a barn.

Methods

Experimental Protocol. I used the same five pairs of ponies that had been used in the first experiment in a crossover experimental design. After baseline pulmonary function was measured, and a histamine dose-response curve was generated (Period A Baseline), each pair of ponies was treated with either flunixin meglumine (Banamine; Schering, Kenilworth, NJ) (1.1 mg/kg intramuscularly, 3 times daily) or a similar volume of 0.9% sodium chloride solution. Treatment continued while the ponies remained at pasture for 48 hours, then the pulmonary function tests and histamine dose-response challenge were repeated to identify any effect of flunixin meglumine on baseline airway reactivity or pulmonary function (Period A Treated). The ponies were then placed in the barn as described in the first experiment and drug treatment was continued until the principal pony developed clinical signs of heaves. At this time, pulmonary function and histamine dose-response measurements were again performed (Period B). Each pair was returned to pasture for an additional two months, after which the protocol was repeated using the alternate treatment.

At a dose of 1.1 mg/kg or less, flunixin meglumine is an effective cyclooxygenase inhibitor.[146,299] To confirm that this dose was effective in inhibiting cyclooxygenase under our experimental conditions, two ponies were

treated with either flunixin meglumine (1.1 mg/kg intramuscularly, 3 times daily) or a similar volume of 0.9% sodium chloride solution for 48 hours. At the end of the treatment period, blood samples were collected for TxB_2 measurement, then both ponies were challenged with intravenously administered endotoxin (Escherichia coli 055:B5; Sigma Chemical Co., St. Louis, MO) (10 μg/kg), a potent stimulus for arachidonate release and metabolism in ponies.[299] Blood samples were collected for TxB_2 measurement 30 minutes after endotoxin administration.

Pulmonary Function Measurements. These were performed using the same methods as those reported in Chapter 1.

Airway Reactivity Measurements. Airway reactivity was determined by generating dose-response curves to aerosol histamine. Histamine diphosphate (Sigma) was dissolved in sterile 0.9% NaCl, and solutions of 0.001, 0.01, 0.1, 0.3, 1.0, 3.0, and 10.0 mg of histamine diphosphate/ml were prepared. These solutions were aerosolized using an ultrasonic nebulizer (Model 65; DeVilbiss, Somerset, PA) that delivers a particle size of 0.5-3.0 μm. The nebulizer was connected to a two-way nonrebreathing valve that was then attached to the endotracheal tube. Ponies breathed the aerosol spontaneously for two minutes, then the two-way valve was disconnected from the endotracheal tube and replaced with the pneumotachograph. Beginning one minute after the end of each aerosol challenge, transpulmonary pressure, tidal volume, and airflow were recorded for two minutes and used to calculate dynamic compliance and pulmonary resistance. Exactly four minutes after the end of each challenge, another

aerosol challenge was begun. The measurement sequence consisted of a baseline reading followed by saline aerosol, then successive doses of histamine diphosphate solution. Increasing concentrations of histamine were administered until dynamic compliance decreased to 50% of the baseline value.

Dose-response curves of dynamic compliance and pulmonary resistance were plotted as a function of histamine concentration in the aerosol. By interpolation between points on the dose-response curve, I calculated the dose of histamine required to decrease dynamic compliance to 65% of the baseline value ($ED_{65}Cdyn$). I also calculated the change in pulmonary resistance induced by administration of 0.1 mg/ml histamine ($\Delta R_L[0.1]$).

Blood Sampling and TxB_2 Measurement. Blood samples were collected from the external jugular vein using an 18-gauge needle and 20-ml plastic syringe containing 0.2 ml of 15% EDTA solution and 0.3 mg of indomethacin. Syringes were capped, gently inverted to mix inhibitor and anticoagulant, and then placed in ice. Extractions and radioimmunoassay were performed as described in Chapter 1.

Statistical Analysis. The effects of treatment, barn exposure, and pasture were analyzed using a split-plot factorial analysis of variance. When F values were significant at $p < 0.05$, means from each measurement period were compared using Tukey's omega procedure.

Results

Pulmonary Function Measurements. Pulmonary resistance, dynamic compliance, and arterial oxygen tension were not significantly different in principals and controls at A baseline (Table 3-1). Treatment with flunixin meglumine of saline for 48 hours while ponies were kept at pasture produced no alteration in pulmonary resistance, dynamic compliance, or arterial oxygen tension (A treatment) (Table 3-1). The increase in pulmonary resistance and decreases in dynamic compliance and arterial oxygen tension seen in principal ponies housed in the barn were similar for saline- and flunixin meglumine-treated ponies (Table 3-1). Although the magnitude of the pulmonary function changes was not affected by flunixin meglumine treatment, the speed with which these changes developed was increased by cyclooxygenase inhibition. The average time taken for principal ponies to develop clinical signs was reduced from 12.2 to 5.1 days. This reduction, while marked, was not significant due to the small sample size.

Table 3-1 Pulmonary function measurements in principal and control ponies. Measurements were made at pasture (A-Baseline), after treatment at pasture for 48 hours with flunixin meglumine or saline (A-Treatment), and when principal ponies had acute airway obstruction (B).

		A-Baseline			A-Treatment			B		
Group	Treatment	C_{dyn}	R_L	PaO_2	C_{dyn}	R_L	PaO_2	C_{dyn}	R_L	PaO_2
Principal	Saline	0.79 ± 0.15	0.73 ± 0.10	84.1 ± 4.9	0.83 ± 0.13	0.68 ± 0.09	89.5 ± 5.4	0.25*+ ± 0.04	3.74*+ ± 0.69	64.0*+ ± 3.7
	Flunixin	0.92 ± 0.26	0.80 ± 0.15	82.4 ± 3.3	1.04 ± 0.28	0.85 ± 0.07	89.6 ± 2.1	0.32*+ ± 0.12	3.51*+ ± 0.32	69.4*+ ± 2.2
Control	Saline	0.65 ± 0.09	0.53 ± 0.12	87.0 ± 3.0	0.58 ± 0.08	0.66 ± 0.20	88.7 ± 5.6	0.66 ± 0.10	0.65 ± 0.10	92.3 ± 4.4
	Flunixin	0.94 ± 0.22	0.55 ± 0.14	90.0 ± 5.4	0.76 ± 0.09	0.57 ± 0.07	88.4 ± 3.4	0.69 ± 0.14	0.99 ± 0.10	89.9 ± 2.6

Values are mean ± SEM

* = significant difference from A-Baseline and A-Treatment ($p < 0.05$)

+ = significant difference from control ponies at B ($p < 0.05$)

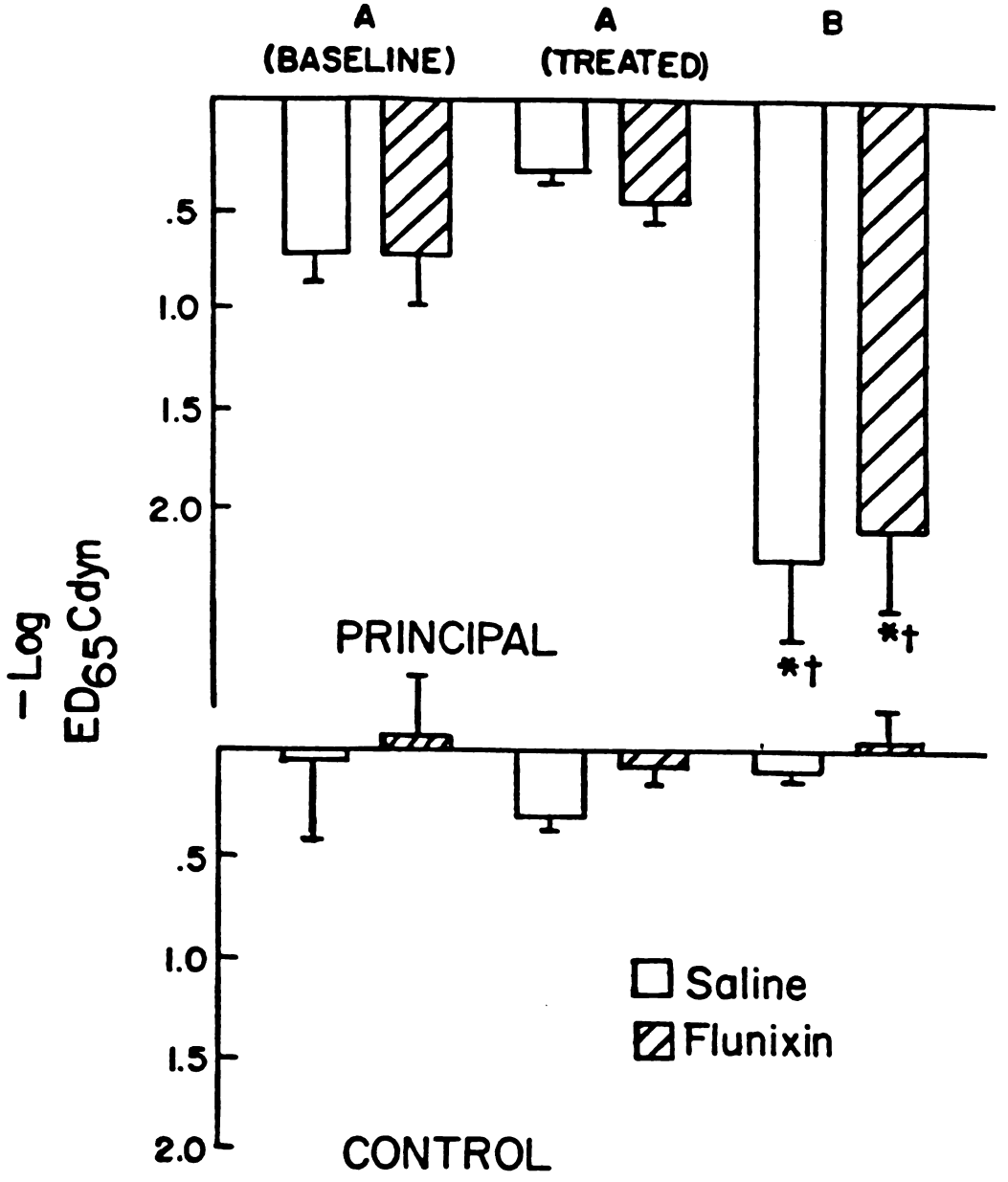


Figure 3-1 Log dose of histamine resulting in a decrease in dynamic compliance to 65% of baseline value ($ED_{65}Cdyn$) in saline- or flunixin meglumine-treated ponies. Measurements were made at pasture (A Baseline), after treatment at pasture for 48 hours with either flunixin meglumine or saline (A Treated), and when principal ponies had acute airway obstruction (B).

Values are mean $\pm$ SEM, n = 5.
* = significant difference from A Baseline and A Treated ($p < 0.05$)
+ = significant difference from control ponies ($p < 0.05$)

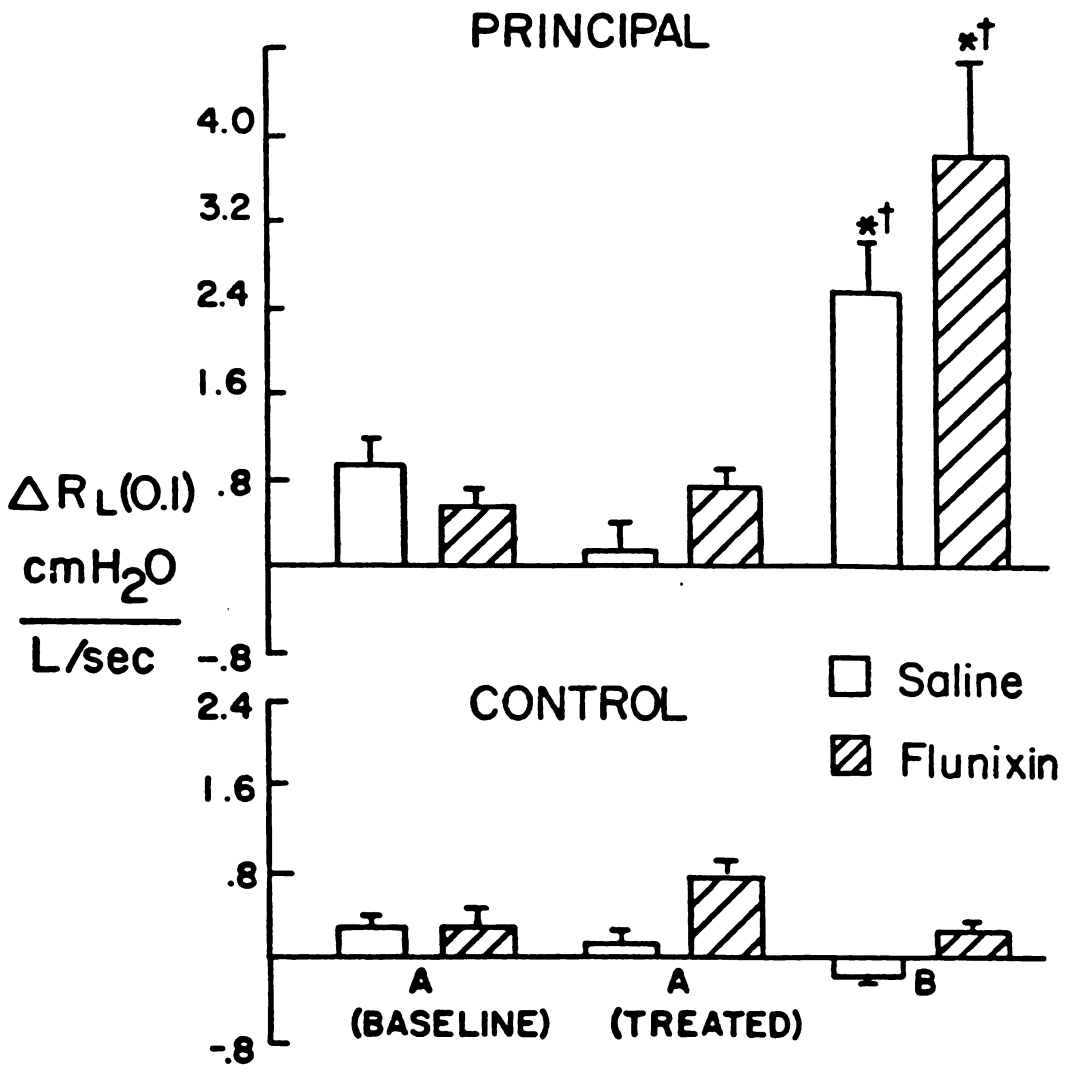


Figure 3-2 Change in pulmonary resistance in response to an aerosol of 0.1 mg/ml histamine diphosphate ($\Delta R_L[0.1]$) in saline- and flunixin meglumine-treated ponies. Measurements were made at pasture (A Baseline), after treatment at pasture for 48 hours with either flunixine meglumine or saline (A Treated), and when ponies had acute airway obstruction (B).

Values are mean $\pm$ SEM, n = 5.
* = significant difference from A Baseline and A Treated ($p < 0.05$).
+ = significant difference from control ponies at B ($p < 0.05$).

Airway Reactivity Measurements. No significant differences in $ED_{65}Cdyn$ and $\Delta R_L[0.1]$ between principal and control ponies were seen at the A baseline or A treated time periods. Within each group, there were no significant differences in $ED_{65}Cdyn$ or $\Delta R_L[0.1]$ after 48 hours of treatment with either flunixin meglumine or saline. At Period B, $ED_{65}Cdyn$ decreased significantly in the principal group but was unchanged in the control group (Figure 3-2). A significant increase in $\Delta R_L[0.1]$ occurred in the principals but not in the controls (Figure 3-3). Treatment with flunixin meglumine did not alter these results.

Demonstration of Effective Flunixin Meglumine Dosage. In the control pony, plasma TxB_2 increased from 140 pg/ml at baseline to greater than 5,000 pg/ml at 30 minutes after endotoxin administration. In the flunixin meglumine-treated pony, plasma TxB_2 was 100 pg/ml at baseline and 78 pg/ml 30 minutes after endotoxin challenge.

Discussion

Increased plasma TxB_2 concentrations were associated with the occurrence of heaves in the first experiment, but in this second experiment, ponies treated with a cyclooxygenase inhibitor to prevent TxB_2 production still developed airway obstruction and hyperresponsiveness to histamine when housed in the barn. The alterations in pulmonary function and reactivity that occurred at period B were similar to those previously reported,[6,40,82] and were unaffected by cyclooxygenase blockade. These results are different from those

reported for the dog, where TxA_2 inhibition with both a cyclooxygenase inhibitor and a thromboxane synthase inhibitor reversed airway hyperresponsiveness to acetylcholine induced by ozone exposure or aerosol challenge with platelet activating factor or LTB_4.[55,259,260]

Thromboxane A_2 is said to be one of the most important bronchoconstrictive arachidonic acid metabolites in human lung.[365] My results do not support such a role for TxA_2 in the airway hyperreactivity and obstruction seen in heaves, because treatment with flunixin meglumine did not alter the occurrence of these signs. It is possible that in heaves, TxA_2 is acting as a non-specific indicator of an activation process that releases several mediators, and that TxA_2, although increased, is not involved in development of the disease. Platelets are a major source of TxA_2 and there is evidence that platelets and substances affecting them, such as platelet-activating factor, may be involved in the pathogenesis of pulmonary disease.[246,268,349] Platelet activating factor infusion into isolated guinea pig lungs stimulates release of TxB_2 and PGE_2, while administration of a specific platelet activating factor antagonist can inhibit the release of TxB_2, PGE_2, LTB_4, and LTD_4 from ovalbumin-challenged guinea pig lungs.[307] Horse platelets are extremely sensitive to platelet activating factor, with maximal platelet aggregation observed at concentrations as low as 3.58×10^{-14} M,[319], so the increase in plasma TxB_2 could be an indicator for the involvement of platelet activation or platelet activating factor in the pathogenesis of heaves.

The failure of flunixin meglumine to prevent the development of heaves could result from increased production of bronchoconstrictive 5-lipoxygenase metabolites such as leukotrienes C_4 and D_4, since allergen stimulation in the presence of cyclooxygenase inhibition has been associated with enhanced release of leukotrienes.[97] I did not measure plasma concentrations of leukotrienes in this study and so cannot rule out this possibility. However, a recent study in humans showed that urinary TxB_2 concentrations were increased in asthmatics following allergen challenge, but inhibition of TxB_2 production with a cyclooxygenase inhibitor did not alter the course of the disease. Urine LTE_4 concentrations were measured in that study as an estimate of 5-lipoxygenase activity, and there was no increased production associated with cyclooxygenase inhibition.[308]

The failure of cyclooxygenase blockade to inhibit the development of airway obstruction or airway hyperresponsiveness suggests that TxA_2 does not play a primary role in the development of heaves. However, the ability of cyclooxygenase inhibition to decrease the time taken for affected ponies to develop clinical signs of disease raises several possibilities. As discussed above, one explanation for this action could be that cyclooxygenase inhibition permitted increased metabolism of released arachidonic acid via the 5-lipoxygenase pathway resulting in production of bronchoconstrictive leukotrienes. An alternate explanation is that a cyclooxygenase metabolite/s may have a role in preventing or delaying the onset of heaves. One possible candidate would be PGE_2, which is synthesized by airway epithelium,[15,89,354] is a bronchodilator in

normal and asthmatic humans,[74,183] and relaxes human,[322] and equine[124] airway smooth muscle in vitro. It attenuates endotoxin-induced pulmonary dysfunction in sheep,[38] and endotoxin induced airway hyperreactivity in guinea pigs is associated with decreased epithelial PGE_2 production.[108]

Having determined that TxA_2 production did not appear to play a major role in the pathogenesis of equine COPD, I sought to determine the importance of the increase in plasma 15-HETE concentrations measured in principal ponies during acute airway obstruction in the first experiment.

CHAPTER 4

INCREASED PULMONARY PRODUCTION OF IMMUNOREACTIVE 15-HYDROXYEICOSATETRAENOIC ACID IN PONIES WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Introduction

In the first experiment I demonstrated a significant increase in plasma immunoreactive 15-HETE (i15-HETE) concentrations in jugular blood samples from principal ponies during an episode of acute airway obstruction. While there was a consistent increase in plasma i15-HETE concentrations in the affected animals in these experiments, the actual values were large (in excess of 200 ng/ml plasma for some ponies), suggesting that substances other than 15-HETE might be interfering with the radioimmunoassay. In addition, sampling from the jugular vein did not permit speculation as to the source of the increased i15-HETE production. I reasoned that if 15-HETE had a role in the pathogenesis of equine COPD, the source of this increased production should lie within the lung, and consequently, i15-HETE concentrations in blood draining the lung would be greater than i15-HETE concentrations in blood entering the lung. To approximate pulmonary blood inflow and outflow, I

collected blood samples simultaneously from the right ventricle and carotid artery. To increase the specificity of the assay, all samples were processed by high-performance liquid chromatography prior to radioimmunoassay.

Methods

As in the previous studies, five mixed-breed ponies with a history of heaves (principals) were matched for age and sex with five ponies with no history of heaves (controls). The ponies were fed, housed, and transported together, and studied on the same day.

Experimental Protocol. Pulmonary function tests were performed on ponies following 2 months at pasture, with no exposure to hay, straw, or a barn environment (Period A). They were then housed in a barn, bedded on straw, and fed hay. When the principal ponies developed clinical signs of airway obstruction, pulmonary function tests were repeated in both ponies (Period B). Ponies were then returned to pasture for a minimum of two weeks, and pulmonary function was monitored until it had returned to pre barn-exposure values (Period C). Blood samples for i15-HETE measurement were collected at each time period.

Pulmonary Function Measurements. These were performed as described in the first experiment.

Blood Sample Collection. Forty ml of blood was collected from the right ventricle and carotid artery of each animal after pulmonary function measurements had been performed. To obtain blood from the right ventricle,

a skin incision was made over the external jugular vein, a 10-gauge sterile needle was inserted into the vein and a 7 french, 120-cm, end-hole cardiac catheter (USCI; Billerica, MA) passed through the needle into the jugular vein. The external end of the catheter was connected to a pressure transducer (DP45-34;Validyne) and physiograph (VR12; Electronics for Medicine) via a three-way stopcock. The catheter flushed with sterile heparinized saline then advanced down the jugular vein and into the right ventricle. Correct positioning of the catheter tip was confirmed by the presence of the characteristic right ventricular pressure trace on the oscilloscope. Twenty ml of blood was then withdrawn and discarded, and two, 20-ml blood samples were collected into syringes containing 0.2 ml of a 15% EDTA solution. One hundred microliters of eicosatetraynoic acid solution (48 mg in 2 ml dimethylsulfoxide) was added to each 20 ml of blood, and the syringes were capped and placed on ice. Arterial blood samples were obtained from the exteriorized carotid artery via a 20-gauge needle and handled in the same way.

Sample Extraction. The pairs of 20-ml blood samples were pooled, centrifuged (800 g for 10 min) in a refrigerated centrifuge at 4°C, and the plasma was drawn off and centrifuged (1100 g for 10 min) to ensure that no further cells remained. Samples were acidified to pH 4.0-4.5 with 1 N H_3PO_4 injected onto a preconditioned Sep-Pak C_{18} column (Waters, Milford, MA), washed with 20 ml of distilled water, and eluted with 20 ml of ethyl acetate. The eluate was dried under nitrogen at room temperature, dissolved in methanol (6 ml), divided into three, 2 ml aliquots and stored under nitrogen at

minus 70°C until high-performance liquid chromatographic separations and radioimmunoassay could be performed.

High-Performance Liquid Chromatographic Separation. Reverse-phase high-performance liquid chromatography was used to separate arachidonic acid metabolites prior to quantitation of 15-HETE by radioimmunoassay. High-performance liquid chromatography was performed using dual Waters pumps (Model 510, Waters) operated through an automated gradient controller (Model 680, Waters) at a constant flow rate (1 ml/min). Separations were achieved on an Altex 4.6 mm x 250 mm, 5-μm C_{18} octadecylsilane column contained within a Rainin column heater set at 25°C. A Waters C_{18} precolumn protected the analytical column. The initial mobile phase used was methanol:water:acetic acid, buffered to pH 6.2 with NH_4OH; 60:40:0.08 (vol/vol/vol) for the first forty minutes then 70:30:0.08 for the remainder of the run. Effluent was monitored by ultraviolet absorption at 229 nm (Waters LC Spectrophotometer, model 481). One-minute fractions (1 ml each) were collected (Gilson FC-80 Micro-Fractionator) into separate polypropylene centrifuge tubes and stored at -80°C until further analysis by radioimmunoassay.

Each Sep-Pak extract was individually subjected to reverse phase high-performance liquid chromatography. The extracts were dried under vacuum in a Speed Vac Concentrator (Savant Instruments, Hicksville, NY), resuspended in 300 μL mobile phase, and injected onto the column.

To establish the elution position of 15-HETE, unlabeled 15-HETE (Biomol Research Labs, Plymouth Meeting, PA) and radioactive 15-HETE (New England

Nuclear, Boston, MA) were injected onto the column in separate runs, with appropriate ultraviolet monitoring and liquid scintillation spectrometry. Chromatographic peaks and retention times were displayed by a Waters 740 Data Module. In this system, the elution time for tritiated 15-HETE was 58.8 ± 0.5 min. Although the elution times for biologically derived 15-HETE generally parallel the elution profile of 15-HETE standards, variability in chromatographic conditions can cause the biological samples to deviate ± 2 fractions. To allow for this, two or three fractions on either side of the fraction, corresponding to the retention times of 15-HETE standards (determined on separate runs), were collected (total of 6 fractions per run), pooled, dried under vacuum, and 15-HETE quantitated by radioimmunoassay.

15-HETE Measurement. Fifteen-HETE was quantitated using a commercial radioimmunoassay kit (Amersham, Arlington Heights, IL). The assay was performed according to the manufacturers instructions. Each dried fraction was resuspended in assay buffer (phosphate buffered saline/gelatin) and duplicate 100 μL aliquots were assayed. Specificity of the assay antiserum at 50% B/Bo was 15-HETE 100%, 15-HPETE 41%, 5,15-diHETE 2.0%, all other HETEs < 0.1%. The lower limit of detection for the assay was 8 picogram/tube.

Statistical Analysis. Plasma 15-HETE concentrations in arterial and mixed venous blood from individual animals were compared using Students t-test for paired values, while results from principal and control animals for each site and time were compared using Students t-test for unpaired values. Correlations between i15-HETE and pulmonary resistance, dynamic compliance,

and arterial oxygen tension were performed using the Pearson correlation procedure. Significance was set at $P < 0.05$. Pulmonary function data and 15-HETE concentrations for heavey and control ponies at periods A, B, and C were compared using a single factor repeated measures analysis of variance. Where F values were significant at $p < 0.05$, means from each measurement period were compared using the LSD test.

Results

Pulmonary Function Measurements. At Period A and Period C, pulmonary function measurements were similar in control and principal ponies, except for pulmonary resistance, which was significantly greater in principals at period C (Table 4-1). At Period B, there was no change in pulmonary function in the control group, but in the principal group arterial oxygen tension and dynamic compliance were significantly decreased and pulmonary resistance was significantly increased relative to principals at periods A and C and controls at period B. These changes were consistent with those previously reported.[6,40,82]

15-HETE Concentrations. Plasma i15-HETE concentrations were increased in samples from the carotid artery (arterial) relative to samples from the right ventricle (venous) in principals and controls (Table 4-2). This increase was significant at periods A, B, and C for the principals and at period A for the controls, suggesting that the lung was a source of i15-HETE production.

Table 4-1 Pulmonary function measurements in principal and control ponies at pasture (period A), during (period B), and following (period C) acute airway obstruction (n = 5).

	Period A				Period B				Period C			
	R_L	C_{dyn}	f	PaO_2	R_L	C_{dyn}	f	O_2	R_L	C_{dyn}	f	O_2
Principal	0.77 ±.08	0.99 ± .35	13.1 ± 1.4	86.6 ± 1.4	6.19*+ ± .44	0.14*+ ± .03	25.8*+ ± 5.2	68.1*+ ± 3.0	0.90# ± .07	0.96 ± .30	14.2 ± 4.0	87.5 ± 4.6
Control	0.50 ± .09	0.89 ± .16	13.1 ± 1.4	89.0 ± 4.1	0.52 ± .07	0.91 ± .06	16.0 ± 4.0	95.3 ± 6.1	0.56 ± .09	0.82 ± .08	13.7 ± 1.4	89.3 ± 2.2

PaO_2 = arterial oxygen tension, Cdyn = dynamic compliance, R_L = pulmonary resistance, f = frequency.
Values are means ± SEM.
Significance = $p < 0.05$.
* = signfiicant difference from period A and C ($p < 0.05$)
+ = significant difference from control group at period B ($p < 0.05$)
# = significant difference from control group at period C ($p < 0.05$)

Table 4-2 Individual carotid artery and right ventricle plasma i15-HETE concentrations (pg/ml plasma) in samples from principal and control ponies prior to (Period A), during (Period B), and following (Period C) acute airway obstruction (n = 5). 8 pg lower limit of accurate detection for the assay.

* = significant difference from period A ($p < 0.05$)
+ = significant difference from control value ($p < 0.05$)
# = significant difference from period B ($p < 0.05$)
† = significant difference from corresponding venous value ($p < 0.05$)

Table 4-2

	Period A			Period B			Period C		
Principal	Venous (V)	Arterial (A)	(A-V)	Venous (V)	Arterial (A)	(A-V)	Venous (V)	Arterial (A)	(A-V)
#1	17	53	36	25	55	30	22	47	25
#2	7	107	99	12	134	122	15	58	43
#3	13	106	93	2	109	101	15	77	62
#4	2	90	81	8	104	96	19	65	46
#5	8	109	101	21	145	124	16	88	62
Mean ± SEM	9.4 ± 2.9	93.0* ± 10.6	82* ± 12.0	13.6 ± 4.7	109.4*† ± 15.6	94.6*† ± 17.1	17.4* ± 1.4	67*#† ± 7.2	47.6# ± 6.9
Control									
#6	7	29	21	21	69	48	21	69	48
#7	15	24	9	16	33	17	7	63	55
#8	10	29	19	26	29	3	14	24	10
#9	13	32	19	24	22	0	21	28	7
#10	13	52	39	16	50	34	9	22	13
Mean ± SEM	11.6 ± 1.6	33.2† ± 4.9	21.4 ± 4.9	20.6 ± 2.0	40.6 ± 8.5	20.4 ± 9.2	14.6± 2.8	41.2 ± 10.2	26.6 ± 10.3

No significant differences in venous i15-HETE concentrations between principals and controls occurred at any time period, but arterial i15-HETE concentrations and the difference in arterial and venous (A-V) i15-HETE production were significantly greater in principals than in controls at periods A and B. At period C, arterial i15-HETE concentrations and A-V i15-HETE were also greater in the principals, but not significantly so. In the principals, mean (A-V) i15-HETE increased when animals developed airway obstruction ($p = 0.08$) and decreased significantly when animals were returned to pasture. Arterial and venous i15-HETE concentrations, but not (A-V) i15-HETE, were significantly reduced in principal animals at period C relative to period A.

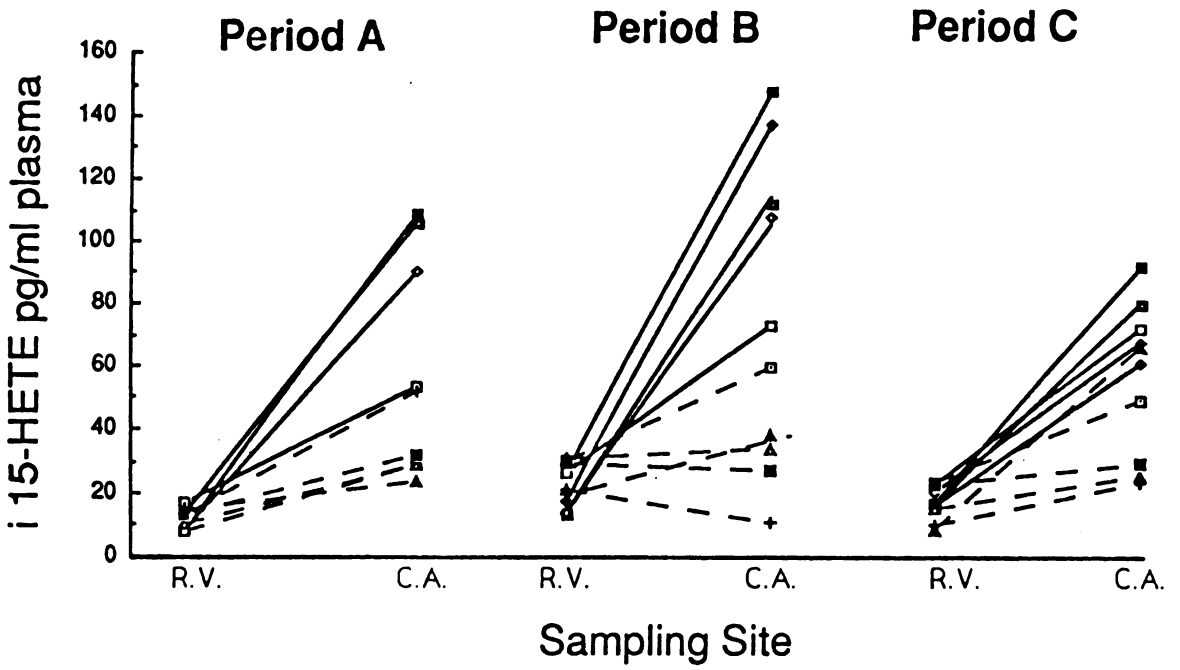


Figure 4-1. Plasma immunoreactive 15-HETE concentrations (pg/ml plasma) in right ventricle and carotid artery samples collected from principal and control ponies prior to (Period A), during (Period B), and following (Period C) acute airway obstruction (n = 5). Solid line = principal, broken line = controls.

There was a significant correlation between changes in i15-HETE production and changes in dynamic compliance measured between Period A and period B (r = -0.78), but no significant correlation with changes in pulmonary resistance or arterial oxygen concentration (Fig. 4-2).

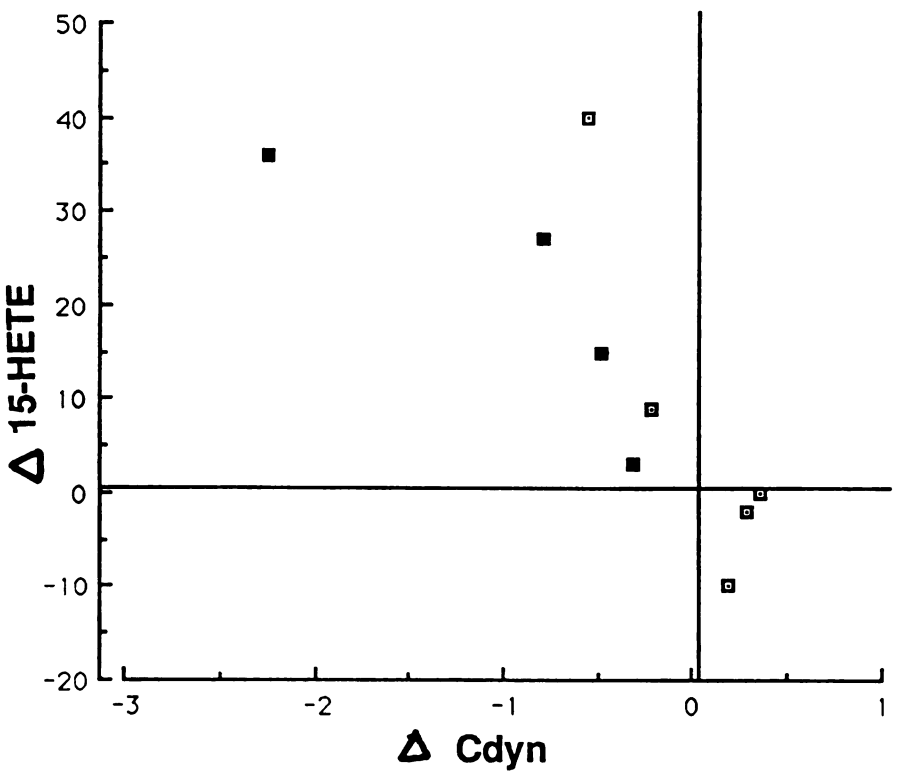


Figure 4-2. Change in dynamic compliance (ΔC_{dyn}) versus the change in i15-HETE concentration (Δi15-HETE) between Period A and Period B for principal and control ponies (n = 9). □ = control; ■ = principal

Discussion

In this experiment I demonstrated that the equine lung was a source of i15-HETE and that pulmonary i15-HETE production was significantly greater in lungs of ponies with COPD compared to control animals. There was also a significant correlation between the change in (A-V) i15-HETE production and alterations in dynamic compliance in ponies at Periods A and B. Previous studies of ponies with heaves have consistently shown that the airway obstruction I measured in this study is accompanied by non-specific airway hyperresponsiveness.[6,40,82,135]

Airway hyperresponsiveness and airway obstruction are characteristics of human asthma, and several studies have reported an association between asthma and 15-HETE.[77,143,192,253] Hamberg et al. identified 15-HETE as a major metabolite of arachidonic acid in homogenates of human lung. Samples of lung tissue from asthmatics and non-asthmatics were studied and it was found that conversion of arachidonic acid to 15-HETE was greatest in lung tissue from asthmatics.[143] A similar result was reported by Dahlen,[77] while other studies identified airway epithelium as the most likely source of 15-HETE in human lung.[167,192] A greater than three-fold increase in bronchoalveolar fluid 15-HETE concentrations has been reported in asthmatics given a local aerosol challenge with *D. pteronyssinus* antigen, but this increase was found to be not significant due to the large variability in measured concentrations and the small sample size.[253]

The role of 15-HETE in the pathogenesis of heaves or asthma is presently unknown. However, in vitro, 15-HETE can potentiate histamine-induced contraction of bronchial smooth muscle[66] and stimulate mucus secretion in the trachea.[178,228] In addition, it can inhibit[128,335] or activate[337] other lipoxygenase pathways and may act to regulate leukotriene biosynthesis. A 15-HETE metabolite (8S,15S-diHETE) causes neutrophil chemotaxis in an in vivo assay system,[187] and Samuelsson et al. have shown that 15-HETE can also be metabolized via the neutrophil 5-lipoxygenase pathway to yield lipoxin A, a recently identified arachidonic acid metabolite with spasmogenic and chemotactic properties.[292]

My observation that pulmonary i15-HETE production is significantly increased in principal animals relative to controls during both remission and clinical disease suggests that if 15-HETE does play a role in the pathogenesis of this condition then it is not as a primary mediator. One mechanism by which 15-HETE could be involved in the pathogenesis of heaves is through its ability to modulate the activity of other cell types.[335,337] Airway inflammation is seen in ponies with heaves,[34,86,215,328] but inflammation per se does not result in altered pulmonary function. Derksen et al. were able to induce a similar inflammatory response in both principal and control ponies challenged with aerosol *M. faenii,* but only principals exhibited impaired pulmonary function.[83] If 15-HETE were able to modulate the activity of inflammatory cells attracted to the airways during disease episodes, or alternatively to alter the responsiveness of airway smooth muscle to inflammatory mediators released by

these cells,[66] this could help explain why elevated pulmonary i15-HETE production during disease remission is not associated with pulmonary dysfunction. The significant difference in plasma i15-HETE concentrations in principal ponies at periods A and C in the absence of significant differences in pulmonary function further supports my suggestion that if i15-HETE is involved in the pathogenesis of heaves, it is not as a primary mediator. When I designed this study I expected that mediator production would follow a similar pattern to the pulmonary function measurements, which are not different at these two periods. It is possible that following an episode of airway obstruction, there is downregulation of i15-HETE production that requires longer to return to pre-barn levels than I allowed.

Possible sources for the observed increase in i15-HETE include eosinophils,[161,332] airway epithelium,[160,161,167] alveolar and intravascular macrophages,[21] and polymorphonuclear leukocytes.[236,336] Of these, eosinophils and airway epithelium are major sources, at least in humans.[161] Increased eosinophil numbers or activation of eosinophils during sample collection could lead to increased i15-HETE production in vivo or ex vivo and account for the observed changes. However, unlike human asthma, airway obstruction in ponies with heaves is not associated with increased numbers of eosinophils in blood or bronchoalveolar fluid samples.[86] To minimize potential ex vivo production of 15-HETE, a lipoxygenase inhibitor was used, and samples were immediately placed in ice and centrifuged within 15 minutes in a refrigerated centrifuge. Airway epithelium is a source of 15-HETE in humans,[160,161,167,192,234] but not in

other species,[145,160] so this is a potential source of 15-HETE in the pony that needs to be examined.

The augmented i15-HETE production seen in principal ponies during episodes of acute airway obstruction may result from stimulation of cells responsible for the basal i15-HETE production, from cells such as neutrophils recruited into the airways, or as a consequence of cell injury. Fifteen-HETE production by neutrophils[236] and bronchial epithelial cells[234] increases following cell injury, and epithelial injury is a feature of both human asthma[94,197] and heaves in horses.[93] Alternatively, if this damage resulted in increased epithelial permeability, metabolites such as 15-HETE produced by neutrophils in the airway lumen could more readily gain access to the subepithelial tissues and the bloodstream.

The observed correlation between changes in i15-HETE production from Period A to Period B and changes in dynamic compliance, while interesting, does not necessarily imply a cause and effect relationship. Heaves is described as affecting primarily small airways,[82,328] and changes in dynamic compliance tend to reflect predominantly changes in small airway caliber. Fifteen-HETE is a potent secretagogue,[178,228] so the changes in dynamic compliance could be the result of increased mucus secretion by epithelial cells lining smaller airways. Such an explanation fails to account for the lack of a significant difference in dynamic compliance between principals and controls at period A despite a significantly greater (A-V) i15-HETE production in the principals, unless

stimulation of mucus secretion in vivo by 15-HETE requires another factor, such as the presence of inflammatory cells.

There is considerable variation in the profile of eicosanoids produced by tissues from different species,[145,160] but to my knowledge, this was the first study to report in vivo i15-HETE production in a species other than humans. My results were especially interesting because I showed the lung to be a source of 15-HETE production in ponies, and also that 15-HETE production is increased in animals affected by a disease with similarities to human asthma. The 1000 fold decrease in plasma i15-HETE concentrations measured in this experiment compared to those from the first experiment confirmed my suspicion that there had been some interfering substance present in the earlier samples.

In the next study, I attempted to identify the source of 15-HETE production within the lung. Eosinophils do not appear to be involved in the pathogenesis of equine COPD, so I chose to study the airway epithelium, since human airway epithelium is a source of 15-HETE, and epithelium from asthmatics produces greater quantities of 15-HETE than does epithelium from control subjects. To do this it was necessary to develop an in vitro technique for studying arachidonic acid metabolism in equine tracheal epithelium, and in Chapter 5 I describe the technique that I developed.

CHAPTER 5

EPITHELIAL STRIPS: A TECHNIQUE FOR EXAMINING ARACHIDONATE METABOLISM IN EQUINE TRACHEAL EPITHELIUM

Introduction

The histological lesions of bronchial asthma are characterized, in part, by airway inflammation, epithelial injury, and mucosal edema.[197,204] The precise factors involved in the genesis of this inflammatory response, however, are yet to be elucidated. Because airway epithelial cells can produce several bioactive arachidonic acid metabolites, a large body of work has focused on the production of these metabolites by epithelial cells from various species, and their potential role in modulating airway inflammation.[58,136,145,153,154,159161,167,354,362] The most common methods for assessing arachidonic acid metabolism by tracheal epithelium utilize suspensions of enzymatically dispersed tracheal epithelial cells,[136,145,153,154,159-161,167] or such cells cultured for various periods of time.[58,354,362] Substantial differences exist in the eicosanoid profile obtained using these two methods and there is debate about which better represents their metabolism in vivo. Several potential problems have been described for each method,

including recent exposure to proteolytic enzymes with cell suspensions[145] and alterations in arachidonic acid metabolism over time with cell cultures.[362] I therefore sought an alternate method for examining epithelial arachidonic acid metabolism that would circumvent these problems.

In this chapter I describe such a method, which consists of obtaining a strip of equine tracheal epithelium attached to a thin elastic lamina, mounting this strip on a glass support, and studying it within 24 hours of removal from the animal. Using this technique I delineated the release and metabolism of endogenous arachidonic acid from normal tracheal epithelial cells in response to calcium ionophore A23187, as well as to the physiological agonists histamine and bradykinin. I also compared these results with those for cell suspensions obtained from the same animal and studied in parallel.

Methods

Tissue Preparation. Tracheas were obtained from 4 ponies euthanized with pentobarbital (100 mg/kg administered intravenously). Tissues were collected and processing begun within 20 minutes of administering the pentobarbital. Twelve-cm lengths of trachea were placed in ice-cold balanced salt solution (NaCl 118.5 mM, KCl 4.7 mM, KH_2PO_4 1.2mM, glucose 11.0 mM, $NaHCO_3$ 24.9 mM), which was bubbled with O_2/CO_2 (95:5) and contained antibiotics (penicillin G 100 U/ml, streptomycin 0.1 mg/ml, amphotericin 0.25 μg/ml - antibiotic:antimycotic, Sigma, St. Louis, MO). Tracheal segments were kept in this solution and bubbled with O_2/CO_2 until ready for further

processing. Each segment was divided in half longitudinally, pinned to a dissecting board with the mucosal surface uppermost, and rinsed with balanced salt solution to remove any blood or mucus and to keep the mucosal surface moist. A transverse incision was made through the epithelial layer into the underlying elastic lamina, taking care to remain superficial to the deeper submucosal tissues. The epithelium was teased back using Allis forceps until 3-mm long tags of epithelium were obtained. Several tags were grasped using half-curved iris dressing forceps (Jarit, Hawthorne, NY) and pulled gently downwards producing strips of epithelium 150-200 μm thick, 1.5 cm wide, and 12 cm long. The strips were placed in chilled Dulbecco's MEM/F-12 HAM medium (D/F12) (Sigma) containing 10% newborn calf serum (Sigma) and antibiotics and kept on ice.

Epithelium-on strips: Strips of epithelium were transferred to a fresh petri dish and divided into 1.2 cm lengths, each of which was then sutured to a cylindrical glass support. Each mounted strip was transferred to a well of a 24-well culture plate (Gibco, Grand Island NY) and 1.5 ml of M199 (Sigma) containing L-glutamine (Sigma), 10% newborn calf serum, and antibiotics was added to each well. The culture plate was then placed in an incubator until the strips were labelled or stimulated.

Epithelium-off (control) strips: These consisted of strips made from tracheal segments that had had the epithelial cell layer removed by first scraping across the mucosal surface with the flat edge of a scalpel blade and then rubbing

firmly with a gauze swab. The resulting denuded strip was handled in the same manner as the intact epithelial strip.

Cell suspensions: Strips of epithelium were chopped into 2 x 2 mm pieces, rinsed 3 times with balanced salt solution, and incubated in D/F12 containing 1 mg/ml pronase E Type XIV (Sigma) and 10% newborn calf serum for 16 hours at 4°C. The enzyme solution was decanted, replaced with enzyme-free D/F12 warmed to 37°C, and the epithelial pieces gently agitated for 10 minutes at 37°C in a heated water bath. The resultant cell suspension was filtered through a triple layer of gauze to remove cell clumps, centrifuged, and resuspended in fresh D/F12 with 10% newborn calf serum. Cells were counted using a hemocytometer and viability assessed by trypan blue dye exclusion. The cell suspension was placed on ice until the agonist or label was added.

Morphologic Evaluation of Preparations. Epithelium-on and epithelium-off strips were placed in buffered 4% glutaraldehyde for 6 hours, then transferred to 0.1 M phosphate buffer. Tissues were post-fixed in osmium, dehydrated in ethanol, embedded in epon-araldite, and one micron sections were cut and mounted for examination by light microscopy. Aliquots of the cell suspensions were cytocentrifuged (Cytospin; Shandon Southern Instruments, Sewickley, PA). The preparations were stained with Wright-Giemsa stain and cell types identified by their morphological characteristics under light microscopy. Percent distribution of cell types was based on counts of 300 cells. The epithelial origin of these cells was confirmed using immunofluorescent staining for cytokeratin. Cytospin preparations were fixed in methanol (-20°C)

for 20 minutes, blocked with non-immune goat serum (Sigma) at a 1:10 dilution, incubated with monoclonal anti-cytokeratin K8.13 (Sigma)(1:20) for 30 minutes at 37°C, then incubated with goat anti-mouse IgG FITC conjugate (Sigma) (1:16) for 30 minutes at 37°C.

[^{3}H]Arachidonic Acid Labeling. Epithelial strips and cell suspensions were incubated with tritiated arachidonic acid ([^{3}H]arachidonic acid) (Dupont-New England Nuclear, Boston, MA; 60-100 Ci/mM). For the cell suspension, 2 x 10^6 viable cells per ml were incubated with [^{3}H]arachidonic acid (0.5 μCi/ml) in M199 for 2 hours at 37°C. The cell suspension was then centrifuged (500 g for 10 minutes), the supernatant discarded, and the cell pellet resuspended in fresh M199 with 10% newborn calf serum. The centrifugation/resuspension step was repeated twice to ensure removal of unincorporated [^{3}H]arachidonic acid, and following a final centrifugation the cell pellet was resuspended in medium at a concentration of 2 x 10^6 cells/975 μL for experimental incubations. Aliquots of 975 μL were pipetted into 12 x 75 mm glass tubes and placed in the incubator while the agonists were prepared.

Epithelial strips were incubated with the same concentration of [^{3}H]arachidonic acid at 37°C for 16 hours to ensure adequate incorporation of label. The labeling solution was aspirated and the strips washed with three 2-ml aliquots of M199/10% newborn calf serum to remove unincorporated label. The appropriate stimulation medium was then added to each well (975 μL) and the culture dish returned to the incubator. To characterize eicosanoid products, prelabeled (for high performance liquid chromatography), or unlabeled (for

radioimmunoassay) cells were stimulated in protein-free M199. To estimate free arachidonic acid release using thin layer chromatography, stimulations were performed in M199 containing 1 mg/ml essentially fatty acid free equine serum albumin (Sigma) to bind released arachidonic acid.[275]

Agonist Stimulation. Stock solutions of A23187, histamine, and bradykinin were prepared at a concentration such that a 25 μL aliquot added to 975 μL of media would produce the desired final agonist concentration. Histamine and bradykinin were dissolved in distilled water and A23187 was dissolved in ethanol. The final ethanol concentration in the media did not exceed 0.5%. Histamine and bradykinin concentrations of 50 μM were chosen to allow comparison of results with those reported elsewhere.[145] The A23187 concentration used was 5 μM as this concentration was on the plateau of the dose-response curve (see Figure 5-3) and was not associated with cytotoxicity in preliminary experiments that measured lactate dehydrogenase release. Agonists were prepared immediately prior to their addition to the tissues and kept on ice. After adding the agonists, the strips and cell suspensions were returned to the incubator for 45 minutes. The supernatants from the epithelial strips and from the cell suspensions after centrifugation were collected in polypropylene tubes and stored at -70°C.

Measurement of Free [^{3}H]Arachidonic Acid by Thin Layer Chromatography. Lipids were extracted from the medium with 6 ml of chloroform:methanol (2:1 vol/vol) followed by a wash with 2 ml of chloroform.[275] All organic solvents contained 0.05 mg/ml butylated hydroxytoluene (Sigma) as an antioxidant. The chloroform phases were combined, the total lipid extract evaporated to dryness under nitrogen, and the samples stored at -70°C. Lipid residues were dissolved in 60 μL of chloroform and quantitatively applied to aluminum-backed thin-layer chromatography sheets precoated with Silica Gel 60 (EM Science, Gibbstown, NJ). A solvent system of hexane:diethyl-ether:acetic acid (70:30:2 vol/vol) was used to separate arachidonic acid from other lipids. Spots co-migrating with an authentic arachidonic acid standard (Nu-Chek, Elysian, MN) were identified by exposure to iodine vapor, cut out, eluted with methanol, and their radioactivity quantitated by liquid scintillation spectrometry. The net stimulated concentration of free [^{3}H]arachidonic acid was defined as the difference between the free [^{3}H]arachidonic acid measured in control incubations and stimulated samples.

Determination of Eicosanoid Profiles by High-Performance Liquid Chromatography. To determine the profile of [^{3}H]eicosanoids produced by prelabeled epithelial strips and cell suspensions, eicosanoids were separated by reverse-phase high-performance liquid chromatography, identified by co-elution with authentic standards, and quantitated by liquid scintillation counting of eluate fractions. For these experiments, media from 2 replicate wells were pooled, extracted using Sep-Pak C_{18} cartridges (Waters, Milford, MA), and

subjected to high performance liquid chromatographic separation. Reverse-phase separations were performed using a 30 cm μBondapak C_{18} column (Waters Associates, Milford MA) at a flow rate of 1 ml/min. Ultraviolet absorbance was monitored at 210 nm for cyclooxygenase products and free arachidonic acid, 280 nm for leukotrienes, and 235 nm for monohydroxyeicosatetraenoic acids.[275] Cyclooxygenase standards were a gift from Dr. J. Pike (Upjohn Co., Kalamazoo, MI) and lipoxygenase standards a gift from Dr J. Rokach (Merck Frosst, Inc., Quebec, Canada). The solvent system used was acetonitrile/water/trifluoroacetic acid, which separated cyclooxygenase metabolites isocratically (33:67:0.1) and then sequentially separated leukotrienes, monohydroxyeicosatetraenoic acids, and finally free arachidonic acid with increasing concentrations of acetonitrile.

Measurement of Immunoreactive PGE_2. Media from unlabelled epithelial strips and cell suspensions were stored at -70°C, and PGE_2 concentrations were quantitated by radioimmunoassay. Prostaglandin E_2 antiserum was obtained from Advanced Magnetics (Cambridge, MA), tritiated PGE_2 from Dupont-New England Nuclear (Boston, MA), and authentic PGE_2 standard from Cayman Chemical Co. (Ann Arbor, MI). The cross reactivity of the antisera at 50% B/B_0 was PGE_1 50%, PGA_2 6.0%, PGA_1 3.0%, $PGF_{2\alpha}$ 1.3%, all other eicosanoids 1.0% or less.

Immunocytochemical Staining for PGH Synthase. Cytospin preparations of the cell suspension and frozen sections of epithelial strips were blocked with nonimmune goat serum (Sigma) at a dilution of 1:10 for 30 minutes at 37°C, incubated with rabbit polyclonal anti-PGH synthase (gift of Dr. W. Smith, Dept.

Biochemistry, Michigan State University), 1:10 for 30 minutes at 37°C, then incubated with goat anti-rabbit IgG FITC conjugate (Sigma), 1:40 for 30 minutes at 37°C. Control antibody solution was obtained by incubating anti-PGH synthase antibody with an excess of purified sheep seminal vesicle PGH synthase (Oxford Biomedical Research, Oxford, MI) for 24 hours at 4°C to precipitate anti-PGH synthase activity. The mixture was then centrifuged at 15,000 x g for 10 minutes, and the supernatant used instead of antibody solution for control preparations.

Statistical Analysis. Values obtained from epithelial strips and cell suspensions were compared using Student's t test for paired values. Significance was set at $p < 0.05$.

Results

Tissue Preparation. The epithelial stripping technique produced a strip 150-200 μm thick consisting of a 70-μm thick layer of ciliated, pseudostratified columnar epithelium attached to a layer of connective tissue (Figure 5-1). This epithelial layer could be succesfully removed to produce epithelium-off strips (Figure 5-2). The distribution of cell types in the cell suspension (n = 4) was 66.3 $\pm$ 1.1% basal cells, 10.0 $\pm$ 4.2% ciliated cells, 2.0 $\pm$ 1.0% goblet cells, and 21.3 $\pm$ 4.2% undifferentiated cells, which is similar to that previously reported.[58,145,160,167]

Figure 5-1 Micrograph of a tracheal epithelial strip showing preservation of the normal pseudostratified epithelium (A), basement membrane (B), and connective tissue stroma (C). The granular appearance of the connective tissue stroma is due to the presence of collagen bundles in cross-section.

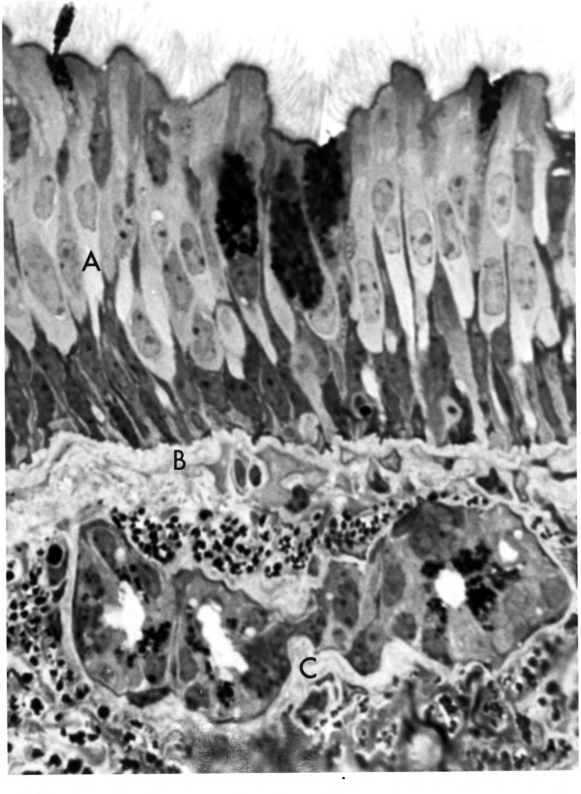
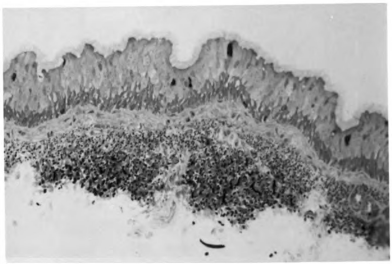

A
B
C

Figure 5-2. Micrograph of an epithelium-off strip (a) and epithelium-on strip (b), confirming removal of the epithelial cell layer.

A



B

Figure 5-2

Cytokeratin staining of cytospin preparations confirmed the epithelial origin of these cell types and the absence of non-epithelial cells apart from inflammatory cells, which constituted less than 2% in all preparations. Viability for the cell suspension was 85 ± 2.0% (n=4) and non-viable cells were predominantly mature ciliated cells.

Incorporation and Release of [^{3}H]Arachidonic Acid. Epithelium-on strips incorporated 40.8 ± 3.6% (n=4) of the added radioactivity after incubation for 16 hours at 37°C, and the cell suspensions incorporated 59.6 ± 1.6% after incubation for 2 hours at 37°C. Dose-response curves to A23187 revealed a dose-dependent stimulation plateau at 5 μM for both preparations (Figure 5-3).

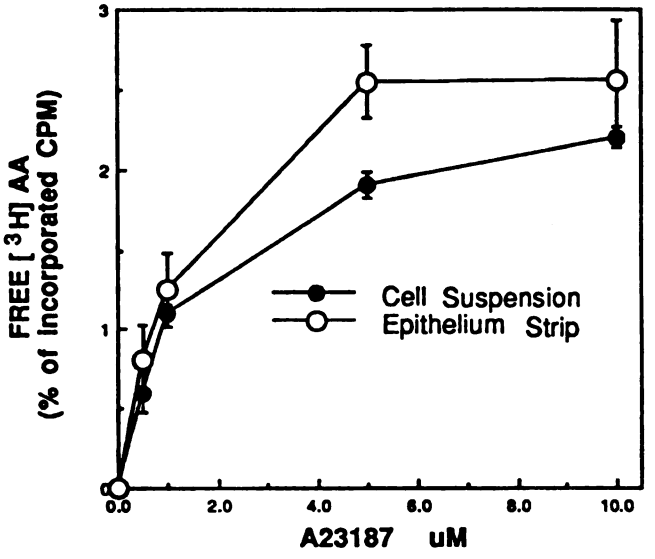


Figure 5-3 Effect of A23187 concentration on [^{3}H]arachidonic acid release from epithelium-on strips and cell suspensions. The amount of radioactivity is expressed as a percentage of the total amount of radioactivity incorporated by each preparation prior to stimulation. Each data point represents the mean ± SEM of four experiments performed in duplicate.

When stimulated with 5 μM A23187, epithelium-on strips released a larger percentage of incorporated radioactivity (stimulated-baseline) than did the cell suspensions (2.55 $\pm$ 0.23% vs 1.90 $\pm$ 0.08%), but the difference was not significant. A release of 2.55% corresponded to 6077 cpm. Although not significantly different, unstimulated (baseline) release of radioactivity was less from epithelium-on strips (1.37 $\pm$ 0.21% vs 1.84 $\pm$ 0.18%). Stimulation with the physiological agonists histamine and bradykinin produced a significant release of arachidonic acid from the epithelial strips only (Table 5-1).

Table 5-1 Free [^{3}H]Arachidonic acid release from epithelium-on strips and cell suspensions stimulated with 50 μM histamine or bradykinin.

Agonist	Epithelium-on Strip	Cell Suspension
Histamine (50 μm)	1.79 $\pm$ 0.22*	1.11 $\pm$ 0.07
Bradykinin (50 μm)	1.50 $\pm$ 0.16*	1.03 $\pm$ 0.03

Values are expressed as the ratio of total cpm released from each preparation after stimulation / total cpm released during control incubations (mean $\pm$ SEM). Zero release would equal 1.0. Control incubations were performed under identical conditions except for the addition of histamine or bradykinin (n = 4). * = significant difference from control values ($p < 0.05$)

Eicosanoid Synthesis by Epithelium-on and Epithelium-off Strips and Cell Suspensions. High-performance liquid chromatography radioactivity profiles of eicosanoids released into the medium from prelabeled epithelium-on strips stimulated with 5 μM A23187 were characterized by peaks that coeluted with cyclooxygenase metabolites. The largest peaks were those coeluting with free arachidonic acid and PGE_2 with smaller peaks coeluting with $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin (Figure 5-4A). The epithelial layer appeared to be the source of the PGE_2 and $PGF_{2\alpha}$, since there was minimal production of these metabolites by the epithelium-off strip (Figure 5-4B). The largest peak in medium from epithelium-off strips coeluted with free arachidonic acid with a smaller peak coeluting with 6-keto-$PGF_{1\alpha}$. Major peaks of radioactivity from the cell suspension corresponded to free arachidonic acid, LTB_4, and 12-HETE. No radioactivity coeluted with PGE_2, $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ standards (Figure 5-4C). To quantitatively confirm the difference in PGE_2 production by epithelial strips and cell suspensions, immunoreactive PGE_2 was determined by radioimmunoassay of supernatants from each preparation after stimulation with 5 μM A23187. Epithelium-on strips produced 10.3 $\pm$ 1.3 ng/strip (n=4), while PGE_2 production/5 x 10^6 cells in suspension was below the detection limit for the assay (100 pg/ml). Despite the lack of PGE_2 production by cell suspensions, immunocytochemical staining for PGH synthase showed positive fluorescence in the majority of the cells present in the suspension (Figure 5-5).

Figure 5-4 Reverse-phase HPLC profiles of [^{3}H]arachidonic acid products released by representative epithelium-on (A), and epithelium-off (B) strips, and epithelial cell suspension (C) obtained from equine trachea. Preparations were stimulated with 5 μM A23187 for 45 minutes, and media was extracted and separated by HPLC. Fractions corresponding with elution times of eicosanoid standards were collected and quantitated by liquid scintillation counting. Results are expressed as a percentage of the total radioactivity released by each preparation.

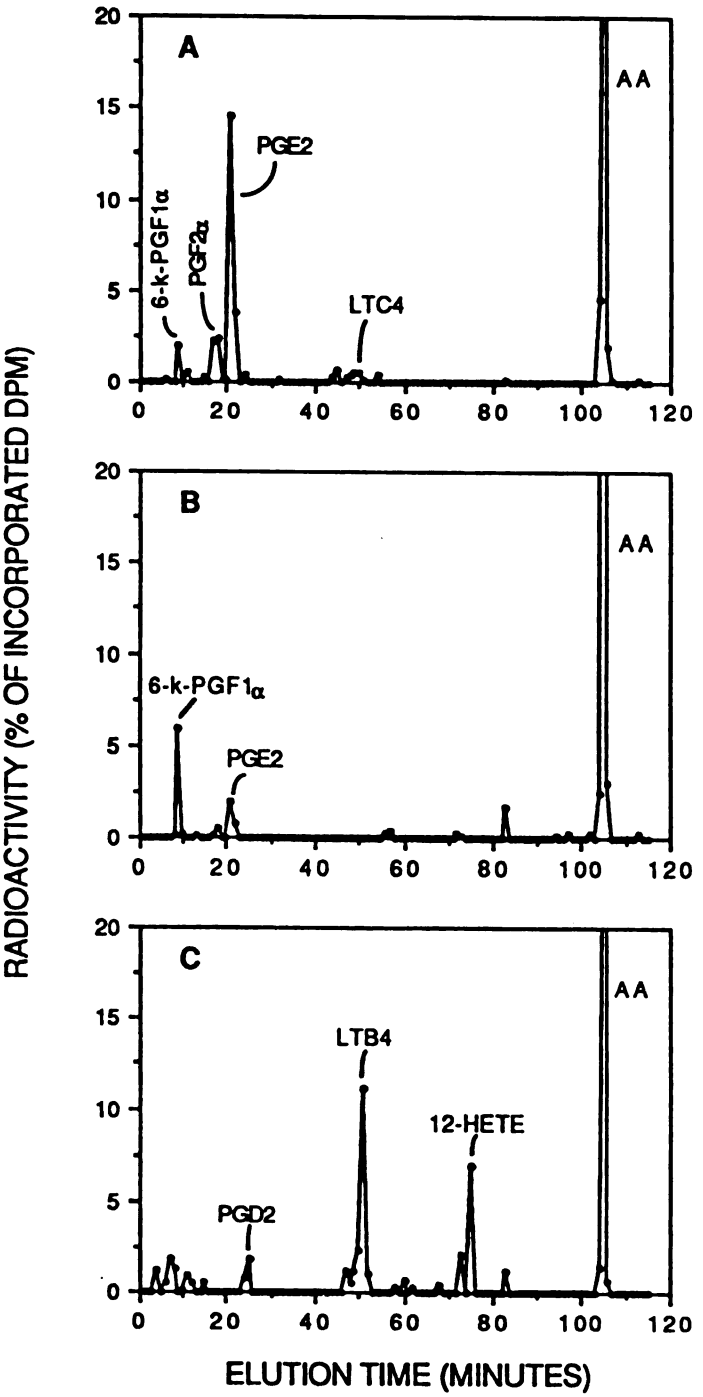
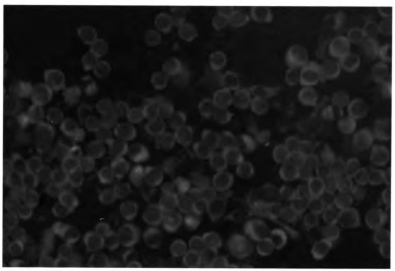
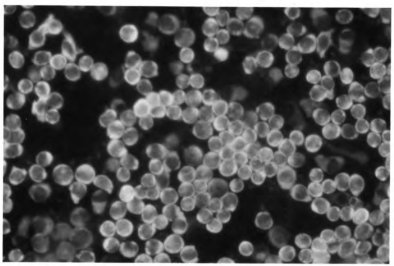

A
B
C
AA
PGE2
6-k-PGF1α
PGF2α
LTC4
6-k-PGF1α
PGE2
LTB4
12-HETE
PGD2
20
15
10
5
0
0
20
40
60
80
100
120
RADIOACTIVITY (% OF INCORPORATED DPM)
ELUTION TIME (MINUTES)

Figure 5-4

Figure 5-5 Immunofluorescent staining of epithelial cell suspensions with a polyclonal antibody to PGH synthase (bar = 8 μM).
A= control supernatant
B = anti-PGH synthase antibody



A



B

Figure 5-5

Discussion

The profile of eicosanoids produced by airway epithelial cells has been the subject of considerable debate. Resolution of discrepancies in reported metabolite profiles is complicated by variations in the species used and the experimental conditions under which the tissues have been studied. Stimulation of intact guinea-pig trachea in vitro induces PGE_2 production that is significantly reduced by epithelium removal.[108] Human tracheal epithelial cells in culture metabolize endogenous arachidonic acid via the cyclooxygenase pathway, yielding predominantly PGE_2 and $PGF_{2\alpha}$,[58,354] which correlates with the reported pattern of cyclooxygenase activity in vivo in the human lower respiratory tract.[267] However, studies utilizing enzymatically dispersed human tracheal epithelium incubated with exogenous arachidonic acid report predominantly lipoxygenase activity, with 15-HETE as the major metabolite.[153,154,160,167] A similar discrepency is seen with canine tracheal epithelium, in which cell culture experiments utilizing endogenous arachidonic acid report production of predominantly PGE_2, 6-keto $PGF_{1\alpha}$, and $PGF_{2\alpha}$,[354] whereas cell suspensions produce PGD_2, LTC_4, and LTB_4 from endogenous arachidonic acid,[101] and PGD_2, 5-HETE, LTB_4, 15-HETE, and 12-HETE when incubated with exogenous arachidonic acid.[159,160]

Criticism has been directed at both cell culture and enzymatically dispersed cell suspensions as a means of examining epithelial arachidonic acid metabolism. Cyclooxygenase activity of tracheal epithelial cells changes with time in culture,[155,362] and can also be altered by variations in culture conditions.[58]

Cell suspensions require tissues to be exposed to proteolytic enzymes, resulting in a selective loss of ciliated cells,[54] and a distribution of cell types[145,167] that is not representative of that seen in vivo.[31,120,358] In addition, cell suspensions fail to respond to stimulation with a variety of physiological agonists.[145]

The epithelial strip technique that I developed has a number of features that make it useful for examining tracheal epithelial arachidonic acid metabolism in vitro. It maintains the normal pseudo-stratified epithelial architecture and consequently the same distribution of epithelial cell types that occurs in vivo. This is important if the epithelium is viewed as a functioning unit rather than a collection of independent cells. There is no requirement for proteolytic enzymes that could potentially alter cell membrane receptor populations and hence responses to physiological agonists. Indeed, tracheal epithelium obtained using this technique was able to respond to physiological agonists as well as to A23187, which is important if inferences about the role of epithelium-derived eicosanoids in pulmonary pathophysiology are to be made. Finally, it allows the epithelium to be studied shortly after removal from the animal, which reduces the potential for alterations in metabolism induced by ex-vivo culture conditions. One criticism of the technique is that there are non-epithelial cells present in the underlying connective tissue support, which could contribute to the measured eicosanoid profile. By studying the responses of epithelium-off strips that consist of the underlying connective tissue stroma only, I could identify any contribution from this potential source of eicosanoids.

Using this technique I found that in intact equine tracheal epithelium, endogenous arachidonic acid was metabolized via the cyclooxygenase pathway, with PGE_2 as the major product and $PGF_{2\alpha}$ as a minor metabolite. Dispersed epithelial cell suspensions from the same animal, collected and stimulated in parallel, exhibited predominantly lipoxygenase metabolism, with LTB_4 and 12-HETE as the major metabolites.

The profile of metabolites produced by epithelium-on strips stimulated within 24 hours of removal from the animal resembled those reported from mature cultures of human and canine tracheal epithelial cells,[58,354] and from freshly isolated canine trachea.[354] The strips also responded to stimulation with histamine and bradykinin, as has been reported for cell cultures and freshly isolated tissue.[354] In contrast, the profile from cell suspensions showed markedly less cyclooxygenase products and more lipoxygenase products relative to the epithelium-on strip, as well as a minimal response to histamine and bradykinin. This profile was consistent with previous reports for tracheal cell suspensions from other species,[145,154,159] as was the lack of response to physiological agonists.[145]

To my knowledge, this is the first study to compare the arachidonic acid profile of enzymatically dispersed tracheal epithelium with that of epithelium obtained from the same animal using an alternate technique. As such it enabled me to address some of the explanations that have been offered for discrepancies between profiles obtained using different methods in separate experiments. If the predominant cyclooxygenase activity of tracheal epithelial cells in culture is an artifact induced by in vitro manipulation, this could be due

to selection of a subset of epithelial cells that survive in culture or to an alteration in the expression of oxygenation pathways during culture in artificial media.[145] Such an explanation does not account for the cyclooxygenase activity demonstrated by the mature tracheal epithelium present in my epithelium-on strips or the results of experiments utilizing freshly isolated tissues.[108,354]

An alternate explanation is that tracheal epithelium normally exhibits cyclooxygenase activity and that deviations from this activity reported in enzymatically dispersed cell suspensions are artifacts induced during the isolation and handling of those cells. The predominant lipoxygenase metabolism exhibited by freshly isolated cells may result from alterations in expression of oxygenation enzymes induced by the isolation process,[145] or from actual cell injury. Increased production of 15-HETE has been associated with cell injury in a variety of cells, especially in the presence of exogenous arachidonic acid.[136,160,234,236] Different profiles of metabolites can also result from the use of exogenous versus endogenous arachidonic acid,[61] or from varying concentrations of exogenous arachidonic acid.[159]

A third explanation is that the two methods are examining arachidonic acid metabolism in different cell populations, and the profiles reported for each preparation reflect the activity of the predominant cell types present in each population. Morphometric analysis of tracheal epithelium from several species shows three principal cell types: ciliated, non-ciliated (PAS-positive), and basal. The percentage of each type varies among species, but the relative proportions are usually ciliated > non-ciliated > basal cells.[31,120,358] In contrast, enzymatic

digestion of the tracheal epithelium to produce cell suspensions causes selective loss of ciliated cells,[54] and can result in preparations that are 65 to 99% basal cells.[145,167] Tracheal epithelium is an example of a cell renewal system where there is a gradual loss of proliferative ability as cells mature and become fully differentiated.[31] Basal cells act as stem cells for tracheal epithelium,[29,36] dividing to form one basal and one superficial cell. Each superficial cell divides to form two superficial cells,[29] which then extend from the basal to the superficial layers and are lost after a variable period.[29] Ciliated cells, which are terminally differentiated, develop from superficial cells and do not undergo further division.[31] Alterations in metabolic activity in vitro could accompany this maturation process, since alterations in arachidonic acid metabolism are associated with differentiation of cells in culture[153,362] and in situ.[155] Basal cells have been proposed as the source of 15-HETE production in suspensions of human tracheal epithelial cells,[167] and such activity is consistent with reports of lipoxygenase activity in other dividing cell types such as fibroblasts, carcinomas, and sarcomas.[313] It is possible that the eicosanoid profile produced from cell suspensions reflects the metabolic activity of the more primitive or stem cell lines present in the epithelium, whereas the profile that we observed from epithelium-on strips better represents the activity of intact tracheal epithelium in vivo, where ciliated cells are the predominant cell type located at the air-lung interface.

The results of high-performance liquid chromatographic and radio-immunoasssay analysis of media from stimulated cell suspensions demonstrated

an absence of cyclooxygenase activity, yet the majority of these cells display positive fluorescence for PGH synthase. This inconsistency could be explained by a defect in arachidonic acid mobilization and release, which would reduce available substrate and hence production of metabolites. This is unlikely since I have shown that arachidonic acid release in response to 5 μM A23187 is similar in both preparations (Figure 5-3), and also because the cell suspensions have adequate substrate to produce lipoxygenase metabolites. An alternate explanation is that basal cells produce an inhibitor of PGH synthase, which prevents activation of the enzyme. Endogenous prostaglandin synthase inhibitors have been isolated from bovine placental tissue,[302] ovine endometrium,[16] rabbit kidney,[326] as well as plasma from several species.[288] A decline in inhibitor production has been proposed to explain the increased placental prostaglandin synthesis associated with parturition in cattle,[302] and could also account for the acquisition of prostaglandin synthetic capability by mature equine tracheal epithelium in this study.

Altered arachidonic acid metabolism can occur in vivo with diseases such as asthma.[172,253,295,325] These changes could be due to mediators such as TxA_2, PGD_2, 15-HETE, or sulphidopeptide leukotrienes produced by epithelial cells or inflammatory cells present in the airways and alveoli. If airway epithelium eicosanoid metabolism is altered in asthma, this may be the result of selective damage and loss of ciliated epithelial cells, which would expose underlying intermediate or basal cells to injurious stimuli and permit release of their characteristic eicosanoid profile. Chronic injury to the airway epithelium may

lead to replacement of ciliated cells with relatively undifferentiated cells[93] in which the pattern of arachidonic acid metabolism could be different from that present in normal epithelium. Alternatively, interaction among different cell types either through exchange of arachidonic acid and other intermediate metabolites such as LTA_4,[113,262] or via release of unidentified cell factors,[188] can alter the type and/or quantity of eicosanoids produced. Such an interaction might occur between epithelium and inflammatory cells present in the airway lumen.

The technique that I developed and described in this chapter appeared to be a useful method for studying arachidonic acid metabolism in equine tracheal epithelium. While there was no evidence of 15-HETE production based on high-performance liquid chromatography profiles, these studies utilized tissues from normal animals and the agonist used for generating the profiles was the non-physiological agonist A23187. To determine whether tracheal epithelium from horses or ponies with COPD could produce 15-HETE I used my epithelial strip technique to study the response of tissues collected from affected horses during acute airway obstruction and stimulated with A23187, histamine, and bradykinin. This was the final experiment that I performed and the results are presented in the next chapter.

CHAPTER 6

PRODUCTION OF IMMUNOREACTIVE 15-HETE AND PGE_2 BY TRACHEAL EPITHELIUM FROM HORSES WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Introduction

In Chapters 2 and 4 I reported that plasma 15-HETE concentrations were increased in ponies with COPD relative to controls, that production of 15-HETE increased during the occurrence of airway obstruction, and that the lung was the source of this increase. One potential pulmonary source of 15-HETE in humans is airway epithelium, and in Chapter 5 I presented a technique that I had developed for examining arachidonic acid metabolism by equine tracheal epithelium in vitro. Therefore, the first purpose of this experiment was to utilize that technique to test the hypothesis that equine airway epithelum is the source of the pulmonary 15-HETE production in ponies with COPD. If this was correct, I expected to detect 15-HETE production by tracheal epithelial strips obtained from horses with COPD.

A number of researchers have reported that airway epithelium produces substances that modulate the responsiveness of underlying airway smooth

muscle to contractile agonists and have coined the term "epithelial-derived relaxant factor" to describe these substances.[106,248,331] Decreased production or release of such a factor could increase the responsiveness of airway smooth muscle to contractile stimuli and thus contribute to the hyperresponsiveness seen in diseases such as equine COPD and asthma. In some species this epithelial-derived relaxant factor appears to be a cyclooxygenase metabolite, most probably PGE_2.[15,44,108] Prostaglandin E_2 is produced by intact airway epithelium in response to a variety of stimuli, including eosinophil derived major basic protein,[174] granulocytes and reduced oxygen molecules,[181] bradykinin, platelet activating factor, and calcium ionophore (A23187).[354] In vivo, PGE_2 is a bronchodilator in both normal and asthmatic humans[74,183] and reverses bronchoconstriction caused by administration of $PGF_{2\alpha}$ [309] In vitro, it relaxes isolated human[322] and equine[124] tracheal smooth muscle, possibly by inhibiting acetylcholine release at a prejunctional site.[15] Decreased production of PGE_2 by tracheal epithelium is also associated with endotoxin-induced hyperreactivity in guinea-pig isolated trachea.[108]

In Chapter 3, I reported that cyclooxygenase inhibition appeared to decrease the time taken for principal ponies to develop airway obstruction when housed in the barn. One explanation for such an effect is that flunixin meglumine further reduced an already depressed capacity for epithelial PGE_2 production in ponies with COPD, thus hastening the onset of clinical signs. Therefore, I planned to use the epithelial strip technique to test the hypothesis that there is decreased production and/or release of PGE_2 from airway

epithelium in horses with COPD. If this was correct, I expected to measure increased PGE_2 release by tracheal epithelial strips from control horses relative to epithelial strips from horses with COPD.

Methods

Experimental Protocol. Six horses with a history of heaves (principals) were obtained and matched for age and sex with six horses with no history of heaves (controls).Prior to the experiments, horses were kept at pasture. Baseline pulmonary function measurements were performed and horses were placed in adjoining stalls in the barn, bedded on straw, and fed hay. When the principal horse developed clinical signs of airway obstruction, pulmonary function tests were performed on both horses. If the results of these tests met my predetermined criteria for airway obstruction, which were a change in pleural pressure greater than 15 cm water during tidal breathing, and an arterial oxygen tension less than 75 torr, the principal animal was euthanized and tracheal epithelium collected for the in vitro studies. The control horse was kept in the barn for a further 48 hours while tissues from the principal horse were processed and pulmonary function measurements repeated. If these indicated no evidence of airway obstruction, the animal was euthanized and tracheal epithelium was collected and processed.

Pulmonary Function Measurements. These measurements were performed using the same techniques as described in Chapter 1, with the following exceptions. The horses used in the study did not have permanent tracheostomas

and therefore a rigid mask fitted with a pneumotachograph (Fleisch No.5; Dynasciences, Blue Bell, PA) was used instead of an endotracheal tube for measurement of airflow. The mask was placed over the horse's nose after the esophageal balloon had been positioned, sealed to prevent any air leakage, and connected to the recording equipment. Also, these horses did not have exteriorized carotid arteries, so arterial blood samples for arterial oxygen tension estimation were obtained by percutaneous puncture of the carotid artery in the lower neck using a #20 needle and syringe. Correct placement of the needle was confirmed by observing high pressure flow of blood from the needle before the syringe was connected and the sample collected.

Isolation and Handling of Tracheal Epithelium. Strips of tracheal epithelium were obtained using the technique that I described in Chapter 5. Briefly, horses were euthanized with pentobarbital (100 mg/kg administered intravenously) and tissues were collected within 15 minutes of pentobarbital administration. Both epithelium-on and epithelium-off strips were prepared for agonist stimulation. Each strip was trimmed to approximately the same size (15 x 10 mm) using a graph paper template attached to the base of the petri dish in which the strips were handled. Before attaching the strips to their glass supports, the surface area of individual strips was measured and recorded, to enable me to express eicosanoid production as picogram per mm^2 of epithelium.

Agonist Stimulation. Stock solutions of A23187, histamine, and bradykinin (Sigma) were prepared at a concentration such that a 25 μL aliquot added to 975 μL of media would produce the desired final agonist concentration. Histamine

and bradykinin were used at a concentration of 50 μM and A23187 at a concentration of 5 μM. These concentrations were chosen because they had been used in developing the technique and because I had shown that these concentrations stimulated arachidonic acid release from epithelial strips. Histamine and bradykinin were dissolved in distilled water and A23187 was dissolved in dimethylsulfoxide. The final dimethylsulfoxide concentration in the media did not exceed 0.5%. Agonists were prepared immediately prior to their addition to the tissues and kept on ice. Two strips were used for each agonist, and stimulation was performed approximately 6 hours after the horse was euthanized. The culture plates containing the epithelial strips were removed from the incubator and the media aspirated and replaced with 975 μL of protein-free M199 solution. Agonists were added to their respective strips and the plates were returned to the incubator for 45 minutes. At the end of the stimulation period, media from each pair of strips were pooled, centrifuged, and the supernatant was divided into 300 μL aliquots and stored in polypropylene tubes at -70°C. After the media were collected, each strip was placed in a desiccator for 24 hours and then weighed to allow eicosanoid production to be expressed as pg per mg dry weight of tissue.

Measurement of Immunoreactive 15-HETE and PGE_2. Samples from each pair of horses were stored at -70°C until the experiment was completed and then all the samples for the respective epithelium-on and epithelium-off strips were analyzed as a single batch.

15-HETE Assay. Immunoreactive 15-HETE in supernatants from epithelium-on strips was measured using a commercial radioimmunoassay kit from Amersham, and the assay was performed according to the manufacturer's recommendations. The antiserum cross-reactivity at 50% B/Bo was 15-HETE 100%, 15-HPETE 41%, 5,15-diHETE 2%, 13-HOOD 0.4%, all other eicosanoids 0.1% or less. The lower limit of accurate quantification for the assay was 8 picogram/tube.

PGE_2 Assay. Immunoreactive PGE_2 production from epithelium-on and epithelium-off strips was assayed using reagents purchased separately rather than a commercial kit. Antiserum was obtained from Advanced Magnetics (Cambridge, MA), tritiated PGE_2 from Dupont-New England Nuclear (Boston, MA), and authentic PGE_2 standard from Cayman Chemical Co. (Ann Arbor, MI). Separation of bound from free trace was achieved with dextran-coated charcoal. The cross-reactivity of the antisera at 50% B/B_o was PGE_2 100%, PGE_1 50%, PGA_2 6.0%, PGA_1 3.0%, $PGF_{2\alpha}$ 1.3%, all other eicosanoids 1.0% or less. Where necessary, samples were diluted with medium to produce values on the straight portion of the standard curve. The lower limit of accurate quantification for the assay was 12 picogram/tube.

Statistical Analysis. Values obtained from epithelium-on and epithelium-off strips for each animal were compared using Student's t test for paired values and epithelium-on and epithelium-off values from principal and control horses were compared using Student's t test for unpaired values. Significance was set at $p < 0.05$.

Results

Pulmonary Function Measurements. In the principal horses, pulmonary resistance, respiratory frequency, change in pleural pressure during tidal breathing, and arterial carbon dioxide tension were significantly increased, and dynamic compliance and arterial oxygen tension significantly decreased at period B relative to period A (Table 6-1). No equivalent changes were present in the control horses. There were no significant differences between pulmonary parameters in the control horses at either of the period B measurement periods, so these values were combined to provide a single period B value for each parameter. At period A, respiratory frequency and the change in pleural pressure were significantly greater in the principals relative to the controls. At period B, pulmonary resistance and the change in pleural pressure were significantly increased, and dynamic compliance and arterial oxygen tension significantly decreased in principals relative to controls.

Table 6-1 Pulmonary function measurements in principal and control horses at pasture (Period A) and during (Period B) airway obstruction (n = 6).

	Period A						Period B					
	P_R	C_{dyn}	R_L	f	PaO_2	$PaCO_2$	P_R	C_{dyn}	R_L	f	PaO_2	$PaCO_2$
Principal	7.6 ± 0.3+	1.23 ± 0.18	0.91 ± 0.09	12.0 ± 1.8	94.8 ± 2.7	38.2 ± 0.9	36.1 ± 4.9*+	0.31 ± 0.06*+	2.43 ± 0.26*+	17.6 ± 2.0*	60.6 ± 3.8*+	45.9 ± 1.4*+
Control	6.3 ± 0.4	1.37 ± 0.28	0.63 ± 0.11	17.2 ± 1.05	95.3 ± 2.8	40.5 ± 1.1	6.7 ± 0.4	1.26 ± 0.15	0.87 ± 0.10	13.9 ± 2.2	95.1 ± 1.7	42.6 ± 1.0

PaO_2 = arterial oxygen tension, $PaCO_2$ = arterial carbon dioxide tension, C_{dyn} = dynamic compliance, R_L = pulmonary resistance, P_R = change in pleural pressure, f = frequency.
Values are means ± SEM.
* = significant difference from period A value ($p < 0.05$)
+ = significant difference from control value ($p < 0.05$)

Immunoreactive 15-HETE and PGE_2 Production by Epithelial Strips.

15-HETE Production. Quantifiable amounts of 15-HETE detected were in epithelium-on strip supernatants from the principals and one control horse (Table 6-2). In strips from a number of other horses, small amounts of 15-HETE were detected. The amounts produced were below the limit for accurate quantitation, and did not increase when strips were stimulated with A23187, histamine or bradykinin.

Table 6-2 Immunoreactive 15-HETE release by epithelial strips from principal and control horses

	Unstimulated	A2317	Bradykinin	Histamine
Principal				
Horse #				
1	0	0	0	0
2	21	11	21	19
3	5	0	6	2
4	12	11	0	0
5	0	0	0	0
6	3	6	0	0
Control				
1	0	0	0	0
2	0	6	0	5
3	0	35	20	14
4	0	3	0	0
5	0	0	0	0
6	4	8	9	2

Values expressed as picogram/100 μL supernatant.
8 picogram = lower limit of accurate quantification for assay

PGE_2 Production. PGE_2 production in response to A23187, bradykinin, and histamine was significantly increased over unstimulated values for both groups. Strips from control animals produced greater quantities of PGE_2 than did strips from principal animals, but the difference was not significant. Expressing PGE_2 production per strip, per mm^2 of epithelium, or per mg-dry weight of epithelial tissue did not alter the interpretation of the data.

Table 6-3 PGE_2 release by epithelium-on strips from principal and control horses

	Unstimulated	A2317	Bradykinin	Histamine
Principal				
Horse #				
1 (25†)	100	426	306	344
2 (24†)	74	242	350	194
3 (29†)	68	480	408	248
4 (8†)	62	96	320	114
5 (12†)	0	144	100	142
6 (7†)	0	128	64	66
	54.7 ± 15.9	252.7 ± 73.0*	258.0 ± 63.0*	184.7 ± 44.8*
Control				
1	0	113	260	39
2	0	266	220	354
3	70	522	462	296
4	100	420	446	212
5	210	986	918	328
6	58	310	422	176
	77 ± 32.9	436.2 ± 135.3*	454.7 ± 111.0*	234.2 ± 52.4*

Values expressed as picogram/100 μL supernatant.
12 picogram = lower limit of accurate detection for assay
† = time to heaves (days)
* = significant difference from unstimulated value ($P < 0.05$)

In principal horses there was a significant correlation between the time taken to develop airway obstruction and the amount of PGE_2 released in response to A23187 ($r = 0.92$, $p = 0.009$) and histamine ($r = 0.86$, $p = 0.03$), but not bradykinin ($r = 0.74$, $p = 0.09$) (Figure 6-1).

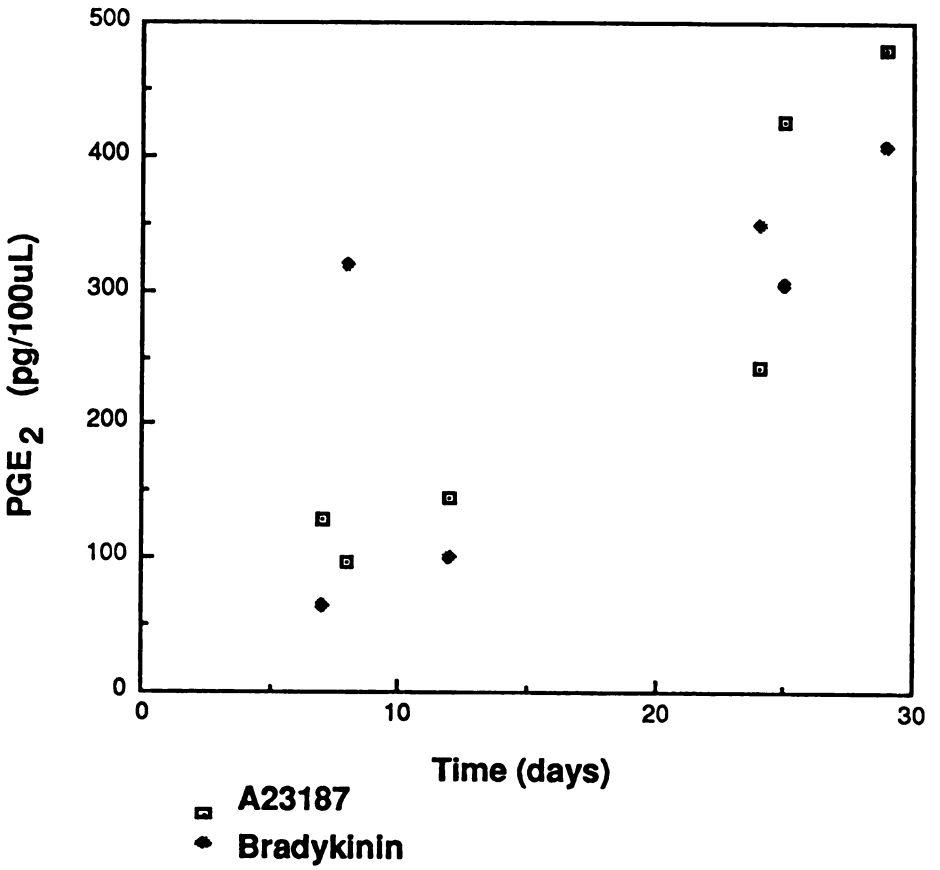


Figure 6.1 Time taken for horses to develop airway obstruction (days) versus PGE_2 production by epithelium-on strips (pg) in response to A23187 and bradykinin ($n = 6$).

There was no difference between principals and controls in PGE_2 production by unstimulated epithelium-off strips, but epithelium-off strips from control horses produced greater quantities of PGE_2 than did strips from principal horses when stimulated with A23187, bradykinin or histamine. This difference was significant for both A23187 and bradykinin, but not for histamine (Table 6-4).

Table 6-4 PGE_2 release by epithelium-off strips from principal and control horses (n = 6)

	Unstimulated	A2317	Bradykinin	Histamine
Principal				
Horse #				
1	0	0	111	79
2	0	0	12	0
3	0	0	0	0
4	20	29	12	52
5	0	5	0	0
6	0	1	8	0
	3.3 ± 3.7	5.8 ± 5.1	23.8 ± 19.3	21.8 ± 15.6
Control				
1	66	81	100	86
2	0	58	111	10
3	1	69	95	283
4	0	0	193	31
5	0	119	32	12
6	6	0	42	85
	12.2 ± 11.8	54.5 ± 21.0*+	95.5 ± 25.8*+	84.5 ± 46.0+

Values expressed as picogram/100 μL supernatant.
12 picogram = limit of detection for assay
* = significant difference from principal value
+ = significant difference from unstimulated value

Discussion

Unlike human tracheal epithelium, equine tracheal epithelium is not a prominant source of 15-HETE. When detected, amounts of i15-HETE produced were one tenth to one twentieth of those measured for PGE_2 production. These results suggest that trachcal epithelium was not the source of the increased pulmonary i15-HETE production measured in the experiment reported in Chapter 4. Since the percentage of total surface area contributed by the trachea is small, this conclusion cannot be extended to include all airway epithelium, unless it is assumed that the capacity of tracheal epithelium to produce eicosanoids is representative of airway epithelium in general. Given the marked variation in the cellular composition of epithelium throughout the lung, this may not be a valid assumption.

Several other cell types may have been the source of the i15-HETE produced in ponies with COPD. Holtzman et al. examined the ability of a wide range of human cell types to produce 15-HETE. Of the cells examined, airway epithelial cells and eosinophils were major sources, producing approximately 5000 and 3000 pmol/10^6 cells, respectively. The next group, comprising dermal and lung fibroblasts and endothelial cells produced 20-50 pmol 15-HETE/10^6 cells. The remaining cells studied included keratinocytes and the HL-60 granulocyte and K-562 erythroblast leukemic cell lines, all of which produced 1 pmol or less/10^6 cells.[161] Eosinophil numbers do not appear to be increased in bronchoalveolar lavage fluid, or peripheral blood samples collected from ponies

with COPD during acute airway obstruction,[86] but increased eosinophil numbers are reported to be present in histological sections of lung tissue from horses and ponies with COPD.[328] In that study, eosinophils were present in 5 of 7 horses with a clinical diagnosis of heaves, and in only 1 of 6 control horses. When present, eosinophils were located around arteries, veins, and bronchioles, so eosinophil-derived mediators would have ready access to the pulmonary vasculature. In human smokers, a comparison of bronchoalveolar lavage fluid cell populations with histological evaluation of airway inflammation revealed no correlation between the percentage of neutrophils present in lavage fluid and the severity of the inflammatory response in peripheral airways.[157] If a similar situation existed in ponies with COPD, then the absence of eosinophils in bronchoalveolar lavage fluid samples would not preclude a role for these cells in the pathogenesis of the disease.

One recently discovered cell type not included in Holtzman's study was the pulmonary intravascular macrophage. Pulmonary intravascular macrophages are located in the pulmonary vasculature of a number of species, including cats, calves, sheep, pigs, and goats.[359] Considerable numbers of these cells can be present in the lungs of species in which they occur. In sheep the surface area of pulmonary intravascular macrophages exposed to circulating blood was estimated to be 15.9 m^2, suggesting that mediators produced by these cells would readily gain access to the circulation.[346] Bertram et al. have reported that porcine pulmonary intravascular macrophages are able to produce both 12-

and 15-HETE in vitro.[21] Unfortunately, there has been no reported attempt to determine whether pulmonary intravascular macrophages are present in the lungs of horses and ponies.

With regard to the PGE_2 data, there was increased production of PGE_2 by epithelium-on strips from control animals, relative to principals, but the difference was not significant. However, there was a marked variation in PGE_2 production by individual principals. A similar variability existed in the time taken for principals to develop airway obstruction (7 to 29 days). If the amount of PGE_2 produced was an important factor in determining the onset of airway obstruction, there should be a correlation between PGE_2 production and the time taken for airway obstruction to develop. Such a correlation existed between PGE_2 production in response to A23187 and bradykinin, and the number of days principals took to develop airway obstruction. Epithelial strips that produced the greatest amount of PGE_2 tended to come from principal horses that took the longest time to develop clinical symptoms, which would fit with the theory that PGE_2 production was one factor involved in opposing the development of airway obstruction, possibly by modulating cholinergic stimulation of airway smooth muscle.

Results for PGE_2 production from the subepithelial connective tissue stroma were interesting. Only two of the six principal horses produced PGE_2 in response to stimulation, whereas all six control horses were able to produce PGE_2. Epithelium-off strips from control horses produced significantly more

PGE_2 than did those from principals when stimulated with A23187 and bradykinin. One possible explanation for the difference was that the epithelial cell layer had not been adequately removed from strips from control animals and that these cells were the source of the measured PGE_2. However, previous histological sections have shown that the stripping technique effectively removed the epithelial cell layer. Even if this was not the case for all strips, it is difficult to accept that such a consistent error could have occurred throughout an experiment that lasted four months where principal and control horses were processed alternately. Also, the cells most likely to remain if epithelial removal was inadequate would be basal cells, and, as described in Chapter 5, these cells produce little if any PGE_2.

Sub-epithelial production of PGE_2 has been reported from airway tissue in guinea pigs[108] and rats.[89] The importance of this subepithelial PGE_2 production is uncertain, since the amounts generated are small relative to that produced by the epithelium. However, mediators released into the subepithelial tissues may interact with airway smooth muscle, blood vessels, nerve endings, and nerve fibers supplying submucosal glands and muscle since many of these are located in, or pass through the subepithelial layer. If modulation of cholinergic innervation of airway smooth muscle by PGE_2 is important in the pathogenesis of COPD, the subepithelial tissue is one logical site for such an interaction to occur. Such a possibility, like so many other aspects of this disease, will only be resolved with further research.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Summary

These experiments were designed to study arachidonic acid metabolism in horses and ponies affected with chronic obstructive pulmonary disease, a respiratory disorder in horses and ponies with similarities to human asthma. Altered arachidonic acid metabolism had been reported to occur with a number of pulmonary diseases, including human asthma, and there has been much speculation as to whether these alterations were important in the pathogenesis of allergic airway disease.

In Chapter 2, I described the first experiment where I measured plasma and bronchoalveolar lavage fluid concentrations of 15-HETE, and metabolites of TxA_2 (TxB_2), PGI_2 (6-keto-$PGF_{1\alpha}$), and PGD_2 (9α,11β-PGF_2) in samples collected from principal and control ponies during remission, and when principal ponies had acute airway obstruction. Plasma 15-HETE and TxB_2 concentrations were increased significantly in the principal ponies during airway obstruction. My next step was to investigate the potential significance of these increases. In Chapter 3, I used the cyclooxygenase inhibitor flunixin

meglumine to study the effect of TxA_2 inhibition on pulmonary function and airway hyperresponsiveness in principal and control ponies. Flunixin meglumine treatment did not prevent the development of airway obstruction or airway hyperresponsiveness in principal ponies, but reduced the average time principal ponies took to develop airway obstruction from 12.2 to 5.1 days. This suggested that TxA_2 production was not important in the development of airway obstruction or airway hyperresponsiveness, but that a cyclooxygenase metabolite could be important in delaying the onset of pulmonary dysfunction. Alternatively, the ability of flunixin meglumine treatment to decrease the time taken to develop airway obstruction could be due to shunting of arachidonic acid into the lipoxygenase pathway, with resultant production of pro-inflammatory or bronchospastic leukotrienes such as LTB_4 and LTC_4/D_4.

In Chapter 4, I sought to determine whether the lung was the site of the increased 15-HETE production. This involved collecting blood samples from the right ventricle (pulmonary inflow) and carotid artery (pulmonary outflow) of principal and control ponies during remission (Period A), when principals had airway obstruction (Period B), and when ponies had returned to pasture (Period C), and measuring 15-HETE concentrations in each. All the samples were subjected to high-performance liquid chromatography prior to radioimmunoassay to improve the specificity of the assay. Plasma 15-HETE concentrations were increased in carotid artery samples compared to right ventricle samples in principal and control ponies at all three time periods, showing that the lung was a source of 15-HETE in ponies. This increase was

significant at periods A,B, and C for the principals, and at period A for the controls. In addition, pulmonary 15-HETE production was significantly greater in principals compared to controls at periods A and B. There was also a significant correlation between the amount of 15-HETE produced and change in dynamic compliance measured between periods A and B.

The next experiments were designed to attempt to localize this 15-HETE production to a particular site within the lung. In humans, airway epithelium had been shown to be a major site of 15-HETE production, and in Chapter 5, I described a technique that I developed to study arachidonic acid metabolism by equine tracheal epithelium in vitro. This involved obtaining strips of intact tracheal epithelium attached to a thin connective tissue layer, mounting these strips on glass supports, and studying their ability to incorporate, release, and metabolize arachidonic acid in response to physiologic and non-physiologic agonists. The pattern of eicosanoids released by these epithelial strips was similar to that reported for cultured tracheal epithelium, with PGE_2 being the major cyclooxygenase metabolite. There was little evidence of 15-HETE production, but these strips were obtained from normal horses with no evidence of pulmonary disease.

I then used this technique to study arachidonic acid metabolism in tracheal epithelium collected from horses with chronic obstructive pulmonary disease when they had acute airway obstruction. In the experiment described in Chapter 6, I collected tracheal epithelium from principal and control horses and measured 15-HETE and PGE_2 release in response to stimulation with

histamine, bradykinin, and A23187. My reason for studying PGE_2 as well as 15-HETE was that PGE_2 has anti-inflammatory properties and can also relax airway smooth muscle, leading several investigators to suggest that decreased epithelial production of PGE_2 may be a factor in airway hyperresponsiveness. There were detectable amounts of 15-HETE released by epithelium from both principal and control horses, but the actual amounts measured were one tenth to one twentieth of those measured for PGE_2. This suggested that equine tracheal epithelium, unlike human tracheal epithelium, was not a major source of 15-HETE. Prostaglandin E_2 was produced by epithelium from both principal and control horses, with production from control horse epithelium tending to be greater. In the principal horses, there was a significant correlation between epithelial PGE_2 release in response to bradykinin and A23187, and the time taken to develop airway obstruction. The principals with the greatest capacity to release PGE_2 tended to take the longest time to develop airway obstruction. There was also a significantly greater production of PGE_2 by subepithelial tissues from control horses compared to principals when these tissues were stimulated with bradykinin and A23187.

Conclusions

The experiments that comprise this thesis show that in horses and ponies, as in other species, the occurrence of pulmonary dysfunction is associated with altered arachidonic acid metabolite production. Whether such alterations are a cause of the airway obstruction and hyperresponsiveness seen in ponies and

horses with chronic obstructive pulmonary disease, or a byproduct of the pathophysiological processes involved in producing the disease, is unknown.

I will discuss the three specific alterations that I have reported in the body of the thesis, together with my evaluation of their potential significance and experiments that I think could help to further define their role in the pathogenesis of equine chronic obstructive pulmonary disease.

These alterations were

i) An increase in plasma TxB_2 concentrations during the occurrence of airway obstruction.

ii) An increase in plasma 15-HETE concentrations in blood leaving the lungs of principal ponies.

iii) A possible decreased capacity for epithelial and subepithelial tissue from principal horses to release PGE_2.

i) Increased TxB_2 production. Ponies with chronic obstructive pulmonary disease, dogs exposed to ozone, and human asthmatics challenged with antigen all have increased TxB_2 production. Unlike the situation in dogs, where inhibition of TXB_2 production prevented airway hyperresponsiveness, TxB_2 production does not appear to be important in the pathogenesis of human asthma or equine chronic obstructive pulmonary disease, since inhibition of its production did not prevent or ameliorate the pulmonary dysfunction seen with these two conditions.

To investigate more fully the role of TxA_2 in equine COPD would require using specific TxA_2 synthase inhibitors or receptor antagonists rather

than the cyclooxygenase inhibitor that I used. However, I think that the alterations in 15-HETE and PGE_2 production rather than TxA_2 are a potentially more interesting area for further study.

ii) Increased 15-HETE production. Human asthma and equine chronic obstructive pulmonary disease are both associated with increased production of 15-HETE. An important point concerning the increased plasma i15-HETE concentrations in principal ponies was that there was significantly greater pulmonary 15-HETE production in principals compared to controls during remission as well as when acute airway obstruction was present. If increased 15-HETE production is important in the pathogenesis of equine COPD, and not merely a byproduct of the process, as TxA_2 appears to be, then it is unlikely to be acting as a primary factor. If this were the case then I would have expected to see some evidence of pulmonary dysfunction during the remission phase when pulmonary 15-HETE production was significantly elevated in principal ponies. Two possibilities that arise from this interpretation are 1) that a metabolite of 15-HETE could be involved in the pathogenesis of the disease, and that this metabolite is produced in greater quantities when animals are housed in a barn and exposed to hay dust; or 2) that 15-HETE potentiates the production of, or response to, some other mediator that is produced during barn housing. These possibilities are not unreasonable since 15-HETE is involved in regulating leukotriene synthesis in a variety of cells and can also potentiate histamine-induced contraction of tracheal smooth muscle. Metabolites of 15-

HETE act as neutrophil chemoattractants or smooth muscle spasmogens and can cause neuronal hypersensitivity.

While I was able to show that the lung was a source of 15-HETE production in ponies, my efforts to identify upper airway epithelium as the site of that production were unsuccessful. I think that further efforts to identify a role for 15-HETE in the pathogenesis of equine COPD would be best directed not towards an exhaustive search for the specific site of production within the lung, but rather towards investigating some of the ways in which 15-HETE could be acting in this disease. The absence of specific 15-lipoxygenase inhibitors means that it is not possible to study the effect of inhibition of 15-HETE production on airway function. One area worth exploring would be whether 15-HETE could be acting pre- or post-synaptically to alter airway smooth muscle responsiveness to other inflammatory mediators such as histamine.

iii) Decreased epithelial PGE_2 production. The data showing a possible relationship between the ability of airway epithelium or subepithelial tissue to produce PGE_2 and the occurrence of COPD is interesting, but tenuous.

One of the assumptions inherent in the interpretation of these data is that the responsiveness of airway smooth muscle from principal and control horses to the relaxant action of PGE_2 is comparable. As yet there are no data to suport such a conclusion.

The correlation between epithelial PGE_2 production and the time taken for horses to develop airway obstruction also requires further study to

determine its significance. These data can be interpreted to mean that epithelial PGE_2 has a role in delaying the development of airway obstruction, and therefore, the principals able to produce the greatest amount of PGE_2 would tend to take the longest time to develop clinical signs. Alternatively, barn exposure itself may stimulate PGE_2 production by airway epithelium, so that epithelium from the animals kept in the barn for the longest time would tend to produce the most PGE_2. These possibilities may be distinguished by determining the capacity of airway epithelium to produce PGE_2 prior to placing horses or ponies in the barn, and correlating PGE_2 production and the time animals take to develop airway obstruction. The epithelial strip technique is obviously unsuited to such an experiment, since it requires that the animal be sacrificed, but an alternative could be to obtain epithelial tissue by bronchial biopsy and study its PGE_2 synthetic capacity either directly, or following in vitro culture.

LIST OF REFERENCES

LIST OF REFERENCES

1. Abraham WM, Stevenson JS, Garrido R. A possible role for platelet activating factor in allergen-induced late responses: modification by a selective antagonist. J Appl Physiol 1989; 66:2351-7.

2. Aizawa H, Miyazaki N, Shigematsu N, Tomooka M. A possible role of airway epithelium in modulating hyperresponsiveness. Br J Pharmacol 1988; 93:139-45.

3. Albert DH, Snyder F. Biosynthesis of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) from 1-alkyl-2-acyl-sn-glycero-3-phosphocholine by rat alveolar macrophages. J Biol Chem 1983; 258:97-102.

4. Antonissen LA, Mitchell RW, Kroeger EA, Kepron W, Tse KS, Stephens NL. Mechanical alterations of airway smooth muscle in a canine asthmatic model. J Appl Physiol 1979; 46:681-7.

5. Armour CL, Lazar NM, Schellenberg RR, Taylor SM, Chan N, Hogg JC, Pare PD. A comparison of in vivo and in vitro human airway reactivity to histamine. Am Rev Respir Dis 1984; 129:907-10.

6. Armstrong PJ, Derksen FJ, Slocombe RF, Robinson NE. Airway responses to aerosolized methacholine and citric acid in ponies with recurrent airway obstruction (heaves). Am Rev Respir Dis 1986; 133:357-61.

7. Babior BM. The respiratory burst of phagocytes. J Clin Invest 1984; 73:599-601.

8. Bachwich PR, Chensue SW, Larrick JW, Kunkel SL. Tumor necrosis factor stimulates interleukin-1 and prostaglandin E_2 production in resting macrophages. Biochem Biophys Res Comm 1986; 136:94-101.

9. Barnes PJ. Neural control of human airways in health and disease. Am Rev Respir Dis 1986; 134:1289-1314.

10. Barnes PJ, Brown MJ, Silverman M, Dollery CT. Circulating catecholamines in exercise and hyperventilation induced asthma. Thorax 1981; 36:435-40.

11. Barnes PJ, Crossman DC, Fuller RW. Cutaneous effects of neuropeptides are dependant upon both histamine and cyclooxygenase products. Br J Pharmacol 1987; 90:100P.

12. Barnes PJ, Dollery CT, MacDermot J. Increased pulmonary alpha-adrenergic and reduced beta-adrenergic receptors in experimental asthma. Nature Lond 1980; 285:569-71.

13. Barnes PJ, Fan Chung K, Page CP. Inflammatory mediators and asthma. Pharmacological Reviews 1988; 40:49-84.

14. Barnes PJ, Rhoden KJ. The effect of oxygen-derived free radicals on airway smooth muscle responses. Br J Pharmacol 1987; 90:142P.

15. Barnett K, Jacoby DB, Nadel JA, Lazarus SC. The effects of epithelial cell supernatant on contractions of isolated canine tracheal smooth muscle. Am Rev Respir Dis 1988; 138:780-3.

16. Basu S. Endogenous inhibition of arachidonic acid metabolism in the endometrium of the sheep. Prostaglandins Leukot Essent Acids 1989; 35:147-52.

17. Beasley CRW, Robinson C, Featherstone RL, Varley JG, Hardy CC, Church MK, Holgate ST. 9α,11β-Prostaglandin F_2, a novel metabolite of Prostaglandin D_2 is a potent contractile agonist of human and guinea-pig airways. J Clin Invest 1987; 79:978-83.

18. Beech J. Principals of treatment. Vet Clin North Am [Large Anim Pract] 1979; 1:73-88.

19. Benveniste J, Chignard M, Le Couedic JP, Vargaftig BB. Biosynthesis of platelet-activating factor (PAF-acether). II. Involvement of phospholipase A_2 in the formation of PAF-acether and lyso-PAF-acether from rabbit platelets. Thromb Res 1982; 25:375-85.

20. Berend N, Armour CL, Black JL. Indomethacin inhibits the increased airway responsiveness to histamine following inhalation of C5a des Arg in rabbits. Agents Actions 1986; 18:468-72.

21. Bertram TA, Overby LH, Danilowicz R, Eling TE, Brody AR. Pulmonary intravascular macrophages metabolize arachidonic acid in vitro. Comparison with alveolar macrophages. Am Rev Respir Dis 1988; 138:936-44.

22. Bethel RA, McClure CL. Cyclooxygenase inhibitors increase canine tracheal muscle response to parasympathetic stimuli in situ. J Appl Physiol 1990; 68:2597-603.

23. Beutler B, Tkacenko V, Milsark I, Krochin N, Cerami A. Effect of gamma interferon on cachectin expression by mononuclear phagocytes. J Exp Med 1986; 164:1791-6.

24. Bianco S, Robuschi M, Cesserani R, Gandolfi C, Kamburoff PL. Prevention of aspecifically induced bronchoconstriction by PGI_2 and 20-methyl-PGI_2 in asthmatic patients. Pharm Res Comm 1978; 10:657-74.

25. Bigby TD, Meslier N. Transcellular lipoxygenase metabolism between monocytes and platelets. J Immunol 1989; 143:1948-54.

26. Bisgaard H, Groth S, Madsen F. Bronchial hyperreactivity to leucotriene D_4 and histamine in exogenous asthma. Br Med J 1985; 290:1468-71.

27. Bjork J, Hugli TE, Smedegard G. Microvascular effects of anaphylatoxins C3a and C5a. J Immunol 1985; 134:1115-9.

28. Black JL, Armour CL, Vincenc KS, Johnson PRA. A comparison of the contractile activity of PGD_2 and $PGF_{2\alpha}$ in human isolated bronchus. Prostaglandins 1986; 32: 25-31.

29. Blenkinsopp WK. Proliferation of respiratory tract epithelium in the rat. Exp Cell Res 1967; 46:144-54.

30. Boot JR, Cockerill AF, Dawson W, Mallen DNB, Osborne DJ. Modification of prostaglandin and thromboxane release by immunological sensitization and successive immunological challenges from guinea-pig lung. Int Archs Allergy appl Immunol 1978; 57:159-64.

31. Boren HG, Paradise LJ. Cytogenetics of lung. In Pathogenesis and Therapy of Lung Cancer. C. C. Harris, editors. Marcel Dekker, New York. 1978; 369-418.

32. Braunstein G, Labat C, Brunelleschi S, Benveniste J, Marsac J, Brink C. Evidence that the histamine sensitivity and responsiveness of guinea-pig isolated trachea are modulated by epithelial prostaglandin E_2 production. Br J Pharmacol 1988; 95:300-8.

33. Bray MA, Ford-Hutchinson AW, Smith MJH. Leukotriene B_4: An inflammatory mediator in vivo. Prostaglandins 1981; 22(2): 213-22.

34. Breeze RG. Heaves. Vet Clin North Am [Large An Pract] 1979; 1:219-30.

35. Breeze RG, Lee H, Grant BD. Toxic lung disease. Mod Vet Pract 1978; 59:301.

36. Breuer R, Zajicek G, Christensen TG, Lucey EC, Snider GL. Cell kinetics of normal adult hamster bronchial epithelium in the steady state. Am J Respir Cell Mol Biol 1990; 2:51-8.

37. Brigham KL. Role of free radicals in lung injury. Chest 1986; 89:859-63.

38. Brigham KL, Serafin W, Zadoff A, Blair I, Meyrick B, Oates JA. Prostaglandin E_2 attenuation of sheep lung responses to endotoxin. J Appl Physiol 1988; 64: 2568-74.

39. Britton JR, Hanley SP, Tattersfield AE. The effect of an oral leukotriene D_4 antagonist L-649,923 on the response to inhaled antigen in asthma. J Allergy Clin Immunol 1987; 79:811-16.

40. Broadstone RV, Scott JS, Derksen FJ, Robinson NE. Effects of atropine in ponies with recurrent airway obstruction. J Appl Physiol 1988; 65:2720-5.

41. Broadstone RV, LeBlanc PH, Derksen FJ, Robinson NE. In vitro responses of airway smooth muscle from horses with recurrent airway obstruction. Pulmonary Pharmacology 1990 (in Press)

42. Buckner CK, Songsiridej V, Dick EC, Busse WW. In vivo and in vitro studies on the use of the guinea pig as a model for virus-provoked airway hyperreactivity. Am Rev Respir Dis 1985; 132:305-310.

43. Busse WW. The precipitation of asthma by upper respiratory infections. Chest 1985; 87:44-8S.

44. Butler GB, Adler KB, Evans JN, Morgan DW, Szarek JL. Modulation of rabbit airway smooth muscle responsiveness by respiratory epithelium. Am Rev Respir Dis 1987; 135:1099-104.

45. Cabezas GA, Graf PD, Nadel JA. Sympathetic versus parasympathetic nervous regulation of airways in dogs. J Appl Physiol 1971; 31:651-5.

46. Campbell AM, Harper ST, Hallam C, Wells E, Mann J, Robinson C. Enhancement of leukotriene C_4 release from primate airway macrophages by cellular interactions. Br J Pharmacol 1989; 98:637-45.

47. Camussi G, Aglietta M, Malavasi F, Bussolino F, Piacibello W, Sanavio F, Tetta C. Release of platelet activating factor from human endothelial cells. In. Platelet Activating Factor. INSERM Symposium No.23, Arnoux B, Beneviste J, Eds, Elsevier Science Publications BV. 1983; pp 83-90.

48. Cartier A, Thomson NC, Frith PA, Roberts R, Hargreave FE. Allergen-induced increase in bronchial responsiveness to histamine: relationship to the late asthmatic response and change in airway caliber. J Allergy Clin Immunol 1982; 70:170-7.

49. Casale TB, Bowman S, Kaliner M. Induction of human cutaneous mast cell degranulation by opiates and endogenous opioid peptides: evidence for opiate and nonopiate receptor participation. J Allergy Clin Immunol 1984; 73:775-81.

50. Castleman WL, Sorkness RL, Lemanske RF, Grasee G, Suyemoto MM. Neonatal viral bronchiolitis and pneumonia induces bronchiolar hypoplasia and alveolar dysplasia in rats. Lab Invest 1988; 59:387-96.

51. Chan-Yeung M, Giclas PC, Henson PM. Activation of complement by plicatic acid, the chemical compound responsible for asthma due to western red cedar (Thuja plicata). J Allergy Clin Immunol 1980; 65:333-7.

52. Chauncey JB, Peters-Golden M, Simon RH. Arachidonic acid metabolism by rat alveolar epithelial cells. Lab Invest 1988; 58:133-40.

53. Chauncey JB, Simon RH, Peters-Golden M. Rat alveolar macrophages synthesize leukotriene B_4 and 12-hydroxyeicosatetraenoic acid from alveolar epithelial cell-derived arachidonic acid. Am Rev Respir Dis 1988; 138:928-35.

54. Chilton BS, Kennedy JR, Nicosia SV. Isolation of basal and mucous cell populations from rabbit trachea. Am Rev Respir Dis 1981; 124:723-7.

55. Chung KF, Aizawa H, Leikauf GD, Ueki IF, Evans TW, Nadel JA. Airway hyperresponsiveness induced by platelet-activating factor: role of thromboxane generation. J Pharm Exp Ther 1986; 236:580-4.

56. Chung KF, Evans TW, Graf PD, Nadel JA. Modulation of cholinergic neurotransmission in canine airways by thromboxane-mimetic U46619. Eur J Pharmacol 1985; 117:373-5.

57. Church MK, Cushey MJ, Holgate ST. Adenosine, a positive modulator of the asthmatic response. In: Asthma: Basic mechanisms and clinical management. Barnes PJ, Rodger IW, Thomson NC, Eds. Academic Press, San Diego. 1988; pp 273-281.

58. Churchill L, Chilton FH, Resau JH, Bascom R, Hubbard WC, Proud D. Cyclooxygenase metabolism of endogenous arachidonic acid by cultured human tracheal epithelial cells. Am Rev Respir Dis 1989; 140:449-59.

59. Clancy RM, Dahinden CA, Hugli TE. Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by N-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. Proc Natl Acad Sci USA 1983; 80: 7200-4.

60. Cloud ML, Enas GC, Kemp J, Platts-Mills T, Altman LC, Townley R, Tinkelman D, King T Jr., Middleton E, Sheffer AL, Mcfadden ER Jr., Farlow DS. A specific LTD_4/LTE_4-receptor antagonist improves pulmonary function in patients with mild, chronic asthma. Am Rev Respir Dis 1989; 140:1336-9.

61. Coene MC, Van Hove C, Claeys M, Herman AG. Arachidonic acid metabolism by cultured mesothelial cells. Different transformations of exogenously added and endogenously released substrate. Biochim Biophys Acta 1988; 710:437-45.

62. Cohen P, Derksen A. Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. Br J Haematol 1969;17:359-71.

63. Coles SJ, Neill KH, Reid LM, Austen KF, Nii Y, Corey EJ, Lewis RA. Effects of leukotrienes C_4 and D_4 on glycoprotein and lysozyme secretion by human bronchial mucosa. Prostaglandins 1983; 25:155-70.

64. Coles SJ, Neill KH, Reid LM. Potent stimulation of glycoprotein secretion in canine trachea by substance P. J Appl Physiol 1984; 57:1323-7.

65. Cook WR. Chronic bronchitis and alveolar emphysema in the horse. Vet Rec 1976; 99:448-51.

66. Copas JL, Borgeat P, Gardiner PJ. The actions of 5, 12 and 15-HETE on tracheobronchial smooth muscle. Prostaglandin Leukotriene Med 1982; 8:105-14.

67. Corrigan CJ, Hartnell A, Kay AB. T lymphocyte activation in acute severe asthma. Lancet 1988; 1:1129-32.

68. Cotran RS, Kumar V, Robbins SL. Inflammation. In Pathologic basis of Disease. Cotran RS, Kumar V, Robbins SL eds. WB Saunders, Philadelphia, PA. 1989; pp 45-62.

69. Cotran RS, Kumar V, Robbins SL. Diseases of Immunity. In Pathologic basis of Disease. Cotran RS, Kumar V, Robbins SL eds. WB Saunders, Philadelphia, PA. 1989; pp 163-9.

70. Curry JJ. The action of histamine on the respiratory tract in normal and asthmatic subjects. J Clin Invest 1946; 25:785-1.

71. Cushley MJ, Tattersfield AE, Holgate ST. Adenosine-induced bronchoconstriction in asthma: antagonism by inhaled theophylline. Am Rev Respir Dis 1984; 129:380-4.

72. Cushley MJ, Tattersfield AE, Holgate ST. Inhaled adenosine and guanosine on airway resistance on airway resistance in normal and asthmatic subjects. Br J Clin Pharmacol 1983; 15:161-5.

73. Cuss FM, Dixon CMS, Barnes PJ. Effects of inhaled platelet activating factor on pulmonary function and bronchial responsiveness in man. Lancet 1986; 2:189-91.

74. Cuthbert MF. Bronchodilator activity of aerosols of prostaglandins E_1 and E_2 in asthmatic subjects. Proc roy Soc Med 1971; 64:15-6.

75. Dahinden CA, Zingg J, Maly FE, de Weck AL. Leukotriene production in human neutrophils primed by recombinant granulocyte/macrophage colony-stimulating factor and stimulated with the complement component C5a or fMLP as second signals. J Exp Med 1988; 167:1281-95.

76. Dahl R, Venge P, Fredens K. Eosinophils. In Asthma: Basic mechanisms and clinical management. Barnes PJ, Rodger IW, Thomson NC. eds. Academic Press, San Diego, CA. 1988; pp 115-129.

77. Dahlen SE. Pulmonary effects of leukotrienes. Acta Physiol Scand 1983; Suppl 512.

78. Dahlen S-E, Hansson G, Hedqvist P, Bjorck T, Granstrom E, Dahlen B. Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with the release of leukotrienes C_4, D_4 and E_4. Proc Nat Acad Sci USA 1983; 80:1712-6.

79. Dahlen S-E, Hedqvist P, Hammarstrom S, Samuelsson B. Leukotrienes are potent constrictors of human bronchi. Nature 1980; 288:484-6.

80. Davidson AB, Lee TH, Scanlon PD, Solway J, McFadden ER Jr, Ingram RH Jr, Corey EJ, Austen KF, Drazen JM. Bronchoconstrictor effects of leukotriene E_4 in normal and asthmatic subjects. Am Rev Respir Dis 1987; 135:333-7.

81. De Monchy JGR, Kauffman HF, Venge P, Koeter GH, Jansen HM, Sluiter HJ, de Vries K. Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. Am Rev Respir Dis 1985; 131:373-6.

82. Derksen FJ, Robinson NE, Armstrong PJ, Stick JA, Slocombe RF. Airway reactivity in ponies with recurrent airway obstruction (heaves). J Appl Physiol 1985; 58:598-604.

83. Derksen FJ, Robinson NE, Scott JS, Stick JA. Aerosolized Micropolysporum faeni antigen as a cause of pulmonary dysfunction in ponies with recurrent airway obstruction (heaves). Am J Vet Res 1988; 49:933-8.

84. Derksen FJ, Robinson NE, Slocombe RF. Ovalbumin-induced lung disease in the pony: role of vagal mechanisms. J Appl Physiol 1982; 53:719-25.

85. Derksen FJ, Robinson NE, Slocombe RF, Hill RE. 3-Methylindole-induced pulmonary toxicosis in ponies. Am J Vet Res 1982; 43:603-7.

86. Derksen FJ, Scott JS, Miller DC, Slocombe RF, Robinson NE. Bronchoalveolar lavage in ponies with recurrent airway obstruction (Heaves). Am Rev Respir Dis 1985; 132:1066-70.

87. Derksen FJ, Scott D, Robinson NE, Slocombe RF, Armstrong PJ. Intravenous histamine administration in ponies with recurrent airway obstruction (heaves). Am J Vet Res 1985; 46:774-777.

88. Derksen FJ, Scott JS, Slocombe RF, Robinson NE. Micropolyspora faeni causes airway inflammation but not hyperresponsiveness in sensitized ponies. J Appl Physiol 1987; 62:1398-1404.

89. Devillier P, Acker M, Advenier C, Regoli D, Frossard N. Respiratory epithelium releases relaxant prostaglandin E_2 through activation of substance P (NK1) receptors. Am Rev Respir Dis 1989; 139:A351.

90. Diaz P, Gonzales MC, Galleguillos FR, Ancic P, Cromwell O, Shepherd D, Durham SR, Gleich GJ, Kay AB. Leukocytes and mediators in bronchoalveolar lavage during allergen-induced late-phase asthmatic reactions. Am Rev Respir Dis 1989; 139:1383-9.

91. Dolovich J, Back N, Arbesman CE. Kinin-like activity in nasal secretions of allergic patients. Int Arch Allergy 1970; 38:337-44.

92. Drazen JM, Austen KF. Pulmonary response to antigen infusion in the sensitized guinea pig: modification by atropine. J Appl Physiol 1975; 39:916-9.

93. Drommer W, Kaup FJ, Deegen E. Transmission and scanning electron microscopic findings in the tracheobronchial tree of horses with chronic obstructive pulmonary disease. In: Lung function and respiratory diseases in the horse. Proceedings, International Symposium, Hannover, Germany Deegen E, Beadle RE eds, Hippiatrika, Stuttgart, Germany. 1985; pp 16-9.

94. Dunhill MS. The pathology of asthma with special reference to changes in the bronchial mucosa. J Clin Pathol 1960; 13:27-33.

95. Durham SR, Kay AB. Eosinophils, bronchial hyperreactivity and late-phase asthma reactions. Clin Allergy 1985; 15:411-8.

96. Durham SR, Lee TH, Cromwell O, Shaw RJ, Merrett TG, Merrett J, Cooper P, Kay AB. Immunologic studies in allergen-induced late-phase asthmatic reactions. J Allergy Clin Immunol 1984; 74:49-60.

97. Dworski R, Sheller JR, Wickersham NE, Roberts LJ II, Brigham KL. Cyclooxygenase inhibition enhances leukotriene production in vivo in allergen challenged sheep lung. Clin Res 1987; 35:254A.

98. Edenius C, Haeggstrom J, Lindgren JA. Transcellular conversion of endogenous arachidonic acid to lipoxins in mixed human platelet-granulocyte suspensions. Biochem Biophys Res Comm 1988; 157:801-7.

99. Elias JA, Freundlich B, Kern JA, Rosenbloom J. Cytokine networks in the regulation of inflammation and fibrosis in the lung. Chest 1990; 97:1439-45.

100. Elias JA, Gustilo K, Baeder W, Freundlich B. Synergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor. J Immunol 1987; 138:3812-6.

101. Eling TE, Danilowicz RM, Henke DC, Sivarajah K, Yankaskas JR, Boucher RC. Arachidonic acid metabolism by canine tracheal epithelial cells: Product formation and relationship to chloride secretion. J Biol Chem 1986; 261:12841-9.

102. Elwood RK, Kennedy S, Belzberg A, Hogg JC, Pare PD. Respiratory mucosal permeability in asthma. Am Rev Respir Dis 1983; 128:523-7.

103. Engels F, Oosting RS, Nijkamp FP. Pulmonary macrophages induce deterioration of guinea-pig tracheal beta-adrenergic function through release of oxygen radicals. Eur J Pharmacol 1985; 111:143-4.

104. Ferguson AC, Wong FWM. Bronchial hyperresponsiveness in asthmatic children. Correlation with macrophages and eosinophils in broncholavage fluid. Chest 1989; 96:988-91.

105. Fink JN. Hypersensitivity Pneumonitis. In Immunologic and Infectious Reactions in the Lung. Lung Biology in Health and Disease. Vol 1. Kirkpatrick CH, Reynolds HY eds. Marcel Decker Inc, New York. 1976; pp 229-41.

106. Flavahan NA, Aarhus LL, Rimele TJ, Vanhoutte PM. Respiratory epithelium inhibits bronchial smooth muscle tone. J Appl Physiol 1985; 58:834-8.

107. Flower RJ, Harvey EA, Kingston WP. Inflammatory effects of prostaglandin D_2 in rat and human skin. Br J Pharmacol 1976; 56:229-33.

108. Folkerts G, Engels F, Nijkamp FP. Endotoxin-induced hyperreactivity of the guinea-pig isolated trachea coincides with decreased prostaglandin E_2 production by the epithelial layer. Br J Pharmacol 1989; 96:388-94.

109. Folkerts G, Henricks PAJ, Nijkamp FP. Inflammatory reactions in the respiratory airways of the guinea pig do not necessarily induce bronchial hyperreactivity. Agents Actions 1988; 23:94-6.

110. Ford-Hutchinson AW. The neutrophil and lymphocytes. In Asthma: Basic mechanisms and clinical management. Barnes PJ, Rodger IW, Thomson NC. eds. Academic Press, San Diego, CA. 1988; pp 131-42.

111. Ford-Hutchinson AW. Leukotrienes: their formation and role as inflammatory mediators. Federation Proc 1985; 44:25-9.

112. Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJH. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature 1981; 286:264-5.

113. Fradin A, Zirrolli JA, Maclouf J, Vausbinder L, Henson PM, Murphy RC. Platelet-activating factor and leukotriene biosynthesis in whole blood. A model for the study of transcellular arachidonate metabolism. J Immunol 1989; 143:3680-5.

114. Fredholm BB, Sydbom A. Are the anti-allergic actions of theophylline due to antagonism at the adenosine receptor? Agents Actions 1980; 10:145-7.

115. Friedman MC, Kaliner MA. Human mast cells and asthma. Am Rev Respir Dis 1987; 135:1157-64.

116. Fujimura M, Sasaki F, Nakatsumi Y, Takahashi Y, Hifumi S, Taga K, Mifune J, Tanaka T, Matsuda T. Effects of a thromboxane synthetase inhibitor (OKY-046) and a lipoxygenase inhibitor (AA-861) on bronchial responsiveness to acetylcholine in asthmatic subjects. Thorax 1986; 41:955-9.

117. Fuller RW, Dixon CMS, Cuss FMC, Barnes PJ. Bradykinin-induced bronchoconstriction in man: mode of action. Am Rev Respir Dis 1987; 135:176-80.

118. Fuller RW, Dixon CMS, Dollery CT, Barnes PJ. Prostaglandin D_2 potentiates airway responses to histamine and methacholine. Am Rev Respir Dis 1986; 133:252-4.

119. Gardiner PJ, Collier HOJ. Specific receptors for prostaglandins in airways. Prostaglandins 1980; 19:819-41.

120. Gatto LA. Cell distribution in tracheal surface epithelium and the effects of long- term pilocarpine and atropine administration. Anatomical Rec 1989; 225:133-8.

121. Gerber H. Chronic pulmonary disease in the horse. Equine Vet J 1973; 5:26-32.

122. Gerberick GF, Jaffe HA, Willoughby JB, Willoughby WF. Relationships between pulmonary inflammation, plasma transudation, and oxygen metabolite secretion by alveolar macrophages. J Immunol 1986; 137:114-21.

123. Gibson PG, Dolovich J, Denburg J, Ramsdale EH, Hargreave FE. Chronic cough: eosinophilic bronchitis without asthma. Lancet 1989; 1:1346-8.

124. Gill KK, Kroeger EA. Effect of indomethacin on neural and myogenic components in equine airway smooth muscle. J Pharm Exp Therap 1990; 252:358-64.

125. Gleich GJ, Frigas E, Loegering DA, Wassom DL, Steinmuller D. Cytotoxic properties of the eosinophil major basic protein. J Immunol 1979; 123:2925-7.

126. Glynn AA, Michaels L. Bronchial biopsy in chronic bronchitis and asthma. Thorax 1960; 15:142-53.

127. Godard P, Chaintreuil J, Damon M, Coupe M, Flandre O, Crastes de Paulet A, Michel FB. Functional assessment of alveolar macrophages: comparison of cells from asthmatics and normal subjects. J Allergy Clin Immunol 1982; 70:88-93.

128. Goetzl EJ. Selective feed-back inhibition of the 5-lipoxygenation of arachidonic acid in human T-lymphocytes. Biochem Biophys Res Commun 1981; 101:344-50.

129. Goetzl EJ, Foster DW, Payan DG. A basophil-activating factor from human T lymphocytes. Immunology 1984; 53:227-34.

130. Goetzl EJ, Pickett WC. Novel structural determinants of the human neutrophil chemotactic activity of leukotriene B. J Exp Med 1981; 153:482-7.

131. Gold WM, Kessler GF, Yu DYC. Role of vagus nerves in experimental asthma in allergic dogs. J Appl Physiol 1972; 33:719-25.

132. Gonzalez MC, Diaz P, Galleguillos FR, Ancic P, Cromwell O, Kay AB. Allergen-induced recruitment of bronchoalveolar helper (OKT4) and suppressor (OKT8) T-cells in asthma. Am Rev Respir Dis 1987; 136:600-4.

133. Granstrom E. Methodology in prostaglandin and thromboxane assay. Prog Lipid Res 1986; 25:119-27.

134. Granstrom E, Kumlin M. Assay of thromboxane production in biological systems: Reliability of TXB_2 versus 11-dehydro-TXB_2 as targets for measurement. Adv Prostaglandin Thromboxane Leukotriene Res 1987; 17:587-94.

135. Gray PR, Derksen FJ, Robinson NE, Carpenter-Deyo LJ, Johnson HG, Roth RA. The role of cyclooxygenase products in the acute airway obstruction and airway hyperreactivity of ponies with heaves. Am Rev Respir Dis 1989; 140:154-60.

136. Green FA. Lipoxygenase activities of the epithelial cells of the human buccal cavity. Biochem Biophys Res Commun 1989; 160:545-51.

137. Green K, Hedqvist P, Svanborg N. Increased plasma levels of 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ after allergen-provoked asthma in man. Lancet 1974; 2:1419-21.

138. Greer IA, Winter JH, Gaffney D, McLoughlin K, Belch JJ, Boyd G, Forbes CD. Platelet activation in allergic asthma. Thrombosis Haemostasis 1985; 53:438.

139. Gross NJ. Anticholinergic agents in COPD. Chest 1987; 91(Suppl.5):52-7S.

140. Guyton AC. The Autonomic Nervous System. In. Textbook of Medical Physiology. WB Saunders, Philadelphia. 1986; pp 90-2.

141. Halliwell REW, Fleischman JB, Mackay-Smith M, Beech J, Gunson DE. The role of allergy in chronic pulmonary disease of horses. J Am Vet Med Assoc 1979; 174:277-81.

142. Halonen M, Palmer JD, Lohman IC, McManus LM, Pinckard RN. Respiratory and circulatory alterations induced by acetyl glyceryl ether phosphorylcholine, a mediator of IgE anaphylaxis in the rabbit. Am Rev Respir Dis 1980; 122:915-24.

143. Hamberg M, Hedqvist P, Radegran K. Identification of 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) as a major metabolite of arachidonic acid in human lung. Acta Physiol Scand 1980; 110:219-21.

144. Hanna CJ, Bach MK, Pare PD, Schellenberg RR. Slow-reacting substances (leukotrienes) contract human airway and pulmonary vascular smooth muscle in vitro. Nature 1981; 290:343-4.

145. Hansbrough JR, Atlas AB, Turk J, Holtzman MJ. Arachidonate 12-lipoxygenase and cyclooxygenase:PGE isomerase are predominant pathways for oxygenation in bovine tracheal epithelial cells. Am J Respir Cell Mol Biol 1989; 1:237-44.

146. Hardee MM, Moore JN. Effects of flunixin meglumine, phenylbutazone and a selective thromboxane synthetase inhibitor (UK-38,485) on thromboxane and prostacyclin production in healthy horses. Res Vet Sci 1986; 40:152-6.

147. Hardy C, Robinson C, Bradding P, Holgate ST. Prostacyclin: a functional antagonist of prostaglandin D_2 induced bronchoconstriction. Thorax 1984; 39:696.

148. Hardy C, Robinson C, Lewis RA, Tattersfield AE, Holgate ST. Airway and cardiovascular responses to inhaled prostacyclin in normal and asthmatic subjects. Am Rev Respir Dis 1985; 131:18-21.

149. Hardy CC, Robinson C, Tattersfield AE, Holgate ST. The bronchoconstrictor effect of inhaled prostaglandin D_2 in normal and asthmatic men. N Engl J Med 1984; 311:209-13.

150. Hay DWP, Raeburn D, Farmer SG, Fleming WW, Fedan JS. Epithelium modulates the reactivity of ovalbumin-sensitized guinea pig airway smooth muscle. Life Sciences 1986; 38:2461-8.

151. Heffner JE, Sahn SA, Repine JE. The role of platelets in the adult respiratory distress syndrome. Am Rev Respir Dis 1987; 135:482-92.

152. Henderson WR Jr. Eicosanoids and lung inflammation. Am Rev Respir Dis 1987; 135:1176-85.

153. Henke DC, Boucher R. Arachidonic acid metabolism by human airway epithelium. Am Rev Respir Dis 1989; 139:A409.

154. Henke D, Danilowicz RM, Curtis JF, Boucher RC, Eling TE. Metabolism of arachidonic acid by human nasal and bronchial epithelial cells. Archives Biochem Biophys 1988; 267:426-36.

155. Henke D, Danilowicz RM, Eling TE. Arachidonic acid metabolism by isolated epidermal basal and differentiated keratinocytes from the hairless mouse. Biochim Biophys Acta 1986; 876:271-9.

156. Herxheimer H, Stresemann E. The effect of bradykinin aerosol in guinea-pigs and in man. J Physiol 1961; 158:38P.

157. Hobson JE, Wright JL, Wiggs BR, Hogg JC. Comparison of the cell content of lung lavage fluid with the presence of emphysema and peripheral airways inflammation in resected lung. Respiration 1986; 50:1-8.

158. Holgate ST, Burns GB, Robinson C, Church MK. Anaphylactic and calcium-dependant generation of prostaglandin D_2 (PGD_2), thromboxane B_2 and other cyclooxygenase products of arachidonic acid by dispersed lung cells and relationship to histamine release. J Immunol 1984; 133: 2138-44.

159. Holtzman MJ, Aizawa H, Nadel JA, Goetzl EJ. Selective generation of leukotriene B_4 by tracheal epithelial cells from dogs. Biochem Biophys Res Commun 1983; 114:1071-6.

160. Holtzman MJ, Hansbrough JR, Rosen GD, Turk J. Uptake, release and novel species-dependent oxygenation of arachidonic acid in human and animal airway epithelial cells. Biochim Biophys Acta 1988; 963:401-13.

161. Holtzman MJ, Pentland A, Baenziger NL, Hansborough JR. Heterogeneity of cellular expression of arachidonate 15-lipoxygenase: implications for biological activity. Biochim Biophys Acta 1989; 1003:204-8.

162. Hoover RL, Karnovsky MJ, Austen KF, Corey EJ, Lewis RA. Leukotriene B_4 action on endothelium mediates augmented neutrophil/endothelial adhesion. Proc Natl Acad Sci USA 1984; 81:2191-3.

163. Horn BR, Robin ED, Theodore J, Van Kessel A. Total eosinophil counts in the management of bronchial asthma. N Eng J Med 1975; 292:1152-5.

164. Hsueh W, Kuhn C III, Needleman P. Relationship of prostaglandin secretion by rabbit alveolar macrophages to phagocytosis and lysosomal enzyme release. Biochem J 1979; 184:345-4.

165. Hubscher T. Role of the eosinophil in the allergic reactions: 11. Release of prostaglandins from human eosinophilic leukocytes. J Immunol 1975; 114:1389-93.

166. Humphrey DM, McManus LM, Satouchi K, Hanahan DJ, Pinckard RN. Vasoactive properties of acetyl glyceryl ether phosphorylcholine and analogues. Lab Invest 1982; 46:422-7.

167. Hunter JA, Finkbeiner WE, Nadel JA, Goetzl EJ, Holtzman MJ. Predominant generation of 15-lipoxygenase metabolites of arachidonic acid by epithelial cells from human trachea. Proc Nat Acad Sci 1985; 82:4633-7.

168. Hutchcroft BJ, Guz A. Levels of complement components during allergen-induced asthma. Clin Allergy 1978; 8:59-64.

169. Ind PW, Causon RC, Brown MJ, Barnes PJ. Circulating catecholamines in acute asthma. Br Med J 1985; 290:267-9.

170. Irvin CG, Berend N, Henson PM. Airways hyperreactivity and inflammation produced by aerosolization of human C5a des Arg. Am Rev Respir Dis 1986; 134:777-83.

171. Ishida K, Thomson RJ, Beattie LL, Wiggs B, Schellenberg RR. Inhibition of antigen-induced airway hyperresponsiveness, but not acute hypoxia nor airway eosinophilia, by an antagonist of platelet-activating factor. J Immunol 1990; 144:3907-11.

172. Isono T, Koshihara Y, Murota S, Fukuda Y, Furukawa S. Measurement of immunoreactive leukotriene C_4 in blood of asthmatic children. Biochem Biophys Res Comm 1985; 130:486-92.

173. Jacoby DB, Tamaoki J, Borson DB, Nadel JA. Influenza infection causes airway hyperresponsiveness by decreasing enkephalinase. J Appl Physiol 1988; 64:2653-8.

174. Jacoby DB, Ueki IF, Widdicombe JH, Loegering DA, Gleich GJ, Nadel JA. Human eosinophil major basic protein stimulates prostaglandin E_2 production by dog tracheal epithelium. Am Rev Respir Dis 1987; 135:A316.

175. Jefferey PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An ultrastructural quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989; 140:1745-53.

176. Johnson AR, Revtyak G, Campbell WB. Arachidonic acid metabolites and endothelial injury: studies with cultures of human endothelial cells. Federation Proc 1985; 44:19-24.

177. Johnson HG, McNee ML. Secretagogue responses of leukotriene C_4, D_4: comparison of potency in canine trachea in vivo. Prostaglandins 1983; 25:237-43.

178. Johnson HG, McNee ML, Sun FF. 15-hydroxyeicosatetraenoic acid is a potent inflammatory mediator and agonist of canine tracheal mucus secretion. Am Rev Respir Dis 1985; 131:917-22.

179. Jose PJ, Forrest MJ, Williams TJ. Human C5a des Arg increases vascular permeability. J Immunol 1981; 127:2376-80.

180. Kadowitz PJ, Chapnick BM, Feigen LP, Hyman AL, Nelson PK, Spannhake EW. Pulmonary and systemic vasodilator effects of the newly discovered prostaglandin PGI_2. J Appl Physiol 1978; 45:408-13.

181. Kasura JW, Terada L, Douglas JG. Regulation of respiratory epithelium prostaglandin production by granulacytes and reduced oxygen molecules. Clin Res 1987; 35:631A.

182. Kauffman HF, Van der Heide S, deMonchy JGR, de Vries K. Plasma histamine concentrations and complement activation during house dust mite-provoked bronchial obstructive reactions. Clin Allergy 1983; 13:219-28.

183. Kawakami Y, Uchiyama K, Irie T, Murao M. Evaluation of aerosols of prostaglandins E_1 and E_2 as bronchodilators. Eur J Clin Pharmacol 1973; 6:127-32.

184. Kay AB. Inflammatory cells in bronchial asthma. J Asthma 1989; 26:335-44.

185. Kennedy SM, Elwood RK, Wiggs BJR, Pare PD, Hogg JC. Increased airway mucosal permeability of smokers. Relationship to airway reactivity. Am Rev Respir Dis 1984; 129:143-8.

186. Kimman TG, Terpstra GK, Daha MR, Westenbrink F. Pathogenesis of naturally acquired bovine respiratory syncytial virus infection in calves: Evidence for the involvement of complement and mast cell mediators. Am J Vet Res 1989; 50:694-700.

187. Kirsch CM, Sigal E, Djokic TD, Graf PD, Nadel JA. An in vivo chemotaxis assay in the dog trachea: evidence for chemotactic activity of 8,15-diHETE. J Appl Physiol 1988; 64:1792-5.

188. Kloprogge E, de Leeuw AJ, de Monchy JGR, Kauffman HF. Cellular communication in leukotriene C_4 production between eosinophils and neutrophils. Int Arch Allergy Appl Immunol 1989; 90:20-3.

189. Knauer KA, Lichtenstein LM, Adkinson NF Jr, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. N Engl J Med 1981; 304:1404-7.

190. Kordansky D, Adkinson NF, Norman PS, Rosenthal RR. Asthma improved by non steroidal anti-inflammatory drugs. Annals of Internal Medicine 1978; 88:508-11.

191. Korn JH. Fibroblast prostaglandin E_2 synthesis. Persistence of an abnormal phenotype after short-term exposure to mononuclear cell products. J Clin Invest 1983; 71:1240-46.

192. Kumlin M, Hamberg M, Granstrom E, Bjorck T, Dahlen B, Matsuda H, Zetterstrom O, Dahlen SE. 15(S)-Hydroxyeicosatetraenoic acid is the major arachidonic acid metabolite in human bronchi: Association with airway epithelium. Arch Biochem Biophys 1990; 282:254-62.

193. Kunkel SL, Chensue SW, Phan SH. Prostaglandins as endogenous mediators of interleukin 1 production. J Immunol 1986; 136:186-92.

194. Kunkel SL, Wiggins RC, Chensue SW, Larrick J. Regulation of macrophage tumor necrosis factor production by prostaglandin E_2. Biochem Biophys Res Comm 1986; 137:404-10.

195. Kurimoto Y, de Weck AL, Dahinden CA. Interleukin 3 dependent mediator release in basophils triggered by C5a. J Exp Med 1989; 170:467-79.

196. Lachmann PJ, Peters DK. Complement. In: Clinical Aspects of Immunology, 4th edn. Oxford, Blackwell Scientific 1982; pp 18-49.

197. Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T. Damage of the airway epithelium and bronchial reactivity in patients with asthma. Am Rev Respir Dis 1985; 131:599-606.

198. Laitinen LA, Laitinen A, Widdicombe JG. Effects of inflammatory and other mediators on airway vascular beds. Am Rev Respir Dis 1987; 135:S67-S70.

199. Lands CG, Crawford WEM. Enzymes of membrane phospholipid metabolism in animals. In. The Enzymes of Biological Membranes. Martonosi A. Ed. Plenum, New York, Vol.2, pp3-85.

200. Lands WEM. The biosynthesis and metabolism of prostaglandins. Ann Rev Physiol 1979; 41:633-52.

201. Lands WE, Samuelsson B. Phospholipid precursors of prostaglandins. Biochim Biophys Acta 1968;164:426-9.

202. Larsen GL, Wilson MC, Clark RAF, Lyn Behrens B. The inflammatory reaction in the airways in an animal model of the late asthmatic response. Federation Proc 1987; 46:105-12.

203. Lawson GHK, McPherson EA, Murphy JR, Nicholson JM, Wooding P, Breeze RG, Pirie HM. The presence of precipitating antibodies in the sera of horses with chronic obstructive pulmonary disease (COPD). Eq Vet J 1979; 11:172-6.

204. Lee TA. Interactions between alveolar macrophages, monocytes, and granulocytes: Implications for airway inflammation. Am Rev Respir Dis 1987; 135:S14-7.

205. Lee TC, Lenihan DJ, Malone B, Roddy LL, Wasserman SI: Increased biosynthesis of platelet activating factor in activated human eosinophils. J Biol Chem 1984; 259:5526-30.

206. Lees P, Higgins AJ. Clinical pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. Equine Vet J 1985; 17(2):83-96.

207. Lehnert BE, Valdez YE, Holland LM. Pulmonary macrophages: Alveolar and interstitial populations. Exp Lung Res 1985; 9:177-90.

208. Leikauf GD, Ueki IF, Nadel JA, Widdicombe JH. Bradykinin stimulates Cl secretion and prostaglandin E_2 release by canine tracheal epithelium. Am J Physiol 1985; 248:F48-F55.

209. Lemanske RF Jr, Dick EC, Swenson CA, Vrtis RF, Busse WW. Rhinovirus upper respiratory infection increases airway hyperreactivity and late asthmatic reactions. J Clin Invest 1989; 83:1-10.

210. Lemen RJ, Quan SF, Witten ML, Sobonya RE, Ray CG, Grad R. Canine parainfluenza type 2 bronchiolitis increases histamine responsiveness in beagle puppies. Am Rev Respir Dis 1990; 141:199-207.

211. Lewis RA, Austen KF. The biologically active leukotrienes. Biosynthesis, metabolism, receptors, function and pharmacology. J Clin Invest 1984; 73:889-97.

212. Liu MC, Bleecker ER, Niv Y, Kagey-Sobotka A, McLemore TL, Permutt S, Lichtenstein LM, Proud D, Hubbard WC. Asthma is characterized by elevated levels of bronchospastic prostaglandins and histamine in airway fluids obtained by bronchoalveolar lavage. FASEB Journal 1988; 2(6):A1698.

213. Lopez-Vidreiro MT, Das I, Smith AP, Picot R, Reid L. Bronchial secretion from normal human airways after inhalation of prostaglandin $F_{2\alpha}$, acetylcholine, histamine, and citric acid. Thorax 1977; 32:734-9.

214. Lotner GZ, Lynch JM, Betz SJ, Henson PM. Human neutrophil-derived platelet activating factor. J Immunol 1980; 124:676-84.

215. Lowell FC. Observations on heaves. An asthma-like syndrome in the horse. Am Rev Respir Dis 1964; 35:322-30.

216. Lundberg JM, Saria A, Lundblad L, Angaard A, Martling CR, Theodorsson-Norheim E, Stjarne P, Hokfelt T. Bioactive peptides in capsaicin-sensitive C-fiber afferents of the airways: functional and pathophysiological implications. In: The Airways: Neural control in health and disease. Kaliner MA, Barnes PJ, eds. Marcel Dekker, New York. 1987; pp 417-445.

217. Lunell E, Andersson KE, Persson CG, Svedmyr N. Intravenous enprofylline in asthma patients. Eur J Respir Dis 1984; 65:28-34.

218. MacDermot J, Fuller RW. Macrophages. In Asthma: Basic mechanisms and clinical management. Barnes PJ, Rodger IW, Thomson NC. eds. Academic Press, San Diego, CA. 1988; pp 97-113.

219. MacDermot J, Kelsey CR, Waddell KA, Richmond R, Knight RK, Cole PJ, Dollery CT, Landon DN, Blair IA. Synthesis of leukotriene B_4 and prostanoids by human alveolar macrophages: analysis by gas chromatography/mass spectrometry. Prostaglandins 1984; 27:163-79.

220. MacDonald AJ, Moqbel R, Wardlaw AJ, Kay AB. Platelet activating factor (Paf-acether) enhances eosinophil cytotoxicity in vitro. J Allergy Clin Immunol 1986; 77:227.

221. Maclouf J, Murphy RC, Henson PM. Platelets and endothelial cells contribute to the production of LTC_4 by transcellular metabolism with neutrophils. Adv Prost Thromb Leukotriene Res 1989; 19:259-62.

222. Malik AB, Perlman MB, Cooper JA, Noonan T, Bizois R. Pulmonary microvascular effects of arachidonic acid metabolites and their role in lung vascular injury. Federation Proc 1985; 44:36-42.

223. Mann JS, Holgate ST, Renwick AG, Cushley MJ. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. J Appl Physiol 1986; 61:1667-76.

224. Mann JS, Renwick AG, Holgate ST. Release of adenosine and its metabolites from activated human leukocytes. Clin Sci 1986; 70:461-8.

225. Marcus AJ, Weksler BB, Jaff EA. Enzymatic conversion of prostaglandin endoperoxide H_2 and arachidonic acid to prostacyclin by cultured human endothelial cells. J Biol Chem 1978; 253:7138-41.

226. Marom Z, Shelhamer JH, Bach MK, Morton DR, Kaliner M. Slow reacting substances leukotrienes C_4 and D_4 increase the release of mucus from human airways in vitro. Am Rev Respir Dis 1982; 126:449-51.

227. Marom Z, Shelhamer JH, Kaliner M. Effects of arachidonic acid, monohydroxyeicosatetraenoic acid, and prostaglandins on the release of mucous glycoproteins from human airways in vitro. J Clin Invest 1981; 67:1695-1702.

228. Marom Z, Shelhamer JH, Sun F, Kaliner M. Human airway monohydroxyeicosatetraenoic acid generation and mucus release. J Clin Invest 1983; 72:122-7.

229. Marquardt DL, Gruber HE, Wasserman SI. Adenosine release from stimulated mast cells. Proc Natl Acad Sci USA 1984; 81:6192-6.

230. Marsh WR, Irvin CG, Murphy KR, Behrens BL, Larsen GL. Increases in airway reactivity to histamine and inflammatory cells in bronchoalveolar lavage after the late asthmatic response in an animal model. Am Rev Respir Dis 1985; 131:875-9.

231. Martin TR, Altman LC, Albert RK, Henderson WR. Leukotriene B_4 production by the human alveolar macrophage: a potential mechanism for amplifying inflammation in the lung. Am Rev Respir Dis 1984; 129:106-11.

232. Mathe AA, Hedquist P. Effect of prostaglandins $F_{2\alpha}$ and E_2 on airway conductance in healthy subjects and asthmatic patients. Am Rev Respir Dis 1975; 111:313-20.

233. Mathe AA, Hedqvist P, Holmgren A, Svanborg N. Bronchial hyperreactivity to prostaglandin $F_{2\alpha}$ and histamine in patients with asthma. Br Med J 1973; 1:193-6.

234. Mattoli S, Masiero M, Calabro F, Mezzetti M, Plebani M, Allegra L. Eicosanoid release from human bronchial epithelial cells upon exposure to Toluene Diisocyanate in vitro. J Cell Physiol 1990; 142:379-85.

235. McCarthy K, Henson PM. Induction of lysosomal enzyme secretion by alveolar macrophages in response to the purified complement fragments C5a and C5a des Arg. J Immunol 1979; 123:2511-7.

236. McGuire J, McGee J, Crittenden N, Fitzpatrick F. Cell damage unmasks 15-lipoxygenase activity in human neutrophils. J Biol Chem 1985; 260:8316-9.

237. McKiernan BC, Koritz GD, Scott JS, Berney C, Robinson NE. Plasma theophylline concentration and lung function in ponies with recurrent obstructive respiratory disease. Equine Vet J 1990; 22:194-7.

238. McPherson EA, Lawson GHK, Murphy JR, Nicholson JM, Breeze RG, Pirie HM. Chronic obstructive pulmonary disease (COPD): factors influencing the occurrence. Equine Vet J 1979; 11:167-71.

239. McPherson EA, Lawson GH, Murphy JR, Nicholson JM, Breeze RG, Pirie HM. Chronic obstructive pulmonary disease (COPD) in horses: aetiological studies: responses to intradermal and inhalation antigenic challenge. Equine Vet J 1979; 11:159-66.

240. Mencia-Huerta JM, Lewis RA, Razin E, Austen KF. Antigen-initiated release of platelet-activating factor (PAF-acether) from mouse bone marrow-derived mast cells sensitised with monoclonal IgE. J Immunol 1983; 131:2958-64.

241. Metzger WJ, Zavala D, Richerson HB, Moseley P, Iwamota P, Monick M, Sjoerdsma K, Hunninghake GW. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. Am Rev Respir Dis 1987; 135:433-40.

242. Mita H, Yui Y, Taniguchi N, Yasueda H, Shida T. Increased activity of 5-lipoxygenase in polymorphonuclear leukocytes from asthmatic patients. Life Sciences 1985; 37:907-14.

243. Mizuno K, Yamamoto S, Lands WEM. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. Prostaglandins 1982; 23:743-57.

244. Molina C, Brun J, Coulet M, Betail G, Delage J. Immunopathology of the bronchial mucosa in "late onset" asthma. Clin Allergy 1977; 7:137-45.

245. Moreno RH, Hogg JC, Pare PD. Mechanics of airway narrowing. Am Rev Respir Dis 1986; 133:1171-80.

246. Morley J, Page CP, Paul W. Inflammatory actions of platelet activating factor (Paf-acether) in guinea pig skin. Br J Pharmacol 1983; 80:503-9.

247. Morley J, Sanjar S, Page CP. The platelet in asthma. Lancet 1984; Nov 17; 2:1142-4.

248. Morrison KJ, Gao Y, Vanhoutte PM. Epithelial modulation of airway smooth muscle. Am J Physiol 1990; 258:L254-62.

249. Movat HZ, Rettl C, Burrowes CE, Johnston MG. The in vivo effect of leukotriene B_4 on polymorphonuclear leukocytes and the microcirculation. Comparison with activated complement (C5a des Arg) and enhancement by prostaglandin E_2. Am J Pathol 1984; 115:233-44.

250. Murlas CG, Roum JH. Sequence of pathologic changes in the airway mucosa of guinea pigs during ozone-induced bronchial hyperreactivity. Am Rev Respir Dis 1985; 131:314-20.

251. Murphy KR, Wilson MC, Irvin CG, Glezen LS, Marsh WR, Haslett C, Henson PM, Larsen GL. The requirement for polymorphonuclear leukocytes in the late asthmatic response and heightened airways reactivity in an animal model. Am Rev Respir Dis 1986; 134:62-8.

252. Murray JF. The Normal Lung. WB Saunders, Philadelphia. 1986; pp 69-73.

253. Murray JJ, Tonnel AB, Brash AR, Roberts LJ 11, Gosset P, Workman R, Capron A, Oates JA. Release of prostaglandin D_2 into human airways during acute antigen challenge. N Engl J Med 1986; 315:800-4.

254. Nathan CF. Secretory products of macrophages. J Clin Invest 1987; 79:319-26.

255. Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB. Arachidonic acid metabolism. Ann Rev Biochem 1986; 55:69-102.

256. Nijkamp FP, Henricks PAJ. Free radicals in pulmonary disease. In: Asthma: Basic mechanisms and clinical management. Barnes PJ, Rodger IW, Thomson NC, Eds. Academic Press, San Diego. 1988; pp 315-23.

257. Norris AA, Jackson DM. Sulphur dioxide-induced airway hyperreactivity and pulmonary inflammation in dogs. Agents Actions 1989; 26:360-6.

258. O'Byrne PM, Aizawa H, Bethel RA, Chung KF, Nadel JA, Holtzman MJ. Prostaglandin $F_{2\alpha}$ increases responsiveness of pulmonary airways in dogs. Prostaglandins 1984; 28:537-43.

259. O'Byrne PM, Leikauf GD, Aizawa H, Bethel RA, Ueki IF, Holtzman MJ, Nadel JA. Leukotriene B_4 induces airway hyperresponsiveness in dogs. J Appl Physiol 1985; 59:1941-6.

260. O'Byrne PM, Walters EH, Aizawa H, Fabbri LM, Holtzman MJ, Nadel JA. Indomethacin inhibits the airway responsiveness but not the neutrophil influx induced by ozone in dogs. Am Rev Respir Dis 1984; 130:220-4.

261. O'Byrne PM, Walters EH, Gold BD, Aizawa HA, Fabbri LM, Alpert SE, Nadel JA, Holtzman MJ. Neutrophil depletion inhibits airway hyperresponsiveness induced by ozone exposure in dogs. Am Rev Respir Dis 1984; 130:214-9.

262. Odlander B, Jakobsson PJ, Rosen A, Claesson HE. Human B and T lymphocytes convert leukotriene A_4 into leukotriene B_4. Biochem Biophys Res Commun 1988; 153:203-8.

263. O'Flaherty JT, Wykle RL, Miller CH, Lewis JC, Waite M, Bass DA, McCall CE, De Chatelet LR. 1-0-alkyl-sn-glyceryl-3-phosphorylcholines: A novel class of neutrophil stimulants. Am J Pathol 1981; 103:70-9.

264. Ogushi F, Ozaki T, Kawano T, Yasuoka S. PGE_2 and $PGF_{2\alpha}$ content in bronchoalveolar lavage fluid obtained from patients with eosinophilic pneumonia. Chest 1987; 91:204-6.

265. Ohki S, Ogino N, Yamamoto S, Hayaishi O. Prostaglandin hydroperoxidase, an integral part of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. J Biol Chem 1979; 254:829-36.

266. Olenchock SA, Mull JC, Boehlecke BA, Major PC. Cotton dust and complement in vivo. Chest 1981; 79:53-5S.

267. Ozaki T, Rennard SI, Crystal RG. Cyclooxygenase metabolites are compartmentalized in the human lower respiratory tract. J Appl Physiol 1987; 62:219-22.

268. Page CP, Paul W, Morley J. Platelets and bronchospasm. Int Archs Allergy appl Immun 1984; 74:347-50.

269. Parish WE, Luckhurst E. Eosinophilia. VI. Spontaneous synthesis of chemokinetic, chemotactic, complement receptor-inducing activities for eosinophils by bronchial T-lymphocytes of asthmatic-bronchitic patients. Clin Allergy 1982; 12:475-88.

270. Patterson R, Harris KE. The activity of aerosolized and intracutaneous synthetic platelet activating factor (AGEPC) in rhesus monkeys with IgE-mediated airway responses and normal monkeys. J Lab Clin Med 1983; 102:933-8.

271. Patterson R, Harris KE, Greenberger PA. Effect of prostaglandins D_2 and I_2 on the airways of rhesus monkeys. J Allergy Clin Immunol 1980; 65:269-73.

272. Payan DG, Levine JD, Goetzl EJ. Modulation of immunity and hypersensitivity by sensory neuropeptides. J Immunol 1984; 132: 1601-4.

273. Peck MJ, Piper PJ, Williams TJ. The effect of leukotrienes C_4 and D_4 on the microvasculature of guinea pig skin. Prostaglandins 1981; 21:315-21.

274. Peters SP, MacGlashan DW Jr, Schleimer RP, Hayes EC, Adkinson NF, Lichtenstein LM. The pharmacologic modulation of the release of arachidonic acid metabolites from purified human lung mast cells. Am Rev Respir Dis 1985; 132:367-73.

275. Peters-Golden M, Bathon J, Flores R, Hirata F, Newcombe DS. Glucocorticoid inhibition of zymosan-induced arachidonic acid release by rat alveolar macrophages. Am Rev Respir Dis 1984; 130:803-9.

276. Pincus SH, Schooley WR, DiNapoli AM, Broder S. Metabolic heterogeneity of eosinophils from normal and hypereosinophilic patients. Blood 1981; 58:1175-81.

277. Powell WS. Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica. Prostaglandins 1980; 20(5):947-57.

278. Propst K, Millen JE, Glauser FL. The effects of endogenous and exogenous histamine on pulmonary alveolar membrane permeability. Am Rev Respir Dis 1978; 117:1063-8.

279. Rafferty P, Beasley R, Holgate ST. The contribution of histamine to immediate bronchoconstriction provoked by inhaled allergen and adenosine 5-monophosphate in atopic asthma. Am Rev Respir Dis 1987; 136:369-73.

280. Rankin JA. The contribution of alveolar macrophages to hyperreactive airway disease. J Allergy Clin Immunol 1989; 83:722-9.

281. Rich B, Peatfield AC, Williams IP, Richardson PS. Effects of prostaglandins E_1, E_2, and $F_{2\alpha}$ on mucin secretion from human bronchi in vitro. Thorax 1984; 39:420-3.

282. Richardson J, Beland J. Nonadrenergic inhibitory nervous system in human airways. J Appl Physiol 1976; 41:764-71.

283. Robinson C, Holgate ST. Mast cell-dependant inflammatory mediators and their putative role in bronchial asthma. Clin Sci 1985; 68:103-12.

284. Robinson NE. Functional abnormalities caused by upper airway obstruction and heaves: Their relationship to the etiology of epistaxis. Vet Clinics of North America [Large An Pract] 1979; 1:17-34.

285. Roth GJ, Machuga ET, Ozols J. Isolation and covalent structure of the aspirin-modified, active-site region of prostaglandin synthetase. Biochemistry 1983; 22:4672-5.

286. Roth GJ, Stanford N, Majerus PW. Acetylation of prostaglandin synthase by aspirin. Proc Natl Acad Sci USA 1975; 72:3073-6.

287. Rouzer CA, Scott WA, Hamill AL, Cohn ZA. Synthesis of leukotriene C and other arachidonic acid metabolites by mouse pulmonary macrophages. J Exp Med 1982; 155:720-33.

288. Saeed SA, McDonald-Gibson WJ, Cuthbert J, Copas JL, Schneider C, Gardiner PJ, Butt NM, Collier HO. Endogenous inhibitor of prostaglandin synthetase. Nature 1977; 270:32-6.

289. Saito H, Hayakawa T, Mita H, Yui Y, Shida T. Augmentation of leukotriene C_4 production by gamma interferon in leukocytes challenged with an allergen. Int Arch Allergy Appl Immunol 1988; 87:286-93.

290. Salari H, Borgeat P, Fournier M, Herbert J, Pelletier G. Studies on the release of leukotrienes and histamine by human lung parenchymal and bronchial fragments upon immunologic and nonimmunologic stimulation. J Exp Med 1985; 162:1904-15.

291. Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science 1983; 220:568-75.

292. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. Science 1987; 237:1171-6.

293. Schleimer RP, MacGlashan DW Jr, Peters SP, Pinkard RN, Adkinson NF Jr, Lichtenstein LM. Characterization of inflammatory mediator release from purified human lung mast cells. Am Rev Respir Dis 1986; 133:614-7.

294. Schulman ES, Liu MC, Proud D, MacGlashan DW Jr, Lichtenstein LM, Plaut M. Human lung macrophages induce histamine release from basophils and mast cells. Am Rev Respir Dis 1985; 131:230-5.

295. Schwartzberg SB, Shelov SP, Van Praag D. Blood leukotriene levels during the acute asthma attack in children. Prost Leuk and Medicine 1987; 26:143-55.

296. Scott JS, Broadstone RV, Derksen FJ, Robinson NE. β-Adrenergic blockade in ponies with recurrent airway obstruction. J Appl Physiol 1988; 64:2324-8.

297. Scott JS, Garon H, Broadstone RV, Derken FJ, Robinson NE. α_1-Adrenergic-induced airway obstruction in ponies with recurrent pulmonary disease. J Appl Physiol 1988; 65:687-92.

298. Seibert K, Sheller JR, Roberts LJ II. 9α,11β-Prostaglandin F_2: Formation and metabolism by human lung and contractile effects on human bronchial smooth muscle. Adv Prostaglandin Thromboxane Leukotriene Res 1987; 17:189-91.

299. Semrad SD, Hardee GE, Hardee MM, Moore JN. Low dose flunixin meglumine: Effects on eicosanoid production and clinical signs induced by experimental endotoxaemia in horses. Equine Vet J 1987; 19(3):201-6.

300. Shaw RJ, Walsh GM, Cromwell O, Moqbel R, Spry CJF, Kay AB. Activated human eosinophils generate SRS-A leukotrienes following IgG-dependent stimulation. Nature 1985; 316:150-2.

301. Shelhamer JH, Marom Z, Kaliner M. Immunologic and neuropharmacologic stimulation of mucous glycoprotein release from human airways in vitro. J Clin Invest 1980; 66:1400-8.

302. Shemesh M, Hansel W, Strauss JF III. Modulation of bovine placental prostaglandin synthesis by an endogenous inhibitor. Endocrinology 1984; 115:1401-5.

303. Shulman ES, Newball HH, Demers LM, Fitzpatrick FA, Adkinson NF Jr. Anaphylactic release of thromboxane A_2, prostaglandin D_2 and prostacyclin from human lung parenchyma. Am Rev Respir Dis 1981; 124:402-6.

304. Silberstein DS, David JR. Tumor necrosis factor enhances eosinophil toxicity to Schistosoma mansoni larvae. Proc Natl Acad Sci USA. 1986; 83:1055-9.

305. Simonsson BG, Skoogh BE, Bergh NP, Andersson R, Svedmyr N. In vivo and in vitro effect of bradykinin on bronchial motor tone in normal subjects and in patients with airways obstruction. Respiration 1973; 30:378-88.

306. Sirois P, Borgeat P, Jeanson A, Roy S, Girard G. The action of leukotriene B_4 (LTB_4) on the lung. Prostaglandins Med 1980; 5:429-44.

307. Sirois P, Harczy M, Braquet P, Borgeat P, Maclouf J, Pradelles P. Inhibition of the release of prostaglandins, thromboxanes and leukotrienes from anaphylactic lungs by a platelet-activating factor (PAF) antagonist and its stimulation by PAF. Adv Prost Throm Leuk Res 1987; 17:1033-7.

308. Sladek K, Dworski R, Fitzgerald GA, Buitkus KL, Block FJ, Marney SR Jr, Sheller JR. Allergen-stimulated release of thromboxane A_2 and leukotriene E_4 in humans. Am Rev Respir Dis 1990; 141:1441-5.

309. Smith AP, Cuthbert MF, Dunlop LS. Effects of inhaled prostaglandins E_1, E_2 and $F_{2\alpha}$ on the airway resistance of healthy and asthmatic man. Clin Sci Mol Med 1975; 48:421-30.

310. Smith LJ, Greenberger PA, Patterson R, Krell RD, Bernstein PR. The effect of inhaled leukotriene D_4 in humans. Am Rev Respir Dis 1985; 131:368-72.

311. Smith WL, Borgeat P. The Eicosanoids: Prostaglandins, Thromboxanes, Leukotrienes and Hydroxy-eicosaenoic acids. In: Vance DE, Vance JE, eds. Biochemistry of Lipids and Membranes. Menlo Park, CA: Benjamin Cummings, 1985; 325-60.

312. Spector AA. Fatty acid binding to plasma albumin. J Lipid Res 1975; 16:165-79.

313. Spector AA, Gordon JA, Moore SA. Hydroxyeicosatetraenoic acids (HETEs). Prog Lipid Res 1988; 27:271-323.

314. Spector AA, Hoak JC, Warner ED, Fry GL. Utilization of long-chain free fatty acids by human platelets. J Clin Invest 1970; 49:1489-96.

315. Srivastava N, Gupta SP, Srivastava LM. Effect of house dust mite on serum complement activation and total haemophylic activity in vitro in normal subjects and in patients with bronchial asthma. Clin Allergy 1983; 13:43-50.

316. Stalenheim G, Machado L. Late allergic bronchial reactions and the effect of allergen provocation on the complement system. J Allergy Clin Immunol 1985; 75:508-12.

317. Stewart RM, Weir EK, Montgomery MR, Niewoehner DE. Hydrogen peroxide contracts airway smooth muscle: a possible endogenous mechanism. Respir Physiol 1981; 45:333-42.

318. Stimler NP. Spasmogenic activity of C5a des Arg anaphylatoxin on guinea pig lung parenchymal strips: Sensitivity of the leukotriene-mediated component to cyclooxygenase inhibitors. Biochem Biophys Res Comm 1984; 125:852-8.

319. Suquet CM, Leid RW. Aggregation of equine platelets by PAF (platelet-activating factor). Inflammation 1983; 7:197-203.

320. Svanborg N, Hedqvist P, Green K. Aspects of prostaglandin action in asthma. Adv Prost Throm Res 1976; 1:441-7.

321. Svensson J, Strandberg K, Tuvemo T, Hamberg M. Thromboxane A_2: effects on airway and vascular smooth muscle. Prostaglandins 1977; 14:425-6.

322. Sweatman WJF, Collier HOJ. Effects of prostaglandins on human bronchial muscle. Nature 1968; 217:69.

323. Szentivanyi A. The beta adrenergic theory of the atopic abnormality in bronchial asthma. J Allergy 1968; 42:203-32.

324. Taylor SM, Pare PD, Schellenberg RR. Cholinergic and nonadrenergic mechanisms in human and guinea pig airways. J Appl Physiol 1984; 56:958-65.

325. Taylor GW, Taylor I, Black P, Maltby NH, Turner N, Fuller RW, Dollery CT. Urinary leukotriene E_4 after antigen challenge and in acute asthma and allergic rhinitis. Lancet 1989; 1:584-8.

326. Terragno NA, Terragno A, Early JA, Roberts MA, McGiff JC. Endogenous prostaglandin synthesis inhibitor in the renal cortex. Effects on production of prostacyclin by renal blood vessels. Clin Sci Mol Med 1978; 55(Suppl 4):199-202.

327. Thorsen J, Willoughby RA, McDonell W, Valli VEO, Viel L, Bignell W. Influenza hemagglutination inhibiting activity in respiratory mucus from horses with chronic obstructive pulmonary disorders (heaves syndrome). Can J Comp Med 1983; 47:332-335.

328. Thurbeck WM, Lowell FC. Heaves in horses. Am Rev Respir Dis 1964; 89:82-8.

329. Tiffany CW, Burch RM. Bradykinin stimulates tumor necrosis factor and interleukin-1 release from macrophages. FEBS 1989; 247:189-92.

330. Tizard I. An Introduction to Veterinary Immunology. Second edition. WB Saunders, Philadelphia, PA. 1982.

331. Tschirhart E, Frossard N, Bertrand C, Landry Y. Arachidonic acid metabolites and airway epithelium-dependent relaxant factor. J Pharm Exp Therap 1987; 243:310-5.

332. Turk J, Maas RL, Brash AR, Roberts LJ 11, Oates JA. Arachidonic acid 15-lipoxygenase products from human eosinophils. J Biol Chem 1982; 257:7068-76.

333. Utting JA. Alpha-adrenergic blockade in severe asthma. Br J Dis Chest 1979; 73:317-8.

334. Van Inwegen RG, Khandwala A, Gordon R, Sonnino P, Coutts S, Jolly S. REV 5901: an orally effective peptidoleukotriene antagonist, detailed biochemical/pharmacological profile. J Pharmacol Exp Ther 1987; 241:117-24.

335. Vanderhoek JY, Bryant RW, Bailey JM. Inhibition of leukotriene biosynthesis by the leukocyte product 15-hydroxy-5,8,11,13-eicosatetraenoic acid. J Biol Chem 1980; 255:10064-5.

336. Vanderhoek JY, Karmin MT, Ekborg SL. Endogenous hydroxyeicosatetraenoic acids stimulate the human polymorphonuclear leukocyte 15-lipoxygenase pathway. J Biol Chem 1985; 260:15482-7.

337. Vanderhoek JY, Tare NS, Bailey JM, Goldstein AL, Pluznik DH. New role for 15-hydroxyeicosatetraenoic acid. Activator of leukotriene biosynthesis in PT-18 mast/basophil cells. J Biol Chem 1982; 257:12191-5.

338. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature (London) New Biol 1971; 231:232-5.

339. Vanselow NA, Smith JR. Bronchial asthma induced by indomethacin. Annals of Internal Medicine 1967; 66(3):568-72.

340. Vargaftig BB, Chignard M, Benenviste J, Lefort J, Wal F. Background and present status of research on platelet activating factor (PAF-acether). Ann NY Acad Sci 1981; 370:119-37.

341. Veith MC, Butterworth AE. Enhancement of human eosinophil-mediated killing of Schistosoma mansoni larvae by mononuclear cell products in vitro. J Exp Med 1983; 157:1828-43.

342. Vincenc KS, Black JL, Yan K, Armour CL, Donnelly PD, Woolcock AJ. Comparison of in vivo and in vitro responses to histamine in human airways. Am Rev Respir Dis 1983; 128:875-9.

343. Walters EH, O'Byrne PM, Fabbri LM, Graf PD, Holtzman MJ, Nadel JA. Control of neurotransmission by prostaglandins in canine trachealis smooth muscle. 1984; 57:129-34.

344. Wardlaw AJ, Dunnette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in mild asthma. Relationship to bronchial hyperreactivity. Am Rev Respir Dis 1988; 137:62-9.

345. Wardlaw AJ, Moqbel R, Cromwell O, Kay AB. Platelet-activating factor: A potent chemotactic and chemokinetic factor for human eosinophils. J Clin Invest 1986; 78:1701-6.

346. Warner AE, Barry BE, Brain JD. Pulmonary intravascular macrophages in sheep: morphology and function of a novel constituent of the mononuclear phagocyte system. Lab Invest 1986; 55:276-88.

347. Wasserman MA, Ducharme DW, Wendling MG, Griffin RL, Degraaf GL. Bronchodilator effects of prostacyclin (PGI_2) in dogs and guinea-pigs. Eur J Pharmacol 1980; 66:53-63.

348. Wedmore CV, Williams TJ. Platelet-activating factor (PAF), a secretory product of polymorphonuclear leukocytes, increases vascular permeability in rabbit skin. Br J Pharmacol 1981; 74: 916-7P.

349. Wedzicha JA, Cotter FE, Empey DW. Platelet size in patients with chronic airflow obstruction with and without hypoxaemia. Thorax 1988; 43:61-4.

350. Weiss JW, Drazen JM, Coles N, McFadden ER, Weller PW, Corey EJ, Lewis RA, Austen KF. Bronchoconstrictor effects of leukotriene C in humans. Science 1982; 216: 196-8.

351. Weller PF, Lee CW, Foster DW, Corey EJ, Austen KF, Lewis RA. Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: predominant production of leukotriene C_4. Proc Natl Acad Sci USA 1983; 80:7626-30.

352. White MV, Kaliner MA. Histamine. In Asthma: Basic mechanisms and clinical management. Barnes PJ, Rodger IW, Thomson NC. eds Academic Press Ltd, San Diego, CA. 1987; pp 231-57.

353. White MV, Kaliner MA, Baer H. Stimulated neutrophils release a histamine releasing factor. J Allergy Clin Immunol 1986; 77:132.

354. Widdicombe JH, Ueki IF, Emery D, Margolskee D, Yergey J, Nadel JA. Release of cyclooxygenase products from primary cultures of tracheal epithelia of dog and human. Am J Physiol 1989; 257:L361-5.

355. Williams TJ, Morley J. Prostaglandins as potentiators of increased vascular permeability in inflammation. Nature (Lond.) 1973; 246:215-7.

356. Willoughby RA, McDonell WN. Pulmonary function testing in horses. Vet Clin North Am [Large Anim Pract] 1979; 1:171-96.

357. Wilson DB, Prescott SM, Majerus PW. Discovery of an arachidonoyl coenzyme A synthetase in human platelets. J Biol Chem 1982; 257:3510-15.

358. Wilson DW, Plopper CG, Hyde DM. The tracheobronchial epithelium of the Bonnet monkey (Macaca radiata): A quantitative ultrastructural study. Am J Anatomy 1984; 171:25-40.

359. Winkler GC. Pulmonary intravascular macrophages in domestic animal species: Review of structural and functional properties. Am J Anatomy 1988; 181:217-34.

360. Woodward DF, Weichman BM, Gill CA, Wasserman MA. The effect of synthetic leukotrienes on tracheal microvascular permeability. Prostaglandins 1983; 25: 131-42.

361. Woolcock AJ, Salome CM, Yan K. The shape and the dose-response curve to histamine in asthmatic and normal subjects. Am Rev Respir Dis 1984; 130:71-5.

362. Xu GL, Sivarajah K, Wu R, Nettesheim P, Eling T. Biosynthesis of prostaglandins by isolated and cultured airway epithelial cells. Exp Lung Res 1986; 10:101-14.

363. Yamashiro S, Viel L, Bast T, Harris W. The morphological study of airways and cells recovered by bronchoalveolar lavage in a horse with chronic obstructive pulmonary disease. Anatomical Record 1985; 211:219A.

364. Zheutlin LM, Ackerman SJ, Gleich GJ, Thomas LL. Stimulation of basophil and rat mast cell histamine release by eosinophil granule-derived catioic proteins. J Immunol 1984; 133:2180-5.

365. Zijlstra FJ, Naaktgeboren M, Mons H, Vincent JE. Formation of prostaglandins and leukotrienes by human lung tissue in vitro after activation by the calcium ionophore A23187. Eur J Clin Invest 1987; 17:325-9.