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Inflammatory Mediators, Activated Neutrophils, and Bronchospasm in Equine Small Airways: Implications for Inflammation in the Development of Recurrent Airway Obstruction

presented by

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has been accepted towards fulfillment
of the requirements for

PhD degree in Large Animal Clinical Sciences

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Date Dec 1st '97

INFLAMMATORY MEDIATORS, ACTIVATED NEUTROPHILS, AND BRONCHOSPASM IN EQUINE SMALL AIRWAYS: IMPLICATIONS FOR INFLAMMATION IN THE DEVELOPMENT OF RECURRENT AIRWAY OBSTRUCTION

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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

1997

ABSTRACT

INFLAMMATORY MEDIATORS, ACTIVATED NEUTROPHILS, AND BRONCHOSPASM IN EQUINE SMALL AIRWAYS: IMPLICATIONS FOR INFLAMMATION IN THE DEVELOPMENT OF RECURRENT AIRWAY OBSTRUCTION

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Recurrent airway obstruction (RAO) is a common syndrome in horses, in which delivery of oxygen into the lung is compromised by narrowing of the airways. Due to cholinergically mediated bronchospasm, the mechanism initializing cholinergic bronchospasm in RAO remained unknown. In vivo, isolated airway tissues from horses with RAO demonstrate decreased responses to exogenous ACh and lack of evidence of increased ACh release from airway parasympathetic nerves. This peculiarity could be explained by the fact that during study in vitro, tissues are removed from the inflammatory milieu that surrounds the airway smooth muscle (ASM) in vivo. Therefore I hypothesize that inflammatory mediators present within the airways during exacerbation of RAO are responsible for increased airway cholinergic tone. Concurrently with the development of airway obstruction: 1) neutrophils are recruited and become activated within the airways; and 2) activated mast cells release vasoactive amines and lipid mediators. In order to test my hypothesis, I examined the effect of several mediators and activated neutrophils on cholinergic responses in the airways. I developed a small airway

(SA) preparation, since SA are predominantly altered in the course of RAO. In SA I found that contractile response to electrical field stimulation, but not to exogenous acetylcholine or methacholine is subject to strong modulation by inflammatory mediators, and that histamine, serotonin, and leukotriene D_4, the mediators of anaphylaxis, may strongly increase the response to nerve stimulation, particularly in the physiological frequency range. Furthermore, these different classes of mediators produced a similar magnitude of augmentation.

Activated neutrophils did not affect cholinergic responses in SA and one of the neutrophil products, namely, hydrogen peroxide, decreased rather than increased cholinergic responses presumably due to promotion of inhibitory prostanoid production in isolated trachealis strips. These results may explain the mechanisms of increased cholinergic airway tone in RAO through the effects of a variety of inflammatory mediators on endogenous cholinergic responses of ASM. My data particularly favor allergic reaction as the source of this mechanism, rather than neutrophilic inflammation, since mediators traditionally associated with mast cells but not activated neutrophils or hydrogen peroxide contributed to increased response to cholinergic nerve stimulation in isolated equine SA.

This dissertation is dedicated to my wife, Amy Helen Olszewski,
for her love, support, and help to reach this goal.

ACKNOWLEDGMENTS

It is the glory of God to conceal a matter;

to search out a matter is the glory of kings.

Proverbs 25:2

I would like to express my gratitude to all who helped me during my doctorate work.

For guidance and teaching, my thanks go to Dr. N. Edward Robinson. He is an excellent mentor, scientist, and most of all a wonderful person and friend—everything a graduate student could wish to have in a major professor.

I would like to acknowledge technical assistance and intellectual input of Cathy Berney, Deborah Boehler, Feng-Xia Zhu, Xiang-Yang Zhang, Patti Tithof, Sue Eberhart, John Buhweitz, Mingfu Yu, and Zhaowen Wang. Without their support, this work would not be possible. Dr. Victoria Hoelzer-Maddox and MaryEllen Shea were instrumental in their help with preparation of the manuscript of this dissertation. Finally, I would like to extend my gratitude to all the members of my guidance committee: Drs. Thomas G. Bell, Frederik J. Derksen, Lana Kaiser, Robert A. Roth, and John A. Szarek.

Most of all, for constant support from the beginning, I am indebted to my mother, Sylwia. My father Miroslaw, and my in-laws Rudy and Myra Hirzel have always also given me help during this difficult time.

Last but not least, thank you to my wife, Amy, and to my three children—Mirabella, Veronika, and Adam—for their love and support.

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KEY TO ABBREVIATIONS

ΔPpl	maximal difference in pleural pressure
5-, 12-, 15-LO	5-, 12-, 15-lipoxygenase
5-HT	serotonin, 5-Hydroxytryptamine
7-TM	7-transmembrane
A23187	calcium ionophore
AA	arachidonic acid
ACh	acetylcholine
AHR	airway hyperresponsiveness
ASM	airway smooth muscle
ATR	atropine
BAL	bronchoalveolar lavage
$[Ca^{2+}]_i$	intracellular calcium
C5a	5a complement fragment
CaM	calmodulin
CB	cytochalasin B
C_{dyn}	dynamic compliance
COPD	chronic obstructive pulmonary disease
CysLTs	cysteinyl leukotrienes
DAG	diacylglycerol
E_{dyn}	dynamic elastance
EFS	electrical field stimulation
eNANC	excitatory nonadrenergic noncholinergic
EpDRF	epithelium-derived relaxing factor
FEV_1	forced expiratory volume per second
fMLP	formylmethionyl-peptide, N-Formyl-Met-Leu-Phe
G_i, G_p, G_s G_q	GTP-binding protein proteins
GM-CSF	granulocyte-monocyte colony stimulating factor
H_2O_2	hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HETE	hydroxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
hrC5a	human recombinant C5a complement fragment
IF-γ	interferon-γ
IgA, IgE, IgG	immunoglobulin A, immunoglobulin E, immunoglobulin G
IL-	interleukin-
iNANC	inhibitory nonadrenergic noncholinergic

IP_3	inositol triphosphate
ISO	isoproterenol
K-H	Krebs-Henseleit
LC_{20}	20 kDa myosin light chain
LNNA	*N*ω-nitro-L-arginine
LP	lung parenchyma
LPS	lipopolysacharid
LT	leukotriene
M	muscarinic
MAPK	mitogen-activating protein kinase
MCh	methacholine
MEC	meclofenamate
MEK-1	mitogen-activating protein kinase kinase
MLCK	myosin light chain kinase
MPO	myeloperoxidase
NANC	nonadrenergic-noncholinergic
NE	norepinephrine
NO	nitric oxide
NS	not significant
$O_2^{-}\cdot$	superoxide anion
OH·	hydroxyl radical
OZ	opsonized zymosan
PAF	platelet-activating factor
PG	prostaglandin
PIP_2	phospholipid phosphatidylinositol-biphosphate
PKC	protein kinase
PLC	phospholipase C
PMA	phorbol ester, phorbol myristate acetate
RAO	recurrent airway obstruction
R_L	pulmonary resistance
ROS	reactive oxygen species
SA	small airways
SOD	superoxide dismutase
T_H	T helper cells
TNF	tumor necrosis factor
TTX	tetrodotoxin
TX	thromboxane
V	volume
$\dot{V}$	flow
VIP	vasointestinal inhibitory peptide

INTRODUCTION

Recurrent airway obstruction (RAO) is an allergic respiratory syndrome in horses (known as "heaves"), in which the delivery of oxygen into the lung is compromised by narrowing of airways. The recurrent and progressive nature of this syndrome is defined by two features: induction of the clinical disease in response to contact with airborne antigens, and the development of more severe forms of airway obstruction when the antigen exposure is long-lasting or periodically repeated. In the mild form, airway obstruction manifests itself by intermittent cough and decreased exercise tolerance. In more advanced stages, as the hypoxemia becomes apparent, affected animals change their breathing pattern, showing a high level of expiratory dyspnea with a strong recruitment of the abdominal muscles in respiratory work, high peaks of expiratory flow, and wheezing. At the most severe stage, animals become respiratory cripples unable to perform a minimal physical effort. The clinical signs of airway obstruction gradually reverse when allergens are removed from the environment of susceptible horses, and they return upon subsequent exposures, often with higher intensity.[81, 226]

At the turn of the century, clinicians describing this disease suggested that RAO is both neural and inflammatory in nature. Today, when a much greater database of scientific information about RAO is available, it is apparent that the clinical hunch from a century ago was entirely correct. The bronchospasm responsible for airway obstruction

in RAO is mediated by the parasympathetic nervous system, and airway inflammation is an integral part of acute bouts of RAO.[34, 58, 183]

These two pieces of information are crucial for the clinical management of RAO. Therapy with bronchodilators and anti-inflammatory drugs, largely corticosteroids, is among the most frequently recommended and of the greatest benefit.[22, 154] However, it is unknown at present to what degree bronchospasm in RAO is a result of the inflammatory process, and why and how the inflammation interferes with the neuromuscular control of airway tone. This information is crucial for the therapeutic management and prevention of airway obstruction in horses. Therefore, studying the association between airway inflammation and airway responses was at the core of my investigation.

Even though, beyond any doubt, bronchospasm is responsible for the majority of the airway obstruction in RAO and this bronchospasm is cholinergic, i.e., mediated via activation of M receptors on the ASM, in vitro studies have excluded the possibility of intrinsic hyperresponsiveness of ASM in horses with RAO.[33, 308] Airways from throughout all the respiratory tract, isolated from horses in the acute phase of RAO, are hyporesponsive to ACh. Also a measurement of the ACh release in vitro, by high-performance liquid chromatography (HPLC), coupled with electrochemical detection, has failed to demonstrate increased ACh release in the trachea and bronchi of horses with RAO in response to nerve stimulation.[285] This apparent discrepancy between in vivo and in vitro results leads me to the conclusion that, in the experimental situation, tissue removed from the neuro-humoral environment behaves differently than in vivo. In other words, some factors responsible for the increased cholinergic responses in vivo are no

longer present in the tissue baths. This would certainly be the case if inflammatory mediators were responsible for altered cholinergic responses in RAO. After hours of tissue preparation and equilibration, tissues must be largely depleted of soluble factors involved in inflammation due to multiple changes of buffer solutions. The potential effects of inflammatory mediators are 1) a direct contractile action on ASM, 2) synergism with cholinergic responses at the level of ASM, and 3) facilitation of ACh release from parasympathetic nerves that innervate ASM. The two latter mechanisms are particularly likely to be at play in the development of RAO, since the mechanism of airway obstruction is cholinergic.[34, 154]

To investigate the effects of inflammatory mediators on in vitro responses of airways, I developed models that utilize mediators that have been reported to increase in the course of RAO. In the presence of inflammatory mediators, I have studied cholinergic responses of airways in vitro. My final approach was to use activated inflammatory cells to "supplement" the missing component of inflammatory response in vitro. Two groups of inflammatory mediators were studied. Because of the allergic nature of the disease I have tested effects of mediators traditionally associated with type I allergic response, i.e., those derived from the mast cells that are the primary effector cells in this type of allergy.[207] Mast cell degranulation leads to release of vasoactive amines such as histamine or serotonin (5-HT) and is accompanied by release of lipid mediators including cysteinyl leukotrienes (LT) and prostaglandin (PG)D_2, which are capable of contracting ASM.[94] In other species, some of these mediators have been reported to increase cholinergic responses or act via vagal mechanisms in airways or other types of smooth muscle.[197] In the course of RAO histamine, LTE_4 and 5-HT have

been reported to increase in airway lavage, urine, and plasma of horses, respectively.[70, 81, 182] In the course of my PhD work I have tested cholinergic responses of ASM in the presence of these inflammatory mediators to determine if cholinergic responses of equine smooth muscle can be regulated by these autacoids.

Neutrophils were also very interesting to me, since it is well accepted that neutrophils are the only inflammatory cell that consistently increases in the airway secretion during acute bouts of airway obstruction. Moreover, neutrophil number in airway lavage increases with severity of airway obstruction in horses, and the time course of its development generally follows neutrophil influx into the airways.[59, 64, 84, 183] My earlier study had also indicated that neutrophils washed from airways of horses with RAO are strongly activated in comparison with peripheral blood neutrophils.[208] For these reasons and because products released by activated neutrophils such as ROS (ROS) and lipid mediators are capable of changing responses of ASM, neutrophils have been long postulated to be involved in the pathogenesis of RAO.

To shed light on the role of neutrophils in development of airway obstruction in horses, during my PhD research I designed experiments in which I tested whether neutrophils affect responses of isolated airways in vitro. To do this, I first developed techniques to isolate neutrophils from peripheral blood and studied their activity when treated with the compounds known to activate neutrophils in horses or in other animal species. In the final approach, I measured responses of airways in the presence of activated neutrophils to determine their effects. To account for the possibility of a differential response of neutrophils to activation and for differential responses of airways to the neutrophil-derived products and the nerve stimulation between horses with RAO

and the controls, I have used a cross-over design that involved all combinations of tissue and neutrophils from healthy and affected animals. I also examined the effect of a specific neutrophil product, hydrogen peroxide (H_2O_2), which is one of the ROS, testing its effect on cholinergic responses in equine trachealis.

My experiments have shown that the endogenous cholinergic responses of equine airways are subject to strong modulation by inflammatory mediators and that pro-inflammatory mediators may strongly increase the response to nerve stimulation, particularly at physiological frequencies. Moreover, different classes of mediators are capable of producing a similar magnitude of augmentation. I have also found that activated neutrophils do not affect cholinergic responses in SA, and that decreased rather than increased cholinergic responses, presumably due to promotion of inhibitory prostanoid production in isolated trachealis strips. These results may explain the mechanisms of increased cholinergic airway tone in RAO via effects of a variety of inflammatory mediators on endogenous cholinergic responses of ASM. My data particularly favor a type I allergic reaction as the source of this mechanism rather than the neutrophilic inflammation, since mediators traditionally associated with mast cells, but not activated neutrophils or H_2O_2, contributed to increased cholinergic responses of isolated airways to EFS.

Chapter 1

LITERATURE REVIEW: MAMMALIAN AIRWAYS IN HEALTH AND DISEASE

Introduction

The purpose of this review is to present the state of our knowledge about RAO horses and the background of current information about the physiology and pathology of airways. The mechanisms responsible for airway regulation that were hypothesized to be involved in the development of RAO and studied during my PhD work are discussed in detail in this review. These include structural and functional features of airways in the horse; ASM and its autonomic regulation; bronchospasm and airway hyperresponsiveness (AHR); inflammation and function of inflammatory cells; and mediators and their influences on airway regulatory mechanisms in healthy and RAO horses.

The main function of airways is to conduct the air into and from the alveoli, where gas exchange takes place. The process of ventilation relies on the appropriate function of airways, thus several accessory mechanisms are crucial to maintain ventilation undisturbed. These functions include: 1) air-conditioning to optimize temperature and humidity of the air delivered into the alveoli, and 2) protection of the respiratory tract from both biological and toxic assault and the occlusion of airways, by endogenous and

exogenous material. The anatomy and physiology of the airways are perfectly adapted to fulfill these functions with the greatest efficiency.

Tracheobronchial tree

The airway tree is formed by the trachea and bronchi dividing into multiple generations of branched tubes with decreasing diameter. In equine bronchi the branching pattern is mainly asymmetric so that airway size does not necessarily reflect airway generation. Even though the general structural components such as mucous membrane, lined by respiratory epithelium, ASM, cartilage rings, and connective tissue surrounding the airways are similar throughout most of the respiratory tract, there are significant differences between parts of the tracheobronchial tree in the size, organization of structural components, and mechanical properties of the airway walls. In the trachea a sheet of ASM connects the terminals of the horseshoe-shaped cartilage rings in their internal aspects. These cartilage rings, especially in large animals, are extremely rigid, which in combination with the ASM position strongly limit variability in the caliber of trachea. In bronchi, cartilage plates embedded in the connective tissue overlap, creating a complete ring to which the smooth muscle layer is relatively loosely attached via sparse connective tissue with fairly high compliance.[252] Thus in bronchi, external size is only slightly modified, while in the bronchioles the lumen may narrow "to almost nothing" in response to ASM contraction.[252] With a decrease in size and a smaller amount of cartilage in peripheral airways (small bronchi), compliance of the airway increases and alveolar septa grow directly out from the wall of the airway. Even though the amount of ASM is smaller in these airways, plasticity of airway diameter can be very large because

mechanisms other than just smooth muscle tone, e.g., tissue interdependence forces, have a stronger effect on airway caliber. Finally, bronchioli do not possess cartilaginous elements and the only force opposing smooth muscle tone and preventing SA from collapse is the elastic force of lung parenchyma (LP). These relationships have to be considered not only when pulmonary functions are measured in vivo but also when different generations of airways are used to study their tension responses in vitro.

Outline of airway anatomy and organization

The airway wall consists of well-vascularized mucous membrane, which protects and cleanses the lung and conditions the air. Airway mucosa is composed of epithelium and lamina propria. It is surrounded by a layer of smooth muscle, supported from outside by cartilage rings that are present up to the level of bronchioli.

Epithelium

In the equine conducting airways, like in the other mammals, epithelium is pseudostratified and composed of ciliated, secretory, intermediate, and basal cells. Epithelial cells are attached to a basement membrane, largely through contact with basal cells. Ciliated cells are most numerous and extend through the entire thickness of the epithelium. On the apical surface these cells possess 50–100 cilia and some microvilli. Several types of secretory cells are also present in the epithelium, including mucous (goblet) cells, serous cells, Clara cells, and sero-mucous cells, which possess both mucous and serous cell characteristics. Ciliary motions and the appropriate quantity of

the airway fluids are both necessary for correct function of the so-called mucociliary escalator (see defense mechanisms). In addition structural and mechanical functions, the epithelium also plays a regulatory role. Factors that regulate ASM tone (such as epithelium-derived relaxing factor [EpDRF]), and mediators of immune response (such as interleukins [IL]s and chemokines) are released from epithelium.[304] Intracellular transport of antibodies into the airway lumen by the epithelium also plays an important role. In the transepithelial clefts some non-epithelial cells are also present, e.g., mast cells, antigen-presenting dendritic cells,[118] and neuroepithelial cells. These cells are part of the airway alarm system, which is responsible for recognition of antigens and nonspecific irritants, and switching on the defense mechanisms.

Lamina propria

The lamina propria connects the basement membrane with the airway wall (smooth muscle layer). It contains several layers of connective tissue, with an extensive capillary bed and mucous glands. Due to the very good blood supply, presence of sensory nerves, and residual inflammatory cells, this part of the airway wall changes dramatically in response to irritation or assault. The lamina propria can expand dramatically due to swelling, become infiltrated with inflammatory cells, and increase secretion of mucus from glands. These processes, by changing airway geometry and possibly exerting effects on ASM responses, may be very important in the development of airway obstruction.

Airway smooth muscle

Smooth muscle is present throughout all levels of the conducting airways. In the equine trachea approximately 1/4–1/3 area of its wall is underlined by trachealis muscle, which spans between the internal perimeter of the cartilage. In larger bronchi, a well-defined muscle layer in the airway wall forms a compact ring, whereas in small peripheral airways, small groups of muscle are more loosely scattered.[158] Even though muscle layers are less complete in peripheral airways, Lei's data provide evidence that in the terminal airways (bronchioli), the vast majority of smooth muscle lies perpendicularly to the airway axis. This position ensures that smooth muscle contraction has a direct effect on airway diameter, rather than on the other airway dimensions[157] and indicates that even in the terminal bronchioli, narrowing of the airway lumen (bronchoconstriction) is a predominant function of ASM. The overall tone in the airways depends on a balance between the factors causing ASM contraction (excitatory) and the inhibitory (relaxing) stimuli. These factors involve neurotransmitters, circulating hormones, and autacoids-inflammatory mediators.

Airway innervation

Both afferent and efferent airway innervations play an important role in the regulation of airway tone, secretion, and local circulation. In addition, neurons can contribute directly to the process of inflammation.[274] The afferent branch conducts impulses into the central nervous system, with sensory impulses arising from receptors within the airways. The afferent pathway is composed of sensory nerves, predominantly

unmyelinated C fibers, and some myelinated nerves arising from the irritant and stretch receptors.

Efferent innervation, composed of autonomic nerves, conducts stimuli from the central nervous system into the ASM, blood vessels, and glands. The efferent, autonomic nerves are represented in the airway by cholinergic (parasympathetic), adrenergic (sympathetic), and nonadrenergic-noncholinergic (NANC) nerves.[213, 251]

With regard to nerve distribution and abundance in the airways, there are marked variations among the species. Generally, however, parasympathetic, cholinergic nerves provide the predominant excitatory neural input onto ASM.

Neuromuscular control of airway tone

General information

The ASM tone is crucial to development of bronchospasm and airway obstruction, thus the physiological mechanisms regulating airway tone will be presented in the following section. The ASM tone is determined by a variety of extracellular signals, including neurotransmitters, circulating hormones, and locally produced autacoids. The latter are produced by epithelial cells and other cells of the airway mucosa, such as residual or recruited inflammatory cells and fibroblasts. Among all types of cells, neurons are particularly specialized to perform the function of airway control. Activation of either the afferent, efferent, or both pathways can affect ASM tone. Afferent nerves may initiate a central reflex, in which impulses generated within the airways return via the central nervous system and the efferent pathway into the ASM. The afferent nerves can also generate a local reflex, in which stimulated sensory nerves become

autoactivated, spreading the excitatory wave via antidromic propagation.[247] The efferent pathway itself may be activated or inhibited due to effects of mediators on ganglionic cells, or via prejunctional modulation of neurotransmitter releases from postganglionic nerve terminals (see below). The efferent pathway seems to be predominantly involved in equine RAO, and here I will focus predominantly on this pathway.[62, 205]

Efferent excitatory nerves: parasympathetic pathway

In mammalian airways, stimulation of parasympathetic nerves contracts ASM. This dominant airway excitatory pathway originates from the dorsal motor nucleus of the vagus and nucleus ambiguus in the brain stem. Preganglionic fibers descend in the vagus nerve, enter the thorax and synapse on ganglia located within the airway walls.[213] Covering the short distance from the ganglion to the smooth muscle cells, the postganglionic fibers form multiple varicosities, from which stored neuromediator is released. Acetylcholine (ACh) is the universal neurotransmitter throughout the parasympathetic system, activating a nicotinic receptor within the ganglionic synapse, and the muscarinic (M) receptors on the ASM. Therefore the contraction induced by vagal stimulation may be abolished by hexamethonium, a nicotinic receptor blocker, while the M receptor antagonist, ATR, eliminates responses of ASM to postganglionic nerve stimulation. Released ACh either free or bound to receptors is rapidly inactivated via the specific ACh-esterase enzyme, and the free ACh does not accumulate in the tissues. Thus at any given moment, the amount of ACh released from parasympathetic nerve terminals dynamically modulates the cholinergic airway tone. The ACh release is determined by the frequency at which postganglionic nerves fire in the airways and the

overall amount of ACh release from the nerve per pulse. The mechanisms of ACh release by parasympathetic nerve is controlled at least at three levels: the frequency of preganglionic stimulation from the central nervous system, the process of ganglionic filtration, and the modulation exerted via prejunctional receptors present on postganglionic nerves. The two latter mechanisms take place directly within the airway wall so they can be affected via mediators released within the airways. The change of ganglionic filtration may enhance or suppress the efficacy of synaptic transmission in parasympathetic ganglia, independently from central nervous system.[274] The prejunctional effects may be excitatory or inhibitory, i.e., increasing or decreasing ACh release from airway parasympathetic nerves. Endogenous substances such as neurotransmitters, hormones, and also some of the inflammatory mediators can modulate ACh release via action on the specific prejunctional receptors (see histamine, 5-HT, PGE_2, etc.), or via interaction at the cholinergic autoreceptor on airway parasympathetic nerves (major basic protein released by eosinophils).

Excitatory noncholinergic nerves

Part of the neurally mediated contractions of airways that cannot be experimentally abolished with ATR is attributable to the excitatory nonadrenergic, noncholinergic eNANC response.[14] This response in many species, including in humans, is believed to be mediated by neuropeptides, the putative spasmogenic neurotransmitter in eNANC. Occasionally also, inhibitory effects of sensory neuropeptides are observed.[213, 258] The unmyelinated sensory nerves, or C fibers, carry neuropeptides such as the tachykinins: substance P and neurokinins A and B, and calcitonin gene-related peptide. The terminals

of sensory nerves are within and just below the epithelium. This location facilitates detection of noxious stimuli within the airways. The activation of airway sensory nerves occurs in response to inhaled irritants such as dry air, noxious gases (NH_3 and SO_2), smoke, dust, and other factors such as capsaicin, a component of chili pepper. When sensory nerves are stimulated, antidromic propagation of the neural impulse causes release of sensory neuropeptides within the airway wall, and in this respect, these afferent nerves play a role similar to efferent nerves.[213, 274] This action is thought to amplify the sensation of irritant within the airway and turn on the defense mechanisms in order to remove the irritant from the airways. These mechanisms involve coughing, sneezing, bronchospasm, increased mucus secretion, and promoting the immediate local inflammatory response, presumably to facilitate repair in the site of eventual injury and to protect it from infection (see neurogenic inflammation).[237] In the airways of adult horses, the role of sensory neuropeptides is unclear. Nerves immunoreactive to substance P and calcitonin gene-related peptide are predominantly present in the central airways, but most of them are associated with bronchial blood vessels and epithelium.[248] In the horse trachea, these mediators cause neither contraction (Yu, personal communication) nor relaxation,[310] and the prejunctional effects of neuropeptides in horses have been not studied.

Inhibitory innervation: sympathetic nerves

Sympathetic nerves originate from thoracic segments of spinal cord. The preganglionic nerves synapse at the last few cervical and first four thoracic paravertebral ganglia. Thus, only the postganglionic nerves enter the thorax and ASM. The

preganglionic nerves release ACh, which activates nicotinic receptors in the ganglion, and the postganglionic nerve terminals release norepinephrine (NE). The NE acts on α- and β-adrenergic receptors. The α_1-adrenergic receptors predominantly mediate contraction of vascular smooth muscle, but in the airways, α_1-mediated contraction can be demonstrated only in certain species and only in certain conditions.[17, 234] Because, in ASM, relaxation is afforded predominately by β_2-receptors, to which NE affinity is low, the inhibitory role of NE is probably mediated by activation of α_2-prejunctional inhibitory receptors on airway parasympathetic nerves rather than by actions on ASM. Indeed EFS of precontracted equine airways evokes endogenous adrenergic relaxation only in the upper part of the trachea. This suggests that the major component of the adrenergic inhibitory action of the lung is produced by circulating catecholamines, predominantly epinephrine, which avidly binds to β_2-receptors. Still, prejunctional mechanisms of circulating epinephrine in the horse most likely predominate over postjunctional effects. Recently LeBlanc, Yu, Zhang, and their collaborators have shown that in the levels approached in circulation during exercise, epinephrine and NE exert their inhibitory effects on the EFS-stimulated airways. Effect of epinephrine and NE is mediated predominantly via inhibitory α_2-adrenergic, prejunctional receptors, and in small part via β_2-receptors located on ASM.[155, 306, 309]

Nonadrenergic inhibitory system

In vitro studies of airways in many species have revealed the presence of an endogenous, non-adrenergic, relaxant response, mediated by postganglionic nerves. Since putative inhibitory nonadrenergic noncholinergic (iNANC) neurotransmitters,

described in this paragraph, have been colocalized predominantly with ACh in parasympathetic efferent nerves, existence of the distinct neural pathway responsible for iNANC responses is questionable.[16, 213] In the guinea pig, iNANC response is attributable to vasointestinal inhibitory peptide (VIP), peptide histidine-methionine (PHM), and nitric oxide (NO). Both inhibitory peptides, VIP and PHM, are structurally and functionally related and relax both airway and vascular smooth muscles by increasing intracellular cAMP. In guinea pig airways, these peptides cause significant relaxation, but in humans their importance has not been confirmed. Similarly to the role in humans, the functional role of VIP in the horse is unknown. In the horse, exogenous VIP induces profound relaxation of tracheal smooth muscle but there is no evidence of endogenous release of VIP in the equine airways.[310] In contrast to VIP, NO produces a large portion of neurally mediated inhibitory response in airways of both people and horses.[305] Nitric oxide produced in stimulated nerves crosses the cell membranes and binds to the intracellular soluble guanylylcyclase receptor. Relaxation induced by NO is therefore achieved via an increase of intracellular cyclic GMP.[124] Even though the neural origin of NO in EFS-stimulated airways has been confirmed by inhibitory effects of TTX on iNANC responses, this mediator also can be released from many other types of cells, including endothelium, neutrophils, and macrophages.[305]

Neuromuscular junction

The neuroeffector junction at the level of ASM is formed by extensively branching nerve terminals, containing multiple varicosities, from which stored neurotransmitters are released onto the muscle. The distance between varicosities and

the effector cells is variable, so that the level of exposure to the neurotransmitters between the smooth muscle cells is not uniform. For that reason, the spread of the excitation between the adjacent muscle cells most likely contributes to the control of ASM tone. Ultrastructural studies of human airway neuromuscular junctions revealed in the trachea a sparse innervation and moderate frequency of gap junctions between ASM cells, and denser innervation of muscle in bronchi small junctions without identifiable gaps.[56] Based on these morphological differences, Daniel et al. proposed that tracheal muscle is organized for a major myogenic control of activity via spread of stimulation from cell to cell in smooth muscle.[57] In bronchi, however, neural rather than myogenic control could be more important. Additionally, in bronchi, mast cells were found in proximity to both nerve terminals and ASM, suggesting that these cells may play a role in neurally mediated ASM responses. Meyers and Undem proposed that mediators released from these mast cells may significantly contribute to airway tone, not only directly contracting ASM, but also interacting with their neurons.[196, 274] These effects could involve: increase of synaptic efficacy at the airway parasympathetic ganglia, stimulation of sensory fibers with subsequent generation of central reflexes, and the action of histamine on sympathetic nerves. In addition, existence of prejunctional histamine receptors on airway autonomic nerves is possible, even though systematic study of prejunctional effects of histamine has been not yet performed within the respiratory tract.[274]

Receptors on ASM

ASM holds a large diversity of receptors via which extracellular signals are presented to ASM. Thus, regulation of airway tone is a complex mechanism involving responses to many factors simultaneously. This complexity appears even greater because many mediators possess more than one subtype of receptor on the muscle, each of which may mediate a different response. For example, histamine contracts ASM via H_1 receptors, but, when this receptor is blocked, relaxant effects mediated via the H_2 receptor can be unmasked in equine airways.[41] At least two subtypes of M receptors are also present on ASM. The less numerous M_3 receptor mediates the ASM contraction in many species including the horse,[304] while the role of the more numerous M_2 receptor on equine ASM is unclear.[304] Studies of M receptor subtypes in the airways, particularly M_2 are complicated by the presence of both prejunctional inhibitory M_2 autoreceptor on airway parasympathetic nerves, and M receptors in the mucosa/epithelium.[89] In contrast, in many mammalian species the universal inhibitory role of β_2-adrenoceptor on the ASM is generally accepted.

Intracellular signal transduction

Balance between excitatory and inhibitory signals determines the overall tone of smooth muscle. Thus at ASM, all these signals must be integrated at the intracellular level. The effects of most mediators in ASM are exerted via activation of G-protein coupled membrane receptors.[215] The exception from this general rule is NO acting on soluble guanylyl cyclase receptor. The effects of ACh at M_2 and M_3 receptors and β_2-receptor agonists are the best examples with which to discuss the function of G proteins

in ASM. The contraction of ASM induced by M_3 is mediated by G proteins coupled to phospholipase C (PLC), which hydrolyzes the membrane phospholipid phosphatidylinositol-biphosphate (PIP_2) to form inositol triphosphate (IP_3) and diacylglycerol (DAG).[121] The DAG is a protein kinase (PKC) activator, while IP_3 releases of Ca^{2+} from the stores in the endoplasmic reticulum. It appears that the contractile state of the ASM cell is largely determined by the level/activity of these two second messengers (Figure 1), because other spasmogens such as histamine, LTs, or thromboxane (TX) are also coupled to the same signal transduction pathway.[104]

Inhibitory mechanisms lead to a decrease in intracellular calcium ($[Ca^{2+}]_i$) but the mechanism of this decrease is not entirely understood. The effects of β-receptors on ASM are mediated via G_s coupled receptors. In fact most mediators or drugs that relax ASM act via receptors coupled to G_s, which is positively coupled with adenylyl cyclase. Besides β-receptor agonists, the inhibitory prostanoid PGE_2 and VIP are known to increase intracellular cAMP.[216] Accumulation of cAMP in the ASM interferes with Ca^{2+} metabolism by at least two mechanisms. It decreases PIP_2 hydrolysis and therefore prevents increase of free $[Ca^{2+}]_i$. Additionally, via activation of PKA, cAMP prevents the myosin light chain kinase (MLCK)-dependent phosphorylation of 20 kDa myosin light chain (LC_{20}).[193, 218] Similarly, inhibitory effects via changes in $[Ca^{2+}]_i$ distribution are associated with increase of cGMP mediated by NO. The level of cyclic nucleotides is also controlled by phosphodiesterase enzymes, transforming "active" cyclic nucleotides to "inactive" mononucleotides. Activity of these enzymes can be controlled by levels of cAMP or cGMP and by $[Ca^{2+}]_i$. Thus, phosphodiesterases are a very important factor in the integration of the excitatory and inhibitory signal transduction pathways in ASM

cells.[216] Besides phosphodiesterase activities, another way of limiting the inhibitory G_s pathway, is the control of cAMP production by action of G_i protein. Activation of M_2 receptors, coupled to G_i, decreases the efficacy of β-agonists to relax ASM.[288] The mechanism of this functional antagonism involves both direct inhibition of adenylyl cyclase by G_i and decreased binding of G_s to this enzyme. Thus G_i-coupled receptors, inhibiting production of cAMP, should promote intracellular events leading to smooth muscle contraction.[215]

Intracellular contractile apparatus

Contractile elements of ASM are composed of thick, i.e., myosin and thin, i.e., actin/troponin filaments. Each molecule of myosin contains two heavy chains and two pairs of light chains, 20 and 17 kDa light chains. The phosphorylation of the LC_{20} is thought to play a regulatory role in smooth muscle contraction, since it is both sufficient and necessary to contract smooth muscle.[120] Covalent crossbridging due to this reversible phosphorylation of LC_{20} by MLCK stimulates the cyclic actin-myosin interaction necessary for actomyosin-ATPase activity.[194] Hydrolysis of ATP delivers chemical energy, which is then transduced into mechanical work to move the thin and thick filaments past each other (sliding filament model. [193, 218]) Activation of MLCK depends on binding to the Ca^{2+}/calmodulin (CaM) complex.[120] Thus, the level of free $[Ca^{2+}]_i$ is critical for smooth muscle contraction and, because of that, Ca^{2+} is a central second messenger, integrating responses to multiple stimuli that affect smooth muscle tone. Stimuli that increase $[Ca^{2+}]_i$, and in turn saturate CaM, activate MLCK; and those that decrease $[Ca^{2+}]_i$ and cause $[Ca^{2+}]_i$ dissociation from CaM deactivate it. High levels of

$[Ca^{2+}]_i$, LC_{20} phosphorylation, and ATP-ase activity are required for development of smooth muscle tone. Other mechanisms are important for maintenance of ASM in the contracted state. In tonic smooth muscle, initial increase in $[Ca^{2+}]_i$ and LC_{20} phosphorylation is only transient. In spite of this, ASM are capable of maintaining significant contraction, with nearly basal levels of Ca^{2+} and LC_{20} phosphorylation. Horowitz et al. in their review present a large body of evidence that PKC is an important regulatory component in this process, which in this case, involves thin actin/tropomyosin filaments.[120, 194] Recently, Wardle and Gerthoffer, who studied mechanisms of equine and canine trachealis contraction, respectively, concluded that PKC stimulates the intracellular contractile apparatus via mechanisms that increase its Ca^{2+} sensitivity. [96, 218, 287] This situation may play a role in the functional synergism between different mediators contracting ASM. When initial mechanisms of contraction become activated, and the intracellular contractile apparatus is "sensitized," only a small increase in $[Ca^{2+}]_i$ is required to maintain the contractile state of ASM cells. This is in contrast to the initiation of contraction that requires much higher levels of $[Ca^{2+}]_i$ and subsequent phosphorylation of LC_{20}.[96, 120] Therefore, the interaction of many airway spasmogens, coupled to the PLC signal transduction pathway, is a possible way by which sustained bronchoconstriction is evoked in status asthmaticus.

Local defense mechanisms

Introduction

Equine RAO (see Chapter 2) is an example of a syndrome in which the exaggerated responses of local defense mechanisms, rather than noxious effects of the

pathogen itself, are responsible for functional changes and structural lesions. Thus, local defense mechanisms in the airways will be addressed in detail. The predominant defense mechanisms in the airways involve: the airway secretion and the mucociliary escalator, a local immune system functioning as a branch of systemic immunity, and the system of neuromuscular airway control. At present a sufficient amount of evidence is available to conclude that, to some degree, all these mechanisms are involved in the pathogenesis of airway obstructive diseases such as asthma and COPD in humans and RAO in equine species.

Mucociliary escalator

In physiologic conditions, the respiratory tract possesses a double layer of mucus, a sol and gel layer. The mucus lining protects the airways from overdrying, and traps the particles brought with the air in the respiratory tract. Additionally, it carries inflammatory cells and factors involved in airway defense, i.e., antibodies and other proteins with antibacterial, antioxidant, antiprotease and pro- or anti-inflammatory properties. Instant ciliary motion, synchronized between the cells, efficiently propels airway secretions toward the pharynx. This action, called a mucociliary escalator, eliminates excess airway secretions, cell debris, microorganisms, and inhaled particles that have been deposited in the mucus. An appropriate quantity and composition of the airway fluids ensures correct function of the mucociliary escalator and undisturbed function of airways, while in the pathologic situation, mucus hypersecretion and/or stasis may narrow the airway lumen and cause plugging of smaller airways. Several types of cells are responsible for production of respiratory secretion. Mucous cells (goblet)

produce and store thick mucus containing a large quantity of mucin glycoprotein. Serous cells are thought to be the source of the liquid phase of airway secretion as well as a number of proteins that possess a variety of functions in the airways. Sero-mucous cells possess both mucous and serous cell characteristics. It is quite likely that the presence of this intermediate type of cell is associated with adaptation of the epithelium to some types of injury, leading to changes in cell populations. Clara cells present in surface epithelium of the airways are distinct from serous or mucous cells. Their secretory granules contain multiple proteins, such as lysozyme, uteroglobin (both protease and PLA_2 inhibitor), and antielastase as well as other proteins, the functions of which have not been clearly defined. A decreased number, and changes in the morphology of Clara cells are quite obvious in distal airways from horses with RAO, suggesting that the Clara cells are involved in the pathogenesis of RAO.[138]

Bronchospasm

Depending on the species, the ASM is in a completely relaxed state, or possess only a low level of intrinsic airway tone in normal physiological condition. Even in this balanced state, input from tonic excitatory and inhibitory mechanisms is tuned to exert the desired level of airway tone. When the balance between excitatory and inhibitory stimuli shifts toward the former, smooth muscle bronchoconstriction occurs. Contraction of ASM in airways results in narrowing of the airway lumen, apparently without dramatic change of the external diameter.[252] Naturally occurring, or pharmacologically induced bronchospasm results in symptoms of airway obstruction such as dyspnea, increased respiratory sounds, and, in more severe states, hypoxemia. The level of

airway obstruction can be quantified by physiological measurements of transpulmonary pressure, air flow, and volumes during tidal, or forced breathing. The most common parameters calculated based on these measurements are maximal difference in pleural pressure (ΔPpl), airway or pulmonary resistance (R_L), and dynamic compliance (C_{dyn}) or its inverse, dynamic elastance (E_{dyn}). Additionally, in human medicine, measurement of forced expiratory volume per second (FEV_1) is well correlated with airway status, and very easy to perform. The general interpretation of these parameters is that broncho-spasm increases ΔPpl, R_L, and E_{dyn}, and decreases the remaining two parameters. In addition, changes in large airway diameter affect mostly R_L while peripheral airway obstruction causes predominantly decreased C_{dyn}/increased E_{dyn} due to non-uniform and impeded ventilation. ΔPpl, which is determined by both parameters, and additionally affected by changes in air flow due to rapid breathing, is thought to reflect the overall airway status.

Inflammation

The inflammatory process can be seen from two points of view: first as a process that is a source of signs responsible for the course of the clinical picture of disease, and second as a group of mechanisms responsible for elimination of pathogens from the respiratory tract. Because a large group of inflammatory cells and mediators may be involved in different proportions in the response to pathogens, many types of inflam-mation have been described by both clinicians and pathologists. In this summary, I will largely concentrate on the role of inflammatory cells and the action of inflammatory

mediators in order to underline the mechanisms that can be involved in the pathogenesis of airway obstruction.

Pulmonary immunoregulation

Immune mechanisms in the lung, like in the other organs, can involve three phases; an afferent or affector phase, central processing, and the effector phase.[161]

The afferent phase requires antigen-presenting cells, including highly specialized antigen-presenting dendritic cells, macrophages, and B lymphocytes. Endothelial and epithelial cells are also capable of antigen presentation, however much less efficiently.[161] Antigen-processing cells possess the unique capacity to stimulate naive T lymphocytes. Moreover, it has been proposed that these cells, e.g., dendritic cells and macrophages, can transport antigens into regional lymph nodes.

Central processing determines the type of immune response regulating development of the T cell subset. Maturing, early CD4+ cell clones (T_{H0}) may evolve to T_{H1}, which promote predominantly cell-mediated immune response, or to T_{H2}, supporting the response mediated by antibodies. Cytokines secreted by T_{H1} and T_{H2} cells suppress the development of opposite clones of helper cells so that response to antigens is predominantly of one type.[161]

The effector phase, in which effector inflammatory cells are recruited and or activated to eliminate the antigens, provides the final response of the immune system to the antigen. In this phase both immune lymphocytes and nonspecific effector cells such as granulocytes are involved. Mobilization and activation of several groups of inflammatory cells predetermined by events form the central processing phase, and the

immune status of the individual is responsible for the type and course of inflammation and the effectiveness of resolution. Certain elements of the effector phase may be turned on by nonspecific factors such as noxious gases or bacterial endotoxin, without antigen processing. Current evidence supports the hypothesis that both specific and nonspecific immune reactions are involved in the response to natural hay and straw challenges in airways of horses with RAO (see Chapter 2). Even though the first two phases of immune response may be crucial to sensitization of the airways and development of inflammatory response in reactive airway diseases such as RAO, not much available literature can be found about the mechanisms of the first two phases of immunity in the respiratory system, particularly in the horse. Cells and mediators involved in the effector phase are more thoroughly studied, and in Chapter 1, I will mostly focus on the mechanisms of the effector phase that are confirmed or could be potentially involved in pathogenesis of RAO. Neutrophils and mast cells are thought to be the predominant effector cells during the inflammatory response in exacerbations of RAO, and these cells will obtain most attention in this review.

Inflammatory cells

Mast cells

Mast cells are located predominantly in connective tissue of organs throughout the body. In the airway, mast cells are found in airway mucosa, in lamina propria, and some in the epithelium.[288] In horses, mast cells are present throughout the respiratory tract and can be found in airway mucosa and LP (primarily associated with blood vessels, pleura, and SA) and in BAL fluid.[169] Morphologically, mast cells can be distinguished

by cytoplasmic granules, dyed characteristically with histochemical staining. These granules contain inflammatory mediators such as histamine, 5-HT, and proteins like mast cell tryptase and the proteoglycan heparin. Contents of the granules in the mast cells may vary not only between species but also between organs and tissues of individuals.[206] Activation of mast cells can be classically induced by crossbridging of IgE immunoglobulins on the cell surface by specific antigens. Signal transduction in the IgE-induced mast cell activation is not entirely understood, but it appears that cross-linking of these cell-bound antibodies activates yet undefined protein-tyrosine kinase and PLC.[138] The latter step is followed by a cascade of second messengers, including IP_3, leading to increase in cytosolic Ca^{2+}, which is required for mast cell degranulation.[135] Also specific ligands binding to the surface receptors, including complement fragments (C5a and C3a, anaphylatoxins), substance P, or opioids and physical stimuli such as low temperature, cause an increase in the $[Ca^{2+}]_i$, followed by mast cell degranulation. Degranulation of mast cells releases histamine and other mediators capable of ASM contraction and changes in microvascular permeability.[214] The role of mast cells in human allergic airway diseases, such as asthma and rhinitis, was postulated long ago. The number of mast cells in the airway lavage and brush biopsies increases during episodes of the allergic airway diseases.[285] Furthermore, spontaneous histamine release from mast cells obtained by airway lavage is higher in asthmatics than in healthy persons, suggesting that mast cells are activated in asthma.[285] Similarly, in RAO both numbers of mast cells and histamine release increase (see part II). Even though antihistamines seem not to be particularly helpful in the treatment of asthma [214] nor RAO, the amount of histamine in the airways strongly correlates with the sensitivity of the asthmatic to MCh.[39] Since

histamine level is a marker of mast cell activation, mediators released simultaneously with histamine by activated mast cells are most likely also very important in the pathogenesis of allergic airway diseases. Thus, the sole elimination of histamine effects may not be sufficient to reach therapeutic benefits in asthma or RAO.[214]

Beside histamine, activated mast cells generate a large spectrum of pro-inflammatory lipid mediators such as $LTC_4/D_4/E_4$, PGD_2, PAF, and TXA_2. All of these pro-inflammatory lipid mediators exert bronchospastic effects and increase capillary permeability and more or less potently attract neutrophils.[93] In addition to the typical role in the local circulation, current discoveries suggest that activation of mast cells results in production and secretion of multifunctional pro-inflammatory and mitogenic cytokines like IL-1, IL-3, IL-4, IL-5, IL-6, granulocyte-monocyte colony stimulating factor (GM-CSF), interferon-γ (IF-γ), or tumor necrosis factor-α (TNFα).[93] This set of cytokines not only stimulates IgE production important in the early phase reaction, but also hematopoiesis and maturation of granulocytes, immune responses (see lymphocytes), and recruitment of inflammatory cells, all crucial at the late-phase response.[93] Benefits of drugs, like cromolyn sodium, that stabilize the mast cell's membrane, in human asthma and equine RAO [22, 111] provide indirect evidence about the importance of mast cells in the pathogenesis of these diseases.

Basophils

These granulocytes fulfill functions identical with mast cells, and basophils, recruited into the tissues, may be morphologically indistinguishable from the mast cells, because basophil granules stain metachromatically just like the mast cell granules. The

major difference between basophils and mast cells is the presence of chondroitin sulfate in basophils, instead of heparin, which is a major proteoglycan component of mast cell granules. The basophils also lack neutral proteases, characteristic for the mast cells. The mode of activation and profile of mediators released by both types of cells are similar, thus both groups of cells are often discussed together as metachromatic cells.[181]

Neutrophils

This polymorphonuclear leukocyte is one of the first inflammatory cells to appear at the sites of acute inflammation. Neutrophils are highly specialized phagocytic/microbe-destroying cells and are very effective due to their mobility, large variety of receptors for both endogenous and bacterial chemotactic factors, and extensive machinery for intra- and extracellular killing of microbes.[238] The main strategy of neutrophil action is to ingest, neutralize, and destroy pathogens such as bacteria, immune complexes, and cellular debris. A newer concept views neutrophils also as secretory cells.[95, 114] Neutrophils not only discharge their granule contents to the extracellular environment, often independently from phagocytic activity, but they also release a variety of inflammatory mediators such as ROS, lipid mediators, and IL-1.[114, 202] Neutrophils originate from bone marrow and as circulating cells they remain in the blood stream for only a short period of time (half-life of 4–6 hours) until recruited into extravascular space.[238]

Currently, multiple groups of investigators study mechanisms by which neutrophils are recruited from the blood stream, migrate through the vascular endothelium, and enter the tissues. These mechanisms are regulated by chemokines,

e.g., IL-8,[133, 240] and chemotactic ligands, a heterogeneous group of substances attracting neutrophils towards increasing concentration gradients regardless of their remote origin and chemical structure. The best known and most frequently studied are LTB_4,[95] PAF,[87] fMLP,[212] and C5a.[37, 239] In response to these stimuli, both endothelial cells and neutrophils become activated so that leukocytes adhere transiently to the vascular endothelium. Further stimulation leads to neutrophil sticking and diapedesis between endothelial cells and emigration through the wall of the capillary.[156] The adhesive interactions between neutrophils and other cells resulting in "rolling," "sticking," and "diapedesis" are mediated by cell adhesion molecules. The latter are represented by large families: selectins, integrins and Ig-like proteins.[95] Knowledge about molecular properties of cell adhesion molecules, their interactions, expression, and modes of activation has advanced rapidly in recent years; however, in this review only this brief summary is included. For the purpose of my research project it is much more important to review the mechanisms, by which neutrophils can be activated and the types of mediators released upon the activation.

Activation of neutrophils

Mechanisms used for microbial killing are classified as oxygen-dependent and oxygen-independent,[77, 227] and both systems generate several factors that are potentially lethal for the host cells. Thus activation of neutrophils requires precise regulation. Indeed signal transduction in neutrophils is extremely complicated and there are several modes and levels of neutrophil activation.[12] Another feature of neutrophil activation is the phenomenon of priming, in which exposure to some factors such as LPS does not

cause profound stimulation of neutrophils, but, when followed by contact with other stimuli, results in much greater neutrophil stimulation.[26] Initial, low-level activity can be exerted by chemotactic ligands and chemokines, which increase neutrophil motility and may activate secretion, while the profound activation of neutrophils takes place during phagocytosis. Phagocytosis itself is a complex process in which several steps and multiple factors are involved. Recognition of the particle by the cell membrane followed by surface attachment are the initial steps in which membrane receptors are involved. In this stage, opsonins that facilitate the process of phagocytosis are very important and the most effective are antibodies and the C3b complement fragment.[52, 238] The neutrophil membrane possesses both Fc receptors and the C3b receptors that can bind these molecules. Binding to these receptors initiates intracellular events leading to explicit phagocytosis and further activation of antimicrobial mechanisms.[244] These further steps involve particle engulfment by the neutrophil and membrane, creating a phagosome to which lysosomes or granules bind, often even before the entire sealing of the phagosome is established.

Granules

Neutrophils possess up to three types of granules, which have relatively distinct profiles of enzymes. Traditional classification includes the azurophil or primary granules, which are myeloperoxidase (MPO) positive, and specific or secondary granules, which are MPO negative. Recently, a third group was distinguished from the latter, namely gelatinase granules or tertiary granules that do not contain lactoferin.[29] Some of the enzymes in primary granules are bactericidal, such as lysozyme and MPO, and others

such as collagenase, elastase, and cathepsins are important in the pathology of neutrophil-induced tissue injury, because of proteolytic activity.[116, 146] The participation of granules in phagocytosis (fusion of the granules with phagosomes) and use of granule enzyme in both oxygen-dependent and oxygen-independent mechanisms of microbial killing has been well documented. Other enzymes found in the granules, which are not involved in germicidal activity, serve as digestive enzymes to destroy killed microorganisms and other ingested particles inside the phagolysosomes.[238] Granule content is also secreted to the extracellular space by activated neutrophils. This activity occurs not only during frustrated phagocytosis but appears to be a part of a physiological mechanism in which cytoskeleton (actin/myosin) and external plasma membrane are involved.[114] Specific granules are released more readily and to a greater extent than the azurophil ones. The secretory role of neutrophils is currently a subject of several studies and as yet the physiological role of neutrophil secretory activity is not entirely understood. One theory states that enzymes released from neutrophils facilitate their migration through the tissue and create channels through which other types of inflammatory cells may migrate to the site of inflammation.

Oxygen-dependent and oxygen-independent antimicrobial systems

Bacterial killing in neutrophils depends on both oxygen-dependent and oxygen-independent mechanisms. Both mechanisms act synergistically but, depending on the type of microbes, one or the other system is more efficient. The importance of oxygen-dependent mechanisms is underlined by the fact that individuals with deficiency in oxygen-dependent neutrophil functions suffer from a chronic and often fatal chronic

granulomatous disease.[52] Chronic granulomatous disease is characterized by repeated and difficult-to-overcome infections. These infections are predominantly caused by Gram+ bacteria such as *Staphylococci* and *Streptococci,* indicating that the oxygen-dependent microbial killing system is a particularly efficient element of defense against Gram+ infections. On the other hand, oxygen-independent mechanisms can largely compensate for lack of oxygen-dependent mechanisms since in some individuals with complete deficiency of the oxygen-dependent mechanism, severe forms of chronic granulomatous disease may not necessarily develop or are absent for several years of life.[77] The oxygen-dependent system generates ROS and involves an NADPH oxidase system shared by neutrophils and macrophages, and the MPO system found exclusively in neutrophils. The oxygen-independent system involves lysozyme, defensins, cathepsin G, azurocidin, and bacterial permeability-increasing protein.[48, 77]

Respiratory burst

The rapid increase of oxygen consumption by activated phagocytes, i.e "respiratory burst," described by Baldrige and Gerrad in the 1930s, indicates that oxygen-dependent antimicrobial mechanisms have been set in motion. This oxygen is not used by the cell for generation of energy, but is metabolized almost entirely to produce ROS.[227] In the 1960s, H_2O_2 was the first ROS proposed to be generated by activated phagocytes [127] and in early 1970s, Babior et al. postulated that $O_2^{-\cdot}$ is another bactericidal agent in leukocytes. Today almost every handbook of medicine or pathology describes molecular details of respiratory burst, and several reviews, with great precision, picture the structure and function of individual proteins involved in the cascade

generating ROS in activated phagocytes. Briefly, the respiratory burst yields $O_2^{-}\cdot$, which is a precursor molecule in the cascade of ROS formation, from which other generations of ROS are derived (see mediators). Superoxide is produced by univalent reduction of molecular O_2 by the membrane-bound NADPH oxidase complex.[48, 227, 262] The assembly of oxidase upon neutrophil activation requires interaction between membrane-bound components and cytosolic proteins. The core of the membrane-bound NADPH oxidase system is Flavocytochrome b558,[228] which is a heterodimer protein carrying the heme and possessing both FAD- and NADPH- binding domains in middle and carboxyl terminal portions, respectively.[228] This cytochrome has an extremely low redox potential (the lowest of any other mammalian cytochrome) that is sufficiently low to reduce oxygen to $O_2^{-}\cdot$.[272] Additional components of NADPH oxidase involve small GTP binding proteins from the *ras* oncogene superfamily, namely the membrane-bound *rap1* protein, and the cytosolic *rac-1* or *rac-2* proteins, which are thought to be regulators (on and off switches) of NADPH oxidase depending on their GDP/GTP-bound state.[262] Additionally, there are two cytosolic proteins, p47 and p67, which in activated neutrophils are translocated into the membranes, to join the membrane component of oxidase complex.[227, 262, 272] The 47-kD protein is phosphorylated during formation of the oxidase complex, and it binds to the complex, facilitating binding of the p67,[48] which is required for the function of oxidase. Binding of p47 to membrane components depends on its phosphorylation. Because p47 is a substrate for PKC, phosphorylation of p47 by PKC has been postulated to be a mode by which PKC may activate NADPH oxidase.[272]

Generation of ROS

Superoxide ion is only one of the bactericidal ROS. Reactions in which superoxide gives rise to the whole family of ROS is depicted on Figure 2. Two molecules of $O_2^{-}\cdot$ in combination with H^+ ions form H_2O_2 and molecular oxygen..[139, 249, 250] This dismutation reaction can occur spontaneously or may be catalyzed by SOD. H_2O_2 is formed in large amounts by activated macrophages [281] and neutrophils [119] and like $O_2^{-}\cdot$, H_2O_2 is germicidal. However, its reactivity is relatively low and, most likely, rather than being a main weapon, H_2O_2 serves as a substrate to produce more toxic ROS. In the primary granules, neutrophils possess MPO, an enzyme that is released into phagocytic vacuoles when granules fuse with the phagosome. MPO catalyses the reaction during which H_2O_2 and halides such as Cl- form hypohalous acids, e.g., HOCl. This transformation results in increased toxicity since products of halide oxidation are powerful toxins. Additionally, HOCl can be further transformed into bactericidal chloramine, which being an unstable chemical, may generate all spectra of toxic byproducts.[139] The strongest oxidizing activity is exerted by the hydroxyl radical OH·, which is also derived from H_2O_2. In the presence of divalent iron as a reducing agent, H_2O_2 can break down into OH· and OH^- anion or when Fe^{2+} concentration of iron is limiting, OH· generation may occur via the Haber Weiss reaction[249]

$$H_2O_2 + O_2^{-}\cdot \rightarrow O_2 + OH^- + OH\cdot$$

Properties of ROS species and their effects on airway function will be described in the section on mediators.

Signal transduction

Neutrophils treated with high concentrations of chemotactic ligands (LTB_4, PAF, fMLP, C5a) in vitro may undergo rapid activation which results in neutrophil degranulation and activation of the respiratory burst.[95, 131] Receptors for chemotactic ligands belong to the G-protein coupled, seven membrane spanning receptor superfamily.[11, 34, 244] Activation of these receptors results in activation of pertussis toxin-sensitive G_i protein, which in turn activates PLC_β. This PLC cleaves membrane-associated PIP_2 to yield second messenger products, IP_3 and DAG, which in turn mobilize $[Ca^{2+}]_i$ and activate PKC, respectively.[244] Further downstream, signal transduction involves activation of several serine-threonine PKCs such as Raf-1, B-Raf, mitogen-activating protein kinase kinase (MEK-1), and mitogen-activating protein kinase (MAPK) (Figure 3 reproduced from Buhl et al.[34]). This in turn phosphorylates PLA_2 and PLD, leading to their activation.[34, 47] Activation of PLD leads to an accumulation of phosphatidic acid, which appears to be critical in the cellular response of neutrophils.[244] Activated PLA_2 releases arachidonic acid (AA), which is not only released from stimulated neutrophils but also serves as a stimulus directly affecting the cell function of neutrophils.[26, 244] Thus AA acts in the neutrophil as both a second messenger and a substrate for cyclooxygenase and lipoxygenase to yield eicosanoids.[47] As currently shown by Tithof et al.,[266] distinct PLA_2s are activated to release AA for these different purpose.[265] Different functions of neutrophils are regulated by different mechanisms. It has been shown that activation of PKC by PMA or C5a and fMLP causes activation of the respiratory burst [65]; however, PKC is clearly not involved in degranulation/secretion. Degranulation requires elevated levels of cytosolic Ca,[65, 244] but again exocytosis of primary granules is under tighter

control than the release of enzymes from secondary granules.[12] Release of primary granules via the external plasma membrane is most likely regulated by monovalent cations in contrast to the release of secondary granules that may be induced by Ca ionophore. However, neither calcium rise nor PKC activation is required for the shape change caused by activation of the contractile system (migration of neutrophils).[12] This complex regulation of neutrophils prevents premature neutrophil activation and ensures that only the physiological process of phagocytosis will activate the most hazardous mechanisms in neutrophils.

Neutrophils, neutrophil-derived mediators, and their effects in the airways

Activation of neutrophils releases inflammatory mediators that may be critical to lung injury. In both people and animals, oxygen radicals and enzymes released by activated neutrophils are implicated in the fatal disorder, adult respiratory distress syndrome.[43, 117, 141, 250] Similarly, the contribution of neutrophils in the pathogenesis of cystic fibrosis (Suter 1984), farmer's lung disease,[279] or grain-dust induced airway obstruction [44] has been demonstrated. Neutrophils are also a fundamental component of the acute inflammatory responses in the airway obstructive diseases, commonly found in several animal models of allergen-induced inflammation and some types of late asthmatic responses in humans.[44]

It is not unlikely that neutrophils recruited into the airway exert effects on airway responses, because mediators released by activated neutrophils such as TXA_2, LTB_4, PAF, ROS, and elastase affect ASM and may cause bronchospasm or contribute to AHR.[24] Additionally, neutrophil-derived enzymes and ROS, by damage of epithelium,

can promote inflammation, change the epithelial barrier function, and increase mucus secretion.[256] The necessity of circulating granulocytes as mediators of AHR was demonstrated in canine models of bronchoconstriction [24] and neutrophil recruitment is associated with IL-8 induced bronchial hyperresponsiveness in guinea pig.[301] Also in an ozone-induced AHR model, neutrophils were postulated to be the main contributor to the development of AHR.[204] However, more recently, it has been shown that neutrophil recruitment into the airways and AHR resulting from ozone and IL-8 can be dissociated.[158, 203]

The evidence that neutrophils could be involved in human asthma is not very solid either. Several authors have suggested, however, that neutrophils may be involved in this human syndrome. For example, a few studies have demonstrated increased respiratory burst activity (chemiluminescence) of neutrophils isolated from the blood of asthmatics in comparison with control subjects.[129] A few relatively recent studies have also demonstrated that supernatants from activated neutrophils 1) increase response of human bronchi to histamine via cyclooxygenase products, presumably TXA_2,[122] and 2) increase response of human bronchial tissue to both electrical field stimulation and histamine.[107] In asthma, neutrophils are found in sputum and BAL fluid, but their numbers are variable. For example Montefort et al. [191] demonstrated an increased number of neutrophils in bronchial biopsies 4 and 6 hours after antigen challenge, respectively, whereas in another study, endobronchial biopsies and BAL in mild asthmatics 18 hours after allergen bronchoprovocation showed no changes in neutrophil populations.[19] Immunohistopathology studies of slow- and sudden-onset fatal asthma have demonstrated that eosinophils are the predominant cell in slow-onset, while in the

sudden-onset "...a relative paucity of eosinophils in the face of an excess of neutrophils" was found in the airway mucosa.[255] In most cases, neutrophil numbers recovered with airway lavages do not correlate with severity of asthma, which is in contrast with the numbers of eosinophils.[156, 190] Since good correlation between neutrophil presence and severity of asthma cannot be found, Henson and Borish, in discussing possible involvement of neutrophils in human asthma, reached the conclusion that, "...to our eyes, the available information does not support a major role for the neutrophil in causing asthma...."[114] This current understanding of the role of neutrophils in asthma does not entirely rule out neutrophils as a factor of asthma pathogenesis. The role of neutrophil and neutrophil-derived mediators is currently seen in the initiation and promotion of the exacerbation of inflammatory events in the airways.[115]

Eosinophils

Eosinophils are also a bone marrow-derived leukocyte, and their presence in the tissue is associated with some allergic and parasitic diseases. In contrast, with neutrophils, secretion of products accumulated in their granules, rather than phagocytosis, is the main mode of action of eosinophils. Eosinophil granules contain basic proteins such as major basic protein, eosinophil peroxidase, and eosinophil cationic protein and neurotoxin. All three proteins possess profoundly toxic effects on living tissues. In addition to the preformed proteins that are stored in granules, eosinophils are a source of low molecular weight mediators such as PAF and LTC_4, which can be generated upon cell activation. Also, in contrast to neutrophils, for which a role in human asthma have not been strictly defined, eosinophils are a unique feature of airway infiltration in asthma

and in several animal models of this disease. In animal airway diseases, eosinophils in the airways are found in feline asthma, the ascaris antigen-challenged guinea pig model of asthma, and the cynomolgus monkey model of airway obstruction. Also, in rare non-obstructive respiratory disorders such as eosinophilic pneumonia, or in response to parasitic infestations in which larvae migrate through the lungs, eosinophils are the predominant inflammatory cell. In patients with asthma, eosinophils are found in sputum, BAL fluid, and biopsy from the airways.

In several reports, airway reactivity strongly correlates with an increased number of eosinophils in BAL, eosinophil granule proteins in lavage, and number of eosinophils in peripheral blood.[300] Upon activation with IL-5 or PAF, eosinophils undergo degranulation, and cationic proteins are released into the airways. The following activities of these proteins play a role in airway pathology: stimulation of histamine release from the mast cells, major basic protein, eosinophil cationic protein, induction of bronchospasm, and increase of airway reactivity (major basic protein), production of toxic oxygen species (eosinophil peroxidase), and direct cytotoxicity, especially to the airway epithelium (eosinophil cationic protein, major basic protein, eosinophil neurotoxin). The latter effect seems to be quite obvious, since epithelial injury in the airways of asthmatic patients can be colocalized with the "spills" of major basic protein. Epithelial damage may contribute to the airway obstruction via reduced mucus clearance (lack of cilia) and increased reactivity of the airways due to impeded barrier function and/or absence of EpDRFs. In the antigen-challenged guinea pig model of airway obstruction, eosinophils are thought to be involved in dysfunction of prejunctional M_2 receptors on parasympathetic nerves.[91] The M_2 are the autoreceptors on the airway

parasympathetic nerves, where they provide a negative feedback for ACh release. Dysfunction of this M_2 receptor causes bronchospasm due to increase of ACh release in the airways. Fryer and Jacoby and collaborators, in a series of elegant studies have demonstrated that M_2 receptors in pulmonary parasympathetic nerves are dysfunctional after antigen challenge in sensitized guinea pig.[90] In guinea pig challenged with antigens, in which M_2 receptors are dysfunctional, large numbers of eosinophils are associated with nerves. A similar tendency was observed in patients with fatal asthma, where overall eosinophil number in the airway was nearly 60 times greater than in the airways from nonasthmatic individuals, but the number of eosinophils colocalized with nerves was nearly 200 times greater.[50, 300] Although current literature generally supports the view that the eosinophil is a key effector cell in allergy and asthma, few recent reports have shown that eosinophils are neither sufficient nor necessary to cause changes in the airway functions.[13] Suppression of allergen-induced eosinophilia in BAL is not sufficient to inhibit induction of AHR in rats[78] and in some patients with asthma, eosinophils in BAL are not detected until hours after initial signs of airway obstruction.

Blood platelets

These smallest blood cells are not classical inflammatory cells but belong to the blood coagulation system. However, they can contribute to the inflammatory response, because they become activated by inflammatory mediators and tissue injury. Activated platelets aggregate and release factors that influence inflammatory processes. Lipid mediators are the best example, since their production is particularly dynamic in activated platelets and results in release of 12-hydroxyeicosatetraenoic acid (HETE), TXA_2, and

PAF.[238] Degranulation of activated platelets yields 5-HT, a mediator implicated in anaphylactic reaction, and in airway diseases, including RAO. Additionally, platelets are a source of pro-inflammatory cytokines including RANTES MIP-1α.[143] These cytokines promote inflammation and are chemotactic to eosinophils and metachromatic cells, respectively. Additionally, thrombin-activated platelets adhere to monocytes and stimulate them to express IL-8 and MCP-1, factors involved in inflammatory/allergic responses.[293]

Macrophages

Mononuclear phagocytes are the most abundant resident cell in the alveolar space and at the air-surface interface of conducting airways in the normal lung.[217] These versatile and multipotent cells play a significant role in all phases of the immune system response. In other words, macrophages are both regulatory in immune response and a typical effector cell. One of the crucial functions for the immune response is antigen processing and its presentation to the helper cells. Antigen-activated macrophages communicate with T cells and other cells with the chemical language of cytokines. The expression and release of a whole spectrum of regulatory cytokines, mediators, and factors, of which approximately one hundred are known today,[217] affects both the afferent phase and central processing [28, 166] in the local lymphatic system and also stimulate the development, recruitment, and activation of virtually all types of effector cells including macrophages themselves. Multifunctional, pro-inflammatory cytokines like TNFα, IL-1, and IFN-γ promote initial responses of T helper cells, which result in their clonal expansion, and affect many other kinds of inflammatory and noninflammatory cells.

Mononuclear phagocyte-derived cytokines, which can modulate the course of inflammation by recruitment, stimulation, and suppression of different inflammatory cell lines, are IL-6, IL-8, IL-10, IL-12, IL-15, granuloyte monocyte-colony stimulating factor (GM-CSF), and TGFβ. The macrophages produce also at least one histamine releasing factor, e.g., MCP-1, acting on metachromatic cells.[159] As an effector cell, macrophages also possesses a wide spectrum of activities, including 1) phagocytosis and germicidal mechanisms, composed of oxygen-dependent, which is shared with neutrophils (see above), and oxygen-independent systems, 2) cytotoxic effects, and 3) ability to release inflammatory mediators, predominantly bioactive lipids, prostanoids, LTs, HETES, and PAF. These activities and mediators are not distinctive for macrophages and can be produced by other types of inflammatory cells. Thus macrophages, rather than being responsible for a defined set of responses during the effector phase, are thought to play a predominantly regulatory and supplementary role to the other more specialized effector cells. However, it has been clearly shown that macrophages themselves can produce tissue injury by release of ROS[250] and other mediators, and thus are capable of exerting direct effects on the lung tissue. It is very likely that the role of these mononuclear phagocytes is much greater than we currently suspect. Because of the complexity of macrophage function, the role of these cells in the respiratory system is not entirely understood.[218] In contrast to other inflammatory cells, populations of which dynamically change in the course of airway diseases, it is very difficult to conclude whether and to what degree macrophages are involved in inflammatory reactions based solely on the cytological studies. The macrophages are permanently present in the respiratory system, their life span is up to several months, and rather than their numbers, the types and

levels of activities they express determine the overall effects of these cells. Interpretation of the data from measurements of particular activities in macrophages is again very complicated because of the large versatility of these cells and amount of interactions with other cells. Response to the same stimuli may be very different in different cellular and humoral environments. Some of the macrophage-released cytokines, rather than being stimulatory, are inhibitory for immune responses, e.g., a factor inhibiting histamine release from the mast cells, IL-1 inhibitor, natural killer cells inhibitor, and a factor decreasing the influx of Ca^{2+} into stimulated T cells. Although there are no data supporting this hypothesis, lack of certain immunosuppressing activities in macrophages, might also contribute to allergic diseases, for example by lack of inhibitory effect on the T_{H2} response.

Lymphocytes

Lymphocytes consist of a large group of cells that play different, often very distinct, roles in the immune response. The main division lies between B, T, and natural killer cells and only the first two mediate specific immune responses. As a typical effector, B cells are responsible for humoral immunity. Activated immune memory B cells proliferate and mature, forming a clone of plasma cells programmed to produce selective antibodies,[217] such a IgA, IgG, or IgE. All of these classes of antibodies as well as B cells can be found in airways and airway lavage fluids. Increases in the amount of Ig in BAL in the course of respiratory diseases suggest that specific immune responses take place in the airways, and indicate that B cells are involved. Additionally, an increase of IgE (reagins) suggest that an anaphylactic, type I response may be

involved in particular diseases, since this type of allergic response is mediated by IgE associated with mast cells. Switching to the production of IgE takes place when B cells are exposed to IL-4 and IL-13, interleukins associated with T_{H2}-type response and allergic diseases.

T cells can be further subdivided into subsets that play different roles in the immune response. The primary distinction can be made based on expression of CD4 or CD8, two types of function-associated molecules that are expressed in the membranes of two mutually exclusive T cell populations.[51] The CD 8+ cytotoxic/suppressor cells destroy infected or abnormal cells after recognition of cell-bound antigens in context of MHC I antigens. Helper cells, CD4+ T cells, are the major cells to which antigen is presented by antigen-presenting cells in the context of MHC II.[51] The CD4+ cells after antigen recognition release a variety of cytokines that promote an appropriate inflammatory response (see central processing phase) due to their effects on white blood cell hematopoiesis, recruitment, and activation of inflammatory cells. Two types of mature helper cells, T_{H1} and T_{H2},[161] can be distinguished. Each type produces different elastes of cytokines, can be distinguished. The cytokines released during antigen stimulation favor development of naive T_{H0} cells into T_{H1} or T_{H2} subsets, and the predominant group of cytokines inhibits development of the other. T_{H1} cells produce IL-2, IF-γ, and TNF-β, thereby promoting cell-mediated immunity.[28] The T_{H2} cells promote the humoral, antibody-mediated immunity, and the allergic type of inflammation, liberating IL-4, IL-5, IL-6 and IL-10. IL-4 is crucial for T_{H2} development because mice treated with antibody against IL-4 fail to develop a T_{H2} response.[51] This interleukin also suppresses T_{H1} development and is essential for B- and plasma cell production of IgE involved in allergic

asthma and rhinitis. Importance of T cells in airway obstructive diseases is supported by their movement into the airway lumen after exposure to the antigen, observed in both human asthma and equine RAO. Additionally, in asthmatic patients, helper cells, involved in late-phase responses, are found predominantly in the airways, while they decrease in the peripheral blood. In contrast BAL fluid from patients exhibiting only the immediate phase reactions has a greater number of suppressor cells and their numbers are lowered in the blood. The lack of suppressor cell migration during the early-phase response is therefore likely responsible for expression of the late-phase response.[1]

Inflammatory mediators

Chemical mediators that account for inflammatory events, briefly called inflammatory mediators, are found ubiquitously in the body, with a variety of functions not only associated with the inflammatory process, but also with regulation of physiological functions and cell-to-cell communication. For example 5-HT, an inflammatory vasoactive amine, is also an important neurotransmitter in the central nervous system, and PGs are the principal autacoids modulating almost every physiological function in the body. Inflammatory mediators, of which dozens if not hundreds have been already identified, are either released from intracellular stores, synthesized *de novo* by stimulated inflammatory or non-inflammatory cells, or their precursors are found in plasma and become activated when plasma protease cascades are set in motion.[52] Some of the mediators act very locally and have an extremely short half-life, and thus can be found only in the form of metabolites or determined by effects. Some others circulate in the entire system for hours affecting virtually every cell. Interestingly, a variety of inflammatory

cells may release the same types or related inflammatory mediators that exert similar effects. These "universal" and powerful mediators are the best known and their inhibitors are commonly used in the therapy of inflammatory diseases. The best example of such an inhibitor is aspirin, which inhibits only one branch of lipid mediator synthesis, namely prostanoids, and very powerfully inhibits acute inflammatory symptoms. In the case of airway obstructive diseases, therapies that eliminate a singular inflammatory mediator or one family of mediators do not offer such a beneficial effect. Positive effects of such medication are observed inconsistently or only in small subgroups of affected individuals. In contrast the effectiveness of steroid therapy in diseases like asthma, indicates that inflammation plays an important role in syndromes of airway obstruction. Considered together, these clinical observations indicate that dynamically changing combinations of different inflammatory mediators are engaged in each individual, or even each episode of airway obstruction. The purpose of my studies is to determine the effects of inflammatory mediators on equine airway responses, and conclude which of them could be potentially involved in airway obstruction. Therefore, I will introduce the main groups of mediators, particularly emphasizing those that have been reported to be associated with RAO.

Vasoactive amines

Histamine

Histamine is widely distributed through the tissues but its richest source is metachromatic cells. When released from the metachromatic cells, histamine exerts its effects via binding to histamine receptors, which are widely spread throughout the body.

Three types of histamine receptors have been identified (H_1, H_2, and H_3). The first two are the most common and belong to the 7-transmembrane (7TM), G-protein-coupled receptors superfamily, while the structure of H_3, found predominantly in the central nervous system, remains unknown.[103] In the airways, all three receptors have been found expressed in different combinations on smooth muscles, neurons, vascular endothelium, or glands.[15] In tissues, the most dramatic and instant histamine effect is on smooth muscle, causing contraction or relaxation depending on the type of receptors to which it binds. The H_1 receptor is coupled to G_q and PLC and is responsible for smooth muscle contraction. The H_2 receptor is coupled to G_s and adenylyl cyclase, and the less-known H_3-like receptors have been demonstrated to cause relaxation of ASM.[41] Additionally, H_2 receptors enhance release of viscid mucous secretion in the airways of some species.[15, 236] In blood vessels, besides regulation of blood flow by constriction or relaxation of vascular smooth muscle, histamine also has an effect on vascular endothelium. Thus histamine induces simultaneous dilation of arterioles and endothelial gap formation due to rounding of the endothelial cells. This increases local blood flow and capillary permeability, resulting in local redness and edema formation in inflammatory/allergic lesions.[238]

In airways challenged with histamine, bronchoconstriction, swelling of airway mucosa, and increased mucus secretion are characteristic. All of these responses narrow the airways, so that histamine, inhaled or administered systemically, precipitates signs of airway obstruction. In asthmatic patients and in ponies with RAO, histamine is a more potent airway spasmogen than in healthy individuals.[59] Even though this hyperresponsiveness to histamine may be non-specific, in both human asthmatics and

horses with RAO, there is a tendency toward an increase in airway histamine level after antigen challenge.[178] This indicates that histamine is likely involved in both syndromes.

It is noteworthy that in several animal models of histamine-induced airway obstruction, part of the histamine effect can be prevented by use of ATR or vagotomy. Thus, besides direct effects on smooth muscles, cholinergic mechanisms of histamine action have been demonstrated.[106, 115] In most studies these effects are attributable to stimulation of sensory nerves by histamine and activation of the efferent vagal pathway resulting in centrally mediated reflex bronchoconstriction.[68, 115] Currently, mechanisms affecting the efferent vagal pathway have been proposed to explain cholinergically mediated components of histamine-induced bronchospasm. In the airways (as well as in other organs) mast cells are found closely associated with ganglia and nerve terminals.[56] Indeed, the ragweed pollen antigen causes parallel histamine release and the increased response to EFS in small bronchi from sensitized dogs. Furthermore, release of histamine from mast cells, triggered by antigen, has been shown to depolarize the ganglionic neurons in guinea pig.[196] The latter may result in generation of an action potential in ganglion cells or decreased filtration of preganglionic action potentials at the ganglia. Both of these effects could lead to increased frequency of postganglionic parasympathetic nerve firing and thus an increased amount of ACh released to ASM.[196, 293] Additionally, histamine has been shown to modulate prejunctionally neurotransmitter release from airway sympathetic and sensory nerves by activation of excitatory H_1 and inhibitory H_3 receptors, and these mechanisms have been also suggested in airway cholinergic nerves.[67, 115, 241] Even though most of the pro-inflammatory effects and bronchospasm induced by histamine are mediated by H_1 receptors,[52] effects of H_1 antagonists are not

very consistent in therapy of asthma. As demonstrated by Howarth,[121] improvement in allergic asthmatics in response to the newest generation of H_1 antagonists is rather small and not very consistent, although in contrast with older drugs, these compounds reach therapeutic concentrations in the airways without causing systemic side effects. These clinical data confirm earlier theories that histamine alone is not a major factor leading to development of airway obstruction in asthma, and probably important only in the first minutes after antigen challenge.[144]

Serotonin

5-Hydroxytryptamine is widely distributed in a variety of tissues, particularly in enterochromafin cells, platelets and as a neurotransmitter in the central nervous system. It is also found in mast cells of several animal species, mostly rodents, but not in humans.[206] As discussed by Ogunbiyi and Erye,[206] 5-HT is released during anaphylaxis in rabbit, mouse, cat, dog, and human either from mast cells or activated platelets. 5-HT is liberated from mast cells in response to antigen challenge (via action on IgE), anaphylatoxins, and all of the other factors known to activate mast cells. Release from platelets takes place upon stimulation by a variety of PAF such as thrombin, collagen, adenosine and lipid mediators. Platelet activation also takes place during anaphylaxis.[51, 238] In isolated lung fragments from ponies sensitized to bovine plasma, 5-HT is released together with histamine in response to antigen.[35] As different as the cellular sources of 5-HT are the multiple tissue effects of 5-HT, which depend on several types and subtypes of receptors to which 5-HT binds in the effector organs. Currently 7 types and at least

12 subtypes of 5-HT receptors have been described and only some of them have selective agonists or antagonists.[18, 54]

Regardless of the complexity of 5-HT pharmacology, it has been well established that the overall effect of 5-HT administration is bronchospasm. Interestingly, this spasmogenic effect of 5-HT, in some models, can be largely reversed by ATR, indicating strong interaction with the cholinergic system.[106, 167] In anesthetized dogs, the 5-HT-induced increase in airway R_L is reduced by vagal cooling, and in doses not altering baseline R_L, 5-HT augments the increase of R_L induced by electric vagal stimulation.[68, 106] Since in vagotomized dogs 5-HT has a similar effect to that in intact animals, Hahn et al. concluded that 5-HT interacts with the efferent vagus nerve, rather than stimulating sensory afferent nerves. Recently the site of 5-HT cholinergic action was determined to be at the parasympathetic nerve terminals, since only ATR but not bilateral vagotomy or ganglionic blockade with hexamethonium significantly reduced the spasmogenic effect of 5-HT infusion.[167] Additionally, this study demonstrated that the 5-HT_2 receptor mediated both direct constriction of ASM and parasympathetic postganglionic nerve stimulation. It is noteworthy that the amount of endogenous 5-HT released in the airways can be sufficient to alter significantly the response to cholinergic nerve stimulation. Studies by Szarek and Schmidt[257] have clearly demonstrated that 5-HT released in isolated rat bronchi in response to treatment with H_2O_2 potentiates ASM contraction elicited by EFS.

Lipid mediators

The majority of lipid mediators are derived from AA. The most important modified phospholipid mediator is PAF, and this lipid mediator is also associated with

metabolism of AA. When AA is cleaved from the sn-2 position of membrane phospholipids by the action of PLA_2, lysophospholipid is formed, which can be utilized for PAF synthesis. Additionally, a crosstalk between both lipid pathways exists, in the form of positive feedback, in which PAF stimulates release of pro-inflammatory AA metabolites, which in turn may increase release of PAF.

Platelet-activating factor

This mediator is released from a variety of inflammatory cells, including activated platelets, neutrophils, eosinophils, basophils, alveolar macrophages, mast cells, and injured or stimulated endothelial cells.[52, 238] This very potent mediator of acute inflammation is capable of activating, more or less, every type of inflammatory cell: inducing adhesion of leukocytes to endothelium; chemotaxis, degranulation and respiratory burst in neutrophils, monocytes or macrophages; chemotaxis and activation of eosinophils; and most of all stimulation of platelets, i.e., their aggregation and degranulation.[238] In blood vessels, PAF increases venular permeability, and depending on concentration, causes vasodilatation or vasoconstriction. In addition to chemotactic properties and edema formation, PAF potently contracts ASM of many animal species and increases airway mucus secretion.[290] Thus, functional and structural changes characteristic of airway obstructive diseases are reproduced by PAF challenges, including dose-dependent bronchoconstriction, and the long-lasting AHR to MCh and cholinergic nerve stimulation.[24, 53, 261, 290] In ascaris antigen-challenged sheep, and ovalbumin-challenged guinea pig, PAF receptor antagonists are protective against the late, but not the early phase of airway response, and eosinophil infiltration.[160, 235, 246] Direct action on

PAF receptors mediates the increase of vascular permeability, while the effects on ASM seem to be mediated by eicosanoids released from PAF activated cells.[53, 165, 246, 261, 270] Thus, airway effects of PAF in different species depend on PAF-induced eicosanoid profiles and airway responses to these eicosanoids. In primates, the effect of PAF appears not to be as dominant as in rodent or canine models.[149, 209] Similarly to human airways, equine airways seem to be relatively insensitive to PAF.[82, 173]

Arachidonate and its metabolites

An important feature of AA metabolites is their wide distribution and contribution to a large number of functions in every tissue of living organisms.[38] Another feature of these mediators is that they are mostly produced on demand rather than being stored by cells. They are released as they are produced and relatively quickly inactivated. Since AA is commonly found in esterified form in membrane phospholipids and must be mobilized by action of PLA_2 and/or PLC before enzymatic biosynthesis of eicosanoids occurs, virtually every cell upon activation of PLA_2 is capable of producing eicosanoids.[38, 243] The profile and amount of eicosanoids produced by cells is determined by the availability of eicosanoid-synthesizing enzymes, i.e., their expression and cellular activity. These properties, together with transcellular metabolism, determine that eicosanoids are not only important hormones in cell-to-cell communication, but they are involved in the amplification or regulation of almost every physiological and pathological process.

AA serves as a precursor from which a large number of eicosanoids are produced. During synthesis of these mediators, only a minor structural modification may

dramatically alter the physiological properties of theses products, from contractile to relaxant in smooth muscle, or from pro-inflammatory to anti-inflammatory. Metabolism of AA involves three major pathways: cyclooxygenase, lipoxygenase, and epoxidase. The first two pathways deliver mediators that are most important from a physiological and pathological point of view. A detailed description of both pathways of eicosanoid synthesis can be found in many sources.[38, 71, 243] The following pieces of information are important to the issues addressed in my studies.

1. All prostanoids are synthesized in two stages. First, synthesis of intermediate metabolite, PGH_2, occurs via cyclooxygenase enzyme. Then PGH_2 is transformed to different prostanoids by specific enzymes.[243] Blockade of cyclooxygenase with compounds like aspirin, meclofenamate (MEC), and indomethacin entirely and nonselectively stops production of all prostanoids.
2. The cyclooxygenase pathway is substrate-limited, and enzymes do not require special activation except for release of AA from the cell membrane. For that reason some cells can produce prostanoids all the time in order to regulate a variety of physiological functions.[243] Constitutively expressed cyclooxygenase gene COX1 is thought to deliver PGH for "housekeeping" prostanoid synthesis. When demand for prostanoids is higher, e.g., during inflammation, expression of cytokine-inducible COX2 enzyme delivers additional amounts of PGH_2,.[142]
3. 5-Lipoxygenase (5-LO) products are synthesized predominantly by inflammatory cells in response to their activation and they are typical inflammatory mediators.[71]
4. Blockade of one eicosanoid synthesizing enzyme may cause a shift of the substrate to the other enzyme and thus increase production of other eicosanoids.[38]

Cyclooxygenase metabolites

The most important active prostanoids are PGE_2, $F_{2\alpha}$, D_2, and I_2, and the TXA_2. Prostanoids exert their effects by binding to 7TM, G protein-coupled receptors. The diversity of their actions is explained by the large number of receptors that mediate their actions. These receptors are coupled to three different signal transduction pathways, resulting in activation or inhibition of adenylate cyclase and activation of PLC.[38, 103] Five main groups of these receptors have been defined based on the greatest affinity of each prostanoid: EP, FP, DP, IP, and TP. However, there is no absolute selectivity of these receptors, and most prostanoids may crossactivate them with differential order of potencies.[38] Additionally, four subtypes of just EP receptor are known and, among these four, all three signal transduction pathways are represented. Based on a combination of these receptors, tissue responses to prostanoids therefore may significantly vary among the organs, and organ responses among the species. In general, the overall effect of rapid release of prostanoids evokes cardinal symptoms of inflammation in tissues. This may occur in response to biological, chemical, mechanical, or thermal insults. Conversely, inhibition of prostanoid synthesis has a profound anti-inflammatory effect, particularly in the acute inflammatory responses. In the airways, the spastic effects appear to be predominantly mediated by the TP (TX) receptor and the relaxant ones via EP (PGE_2) receptor.

PROSTAGLANDIN E_2

This PG is produced by many organs and plays an important role in their physiological functions. Its general properties are anti-inflammatory, and PGE_2 has

gained a therapeutic use in chronic inflammatory conditions. PGE_2 relaxes ASM, both in vivo and in vitro. Via this broncholytic action it contributes to a protective, antispastic mechanism important in the airway regulation of many species.[9, 49, 175] However, there are reports of a bronchoconstricting action of PGE_2, presumably via activation of TP receptor by relatively high concentrations of PGE_2.[9, 49, 175] Thus, the predominant effects of PGE_2 on the airway tone are inhibitory, opposing the action of spasmogen. It has been shown in several species, including humans, that these effects can be both pre- and postjunctional..[76, 283, 292, 303] In the airways of several species, including humans, inhibitory PGE_2 is released by epithelium, for example in response to activation of neurokinin receptors.[36, 185, 258, 271, 282] Since epithelium produces inhibitory factors and is a source of PGE_2, PGE_2 was a candidate for EpDRF. However, experiments in equine trachea suggested that epithelium and prostanoids inhibit isolated airway responses independently.[303] Even though epithelium may not be a predominant source of PGE_2, lamina propria of the mucous membrane in the equine trachea is a source of a considerable amount of PGE_2.[100, 102] Therefore, regardless of its source, PGE_2 is an inhibitory mucosal factor in airways of mammalian species.[292] Abolition of tonic inhibition, which PGE_2 exerts to oppose bronchospasm, is thought to be the main trigger for aspirin-induced asthma in humans. Misoprostol, a synthetic analog of PGE, largely reverses the post-aspirin bronchoconstriction in aspirin-sensitive asthmatics.[259] A decreased PGE_2 synthesis has also been proposed to contribute to cholinergic airway obstruction in RAO (see Chapter 2).

PROSTAGLANDIN I_2

PGI_2 is primarily associated with vascular endothelium. It can also be produced by ASM and epithelium. The level of PGI_2 increases in the airways after antigen challenge, but its effects are most of all vascular.[238] In ASM, PGI_2 has relaxing and airway protective properties.

PROSTAGLANDIN D_2

Prostaglandin D_2 is a major cyclooxygenase product in the mast cell, and thus it is sometimes called a mast cell-specific PG.[164] Additionally, it can be produced by macrophages and epithelium.[52, 102, 142] This PG is a classic pro-inflammatory mediator as it causes vasodilatation and increased vascular permeability, and is thought to contribute to pain sensation.[52] The contribution of PGD_2 to airway diseases may arise from its pro-inflammatory properties. PGD_2 is also a powerful bronchoconstrictor, about 1–1.5 orders of magnitude more potent than histamine. In the asthmatic airways challenged with PGD_2, bronchospasm to histamine and MCh is potentiated.[142, 291] PGD_2 increases in asthmatic airways challenged with antigen, and its increase is correlated with the increase of histamine.[163] Also, in ovalbumin-challenged guinea pig, and in mouse airway challenged with PAF, PGD_2 increases and, in the latter, this increase is correlated with airway hyperreactivity to ACh.[125, 164] The spasmogenic effects of PGD_2 on isolated ASM are thought to be entirely produced by activation of TP, while the vascular effects are mediated by DP receptors.[132] McKenniff et al. demonstrated in isolated human, ferret, and guinea pig trachea that blockade of TP receptors with the specific antagonist BAY u3405 abolished contractions induced by PGD_2 and $PGF_{2\alpha}$.[186] In vivo,

bronchoconstriction induced by PGD_2 was greatly reversed by TP antagonist but not by vagotomy, DP receptor blockade, or inhibition of COX and 5-LO.[110] Independently of the subtype of the receptor by which PGD_2 contracts the airways, in human and guinea pigs, part of the bronchoconstriction induced by PGD_2 can be abolished by ATR or ipratropium, on the average 60%.[20, 275] Thus, part of the PGD_2 effects in the airways is mediated via cholinergic mechanisms.

PROSTAGLANDIN $F_{2\alpha}$

This pro-inflammatory PG has properties in the airways that closely resemble those of PGD_2. Together with PGD_2, $PGF_{2\alpha}$ increases in airways challenged with antigen.[125] It is an airway spasmogen in many species including humans, and this action is mediated by TP receptors.[186] Similarly to PGD_2, challenges with $PGF_{2\alpha}$ have the greatest effect on lung function in asthmatics, producing bronchospasm and augmenting airway response to other factors. In contrast to PGD_2, the cholinergic reflex is much less important in $PGF_{2\alpha}$-induced bronchospasm.[20, 198, 208] This is probably why $PGF_{2\alpha}$ is approximately 3.5 times less potent as a bronchoconstrictor in vivo, while it contracts isolated airways with a potency greater than PGD_2 does in vitro.[25, 142]

THROMBOXANE A_2

Thromboxane is produced primarily by platelets and also by neutrophils, eosinophils, monocytes macrophages, mast cells, and epithelium.[142, 238] Because of its very short half-life, knowledge of the effects of TXA_2 originates from measurements of its stable metabolite TXB_2 and studies of stable analogs of TXA_2. TXA_2 and its analogs

are strong broncho- and vasoconstrictors and they promote microvascular leakage and platelet aggregation.[9, 231] In vitro, TX analogues cause a dose-dependent contraction of ASM, and, in some species, an increased response to ACh and an increase of ACh release from airway parasympathetic nerves.[9, 260] In response to both antigen challenge and nonspecific irritants, such as cigarette smoke, airway TXB_2 increases in parallel with other pro-inflammatory prostanoids.[163] TXA_2 appears to be an important mediator of AHR induced by oxidative stress, and contributes to ozone-induced asthma. Thus, the decrease in FEV_1 and increased airway resistance following ozone exposure can be substantially blunted by indomethacin.[112] AHR to ACh, similar to that induced by ozone, may be also produced by TXA_2 mimetic, both in vivo and in vitro.[112, 128, 231] Noveral and Grunstein have also proposed that via activation PLA_2 and 5-LO dependent pathway, TXA_2 induces proliferation of cultured ASM, and thus it may contribute to the ASM hyperplasia observed in asthma and chronic airway diseases.[199] Even though TXA_2 is typically a very rapid and short-acting mediator, most, if not all spasmogenic effects produced by different prostanoids in the airways are mediated by TP receptor. Thus, pro-inflammatory prostanoids other than TXA_2 can induce bronchospasm by a common mechanism. Antagonists of TP receptor are currently under intensive clinical trials and recently have been reported to have some beneficial effects in treatment of bronchial asthma.[91]

5-LO metabolites

Mediators derived from AA via the enzyme 5-LO include 5-HETE, the role of which is unclear in airway disease; noncysteinyl LT LTB_4; and the cysteinyl LTs LTC_4, LTD_4, and LTE_4.

LEUKOTRIENE B_4

This mediator has obtained much attention because it possesses strong pro-inflammatory properties and, in experimental models, contributes to airway obstruction. LTB_4 is synthesized by a variety of inflammatory cells such as neutrophils, macrophages, and mast cells. It is thought to be a principal endogenous chemotactic molecule, particularly for neutrophils and eosinophils. These effects are exerted via a 7TM, G-protein-coupled LTB_4 receptor, which is highly expressed in leukocytes, and has just recently been cloned.[302] Activation of this receptor induces leukocyte chemotaxis, aggregation, adhesiveness, migration, secretion, and superoxide production.[238, 291] Upregulation of adhesion molecules such as CD11b integrin, on the surface of neutrophils, observed in response to LTB_4, facilitates both neutrophil adhesion and migration. Additionally, LTB_4 enhances leukocyte superoxide production in response to other chemotactic factors such as fMLP, and its production, via intrinsic 5-LO enzyme in neutrophils, is required for IL-8-mediated neutrophil response.[95] This evidence led to the hypothesis that neutrophils use an autocrine mechanism, i.e., produce LTB_4 to amplify their own activity, upon the stimulation with some agonists.[289]

In the airways, LTB_4 contributes to airway inflammation by recruitment of neutrophils and eosinophils and activation of other cells including macrophages. After

antigen challenge, LTB_4 increases in airways of immunized animals and human asthmatics,[114, 221, 291] and LTB_4-receptor antagonists as well as 5-LO/5-LO-activating protein inhibitors prevent eosinophilia and mucus hypersecretion in antigen-challenged rodents.[114, 192, 221] Because LTB_4 is not an airway spasmogen, it was proposed to contribute to AHR via activation of inflammatory cells and subsequent release of IL-6 and IL-8 in antigen-challenged monkey, or recruitment and activation of neutrophils in ozone-challenged dogs.[253, 269]

CYSTEINYL LEUKOTRIENES

Even before they were identified as the triene AA metabolites from leukocytes, LTC_4, LTD_4, and LTE_4 were studied as a slow-reacting substance of anaphylaxis (SRS-A). In many mammalian species SRS-A is released from sensitized lungs treated with antigen.[206] Cysteinyl LTs (CysLts) are released by antigen-activated mast cells/basophils, activated eosinophils, and macrophages; however, their main cellular source remains unknown.[144, 197, 289, 294] Most likely they are generated via transcellular metabolism, e.g., neutrophils delivering LTA_4, and vascular endothelium and/or platelets further transforming it to LTC_4.[71] CysLTs increase vascular permeability and mucus secretion, and produce prolonged bronchoconstriction in many species including human beings.[144, 206] These pro-inflammatory effects are mediated via cysteinyl LT_1 and cysteinyl LT_2 receptors. There is some evidence that ASM possesses only the cysteinyl LT_1 receptor, which can bind LTD_4 with higher affinity than LTE_4. It remains unclear whether LTC_4 activates this receptor directly, or whether it first needs to be converted to LTD_4.[71, 103] Contraction produced via cysteinyl LT_1 receptor is associated with influx

of extracellular Ca^{2+} into the cells.[74] In guinea pig peripheral airways, the contraction produced by this LT is, in part, mediated by TXA_2, and there is some evidence that these two mediators may share common pathways of activation.[5] In human tissue, cysteinyl LTs cause prolonged airway contraction (in comparison with histamine) and this response is not substantially modulated by epithelium or prostanoids. [198] In human asthma, particularly exacerbation in atopic asthmatics, increases of cysteinyl LTs in BAL fluid and urine are highly significant and correlate with the severity of symptoms.[198, 286] In addition to the direct contractile effects on ASM, LTD_4 and LTE_4 induce slow-onset and long-lasting AHR to inhaled ACh, presumably by release of secondary mediators.[231] The persistent airway eosinophilia and mucus hypersecretion, also induced by cysteinyl LTs, may contribute to the AHR.[66, 192, 276] Even though not all types of asthma respond very well to treatment with LT receptor antagonist and 5-LO/FLAP inhibitors, the potent LTD_4 antagonist ICI-204,219 has been shown to significantly reduce bronchospasm resulting from allergen challenge in both early and the late phases.[71] Clinical efficacy of anti-LT therapy in human asthma was recently reviewed by Drazen. He concluded that this therapy clearly demonstrated the capacity to improve pulmonary function and reduce symptoms in clinical models of asthma.[72] Thus, LTs appear to be critical effector molecules, at least in some patients with asthma.[72]

12- and 15-LO metabolites: 12-HETE, 15-HETE

These two mediators are AA metabolites of 12- and 15-LO respectively. 12-HETE is a major product of platelets and macrophages,[98] while 15-HETE is produced by airway epithelium, endothelium, neutrophils, and macrophages and is a major

metabolite of AA in human lung.[150, 230, 242] Some effects of these mediators overlap, and both mediators possess pro- and anti-inflammatory actions. However, pro-inflammatory properties predominate in 12-HETE, whereas anti-inflammatory properties predominate in 15-HETE. Since 15-HETE interferes with the synthesis and effects of LTs and with the TXA_2 receptor, it may blunt the responses of acute inflammation, bronchospasm, and AHR to which these mediators contribute.[86, 171, 210, 242] In contrast, it promotes events characteristic for chronic airway inflammation such as viscous mucus hypersecretion, and a low degree of cellular infiltration. Since 15-HETE level is higher in the airways of asthmatics, and increases further after antigen challenge, 15-HETE may play a role as a security mechanism preventing excessive inflammatory reaction in asthma and other airway diseases.[147]

Arachidonic acid

AA itself is not only a precursor molecule but is also a mediator and second messenger.[140] Thus, AA activates a variety of cells such as neutrophils and platelets. These effects, rather than being mediated via a specific receptor, are produced by the direct action of AA on the intracellular pathways or by eicosanoids released when AA is metabolized via enzymatic cascade.[6, 26] In neutrophils, AA can activate NADPH oxidase, presumably via activation of a certain subtype of PKC in neutrophils, whereas in stromal cells AA activates a signaling pathway, leading to the transcription of an immediate early gene, *c-jun,* involved in cytokine production.[222, 265, 266]

Reactive oxygen species

Generated in activated inflammatory cells such as neutrophils, eosinophils, and macrophages, ROS are effectors of the germicidal, anti-parasitic, and cytotoxic mechanisms (see neutrophils). Amounts of ROS generated within the inflammatory cells are toxic in contact with bacteria or tumor cells, but their short half-life and large amounts of protective antioxidant mechanisms limit their noxious effects. Still, endogenous ROS have been shown to contribute to airway pathology due to effects on membrane receptors, components of intracellular signal transduction, enzymes, or DNA. Since inflammatory cells such as neutrophils, macrophages, and eosinophils are recruited and activated in response to antigen challenge or nonspecific irritation,[85, 170, 207, 250] ROS has been proposed to contribute to the development of airway obstruction.[129, 134, 229] Both endogenously derived or exogenously delivered ROS can affect airway responses in many animal models via a variety of mechanisms. ROS can contract ASM, suppress β-adrenergic relaxation, or change airway reactivity to spasmogens such as cholinergic agonists, EFS, or substance P.[128, 145, 151, 254, 257]

The character, magnitude, and mechanism of the observed effects depends on the type of ROS, the animal species, and the segment of the airway studied. In guinea pig, sheep, and dogs, ozone, $O_2^{-\cdot}$, or H_2O_2 produce bronchospasm and AHR, by releasing cyclooxygenase products,[128, 152, 220] because ROS stimulate AA metabolism and subsequently cause a release of TXA_2/prostanoids, which in these species play a major role in acute models of bronchospasm and AHR. The relationship between these mechanisms has been demonstrated in studies with xanthine-xanthine-oxidase, and ascaris antigen-challenged sheep by Lansing et al.[151, 152] Generation of superoxide in cat airways

induces vagally mediated bronchospasm, succeeded by AHR to ACh, while ozone challenge induces the AHR that can be reversed by SOD, a superoxide anion scavenger. These models demonstrate another link that exists between inflammatory cells, mediators, and the regulation of airway tone.

In addition to effects on ASM cells, oxidants promote inflammatory responses directly by affecting intracellular signal transduction within inflammatory cells. These effects are mediated via changes in the red-ox state of cellular glutathione, which determines the binding affinity of transcription factors, such as NF-κB and AP, to their cognate DNA. This binding regulates expression of immediate early genes required to induce a specific cellular responses to multifunctional cytokines, such as IL-1 and TNF-α.[188, 216] Thus, low-level oxidative stress promotes inflammation by activation of transcription factors in cells mediating the immune response.

Other inflammatory mediators

This section contains a brief description of inflammatory mediators that may be potentially involved in RAO, but their effects in equine airways were not studied in the course of my work. The reason for that was lack of reports associating these mediators with equine airways and their minor importance in other models of airway obstruction. Still, some of these mediators are capable of modulating airway responses and therefore they may be important in RAO.

Complement

This system of plasma proteins includes nine primary components and eleven cleavage products and presents a principal effector and amplification system in the humoral branch of inflammatory response.[52, 238] The classic pathway of complement activation is initiated by antigen antibody complex interaction with C1 component. The alternative pathway of complement activation does not require immune complexes but is initiated by cleavage of the C3 component in response to bacterial products such as endotoxin.[52] Whenever the complement system becomes activated by either pathway, opsonins, C3b, and anaphylatoxins, C3a and C5a, are generated. The latter mediator induces inflammatory cell chemotaxis and activation, increases vascular permeability, and evokes bronchospasm. In experimental conditions it has been demonstrated that inhalation of aerosolized C5a or its infusion in guinea pig and rabbits causes bronchospasm and airway inflammation. These effects in guinea pig are mediated predominantly via cyclooxygenase products, such as TXA_2, but also by H_1 [88, 219] and, in rabbit, presumably by mediators released from activated neutrophils that influx into the airways challenged with C5a.[8, 126] Since evidence of complement contribution in human asthma is rather weak, it is likely that its local activation promotes inflammation in the airway challenged with antigen.[40, 75, 172, 189] In this way complement might contribute to aggravation of inflammatory response, rather than directly affect pulmonary functions.

Sensory neuropeptides and neurogenic inflammation

This family of mediators can be seen as a link between the nervous and immune systems, as both inflammatory mediators and true neurotransmitters in the sensory

C-fibers.[275] Inhalation of the C-fiber activator capsaicin, induces airway inflammation in guinea pig, rat, and human airways.[176, 211, 247, 273] Neurogenic inflammation is characterized mainly by vascular events such as vasodilatation and plasma leakage.[176, 177] The role of tachykinins in human asthma have been long postulated based mainly on investigation of guinea pig model,[14] but currently Tomaki et al. found nearly 18 times more substance P in sputum from asthmatic patients in comparison to healthy individuals.[267] Thus, a possible role of sensory neuropeptides should be considered in human asthma and its animal models.

Kinins

This group of related small polypeptides arises in plasma via kallikrein-induced cleavage of high molecular weight kininogen.[238] Bradykinin is the prototype kinin, possessing strong sensory effects (i.e., causing pain), contracting the extravascular smooth muscle, and relaxing blood vessels. Activation of kallikreins and liberation of kinins takes place in allergic (anaphylactic) reactions in many species, including the horse.[206] Based on studies with human asthmatics, and animal models of asthma, bradykinin induces bronchospasm and evokes late-phase AHR and airway inflammation.[23, 92, 245] Bradykinin antagonists reduce the increase in R_L, in the ovalbumin-challenged guinea pig, indicating that bradykinin contributes to antigen-induced bronchospasm,[84] and reduces AHR in ascaris antigen-challenged sheep.[245] Bradykinin contracts ASM by acting on B_1- and B_2-receptors that open extracellular Ca channels and mobilize intracellular Ca stores in ASM.[245] Besides these direct effects on ASM, bradykinin releases lipid mediators, such as TXA_2, from the inflammatory cells.[4, 55, 123]

In some antigen-challenged animal models, bradykinin-induced increase in airway resistance can be abolished up to 97% by ATR, indicating that bradykinin evokes a cholinergic reflex in the airways.[3, 123]

Summary

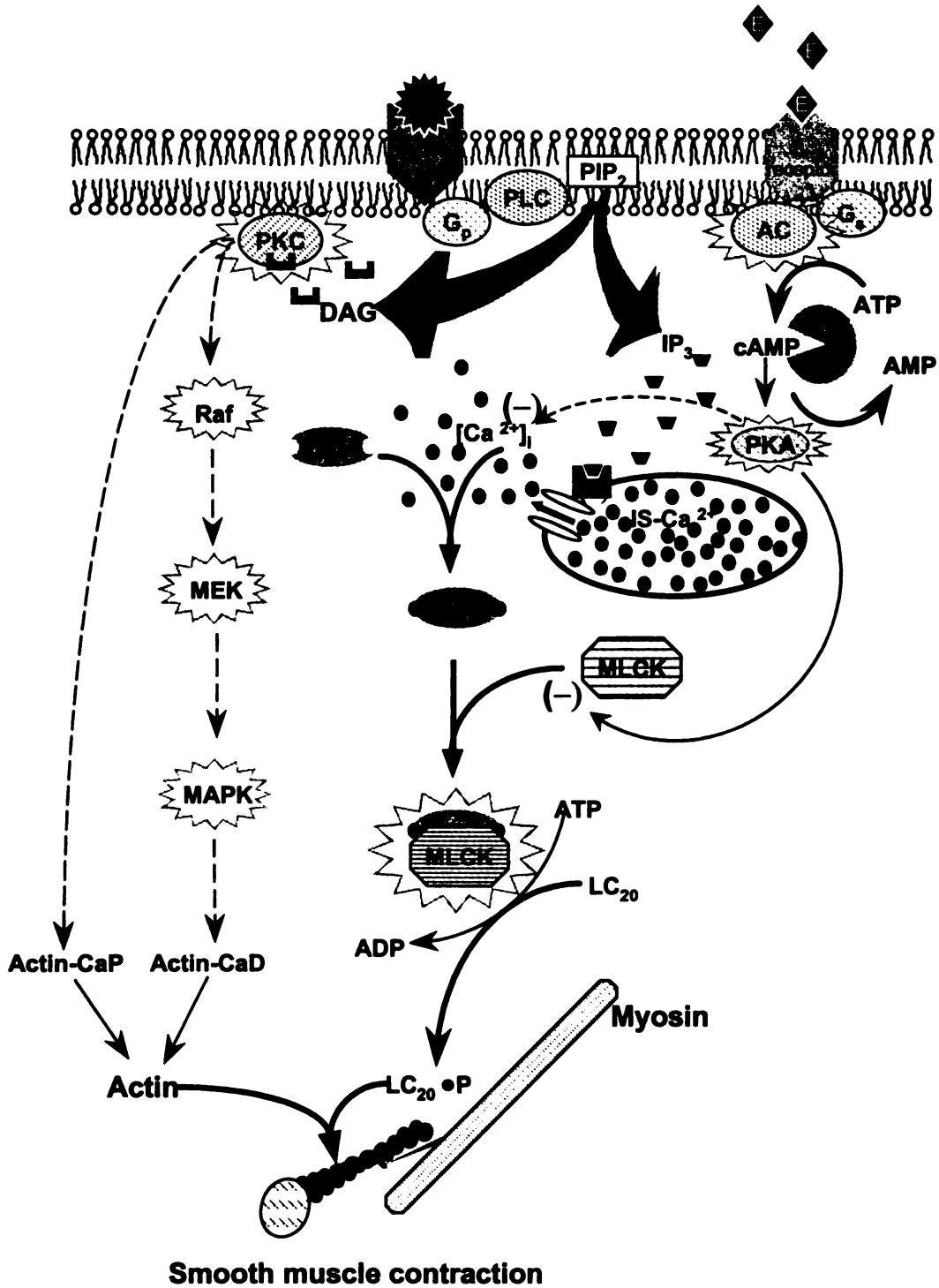
In this part of the literature review, on the background of basic airway anatomy and physiology, I introduced the main features of inflammatory cells and inflammatory mediators. I have focused particularly on mediators, which have proven to play a role in obstructive airway diseases or are capable of altering pulmonary functions and/or airway tone. The physiology of the neutrophil was discussed in depth, since they are particularly important for my hypothesis and are the subject of my studies. From this review two major thoughts have arisen:

1. Many mediators may exert similar effects in the airways, and many of them act in concert. Therefore it is very difficult to determine a major player largely responsible for the pathological process.
2. Inflammatory cells and mediators exist in a network of mutual relationships with each other and with the tissues that they affect. These relationships involve additive effects, synergism, antagonism, positive and negative feedbacks, and sequential relationships.

In Chapter 2, I will introduce the syndrome of RAO in horses and present current knowledge about the mechanisms that are implicated in its pathogenesis. Although much less is known about the role of inflammatory cells and mediators in RAO, similar

mechanisms may be involved in many obstructive airway diseases. Therefore, whenever information from studies of RAO is incomplete or unclear, I will refer to Chapter 1.

Figure 1. Basic intracellular mechanisms regulating airway smooth muscle tone. Activation of muscarinic M_3 receptor by acetylcholine (ACh) is an example of spasmogen action, while binding of epinephrine (E) to β_2-adrenoceptor represents inhibitory mechanism. Two types of heterodimeric GTP binding proteins, G_p and G_s, are coupled to the M_3 and β_2-receptor, respectively. Other components of signal transduction involve: PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3 inositol 1,4,5-trisphosphate; IS-Ca^{2+} intracellular calcium stores, $[Ca^{2+}]_i$, intracellular free calcium; CaM, calmodulin; MLCK, myosin light chain kinase; LC_{20}, 20-kDa myosin light chain; DAG diacylglycerol; PKC, protein kinase C; Raf, Raf kinase; MEK, MAP/ERK kinase; MAPK mitogen-activated protein kinase; CaP, calponine; CaD, caldesmon; AC, adenylyl cyclase; PDE phosphodiesterase; PKA, protein kinase A. Dashed lines represent pathways that may require other factors.



Smooth muscle contraction

Figure 1

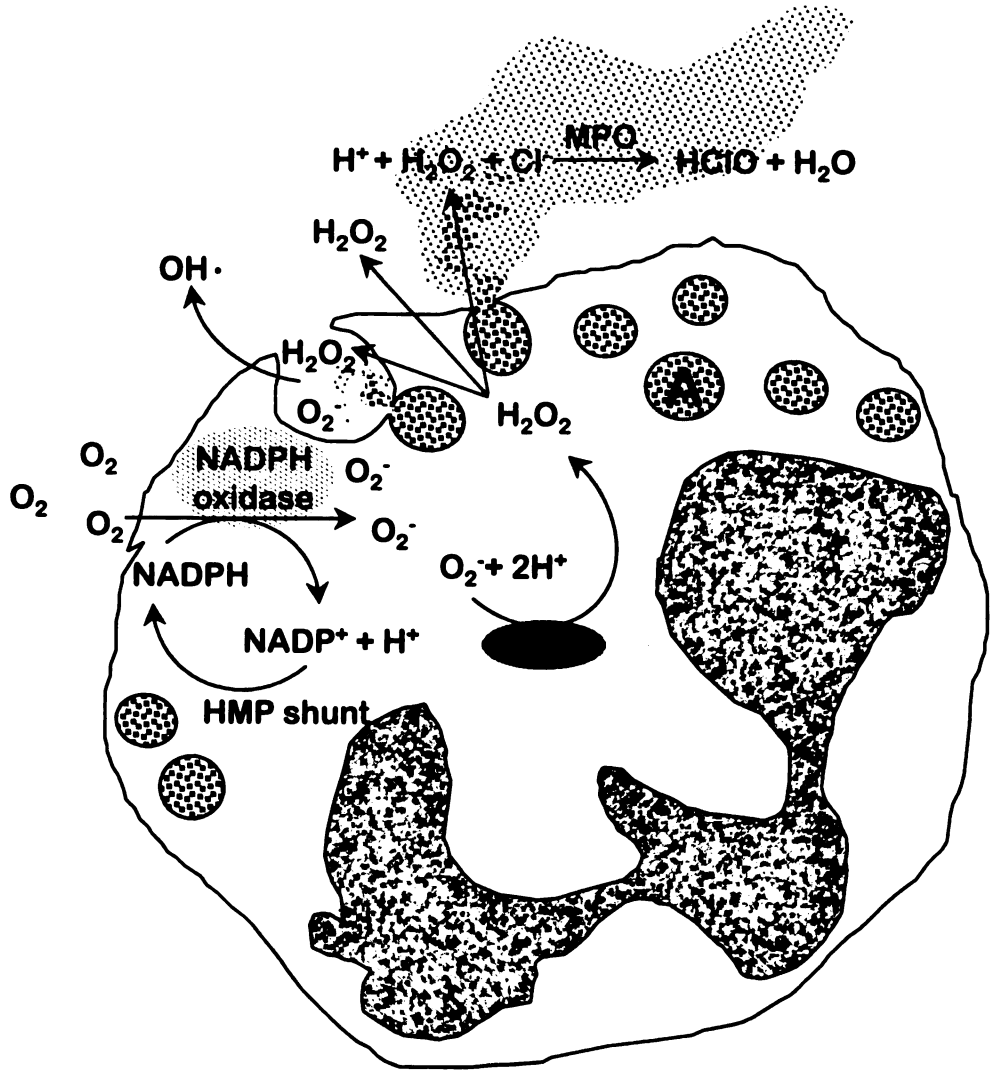


Figure 2. Respiratory burst and the oxygen dependent antimicrobial mechanisms. Respiratory burst activity is driven by hexose monophosphate (HMP) shunt, and the activity of NADPH-oxidase. During this reaction molecular oxygen (O_2) is reduced to superoxide anion ($O_2^{-}\cdot$), from which superoxide dismutase (SOD) forms hydrogen peroxide H_2O_2. Hydrogen peroxide in the presence of myeloperoxidase (MPO), released from azurophil granules (A), can be further metabolized to hypochlorous acid (HCLO), or with the $O_2^{-}\cdot$ may form the extremely reactive hydroxyl radical OH·.

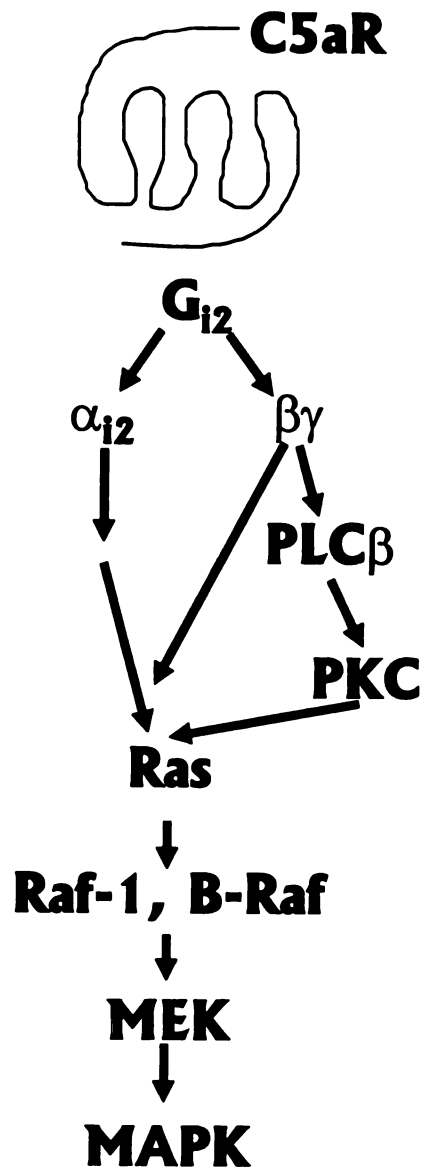


Figure 3. Proposed mechanism for C5a receptor stimulation of the Ras/Raf/MAP kinases cascade. G_{i2}, coupled to phospholipase Cβ (PLCβ), is the predominant G protein responsible for the effects of C5a stimulation in neutrophils. Besides activation of protein kinase C (PKC) via action of diacylglycerol (DAG), PLCβ also promotes intracellular Ca^{2+} mobilization via the liberation of inositoltrisphosphate (IP_3; not shown on this scheme).

Chapter 2

LITERATURE REVIEW: RECURRENT AIRWAY OBSTRUCTION

Introduction

Recurrent airway obstruction is a disease of mature and older horses known since antiquity.[57] The name RAO most correctly describes this syndrome since reversible airway obstruction can be repeatedly induced, in susceptible animals, by stabling and feeding them with poor-quality hay.[80, 162, 225] Other names are colloquial synonyms such as "heaves" or "broken wind," or chronic obstructive lung disease (COLD), chronic obstructive pulmonary disease (COPD), chronic obstructive bronchitis (COB), bronchiolitis, and alveolar emphysema.[225] Often compared to human asthma, RAO is one of just a few naturally occurring models of human airway obstructive diseases such as asthma and COPD. Learning about the genesis of this syndrome is therefore important for the development of new therapeutic strategies in these and other airway diseases, and for a better understanding of basic airway physiology in mammalian species.

In equine RAO, delivery of oxygen into the lung is compromised by narrowing of airways. This impairment of respiratory functions results predominantly in reduced athletic performance. Since both the utility and value of horses are determined by their athletic capabilities, RAO has an important impact on the equine industry and husbandry.

Clinical syndrome

RAO develops gradually over the years and thus several stages of the syndrome can be described. Additionally, among individuals there are differences in the course of the disease, in the predominance of clinical signs, and in response to treatment [79]; therefore the pathological entity of this disease is often questioned. In animals with mild RAO, chronic cough may be the only clinical finding. As the disease progresses, cough becomes more frequent, there is an increase of respiratory sounds, and an increased amount of respiratory secretion in which the number of neutrophils increases. At this stage, horses may start losing their athletic fitness, due to decreased exercise tolerance. During severe, acute bouts of RAO, respiratory sounds are loud, accompanied by wheezing and breathing difficulties. The abdominal muscles are recruited into laborious expiratory work, which results in the characteristic "heaving." Airway secretion is usually very thick and rich in neutrophils. At this phase of diffuse airway obstruction, severe hypoxia develops in arterial blood, which dramatically reduces the animal's capacity to move.

Epidemiology

Because exacerbations of RAO are induced by inhalation of hay dust, this syndrome is common in countries with a colder climate where animals are stabled for a long period of time and fed hay. In hot and humid summers, particularly in the southeastern part of U.S., horses kept on pasture may also develop airway obstruction. This so-called "summer pasture associated obstructive pulmonary disease" is most likely a response to infestation of fungi on grasses and the presence of their spores in the air.

Most of the countries from the northern hemisphere have reported cases of RAO, with as high as 37% prevalence in western Europe and 12% in some parts of the U.S.[225] In African countries and Australia, RAO is very rare and almost completely unknown.

Etiology

Two elements are necessary for the development of clinical signs of RAO: an environmental component and an individual susceptibility. The necessity of both factors is underlined by: 1) recovery of horses, even with the severe airway obstruction, within a few days or weeks after they are moved into an antigen-free environment, and 2) the development of airway obstruction only in a subgroup of susceptible horses, while normal individuals kept in identical conditions are unaffected.

Environmental effect

The environmental effect is a critical factor for the RAO epidemiology and will be described first. Because airway obstruction can be repeatedly induced by stabling animals, especially when they are fed hay and bedded on straw, it is generally accepted that inhaled antigens, from hay and straw, are the pathogens that induce acute bouts of RAO. Hay and straw are a rich source of organic dust particles that can be inhaled into the lung.[46, 148, 299] The storage of hay in humid conditions promotes growth of molds, and poorly cured hay contains large amounts of fungal products, including mold spores. Among many types of spores found in moldy hay, the best known are *Aspergillus fumigatus, Fenia rectivulgaria,* and *Thermoactinomyces vulgaris.* Additionally a large amount of bacterial endotoxin, pollens, mites, and other antigens may be found in poor-quality

hay.[45, 148] The particular antigen or biochemical factor inducing RAO is unknown, and it is very likely that different combinations of antigens to which animals become sensitized with other pro-inflammatory factors, such as endotoxin, are the most effective inducers of both airway inflammation and obstruction. This opinion came from the comparison of the responses to natural hay and straw challenge, with the responses to inhalation of different types of isolated mold spores by animals with RAO. The selected types of spores produce respiratory distress and airway inflammation in a time frame similar to the occurrence of clinical signs in horses exposed to natural challenge. Still, their effect on pulmonary function is never as profound as the natural challenge.[61, 182]

Individual susceptibility

Referring to the information presented above, only susceptible horses develop the clinical syndrome in response to the environmental factor. Both the genetic and the acquired components of this susceptibility are currently thought to be important.

Genetic predisposition

A genetic susceptibility was long speculated in RAO, by analogy with other allergic diseases, particularly human asthma. The impact of a genetic component on the incidence of RAO was demonstrated in the population study of Marti et al. 1991, who observed that incidence of RAO in the offspring of RAO-affected horses was greater than in the offspring of unaffected parents.[174] These authors concluded that RAO is a multifactorial disease with a genetic basis. To date, the genes and/or phenotypes that are linked to the increased incidence or higher risk to develop airway obstruction remain

completely unknown. It is very clear, however, that genetic susceptibility is not sufficient for airway obstruction to develop.

Acquired airway susceptibility

The theory that susceptibility to RAO may be a result of acquired changes in airway physiology and structure is supported by two facts: 1) the development of clinical disease is not very rapid and animals with RAO are rarely younger than 5 years old; 2) the progress of the disease is largely modulated by conditions in which animals are kept. Even though some hypotheses have been formed and some data more or less strongly support theories lying behind them, the initial triggers that cause the development of the disease remain unknown. Among proposed mechanisms are bad hygiene of the air, viral and bacterial respiratory tract infections during the first years of life, or some other events in the juvenile period when active immunity develops in horses.[225, 264] Alternatively, rather than a singular disease in the young animal, lifelong exposure and sensitization of the individual's immune system to progressively greater number of antigens, and changes in local immunity may play a crucial role in the development of RAO. The establishment of chronic inflammation in the airways, via repeated irritation of airways with inhaled pathogens, appears to be particularly important.[232, 297] This increasing sensitivity to allergens, and the changes in function and structure of the airway most likely determine the progress of the disease.[108, 297]

Allergy

Although the initial factors that determine the development of pathological process within the airways remain unknown, it is generally accepted that RAO is an allergic disease. Scientific data supporting this view were collected by several groups.[61, 108, 179, 182] These studies demonstrated that RAO fulfills the criteria of an allergic disease, since the inhalation of thermophilic spores, a putative allergen in RAO, found in large quantities in hay and straw, reproduces some clinical signs of the disease in susceptible animals and not in the controls. Because each individual allergen does not reproduce clinical signs with the full intensity of the "natural challenge," it is therefore likely that the sensitivity to a larger number of antigens has to develop in order to produce a reaction sufficient to alter airway responses. Consistent with this opinion, Halliwell et al. indicated that indeed the number of positive responses (antigens to which animals are sensitized), and the intensity of allergic reaction were significantly greater in RAO horses than in the group of clinically normal animals. In spite of this, the allergic skin responses are not a very good predictor of the presence or severity of RAO in horses. Therefore, besides specific allergic responses, the effects of nonspecific factors are considered as contributors to development of RAO.

These nonspecific mechanisms promoting airway inflammation may be a good explanation of the powerful effects of natural antigen challenge. One of these hypothetical factors is bacterial endotoxin found in very high concentrations in hay dust. As a nonspecific stimulator of the immune response, endotoxin is a perfect candidate for a factor promoting both the inflammatory reactions and hypersensitivity in the airways.[130, 233, 277] Still, even very large amounts of dust produce only low-grade airway

inflammation but not the airway obstruction in normal horses. Therefore, the presence of endotoxin does not explain the differences in type of airway responses to natural challenge between the RAO-susceptible and unaffected animals.

The type of allergic reaction that predominates in RAO remains undefined. Halliwell et al., investigating the role of allergy in RAO, suggested that both type I and type III are predominantly involved in skin reactions to allergenic extracts in horses with RAO.[108] Other studies and clinical work seem to confirm that predominantly these two types of allergy play a major role in RAO. Increased quantities of IgE and histamine in the airways following antigen challenge in horses with RAO, as well as increased numbers of metachromatic cells isolated from airways of affected animals, support the presence of type I (IgE mediated) reactions in the airway of horses with RAO.[2, 108, 109] Neutrophil influx, increase of antibodies of IgG and IgA classes, and the timing of airway responses (usually 4 hours after initiation of challenge) are consistent with the type III (Arthus) reaction as a predominant mechanism in RAO.[108]

Chronic inflammation

When experimental horses with RAO are brought to the stable more than once, within a short period of time, each consecutive exposure to the dusty environment evokes more severe airway obstruction within shorter periods of time.[80] On the other hand, horses with a history of severe airway obstruction kept in an antigen-free environment (pasture) for months or years require many days or even weeks of stabling to develop airway obstruction. This indicates that in the absence of antigen, some of the pathological changes in the airways induced by antigen do not decline as quickly as the

airway hypersensitivity decreases. The existence of these more chronic changes implies that, following the acute allergic reaction, the pathological process continues in the airways. This chronic process most likely accounts for the progress of the disease. The development of chronic inflammation in the airways following repetitive provocation with antigen, and its effect on the thickness of airway wall [30] and function of mucocilliary apparatus, are implicated in the nonreversible component of airway obstruction in horses. Even during clinical remission, in horses kept on pasture, the presence of chronic inflammatory response in the airways is reflected by an increased level of 15-HETE in peripheral blood, increased numbers of inflammatory cells washed from airways of some individuals, slightly higher R_L and lower pulmonary compliance, and increased levels of mucin in lavage fluid.[33, 99]

Pathomorphological findings

Pathological findings in the lungs of COPD horses are numerous but often inhomogeneously distributed, so that only some areas may be found abnormal while other parts of the lung tissue may not be altered. The general observation was made that, in some horses with RAO, even with quite dramatic changes in pulmonary functions, the morphological findings are often disappointing,[30] perhaps because, although bronchospasm plays a major role in the clinical picture of the disease, it is not detectable histologically upon post mortem examination.[80, 153, 232] On the other hand, morphological findings, particularly in advanced cases of RAO, are very well documented in the literature. One of the most characteristic is the presence of excessive secretion in small bronchi and bronchioli, often occluding their lumen, and containing exudate and

inflammatory cells. The morphological abnormalities within the airway wall include changes in the epithelium such as hyper- and metaplasia,[232] proliferation of mucous cells and sero-mucous cells, and changes at the level of epithelial cell ultrastructure, i.e., structural abnormalities and shedding of the cilia, and degeneration of Clara cells.[136] Also, increased numbers of metachromatic cells (mast cells, or basophils) are found in the airway brush biopsies, or demonstrated histologically, accumulated within the intraepithelial clefts..[73, 298] In SA collected from horses with RAO, a thickening of all layers of mucosa and smooth muscle layer are found as well as cellular peribronchial infiltration, containing predominantly mononuclear cells and some neutrophils.[80] Because eosinophilic infiltrations are found only in sporadic cases, it is difficult to assess if their presence is associated with RAO or with some other concurrent condition.[136, 232]

The dramatic morphological changes in the airway walls are restricted to small bronchi, generally smaller than 2-mm outer diameter, and larger bronchioli.[137, 278, 297] The localization of this inflammatory reaction in the airways of a particular size is most likely determined by high rate of sedimentation of allergenic particles in these airways. This preferential antigen accumulation within the peripheral airways of horses is probably determined by 1) the aerodynamic properties of the particles (spores), i.e., their size of 0.5-5 μm and oval shape; 2) the slower rate of the airflow at the periphery of respiratory tract; and 3) rapid changes of the airway directions in terminal airways in contrast to the relatively unidirectional arrangement of larger airways. Alternatively, because of some specific features of equine airway immunity, the allergic/inflammatory reactions perhaps develop preferentially within the SA in response to natural challenge. This theory could be supported by the larger number of mast cells per microscopic field found in terminal

bronchioli of horses in comparison with the larger airways.[298] The inflammatory lesions often observed at necropsy within the walls and the lumen of the SA are defined by pathologists as bronchiolitis or small airway disease. Even though it is not entirely clear whether small airway disease in horses is always associated with RAO, this general impression comes across from the literature, and thus the terms RAO and small airway disease are often used interchangeably.[80, 225]

Pathophysiology

Even though airway inflammation is a prominent morphologic feature of RAO, airway obstruction and altered gas exchange are the most important clinical observations.[225] Obel and Schmiterlow performed one of the earliest studies of RAO, demonstrating increased variations in the intrathoracic pressure, which was interpreted to be consistent with forced breathing against a high resistance.[205] Since that time, the ΔPpl has become a commonly used diagnostic tool in the clinical management of RAO, and appears to be a good overall measure of the effort of breathing. When volume (V) and flows ($\dot{V}$) of the air are measured at the airway opening concurrently with ΔPpl, other parameters reflecting pulmonary functions can be calculated according to the following equation: $\Delta Ppl = R_L \times \dot{V} + V/C_{dyn}$. The parameters of R_L and C_{dyn} are good methods to estimate the status of larger and smaller airways respectively. Since overall R_L is determined mostly by resistance of larger airways, an increase of R_L is interpreted as an indicator of central airway narrowing. The R_L is a good estimate of the size of airway lumen, because the R_L magnitude is inversely proportional to the radius raised to the 4th power, and thus only a 16% decrease of airway diameter is sufficient to double

the R_L. In contrast, the overall effect of the SA narrowing on R_L is small, and the changes in C_{dyn} better reflect the status of smaller airways. The increase in R_L and the decrease in C_{dyn} are commonly reported in RAO.[33, 59, 97, 194, 195] The relationship between the individual pulmonary function parameters has been also studied in horses. In a recent paper, we have extensively discussed how changes in R_L, C_{dyn}, and air flow determine the magnitude of ΔPpl in horses with RAO as the level of airway obstruction changes throughout all observed ranges of ΔPpl.[224] Airway resistance increases most quickly at the lower ranges of ΔPpl, which can be explained by the fact that the large, and therefore stiffer central airways, can relatively easily reach their limits of constriction (see part I). In contrast, progressive decrease of C_{dyn} is observed throughout all the stages of airway obstruction.[224] This physiological manifestation of smaller airway constriction is supported by in vivo video-image computer analysis, which indicates that smaller airways may narrow their lumen to almost nothing.[252] These observations, together with the prolonged nitrogen washout time, indicate the existence of a diffuse airway obstruction in RAO.[94] In summary, the analysis of changes in lung functions in RAO clearly indicates that the narrowing of both smaller and larger airways contributes to the increased effort of breathing. Furthermore, in the more severe cases, the profound obstruction of smaller airways and alteration of airflow are responsible for the progressive deterioration of pulmonary functions.[224]

Bronchospasm

Narrowing of the airways may result from contraction of the ASM, airway occlusion with endogenous end exogenous material, and structural changes in the airway

walls. Even before scientific evidence of bronchospasm in RAO was presented, bronchodilators were used as a clinical treatment of RAO.[205] A study of horses with RAO by Obel and Smitterlow demonstrated that bronchospasm, rather than lung emphysema, is the best explanation for the clinical and physiological alterations observed in RAO. These authors demonstrated reversal of changes in pleural pressure with two classes of bronchodilators (anti-M and sympathomimetic). Additionally the authors reproduced the signs of the disease via i.v. infusion of histamine in asymptomatic animals, and then reversed it with bronchodilators.[205] A variety of bronchodilators have appeared effective in causing both a good clinical response and an improvement of pulmonary functions independently of their mechanism of action or the route of administration.[31, 33, 60, 79, 187, 223] Among the drugs tested are the β-receptor agonists (particularly β_2), methylxanthines, and M receptor antagonists. Regardless of the type of bronchodilator, the response to bronchodilators is immediate, reversing the increase in ΔPpl and R_L by approximately 2/3. These effects confirm that bronchospasm is a major component of airway obstruction in horses with RAO. When data from the individual horses are analyzed, some horses dilate more, almost to the baseline level, while others have a lesser response.[79, 153]

The irreversible component of airway obstruction indicates that mechanisms other than bronchospasm contribute to narrowing of the airways. Inflammation is the predominant structural finding in RAO, and it mediates structural changes in the airway walls and perhaps also affects neuromuscular control of equine airways. Thus, before I discuss the mechanisms of bronchospasm and state my hypothesis, I will describe the inflammatory process in the course of RAO.

Inflammatory response in the course of RAO

Inflammation is an integral part of the syndrome of RAO. The evidence for this comes not only from pathological, but also clinical studies, which describe the relationship between clinical signs of airway obstruction and airway inflammation. Correlation of pulmonary gas exchange measurements with clinical signs and pathologic evidence of chronic bronchiolitis, therapeutic benefits of glucocorticosteroids, and influx of inflammatory cells into the airways all provide evidence about the crucial role of inflammation in the pathogenesis of RAO.[153, 201] The presence of increased numbers of inflammatory cells in airway lavage in horses with RAO is one of the very basic facts. The diagnosis of RAO (particularly in the early stages) is based on cytological evaluation of inflammatory process in the airways. During remission of RAO, BAL fluid cell population is similar to that of control horses and consists primarily of macrophages and lymphocytes. As the animals with RAO are moved into a dusty barn environment, airway inflammation develops rapidly.

Neutrophils in RAO

One of the first responses to antigen challenge observed within the airways of susceptible horses is the influx of the neutrophils. The first wave of the neutrophil influx can be detected within the airways in 4–5 hours after exposure and it largely parallels the initial changes in pulmonary functions.[61, 83, 182] This high number of neutrophils is found in BAL from RAO horses during the entire period when horses are stabled, and returns toward the baseline value within a few days on pasture. The large number of neutrophils in BAL fluid or respiratory secretions of horses with clinical RAO has been reported

frequently.[58, 63, 105, 168, 178, 200, 268, 295, 296] Furthermore, the percentage of neutrophils increases with the severity of clinical signs, although the variation between horses is considerable.[280] The variability in the BAL neutrophil population can be explained, to a certain degree, by their nonuniform distribution within the lung. The content of neutrophils in BAL fluid collected in different parts of lung of the same horse may vary by 10%.[183] Still, it has become apparent that there is a partial divergence between the number of neutrophils and the severity of clinical signs in horses with RAO. Particularly the occurrence of airway neutrophilia, in response to antigen challenge, is not always associated with the bronchospasm.[61, 64, 104]

An important insight into the role of inflammatory cells has been presented by Fairbairn et al. Their study clearly demonstrates that, in the response to natural challenge, neutrophils but not eosinophils or platelets, are recruited into the lungs of horses with RAO.[83] Even though Fairbairn's study confirmed again that neutrophils are definitely a predominant inflammatory effector cell to be recruited into the lung in RAO horses, partial dissociation was observed between the increase in ΔPpl and the dynamics of neutrophil recruitment. This indicates that the mere presence of neutrophils is neither sufficient nor necessary for changes in pulmonary function to occur.[64, 83] Whether a differential level of neutrophil activation explains the lack of good correlation between numbers of neutrophils in the airways and the changes in pulmonary function remains unresolved.

In an earlier study I investigated the activity of neutrophils washed from the airways of horses with RAO, and I observed that neutrophils in the airways of horses are strongly activated in comparison with those from blood. However, I observed a

considerable variability between individuals. Because at that time measurements of pulmonary functions could not be performed, the correlation between the severity of airway obstruction and the activity of neutrophils was not evaluated.[207]

The equine RAO is not the only model of airway obstructive disease in which neutrophils are considered to play an important role. Examples supporting the hypothesis that neutrophils may alter the responses of airways, and therefore contribute to the airway obstructive diseases in other species, are presented in Chapter 1 of the literature review. Particularly interesting were studies that demonstrated that the suspensions of neutrophils are capable of altering in vitro responses to histamine and EFS in human bronchus.[107, 122] Because the effects of neutrophils on equine airway responses have not yet been, the determination of these effects is an important component of my PhD studies.

Apart from neutrophils, at least two other cell types are considered to be significant in the inflammatory reaction of airways in horses with RAO, namely, the lymphocytes and metachromatic cells.

Lymphocytes

Evidence that lymphocytes may play an important role in RAO is supported by: 1) increased percentages of B lymphocytes in airways of asymptomatic horses with RAO and increased concentrations of specific immunoglobulins in airways of horses with RAO; 2) a decreased percentage of T helper cells in peripheral blood and their increase in BAL fluid after antigen challenge; 3) the decreased tendency of lymphocytes isolated from peripheral blood of RAO susceptible horses to undergo apoptosis in cultures,[109, 180]

With the exception of these few pieces of information, the role of lymphocytes in the development of airway obstruction in horses remains unknown and awaits further elucidation.

Metachromatic cells

Mast cells and basophils, as principal effector cells in the anaphylactic response, have been long proposed to be important players in RAO. This was supported by evidence that histamine infused intravenously in control horses evokes short-term respiratory distress with clinical signs of RAO.[7, 205] However, the low therapeutic efficacy of antihistamines in RAO kept the hypothesis of metachromatic cells being important effectors in RAO out of favor.[21] Evidence of local production of allergen specific IgE and of metachromatic cells activation after antigen challenge in equine airways, as well as the therapeutic benefits of mast cell stabilizers, such as cromolyn sodium, may suggest that these cells are an underappreciated factor in the pathogenesis of RAO.[109, 111, 184, 263] An increased level of histamine in the airways of horses with RAO challenged with hay and straw is an indicator of metachromatic cells degranulation within the airways. It is noteworthy that this elevation of histamine occurs within 4 hours after challenge, which fits into the time frame of the early changes in lung function.[81, 182] Mediators released by metachromatic cells are ASM spasmogens and some may affect nerve function. In this context the localization of mast cells in the proximity of ASM, nerves, and surface of the airways may be important. It is also remarkable that metachromatic cells are quite abundant residual inflammatory cells in SA of horses, and that the number of mast cells is greater in airways of horses with RAO.[298]

Inflammatory mediators in RAO

As discussed in Chapter 1, many inflammatory mediators appear to be involved in airway inflammation and to contribute to airway obstruction. In RAO, as in human asthma, elimination of only one mediator, or its effects, does not induce definite improvement.[21] This suggests that different combinations of inflammatory mediators play an important role in the clinical course of RAO. Since the number of studies of the inflammatory mediators in RAO is limited, only some of them have been reported to increase in the course of this disease. The only group of mediators studied quite extensively in equine airways are the eicosanoids. In a series of publications, Gray et al. clearly demonstrated activation of AA cascade in RAO.[99-101] Antigen challenge results predominantly in elevated TXA_2 production.[101] Even though the effects of TXA_2 on airway responses in the horse have not been studied, blockade of cyclooxygenase enzyme does not affect in vivo airway responses in RAO, suggesting that TXA_2 or other prostanoids are not a major cause of changes in pulmonary function in RAO.

The role of 15-HETE is also unclear. Baseline pulmonary production of 15-HETE is elevated in clinical RAO remission and it further increases when experimental ponies are exposed to antigens. Since 15-HETE is predominantly a mucus cell secretogogue, and the mediator of chronic rather than acute inflammation, it is unlikely that it could change airway responses (see Chapter 1).

Much more likely to be involved in the airway responses in RAO are LTs. The LTE_4, a final metabolite of cysteinyl LTs, is reported to increase in urine of horses with RAO.[69] The importance of 5-LO metabolites in equine RAO is still unclear, but by analogy with the research in other species, the cysteinyl LTs could contribute to the

bronchospasm by directly contracting ASM or via cholinergic mechanisms (Chapter 1). In control and asymptomatic horses with RAO, Marr et al. reported that inhaled LTD_4 causes variable bronchospasm, while noncysteinyl LTB_4 evokes early recruitment of radiolabeled neutrophils into the lung without causing changes in lung function.

Histamine, which is reported to increase in BAL fluid within four hours of antigen challenge in the RAO horses, but not the control, is considered the strongest candidate mediator for this disease. However, treatment with antihistamines is of no value in advanced cases of RAO. Histamine is the most frequently studied mediator in equine airways. This is because, when infused i.v. or administered as an aerosol, it induces clinical signs of RAO in both control and RAO horse. The effects of histamine are more profound in horses during the acute phase of the disease. Obel and Schmiterlow reported that, in RAO horses treated with histamine, the clinical signs of airway obstruction became extremely profound, and they could be largely reduced by ATR. Moreover, small doses of histamine have been demonstrated to induce clinical signs of disease in the RAO horse without obvious clinical signs of disease.[205] Because of the beneficial effect of ATR and adrenaline on pleural pressure in horses with spontaneous-and histamine-induced attacks of RAO, it was suggested that histamine may act synergistically with cholinergic mechanisms of airway control. However, unilateral vagotomy and concurrent blockade of the contralateral vagus nerve does not prevent histamine effects on airway function, indicating that vagal reflex is not a mechanism of histamine action in RAO.[205] Later studies with histamine challenges have further indicated that: 1) the spasmogenic effects of histamine are predominantly associated with peripheral airway constriction; 2) histamine has a larger effect in horses with RAO than

in controls; 3) this hypersensitivity to histamine in RAO is observed predominantly during exacerbations of RAO; and 4) the vagal reflex is not a mechanism of histamine action.[8, 33, 59, 62]

Much less is known about other inflammatory mediators in the course of RAO. In about 50% of the horses with RAO studied by Eyre, the 5-HT level in plasma was increased above the normal range.[80] This mediator is also reported to be released in anaphylactic reactions in the sensitized pony lung.[35] PAF, proposed as a mediator of RAO, causes accumulation of neutrophils over the lung field when infused. When inhaled it has no effect on pulmonary function in control or COPD horses [82]

Mechanism of altered airway responses in RAO

Cholinergic component of airway obstruction

Therapeutic benefits of ATR in RAO were known, even before bronchospasm was proposed to be important factor of this disease, at that time known as emphysema.[205] In RAO the high efficacy of anti-M bronchodilators has been demonstrated by several authors.[33, 79, 153, 205, 223] This indicates that bronchospasm in RAO is mediated primarily via activation of M receptors. The most likely explanation of cholinergic bronchoconstriction would be: 1) the intrinsic hyperresponsiveness of ASM to cholinergic stimulation; or 2) an increased amount of ACh at the neuromuscular junction; or 3) lack of inhibitory mechanisms opposing the cholinergic airway tone.

Intrinsic ASM hyperresponsiveness

Although, in vivo, horses with RAO are more sensitive to MCh challenge, particularly during exacerbation, the hypothesis of intrinsic hyperresponsiveness of ASM in horses with RAO was rejected based on several studies in vitro.[32, 154, 307] In these studies, larger or smaller bronchi and the trachealis muscle isolated from horses with RAO are all hyporesponsive to exogenous ACh when compared with the responses of similar tissues isolated from control horses.[32, 154, 307] These results also excluded the decreased activity of ACh esterase as a possible mechanism of cholinergic bronchospasm in RAO. If this would be the case, the ASM treated with exogenous ACh in vitro should demonstrate hyperresponsiveness in contrast to observed hyporesponsiveness.

Increased ACh release from airway parasympathetic nerves

Since there is no intrinsic hyperresponsiveness within smooth muscle to ACh or decreased breakdown of ACh, augmentation of ACh release from the airway parasympathetic nerves onto ASM could explain cholinergic bronchospasm in RAO. Therefore, responses to EFS were extensively studied in several segments of airways, in order to compare their responses between the control and RAO horses. In most studies, ASM from horses with RAO were hyporesponsive to EFS.[154, 307] Thus, just like response to exogenous ACh, EFS responses in isolated ASM from horses with RAO appear weaker than in tissues from control horses. An exception is a study by Broadstone et al., who observed greater responses of RAO horse trachealis to EFS in comparison with the control.[32] Even though this was not confirmed by later studies, Broadstone's results suggested the possibility of increased release of ACh from parasympathetic nerve

terminals in tissue from horses with RAO upon the EFS. The series of studies by Wang et al., who measured ACh release from airway parasympathetic nerves by HPLC coupled with electrochemical detection, did not confirm this hypothesis. In the trachea and bronchi, the release of ACh in response to EFS in horses with RAO is similar to that of controls.[284]

Loss of inhibitory mechanisms

In healthy horses and in horses with RAO in clinical remission, ATR does not affect pulmonary functions. This observation indicates that, except for the acute exacerbations of RAO, the ASM are in an entirely relaxed state. Thus, the magnitude of excitatory input from cholinergic nerves in healthy horses is not sufficient to overcome endogenous inhibitory mechanisms. In airways of normal horses, the following inhibitory mechanisms can be demonstrated: sympathetic and iNANC nerves, the structurally unknown EpDRF and mucosal PGE_2.[32, 303, 305] In the airways of horses with RAO, β-adrenergic relaxation and the inhibitory effect of epithelium appear to be unaltered.[32, 303] Decreased in RAO is mucosal production of PGE_2, which exerts tonic inhibition in the airways and has been shown to play an important inhibitory function in the trachea and bronchi of horses.[100, 303, 307] Since inhibition of cyclooxygenase augments the responses to cholinergic nerve stimulation in isolated airways of horses, decreased mucosal production of PGE_2 could potentially contribute to increased cholinergic airway tone.[32, 303] However, the cyclooxygenase blocker flunixin meglumine, which is frequently used in equine medicine, does not precipitate bronchoconstriction in either control or

RAO horses.[102] This would most likely occur if a lack of mucosal PGE_2 were a predominant factor leading to cholinergic bronchospasm in RAO.

Also deficient in affected horses is the function of a nitroxidergic iNANC. The iNANC response, present in unaffected horses, is consistently lacking in central bronchi isolated from horses with RAO.[32, 307] It is again unlikely that lack of iNANC plays a major role in the development of cholinergic bronchospasm. Blockade of iNANC response in vitro with NO synthase inhibitors does not augment cholinergic responses in normal equine airways to EFS.[305, 308]

Discrepancy between in vivo and in vitro airway function in RAO

Even though several investigators tested airway responses from RAO horses in vitro, the mechanism of cholinergic bronchospasm in RAO remained unresolved. As discussed above, neither intrinsic hyperresponsiveness of ASM nor evidence of increased ACh release could be confirmed in the airways of horses with RAO. Similarly, there was no evidence of decreased breakdown of ACh, or failure of inhibitory mechanisms that would cause considerable changes in cholinergic airway responses. Three other mechanisms still need to be considered as potential mechanisms contributing to cholinergic airway obstruction: 1) nonspecific AHR, 2) cholinergic reflexes, and 3) altered prejunctional modulation of ACh release by airway parasympathetic nerves.

Airway hyperresponsiveness

The exaggerated response to inhalation of aerosolized MCh is a well-documented finding in horses with RAO.[10, 81] The discrepancy between the hyperresponsiveness to

MCh observed in vivo and the ASM hyporesponsiveness observed in vitro can be explained via the phenomenon of nonspecific AHR. Similarly to human asthmatics, exaggerated narrowing of the airways occurs in horses with RAO, in response to stimuli that do not affect normal individuals.[10, 226] Thus, substances that are innocuous for control horses, such as citric acid or water aerosols, evoke bronchospasm in horses with RAO.[10, 59] Also, subthreshold concentrations of spasmogens, such as inhaled or infused histamine or MCh, induce changes in pulmonary functions in horses with acute exacerbation of RAO.[10, 70, 205] The nonspecific AHR may result from geometric changes within the airways such as thickening of the airway wall and mucus stasis, and also from partial contraction of ASM. Thickening of the airway wall, indicated by morphometric study, may not necessarily result in a dramatic change in baseline pulmonary function, but it may cause exaggerated airway narrowing when muscle contracts around the thickened mucosa.[30, 225] An alternative explanation for the exaggerated responses is decreased baseline airway caliber, which might contribute to the greater airway response. The latter hypothesis was attractive, because AHR is present only during acute bouts of RAO, and wanes after the animals return to the pasture. However, because bronchodilation with ATR does not decrease airway reactivity to inhaled histamine in animals with RAO, and because there is no correlation between airway reactivity and changes in their caliber, this hypothesis was rejected.[10, 33, 59]

Parasympathetic nerves and exacerbations of RAO

The presence of nonspecific AHR may in part account for the discrepancy between hyperresponsiveness to MCh inhalation and the decreased responses of ASM in

vitro. It does not yet explain the presence of cholinergic bronchospasm in horses during the course of RAO.

Cholinergic reflexes in response to airway receptor stimulation, facilitation of ganglionic transmission, and altered prejunctional modulation of ACh release could all be important mechanisms of increased cholinergic airway tone (see Chapter 1).

Cholinergic reflexes have not been studied very thoroughly in horses with RAO. However, from the data available, it appears unlikely that cholinergic reflexes play a major role in the development of RAO.[33, 62, 205] Virtually nothing is known about the function of parasympathetic ganglia in equine airways, while modulation of ACh release by variety of prejunctional receptors has been studied extensively in our laboratory.

Dysfunction of prejunctional receptors on airway parasympathetic nerves has been proposed as a mechanism of cholinergic airway obstruction in animal models of asthma. In guinea pig and monkey airway challenged with antigens, hyperresponsiveness and bronchospasm are associated with dysfunction of prejunctional M_2 receptors. This receptor in physiological conditions provides a negative feedback controlling release of ACh (see Chapter 1). The hypothesis of dysfunction of a prejunctional M receptor in RAO was tested and rejected by Wang.[284] In his study he demonstrated that M receptor antagonists induce an identical level of augmentation of the EFS-induced ACh release in the trachea of both control and RAO horses. However, based on the isolated airway tension study, Wardle suggested that mild dysfunction of prejunctional M receptors may exist in horses with RAO. Also, partial dysfunction of prejunctional inhibitory α_2-receptor, observed by Wang in RAO horses, could theoretically contribute to increased ACh release in RAO. However, concentrations of epinephrine that occur in plasma of

resting animals are too small to activate α_2-adrenoceptor and inhibit ACh release even in control horse airways. Moreover, administration of α_2-agonists to horses with RAO causes dramatic improvement of pulmonary functions.[27] This confirms that the prejunctional α_2-receptor is functional in RAO horses, and indirectly suggests increased ACh release in airways of affected animals.

Summarizing the ACh release study, in equine airways, there is no striking evidence for increased ACh release or abnormality in prejunctional modulation in horses with RAO. However, unaltered baseline release of ACh in airways of affected animals observed in vitro may not reflect the actual amount of ACh released in vivo. Many hormones, mediators, and neurotransmitters that have not been investigated in horses are known to affect cholinergic nerves. Thus, it is still possible that, in vivo, the prejunctional modulation of ACh release from postganglionic parasympathetic nerves in equine airways is critical for increased release of ACh and cholinergic bronchospasm in RAO.

The outcome of the in vitro studies provide no evidence of exaggerated responses to cholinergic stimulation or increased release of ACh, which led to the conclusion that some factors that are present in the airways of affected animals in situ may no longer be present in vitro. This hypothesis is the basis for my doctoral study, during which I studied the factors altering cholinergic airway responses during acute bouts of airway obstruction in horses.

Consequences of inflammation on airway responses: my hypothesis

In Chapter 1, I demonstrated that inflammatory cells and mediators play an important role in airway physiology. In many animal models of airway obstruction and human asthma, inflammation has a large impact on the development of bronchospasm and AHR. In Chapter 2, I reported that although inflammation and cholinergically mediated bronchospasm are cardinal signs of RAO, in vitro studies have not explained the genesis of bronchospasm. Therefore, summarizing this review, I present the hypothesis that: ***mediators released from inflammatory cells in response to antigen challenge, interacting with nerves and/or smooth muscle, are responsible for increased cholinergic response leading to cholinergic bronchospasm.***

The discrepancy between the presence of cholinergic bronchospasm in RAO and decreased response to cholinergic nerve stimulation in airways, observed in earlier studies, can be explained, either by depletion of inflammatory mediators from the tissues of horses with RAO in vitro, and/or by the fact that only larger, presumably less inflamed segments of airways, were studied in vitro.

Depletion of mediators must be seriously considered when the responses of tissues from the airways of affected animals are tested in vitro. Before tension can be recorded, isolated tissues are suspended for at least two hours in physiological buffer solution. During this time, the tissue preparations are dissected, mounted within tissue baths, and then their baseline tension is adjusted. Physiological buffer solution is frequently changed and in these conditions, it is likely that the initial concentration of mediators would dramatically decrease by washout.

The most appropriate segment of airways used in studies designed to reveal the relationship between inflammation and cholinergic airway responses is the smallest airway that still produces cholinergic response. This is because pathological studies of RAO clearly indicate that the major inflammatory reaction occurs in the smallest bronchi and bronchioli. Furthermore, significant differences in responses to antigen and inflammatory mediators have been recently demonstrated between larger and smaller airways in dogs, indicating that the greatest response to antigens occurs at the level of the small bronchi.[42]

Therefore, for my dissertation research:

1. I have selected SA from control and RAO horses to study their responses in vitro;
2. I activated neutrophils in the presence of SA in an attempt to mimic in vitro the inflammatory response in vivo, and in these conditions, I studied cholinergic SA responses;
3. I studied the effects of mediators reported to be increased in RAO on responses in my SA preparation; and
4. I measured responses of equine ASM during oxidative stress induced by addition of ROS.

To conduct these experiments I also had to

1. Isolate neutrophils and determine methods for their activation; and
2. Develop a SA preparation.

The results of my studies are presented in the following chapters:

3. Effects of H_2O_2 on equine trachealis responses
4. Characterization of in vitro responses of equine peripheral airways
5. Study of equine neutrophils isolated from control horses and horses with RAO
6. Effects of activated neutrophils and mediators of anaphylaxis on cholinergic responses in equine SA
7. Summary and conclusions.

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Chapter 3

EFFECTS OF HYDROGEN PEROXIDE ON ISOLATED TRACHEALIS MUSCLE OF HORSES

Summary

During acute bouts of RAO in horses, neutrophils that are capable of increased production of ROS accumulate in the airways. In the study reported here, the effect of H_2O_2 (1 μM to 0.1 M), one of these ROS products, on the responses of isolated trachealis muscle of horses was determined. Before and after incubation with H_2O_2, contractile responses to ACh, EFS, 127 mM KCl, and relaxation responses to isoproterenol and activation of the iNANC response were evaluated. Beginning at 1 mM, H_2O_2 contracted trachealis muscle in a concentration-dependent manner. This contraction was unaffected by ATR (1 μM), tetrodotoxin (TTX) (1 μM), or 1 μM MEC. The contraction of trachealis muscle in response to H_2O_2 is, therefore, not attributable to release of PGs, ACh, or other neurotransmitters. Above 0.1 mM, H_2O_2 depressed the responses to EFS, ACh, and KCl in a concentration-dependent manner. At 0.1 M, H_2O_2 decreased the maximal responses to EFS, ACh, and KCl by 62.7 $\pm$ 7.2%, 60.58 $\pm$ 6.12%, and 37.8 $\pm$ 9.54%, respectively. In the presence of MEC (1 μM), partial but significant protection against 1 to 100 mM H_2O_2 was observed. In tracheal strips contracted with 0.3 μM MCh, H_2O_2 had no effect on the ISO concentration-response

curve. Up to a concentration of 100 mM, H_2O_2 had no effect on iNANC response. However, in the presence of 100 mM H_2O_2, this response was abolished in 2 of 4 horses. We conclude that: high concentrations of H_2O_2 affected the responses of ASM by actions on neurotransmission, M receptors, and downstream from receptors; some of the H_2O_2 effects were in part mediated by cyclooxygenase products; and H_2O_2 had no effect on β-adrenergic- or iNANC-induced relaxation.

Introduction

Recurrent airway obstruction in horses, is characterized by bronchospasm and nonspecific hyperreactivity of airways in response to inhaled allergens.[1, 31] During the onset of disease, inflammatory cells are recruited into the lungs, accumulate in intra- and peribronchial spaces, and can be obtained in large numbers by airway or BAL.[6, 8, 19, 29] Although the importance of inflammation is indicated by the therapeutic benefits of corticosteroids,[1] the function of particular inflammatory mediators in development of airway obstruction is unknown. Cells (predominantly neutrophils) lavaged from airways of horses with heaves have a ten-fold greater ability to reduce nitroblue-tetrazolium than do those from blood, indicating a high level of stimulation and an increased respiratory burst.[29] Also, antigen challenge exposure in horses causes increased superoxide production by circulating neutrophils that is more marked in horses with heaves.[24] This information suggests that large amounts of ROS are released and oxidative stress may have a role in the pathogenesis of heaves.

The ROS are important causes of tissue injury during inflammation.[33] When released from activated inflammatory cells, such as neutrophils, eosinophils, and

macrophages, ROS may affect cellular components, including membrane receptors,[21] enzymes, and DNA.[11, 33] In addition, ROS alter metabolism of AA, leading to release of lipid mediators.[11, 23] Experiments with endogenously derived or exogenously delivered ROS indicate their ability to modulate airway responsiveness in many animals. Contraction[34] or relaxation[9, 13] of ASM, changes in airway reactivity to spasmogens, such as ACh,[16, 17] carbachol,[16, 22, 23] MCh,[20] EFS,[16, 35] or substance P[26]; imbalance between β-adrenergic and M response[20]; and changes in secretion of mucus[25] have been observed in various experimental models after exposure to oxidants. The character and magnitude of observed changes depended on the animal species, segment of the airway studied, and the ROS used.

Derived in substantial amounts from neutrophils and macrophages at the inflammatory site,[12, 15] H_2O_2 is one of the best-studied ROS in the airways of animals. Moreover, H_2O_2 is reported to react with NO,[27] the mediator of the equine airway iNANC response that is lacking in bronchi of horses with heaves.[3, 38] Because the sensitivity of equine airways to H_2O_2 and the role of ROS in the pathogenesis of heaves has not yet been studied, we chose to investigate the response of equine trachealis muscle (hereafter referred to as trachealis) to H_2O_2. Specifically, we determined the direct effect of H_2O_2 on resting tension of tracheal strips, the modulatory effect of H_2O_2 on ASM contraction to EFS, ACh, and KCl, the effect on β-adrenergic and nitroxidergic relaxation, and the role of prostanoids in the responses to H_2O_2.

Methods

Horses

Geldings, mares, and stallions of various breeds, 6.5 ± 1 (mean ± SEM) years old, weighing 374 ± 13.18 kg, and free of signs of respiratory tract disease, were subjects of the study, which was approved by the All-University Committee on Animal Use and Care. Animals were euthanatized by IV administration of a lethal dose of pentobarbital sodium, the rib cage was opened, and heart, lungs, and trachea were excised. With other investigators, who obtained tissues for unrelated research projects, we collected tissues from a total of 31 horses. The trachea from the 25th to the 35th ring proximal to the carina was excised and suspended in K-H solution. For the rest of the day, all tissues used in protocols were kept in K-H solution that was continuously gassed with 95% O_2 and 5% CO_2. Preparations used on the second day after overnight refrigerated storage in a covered dish did not differ in response to H_2O_2 and had response curves to EFS or ACh as did fresh tissues. Therefore, second-day results were included in our analysis.

Tissue preparation

To obtain 2-mm-wide strips of ASM with intact mucosa, the cartilaginous wall of the trachea was removed and the remainder was pinned on a wax-plate under K-H solution, then was cut with a template along the direction of the ASM fiber. By cutting the muscle on either side of 3-0 surgical silk ties placed 10 mm apart, muscle strips were created. Strips were suspended between electrode rings placed in the muscle baths filed with K-H solution (38°C) that was bubbled with 95% O_2 and 5% CO_2 and replaced every

15 minutes. The lower thread was attached to the electrode unit, and the upper one was hooked to a force transducer (Model FT 03, Grass Instrument Co., Quincy, MA) installed on a tension manipulator. The isometric force of tissue preparations was recorded on a polygraph (Model 7D or 7E, Grass Instrument Co., Quincy, MA). Only 1 experimental protocol was conducted on each tissue strip.

During equilibration, EFS (16 Hz, 20 V, 0.5 ms) was applied for 2 minutes at 6- to 8-minute intervals, using a stimulator (Model S88, Grass Instrument Co., Quincy, MA) and stimulus power booster (Stimu-Splitter II, Med Lab Instrument, Loveland, CO). Passive tension was maintained at 2 to 3 g during equilibration; when baseline tension and response to 16 Hz EFS became stable, further adjustments were made to maximize the response to 0.1 Hz.

Protocols

Protocol 1, Direct effect of H_2O_2 on isolated trachealis muscle

Tension was recorded during incubation with H_2O_2 for 30 minutes. Five strips were exposed to 0.001, 0.1, 1, 10, or 100 mM H_2O_2, and an additional strip served as a control. The maximal active tension produced by the tissue during incubation was registered as the direct effect. In addition, we tested the direct effect of H_2O_2 after incubating the tissues for 60 minutes in the presence of one of the following inhibitors: 1 μM ATR, 1 μM MEC, and c) 1 μM TTX.

Protocol 2: Effect of H_2O_2 on the response to KCl

The maximal response to 127 mM KCl was measured. After washing and elimination of the cholinergic component of this response by ATR (1 μM for 60 minutes) a second response to KCl-substituted K-H solution was obtained. The final response to KCl plus ATR was recorded after 30 minutes incubation of smooth muscle with H_2O_2 and subsequent washing.

Protocol 3: Effect of H_2O_2 on ACh-induced contractions

After the standard KCl response was obtained, tissues were washed to baseline tension and cumulative response curves to ACh (0.01 μM to 1 mM) were generated. The ACh concentration was increased in logarithmic increments after the response to the previous concentration reached a plateau. After the ACh was washed out, the tissues were incubated with H_2O_2 for 30 minutes and washed, then concentration-response curves to ACh were repeated. The response to ACh was calculated as percentage of maximal KCl response.

Protocol 4: Effect of H_2O_2 on EFS-induced contraction

Electrical field stimulation (16 Hz, 20V, 0.5 ms) was applied to the muscle preparations at frequencies of 0.1, 0.5, 1, 2, 8, and 16 Hz to create noncumulative frequency-response curves. To minimize the differences caused by time after exposure to H_2O_2 the order of stimulation frequencies was randomized. After 40 minutes incubation with H_2O_2 and subsequent washing, EFS was repeated in the same order of

frequencies as before incubation. Data were expressed as percentage of the response to 16 Hz (EFSmax) obtained during acquisition of the first frequency-response curve.

Protocol 5: Effect of H_2O_2 on EFS-induced contraction in presence of cyclooxygenase blockade

After equilibration, muscle strips were incubated in MEC (1 μM for 60 minutes). In the presence of MEC, frequency-response curves were generated as for protocol 4, before and after incubation in H_2O_2.

Protocol 6: Effect of H_2O_2 on β-agonist-induced relaxation of ASM

Muscle strips were contracted with 0.33 μM MCh and treated with increasing concentrations of ISO (0.001, 0.01, 0.1, 1, and 10 μM) to obtain a cumulative relaxation curve. The concentration of methacholine was chosen because it induced a stable contraction that was entirely eliminated by ISO. Concentration-response curves were again obtained after incubation with H_2O_2 and after a 30-minute washout period. Relaxation obtained in response to each concentration of ISO was expressed as percentage of tension in response to 0.33 μM MCh.

Protocol 7: Effect of H_2O_2 on iNANC-induced relaxation of ASM

To unmask the nitroxidergic iNANC response, muscle strips were incubated with guanethidine (10 μM), ATR (1 μM), and indomethacin (3 μM) for 60 minutes.[38] Histamine (1 to 10 μM) was added to induce a stable contraction of 60 to 70% maximal response to EFS. Frequencies of 0.5, 4, 8, and 16 Hz were used to stimulate relaxation

that was expressed as percentage of the histamine-induced contraction. The experiment was performed in 8 muscle baths on 2 groups of tissues. In one group, EFS was applied in the presence of H_2O_2 (0, 1, 10, and 100 mM), whereas in the second half, EFS was used after 30 minutes incubation with the same concentrations of H_2O_2, followed by washing of the tissues.

Agents

Stock solutions of H_2O_2 in cooled K-H solution were prepared from 30% H_2O_2 (Sigma Chemical Co., St. Louis, MO) and kept in the refrigerator for no longer than 30 minutes before addition to the baths. Concentration of solutions was confirmed spectrophotometrically, using a molar extinction coefficient (43.6) at 240 nm. The H_2O_2 concentrations in the muscle bath had no effect on osmolality and pH of the bath solution. ACh, ATR, indomethacin, ISO, MCh, TTX (all from Sigma Chemical Co., St. Louis, MO) and sodium meclofenamate monohydrate (courtesy of Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI) were dissolved in deionized water, diluted in K-H solution, and added to the muscle baths in a volume of 1%.

Statistics

Data represent arithmetic means ± SEM (Excel 5.0, Microsoft Co., Redmond, WA) for tissue responses after the appropriate treatment, and n represents the number of horses used in each protocol. To exclude effects of time or tachyphylaxis we applied only between-bath comparisons. The direct effect of H_2O_2 and responses to 127 mM KCl plus ATR after incubation in H_2O_2 were analyzed by use of one-way ANOVA (Excel 5.0,

Microsoft C., Redmond, WA). The effects of H_2O_2 on the responses to ACh, EFS, EFS with MEC, and ISO were evaluated by use of a mixed design two-way ANOVA (Excel 5.0, Microsoft Co., Redmond, WA). Tukey's honestly significant difference test was used to detect means significantly different from control. Means were accepted to be significantly different at $P \leq 0.05$.

Results

Beginning at a concentration of 1 mM, H_2O_2 contracted the tissues in a concentration-dependent way (Figure 4). Maximal contraction (27.71 $\pm$ 5.61% of EFSmax, n = 8) was reached 10 to 20 minutes after H_2O_2 was added. The magnitude of contractions was unaffected by ATR (n = 6), MEC (n = 7), or TTX (not shown; n = 5).

Mean maximal response of 36 strips from 6 animals to 127 mM KCl was 34.07 $\pm$ 1.37 g. Addition of ATR to eliminate the cholinergic component of this contraction reduced the response by 24.24 $\pm$ 1.41%. In ATR-treated tissues, H_2O_2 caused a concentration-dependent decrease of KCl-induced contractions (Figure 5).

Up to a concentration of 1 mM, H_2O_2 did not change the response of trachealis to ACh but higher concentrations significantly depressed contraction (Figure 6). The tension in response to 1 mM ACh was depressed by 60.58 $\pm$ 6.12% (n = 6) after exposure to 100 mM H_2O_2.

Concentration-dependent decreases in the response to EFS were also observed after incubation in H_2O_2 (Figure 7). This decrease was significant at virtually all frequencies in strips treated with 10 and 100 mM H_2O_2. The response to 16 Hz EFS was

reduced by 62.7 ± 7.2% (n = 7), after addition of 100 mM H_2O_2. In the absence of H_2O_2, MEC (1 μM) increased the response to 0.1 Hz EFS (compare controls in upper and lower panel of Figure 7). In MEC-treated tissues, H_2O_2 still depressed the response to EFS in a concentration-dependent manner (n = 6), but its effect was attenuated (Figure 7).

In the absence of H_2O_2, ISO caused concentration-dependent relaxation of MCh-contracted tissue, reaching baseline tension at 10 μM ISO. A 30-minute incubation in H_2O_2 (1 to 100 mM) reduced the magnitude of MCh-induced contraction in a concentration-dependent manner, but had no influence on relaxation response to ISO (Figure 8).

In protocol 7, EFS-induced iNANC relaxation was frequency dependent (n = 4). Addition of H_2O_2 (1, 10, and 100 mM) to the histamine-precontracted tissue caused 14 ± 3.77, 38 ± 7.0 and 77 ± 3.77% relaxation, respectively (n = 4). Preincubation with H_2O_2 for 30 minutes had no effect on the iNANC response to EFS. Co-incubation with H_2O_2 up to 100 mM did not decrease the iNANC response (n = 4), but 100 mM H_2O_2 abolished it in 2 of the 4 horses (data not shown).

Discussion

Results of this study indicated that H_2O_2 modulates the reactivity of equine trachealis. Hydrogen peroxide, in comparison with other ROS is a relatively stable oxidant, but it diffuses freely through the cell membranes and damages cellular structures.[9, 25] The concentration of H_2O_2 produced locally by inflammatory cells in the airways of horses with heaves is unknown. However, the concentration formed by

phagocytizing macrophages varies from 1 to 100 mM,[15] and isolated human neutrophils (1.6×10^7) suspended in medium produce up to 0.8 mmol of H_2O_2L/h.[12] Large numbers of neutrophils are present in respiratory tract secretions and in the airway wall infiltrates of horses with heaves[6, 19, 28, 29]; therefore, the concentrations achieved in the proximity of ASM during exacerbations of this disease are most likely to be in the range used in our study.

Administration of H_2O_2 leads to airway obstruction in cats in vivo.[17] Also, administration of other ROS or antigen can lead via H_2O_2-dependent pathways to airway obstruction in sheep and cats.[18, 22] In vitro, H_2O_2 modulates ASM function in rats,[20, 35] guinea pigs,[30] cattle,[34] dogs,[9] rabbits,[13] and ferrets,[25] either by direct actions (contraction, relaxation) or by changing ASM reactivity to spasmogens and relaxing agents. In our series of experiments, we determined the direct effect of H_2O_2 on the tension of equine trachealis and its influence on the individual components of the response to spasmogens including KCl, which causes receptor-independent contraction attributable to membrane depolarization; ACh, which activates M receptors; and EFS, which releases neurotransmitters (principally ACh) from postganglionic nerves.

Equine trachealis contracted in response to 1 to 100 mM H_2O_2. In comparison with other species, the sensitivity of equine trachea to H_2O_2 appears similar to that of ferret[25] but lower than that of other species. Under similar conditions, the contractile effects of H_2O_2 were observed in guinea pig trachea at a concentration of 1 μM,[30] and in bovine trachealis and canine lung parenchyma (LP) at 10 μM.[34] The mechanism of the direct effect of H_2O_2 is not identical among species. Because lipid mediators, especially prostanoids, are involved to variable degrees in H_2O_2-evoked reactions,[5, 9, 13, 23, 30] we

tested the effect of cyclooxygenase blockade on the response to H_2O_2. MEC did not change the contractile response to H_2O_2, indicating a prostanoid-independent mechanism. This is in contrast to guinea pig trachealis and canine bronchus in which prostanoids are responsible for most effects associated with exposure to H_2O_2. Lack of inhibition of this response by TTX as well as by ATR rules out release of neurotransmitters or activation of M receptors as a cause of H_2O_2-induced contractions. Therefore, origin of the observed contractions remains unknown. It is possible that H_2O_2 contracts smooth muscle by directly releasing Ca^{2+} from intracellular stores[5] or indirectly by releasing mucosal factors.[35]

Although, at the resting tension of the muscle, H_2O_2 contracted the trachealis, it also caused concentration-dependent relaxation in tissues contracted with histamine in the presence of M, adrenergic, and prostanoid blockade. The mechanism of this relaxation is also speculative, but 3 possible explanations are: breakdown of histamine by H_2O_2,[14] direct stimulation of ASM guanylate cyclase by H_2O_2,[32] or hyperpolarization of the smooth muscle cell membrane potential[36] attributable to inhibition of time-dependent fast inactivation of voltage-gated K^+ channels.[36]

In contrast to its variable effects on ASM tone, H_2O_2 consistently reduced the tissue contraction to spasmogens. The greatest effect was observed on the EFS response (Figure 10). This concentration-dependent decrease of contraction was observed at all frequencies, beginning with 1 mM H_2O_2. As previously observed, MEC increased the EFS response of trachealis, particularly at lower frequencies via blockade of endogenous (inhibitory) prostanoids.[37] Moreover, MEC attenuated depression of EFS-induced responses caused by H_2O_2 (Figure 7). Because MEC attenuated the action of H_2O_2, the

latter must influence the eicosanoid profile in equine tracheal tissue, and these changes are in part, responsible for the observed ASM hyporesponsiveness to EFS. In bronchi from horses with heaves, the prostanoid profile is less inhibitory than that in control horses,[10, 39] but the changes induced by H_2O_2 via the cyclooxygenase pathway caused inhibition of the response to EFS. These differences suggest that heaves and exposure to H_2O_2 have opposite effects on prostanoid metabolism.

The pathway leading to ASM contraction in response to EFS includes nerve stimulation, release of endogenous neurotransmitters, followed by their coupling to tissue receptors; signal transduction, and activation of the intracellular contractile mechanism. The response to ACh includes all the aforementioned except for neurotransmission, whereas the response to KCl in the presence of ATR is downstream to receptor activation and signal transduction. Therefore, comparison of H_2O_2 effects on EFS, ACh, and KCl responses at similar levels of tissue contraction provides an opportunity to evaluate the reduction by H_2O_2 of each individual component of the ASM response to EFS (Figure 9). The response to KCl was significantly inhibited by H_2O_2, suggesting effects downstream from the receptors. This reduced intrinsic cellular contractility most likely contributed to the decreased tissue contraction caused by H_2O_2 in all protocols. At H_2O_2 concentrations up to 100 mM, the depression of the responses to ACh and KCl was similar, indicating no effects of H_2O_2 on M receptor activation by exogenous ACh. However, 100 mM H_2O_2 had greater effects on the ACh than on the KCl response, suggesting effects on M activation. Compared with KCl and ACh, depression of the EFS-induced contractions was the greatest, indicating additional interactions of H_2O_2 with either neurotransmission or the response of smooth muscle to locally released

neurotransmitter. Furthermore, the additional depression of the EFS response was most likely mediated by prostanoids because it was eliminated by MEC.

Although the M contraction was decreased by H_2O_2, the β-adrenergic relaxation remained unchanged. This response is similar to that of guinea pig trachealis[7] but not that of rat trachea, in which 1 mM H_2O_2 decreased the response to MCh and completely abolished β-adrenergic relaxation.[20]

Incubation for 30 minutes with H_2O_2 and subsequent wash had no effect on the inhibitory iNANC response, indicating that the release of NO from the nerves and the metabolic pathway leading to NO-mediated relaxation of the trachealis remained unaffected by H_2O_2. Furthermore, the presence of H_2O_2 up to 100 mM also did not decrease this relaxation, suggesting that a lack of chemical interaction between H_2O_2 and NO is the cause of the iNANC deficiency observed in the bronchi of horses with heaves.[3, 39]

The hypothesis that H_2O_2 may be involved in the pathogenesis of heaves is not strongly supported by our data. The lower concentrations of H_2O_2 that modulate the responses of airways in many other species had no effect in horses and the higher concentrations that affected horse tissues are in the upper limits of concentrations reported in biological systems.[15] In addition, the changes induced by H_2O_2 did not mimic those reported during acute exacerbation of heaves. In horses with heaves, the in vivo response to inhaled spasmogens is exaggerated, but in our study, H_2O_2 caused hyporesponsiveness of smooth muscle. The bronchospasm of heaves is reversible by ATR,[1, 4] but H_2O_2-induced contraction was ATR resistant. On the other hand, experiments in vitro have indicated predominantly ACh hyporesponsiveness as a feature

of ASM from horses with heaves.[37] It is possible that the latter is the result of oxidative stress similar to that caused by incubation of ASM in high concentrations of H_2O_2. This potential role of H_2O_2 fits the paradigm that a variety of mechanisms are part of the pathogenesis of heaves and that no one single factor is responsible for the development of the disease. However, it is important to realize that this study was conducted on tissues from horses with no history of airway disease. It is possible that responses of tissues from heaves-affected horses may be quite different.

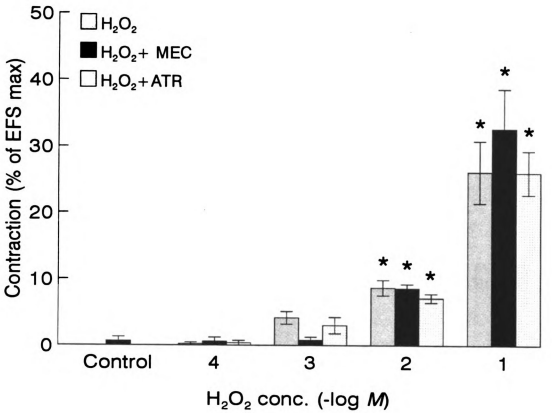

Figure 4. Contraction of equine tracheal strips by H_2O_2, n = 8. Notice the lack of significant effect of ATR (n = 6), and MEC (n = 7). * Significantly different from control ($p \leq 0.05$).

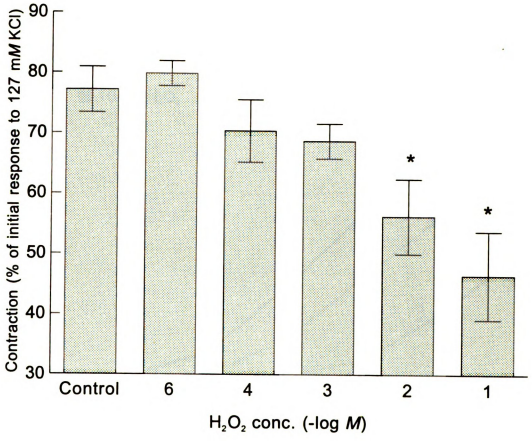

Figure 5. Contractile response of trachealis muscle to K-H solution substituted with 127 mM KCl in the presence of ATR after 30 minutes' exposure to H_2O_2 (n = 6). Response to KCl before incubation with ATR and H_2O_2 served as a 100% standard. * Significantly different from control ($p \leq 0.05$).

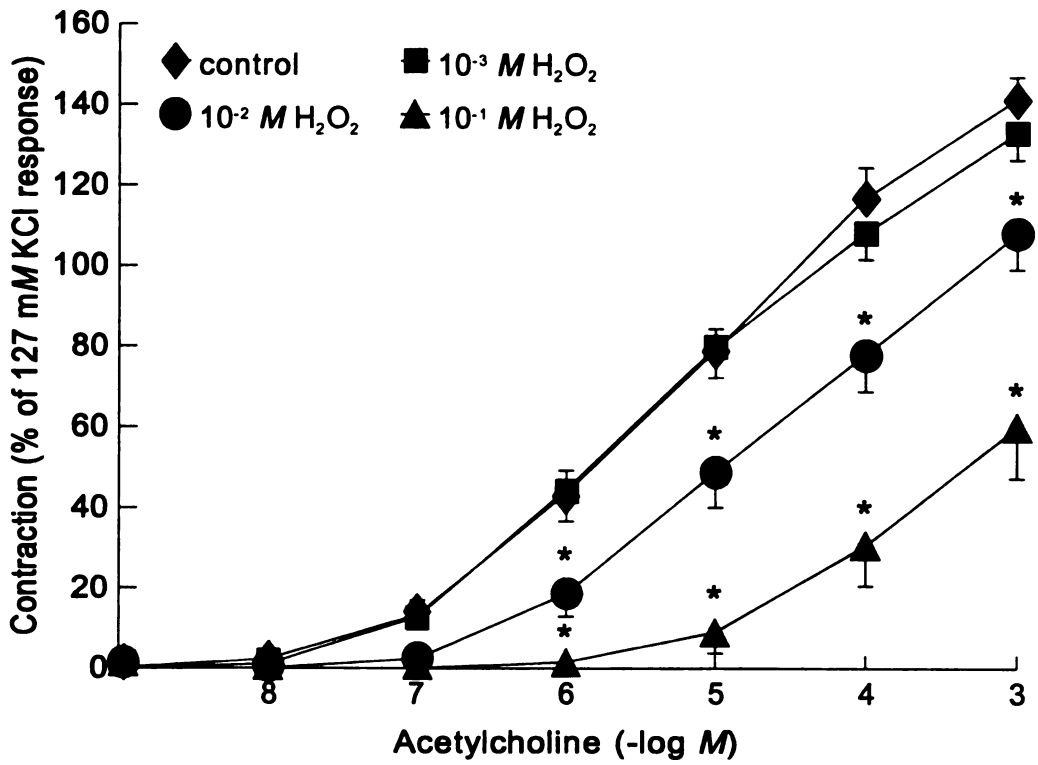

Figure 6. Effect of H_2O_2 on concentration-response curve to ACh (n = 6). * Significantly different from control ($p \leq 0.05$).

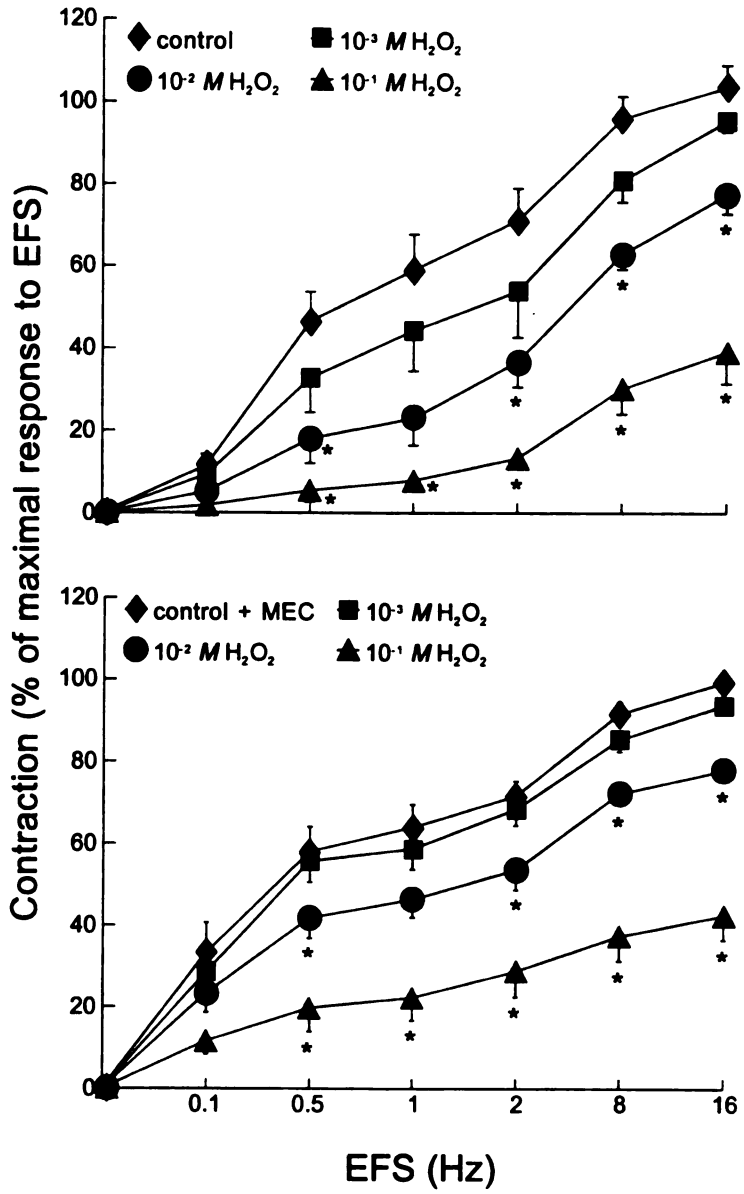

Figure 7. Effect of H_2O_2 on frequency-response curve to EFS (20 V 0.5 ms) in absence (top) and presence (bottom) of MEC (n = 7 and n = 6, respectively). * Significantly different from control ($p \leq 0.05$).

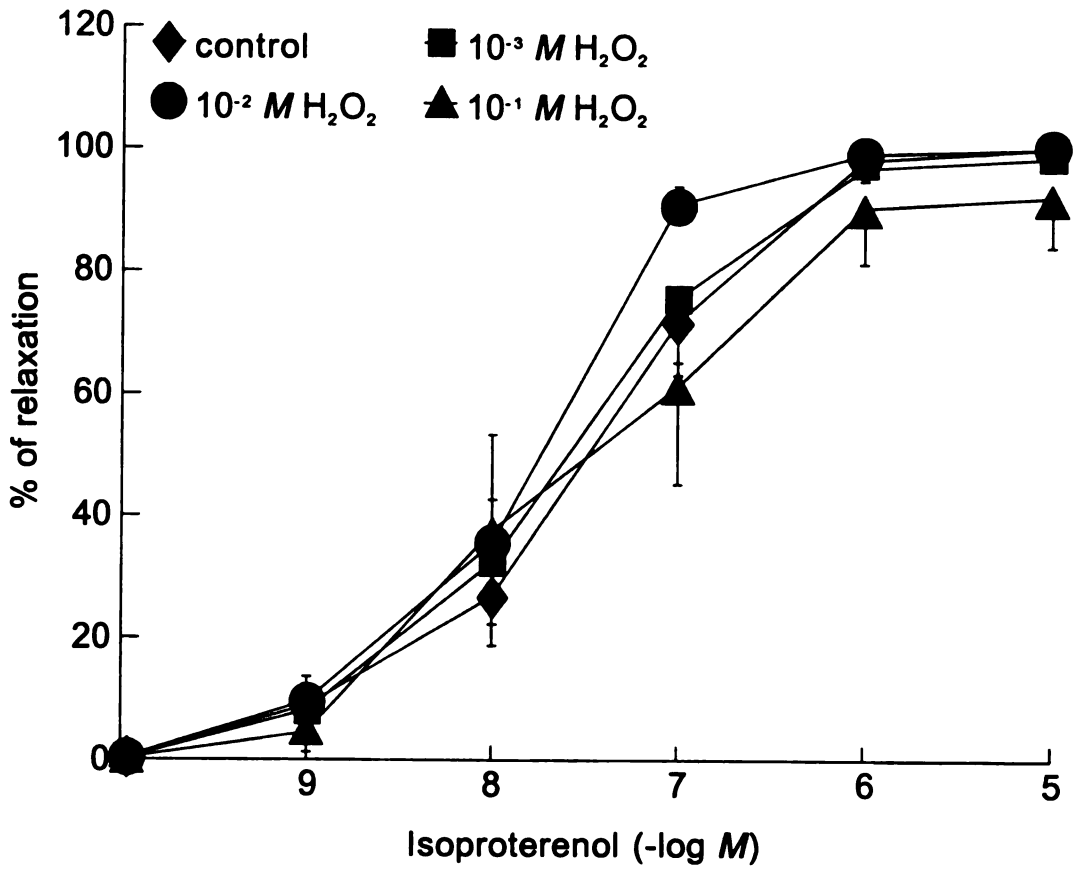

Figure 8. ISO-induced relaxation in trachealis muscle contracted with MCh (n = 5). There was no significant effect of H_2O_2.

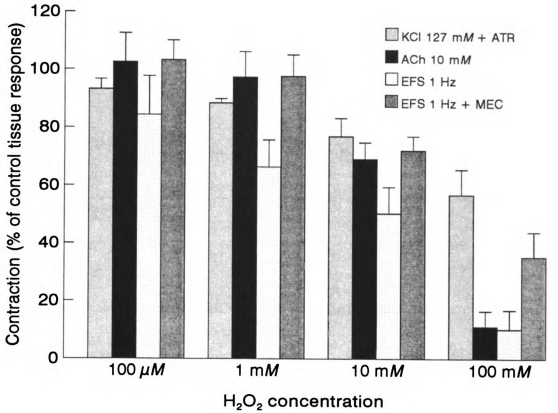

Figure 9. Comparison of H_2O_2 effects on contractions to KCl, 10 μM ACh and 1-Hz EFS in absence and presence of MEC. Each bar represents responses after incubation with H_2O_2 expressed as percentage of primary response of these tissues to the same stimulus.

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Chapter 4

IN VITRO RESPONSES OF EQUINE SMALL AIRWAYS AND LUNG PARENCHYMA

Abstract

In vitro responses of equine peripheral SA and LP were studied. We examined their contractile and relaxant responses and investigated effects of histamine, and endogenous prostanoids as these mediators may play a role in development of RAO in horses. In SA and LP EFS induced respectively nearly maximal and partial frequency-dependent contractions. These contractions were virtually abolished in SA but only partially inhibited in LP by ATR and TTX. Methacholine contracted SA with a 5-fold higher potency and greater maximal tension than LP. In SA but not in LP, a large augmentation of EFS responses at 1-4 Hz was produced by both MEC, a cyclooxygenase blocker, and 3 μM histamine. We conclude that 1) excitatory input from cholinergic nerves largely determines SA tone, but has minor effect in LP 2) in SA endogenous inhibitory prostanoids modulate contractile response to nerve stimulation, and 3) inhibition of cyclooxygenase and histamine greatly potentiate responses to nerve stimulation in SA. These data are consistent with the hypothesis that, in horses with heaves, histamine release and an altered prostanoid profile contribute to cholinergically mediated SA obstruction.

Introduction

Recurrent airway obstruction in horses, "heaves," develops in susceptible animals exposed to inhaled allergens.[17] Influx of inflammatory cells into the airway, and altered pulmonary function, consistently seen in response to antigen inhalation, indicate that two processes, inflammation and bronchospasm, are responsible for airway obstruction.[10, 17, 18] Moreover, development of bronchospasm is thought to be related to the inflammatory process, because a) inflammatory mediators can contract ASM or interfere with mechanisms controlling ASM tone; and b) anti-inflammatory therapy is an effective means of reducing airway obstruction. Bronchospasm in heaves can be most efficiently reversed by use of anticholinergic agents or drugs that decrease ACh release from parasympathetic nerves.[4, 28] Therefore, alterations in cholinergic mechanisms controlling ASM tension are thought to be an important factor leading to airway obstruction in heaves. To confirm such an abnormality in cholinergic, i.e., parasympathetic, regulation of airway caliber, several investigators have compared in vitro airway responses in horses with heaves and in control horses. These studies have not revealed the source of altered responses of the horse respiratory tract in vivo. Comparison of tension responses between control and horses with heaves did not confirm increased responsiveness of the airways to ACh.[29] Also, ACh release from airway parasympathetic nerves, measured in vitro, is not elevated in horses with heaves.[24] For technical reasons it is easier to conduct measurements on larger airways and the latter studies were conducted on trachea and bronchi. However, the most predominant inflammatory response occurs in peripheral airways.[18] Therefore, if the inflammation is a cause of the altered cholinergic mechanisms in the airway, alterations detectable in vitro may be limited to SA.

The aims of the present study were to characterize contractile and relaxant responses in equine terminal airways in vitro. To do this it was first necessary to develop a technique for investigation of the tension responses of SA from horses. Because in heaves there is histamine release in the airways,[15] and prostanoid profile produced by airway mucosa is less inhibitory,[11] we also investigated whether in vitro responses of SA and LP can be modulated by histamine and endogenous prostanoids.

Methods

Horses

Twelve geldings and mares of various breeds, 9.5 ± 2.1 years old, weighing 958.1 ± 24.5 kg, and free of signs of respiratory disease, were subjects of the study, which was approved by the All-University Committee on Animal Use and Care at Michigan State University. Animals were killed by intravenous injection of pentobarbital sodium, the rib cage was opened, and heart, lung, and trachea excised. Immediately after the animal's death the cranial region of the left lung was collected and suspended in Krebs-Henseleit solution (K-H) (composition in mM: 118.4 NaCl, 25.0 $NaHCO_3$, 11.7 Dextrose, 4.7 KCl, 2.6 $CaCl{\cdot}2H_2O$, 1.19 $MgSO_4{\cdot}7H_2O$, and 1.16 KH_2PO_4) saturated with 95% O_2 and 5% CO_2. During dissection and experimental protocols, tissues were kept in K-H that was continuously gassed with 95% O_2 and 5% CO_2.

Tissue preparation

Columns of LP 10 × 3 × 3 mm in size and free of visible blood vessels or airways were cut from the harvested lung within a few minutes after death. Surgical silk

ties were placed on both ends of the columns approximately 8 mm apart. Blocks of parenchyma in which a single SA (1-2.5 mm OD) traversed the central part were also dissected. This SA represents generation 15-20 of bronchi that still contains cartilaginous elements. In SA preparations, blood vessels that often accompanied the SA were eliminated by dissection. Further dissection of parenchyma helped to align the airway and maintain its central position within the narrow column of parenchyma. Silk ties were placed around the top and the bottom of this column approximately 1-2 mm from the edge of the airway. In this way SA was suspended by its parenchymal attachments with the axis of the airway lying perpendicular to the vector of tension measurement. All preparations were suspended between electrode rings placed in the muscle baths filled with K-H (38°C) bubbled with 95% O_2 and 5% CO_2, which was replaced every 15 min. The lower tie was attached to the electrode unit, whereas the upper one was hooked to a force transducer (Model FT 03 Grass Instrument Company, Quincy, MA) installed on a tension manipulator. Isometric force of tissue preparations was recorded on a polygraph (Model 7D or 7E, Grass Instrument Company, Quincy, MA).

During equilibration (2 hours) optimal passive tension was determined by gentle stretching of the tissue and application of EFS (1 Hz, 20 V, 0.5 ms) every 30 min. Optimal tension ranged from 1.5 to 1.6 g or 1.9 to 2.2 g for parenchyma and SA, respectively. Square wave EFS impulses were generated by a stimulator (Model S88, Grass Instrument Company, Quincy, MA) and delivered to the electrodes via a stimulus power booster (Stimu-Splitter II, Med Lab Instrument, Loveland, CO). Experimental protocols were conducted in 8 muscle baths, 4 with SA and 4 with LP preparations.

Protocols

Protocol 1: Characterization of EFS responses

Electrical field stimulation (20 V, 0.5 ms) was applied to the tissue at frequencies of 1, 2, 4, 8, 16, and 32 Hz to create cumulative frequency-response curves. Frequency was increased after the response to lower frequency had reached a plateau. After the first frequency-response curve, tissues from each group, SA and LP, were incubated for 1 hour with one of the following inhibitors: the M receptor antagonist ATR (3 μM), a NO synthase inhibitor *N*ω-nitro-L-arginine (LNNA; 30 μM), a cyclooxygenase inhibitor, MEC (1 μM), or a sodium channel blocker TTX (3 μM). Tissues not incubated with inhibitors served as untreated controls. A second frequency-response curve was generated after 60 min of incubation.

Protocol 2: Responses of the tissue to the cholinergic agonist MCh

Cumulative response curves to MCh (0.01 μM to the 1 mM) were generated. The agonist concentration was increased in logarithmic increments after the response to the previous concentration reached a plateau. MCh response curves involved control strips and strips incubated with inhibitors from protocol 1.

Protocol 3: Effects of histamine

In a similar fashion to protocol 2, cumulative response curves to histamine (0.01 μM to 1 mM) were generated. After repeated washing with buffer for at least 1 hour, tissues were equilibrated for 10 min with 3μM histamine and responses to EFS and MCh were determined as in protocol 1 and 2.

Protocol 4: Relaxation of histamine-contracted tissues by EFS

Tissues were incubated for 60 min with indomethacin (3 μM) and ATR (3 μM) to prevent phasic contraction caused by histamine and to block the cholinergic response, respectively. Histamine was then added in half logarithmic increments to induce stable contraction of 60% KCl response. Electrical field stimulation (1-16 Hz) was then applied in a non-cumulative fashion.

Protocol 5: Relaxation of histamine contracted tissues by ISO

Tissues incubated for 60 min with indomethacin (3 μM) were pre-contracted as in protocol 4. A cumulative concentration-response curve was created by addition of half logarithmic concentrations of ISO, a β-adrenergic agonist.

Agents

On the day of the experiment, ATR, histamine, LNNA, MCh, sodium MEC monohydrate, and TTX (all from Sigma Chemical Company, St. Louis, MO), were dissolved in deionized water to obtain stock solutions (10 or 100 mM). Indomethacin and ISO (both from Sigma Chemical Company, St. Louis, MO) were dissolved in 10 mM sodium carbonate and 0.1% ascorbic acid solutions, respectively. All stock solutions were directly mixed into K-H, except for MCh, histamine, and ISO, which were serially diluted in K-H, and each concentration was added to the muscle baths in a volume of 1%. The concentrations of all drugs were expressed as their final bath concentration.

Statistics

Data ($\bar{x} \pm$ SEM) shown in the text and figures are expressed in grams or as a % of response to 127 mM KCl substituted K-H, and n represents the number of horses used at each protocol. To determine drug effects, we applied between-bath comparisons of treated and control tissues. This excluded any effects of time or tachyphylaxis. Data were calculated and analyzed (Excel 7.0 by Microsoft and SSPS for Windows 7.0 by SSPS Inc., on Gateway 2000 P5-133 computer) using unpaired t-test, one-way ANOVA, or mixed-design 2-way ANOVA as appropriate. Post-hoc Student-Newman-Keuls or Simple Main Effects tests were used to compare means between the treatments and the controls. Means were accepted to be significantly different at $P \leq 0.05$.

Results

All applied spasmogens caused contraction of both SA and LP. The response to 127mM KCl (Table 1) was 4 times greater in SA than in LP, indicating a smaller number of contractile elements in the latter. For this reason, further analysis of responses was based on data expressed as a percentage of the 127 mM KCl response.

Electrical field stimulation contracted both SA and LP in a frequency-dependent manner (Figure 10). In SA, the maximal tension (T_{max}) produced by EFS was 88.2 $\pm$ 2.3% of KCl response, whereas in LP it was only 32.3 $\pm$ 1.9%. The EF_{50} was higher in LP than in SA (Table 1). In SA, the response to EFS was virtually abolished by ATR and TTX, whereas these antagonists only attenuated the response by approximately 50% in LP (Figure 10). In SA but not LP, MEC significantly augmented the EFS response

to the lower stimulus frequencies (Figure 11). Treatment with LNNA had no significant effect on either SA or LP contractile responses (data not shown).

Methacholine contracted SA and LP in a concentration-dependent manner but the latter was nearly five-fold less sensitive to MCh, and its maximal response was 2-fold lower (Table 1, Figure 12). Responses to MCh were blocked by ATR in both SA and LP. Neither MEC nor LNNA significantly altered the responses to MCh in either tissue.

Histamine contracted SA and LP in a concentration-dependent manner. Histamine was 4.6 times more potent in LP than SA but produced a higher maximal response in SA (Table 1, Figure 13). In SA, 3 μM histamine dramatically augmented EFS-induced contractions at lower stimulus frequencies (Figure 14A). Histamine also significantly increased the response to MCh in SA, but the magnitude of this effect was small (Figure 14B). In LP, histamine did not augment the EFS or MCh response.

When preparations were incubated with ATR and contracted by histamine, EFS induced responses only in SA but not in LP. These responses were variable and of little magnitude (not shown). The ISO relaxed both SA and LP in a concentration-dependent manner (Figure 15). Small airways were relaxed entirely at 10-30 μM ISO, while in LP relaxation was variable and reached on average 78.5 $\pm$ 13.5 % of contraction.

Discussion

In recurrent airway disease (heaves) of horses, inflammation of SA is the most prominent morphological feature.[18] In this paper we report for the first time the in vitro responses of equine SA (below generation 15), characterize the extent of endogenous and exogenous cholinergic response, and present evidence that response to nerve stimulation

is subjected to strong modulation by inflammatory mediators. We also compare this SA with LP responses.

Our goal was achieved by development of SA preparations that allowed us to measure accurately and reproducibly the tension produced by ASM. In comparison with spiral or traditional circular preparations that damage smooth muscle or epithelium respectively, our preparation preserves the natural anatomical relationship between tissue elements and there is minimal direct contact between SA tissues and the measurement system.[12] A relatively small amount of parenchyma necessary to support the suspensions is included in the SA preparation, so if there is any contribution of LP to the response of SA it is negligible. This assumption is based on very small magnitude of tension generated by LP-preparation that were constructed exclusively of LP and that at passive tension of 2g in SA preparation parenchyma was entirely stretched, which eliminated possible elastic effects of parenchyma on SA preparation responses.

Even though our LP preparations were free from visible blood vessels, airway and pleural surface, there are certain limits in the interpretation of their tension as the response of terminal bronchioli. Lung parenchyma may contain up to 40 types of cells,[13] including contractile elements other than ASM. Also orientation of this contractile elements is more random than in circular airways, which may further complicate data interpretation. However based on a combination of measurements of contractile responses and histological studies, Drazen and Schneider[9] concluded that the tension produced by LP in vitro reflects the peripheral airway function in vivo and that the alveolar duct smooth muscle contraction is the main component of this response. A pioneer study of the in vitro responses of equine LP to agonist has been published.[8] However, in that

study, maximal responses to agonist produced by slightly larger LP strips were up to 10 times lower than ours, probably associated with the delay between animal death and experimental measurements.

Both SA and LP contracted in response to 127mM KCl, and in spite of relatively smaller amount of the tissue in SA preparations their responses were on average 4 times greater than that of LP. The lower tension produced by LP reflects a smaller number of contractile elements and their scattered orientation, in comparison with SA that possess a well-defined circular muscle layer. These differences between two types of preparations required that tissue responses to spasmogens were standardized as % of the maximal KCl response.

Because RAO has a strong cholinergic component,[4] we were particularly interested in the role of endogenous cholinergic responses in the regulation of ASM. Our study is the first to report that the parasympathetic system extends to the level of LP in the horse. This conclusion is based on our observation of responses to EFS in the presence and absence of TTX or ATR. The TTX-sensitive contraction resulting from EFS is interpreted as the response of smooth muscle to endogenous mediators released from nerve terminals stimulated by electric impulses while inhibition afforded by ATR indicates that ACh released from the nerves is activating cholinergic M receptors. EFS-evoked frequency-dependent contractions in SA reached on average 88.3 $\pm$ 2.4% of KCl and were almost entirely abolished by both TTX and ATR. Thus, even in 1-mm airways, ACh released from cholinergic nerves provides the predominant excitatory input for airway contraction. We were also able to demonstrate functional innervation and M receptors in the LP because EFS evoked frequency-dependent contractions that were

inhibited by TTX and ATR. However, the EFS response reached only 32.3 ± 1.9% KCl and both inhibitors decreased it by approximately half. This relatively low magnitude of response to EFS, of which only 60% results from cholinergic neurotransmission, could suggest sparse cholinergic innervation of LP or that endogenous ACh released from the nerves is not as important in LP as it is in the other segments of the respiratory tract, including SA. Sympathetic α-adrenergically mediated contraction, which was confirmed in the airway of the horse[20] and other animal species,[3] was not the source of the remaining noncholinergic response because TTX and ATR inhibited LP contraction to the same extent. We conclude therefore that in the horse as in the guinea pig and sheep much of the EFS response in LP is not due to nerve stimulation.[19, 22]

MCh efficiently contracted SA, with a potency similar to that in trachea.[8] T_{max} in SA (114.1 ± 4.6) was slightly lower in comparison to the maximal cholinergic responses in trachea and bronchi (approximately 140% KCl).[16, 27] The LP was five-fold less sensitive to MCh than the SA, and MCh induced a nearly 2-fold lower T_{max} (64.6 ± 5.9% KCl). This significantly lower efficacy of cholinergic activation in LP was therefore at least in part responsible for its small response to EFS. It is likely that the decreasing efficacy of cholinergic agonist (ACh, MCh), and EFS response towards peripheral airways is caused by a longitudinal decrease of M receptor density from the trachea to LP. Just as the response to 16 Hz EFS in equine airways decreases from the trachea through the bronchi[16, 27, 29] and SA to LP, reaching approximately 120, 85, 80, and 25% KCl, respectively, cholinergic receptors in the airways of ferret are numerous in the bronchial smooth muscle, sparse in proximal, and almost absent from distal bronchioles.[2] It is also possible that in horses, subtypes of M receptor other than the M_3

reported in larger airways, may predominate in the terminal bronchioli as in pigs, in which Chelala et al. postulated expression of M_4 but not M_3 receptors in LP.[6, 26]

Another possible explanation for the low magnitude of cholinergic response in LP could be the activation of inhibitory mechanisms. Thus activation of M receptors on cells other than smooth muscle could release relaxing factors, which in turn would oppose the contractile action of EFS and MCh in LP. Parenchyma is abundant in both vascular endothelium and epithelium and other types of cells that could release relaxing factors in response to cholinergic or EFS. We used a NO synthase inhibitor LNNA and a cyclooxygenase inhibitor MEC to eliminate two epithelium- and endothelium-derived factors, namely NO and prostanoids. Neither LNNA nor MEC altered the responses of LP and therefore we conclude that endogenous prostanoids and NO do not modulate cholinergic responses in this tissue. Alternatively the profile of prostanoids released by residual cells in LP is neither inhibitory nor excitatory.

In contrast to the situation in LP, MEC significantly augmented SA responses to EFS to an extent similar to that previously observed in bronchi and isolated trachealis muscle.[27] This leftward shift of the frequency-response curve is attributable to the blockade of endogenous prostanoids that tonically inhibit airway contractions.[27] In bronchi from heavey animals, the prostanoid profile is less inhibitory than in control horses and this decreased inhibition of the response to nerve stimulation may contribute to airway obstruction in SA.[11, 29]

In some species it has been observed that the sensitivity of airways to histamine increases towards the periphery[1, 9] and that histamine may alter airway responses via its direct constricting effect[21] or via cholinergic mechanisms.[14, 21, 23] Histamine contracted

both SA and LP in a concentration-dependent fashion and was on average 4.6 times more potent in LP than in SA. In LP, histamine was also more potent than MCh, and T_{max} histamine was greater than T_{max} MCh. In SA, histamine was less potent than MCh but it produced a maximal contraction almost as high as MCh. These results show that the changes in sensitivity to histamine and cholinergic agonists shift in opposite directions along the respiratory tract. While the M response decreases dramatically towards alveoli, airways become more sensitive to histamine. In this respect equine airways resemble those of guinea pig and rabbit.[1, 9, 25]

Histamine not only contracted the SA, but also increased the responses to lower EFS frequencies. It is possible that synergism between M and histaminergic responses in the SA participated in this augmentation, but the small shift in the MCh curve induced by histamine cannot completely explain the dramatic augmentation of SA EFS response. Other possible factors, which require confirmation, could involve H-induced facilitation of ACh release from SA cholinergic nerves, activation of sympathetic α-adrenergic response to EFS[3] and facilitation of the excitation spread through SM cells via gap junctions.[7] In horses with heaves, cholinergically mediated peripheral airway constriction is accompanied by inflammation and histamine is one of the mediators that increases in the airways during acute bouts of heaves. The histamine-induced augmentation of the response to cholinergic nerve stimulation, particularly in physiologic frequency range, i.e., 1–2 Hz could explain how inflammation and cholinergically mediated airway obstruction are linked together. It is unlikely that similar mechanisms are at play in LP, because 1) cholinergic responses were trivial in this tissue, and 2) there was no synergism between EFS and H.

In order to study inhibitory innervation, we blocked the M receptors with ATR and contracted the tissues with histamine. Under these conditions, we were unable to demonstrate a functional inhibitory innervation in LP similar to that observed in larger airways.[30] In SA, small and inconsistent responses indicated that there is little functional inhibitory innervation. It is clear however based on our data that β-receptors are universally present throughout the equine airways and have a bronchodilating function.

In conclusion, we have demonstrated 1) the excitatory (bronchospastic) action of cholinergic nerves and M receptors up to the level of the smallest airways in horses, 2) a decreasing magnitude of cholinergic contraction and increasing sensitivity to histamine towards the terminal airways, and 3) augmentation of the predominantly cholinergic response to EFS in SA by both histamine and an altered profile of eicosanoids. It is likely that the latter mechanisms could be involved in SA constriction in heaves because an altered prostanoid profile[11] and an increase of histamine release[15] occur in this disease.

Table 1: Comparison of contractile responses of small airway and lung parenchyma

Tissue	Small airways			Lung parenchyma		
Spasmogen	T_{max}	pD_2 *or* EF_{50}	*n*	T_{max}	pD_2 *or* EF_{50}	*n*
127 mM KCl	1.68 ± 0.10[a]	NA	11	0.39 ± 0.02[a,i]	NA	11
Histamine	1.75 ± 0.42[b]	4.89 ± 0.08[e,h]	9	0.33 ± 0.04[b,j]	5.67 ± 0.14[e,k]	9
Methacholine	1.64 ± 0.21[c]	5.96 ± 0.09[f,h]	11	0.23 ± 0.03[c,i,j]	5.23 ± 0.12[f,k]	11
EFS (32 Hz)	1.47 ± 0.14[d]	2.99 ± 0.32[g]	11	0.12 ± 0.01[d,i,j]	7.85 ± 0.78[g]	11

T_{max} = maximal tension evoked by spasmogen (g)
PD_2 = concentration of histamine and MCh evoking 50% of maximal response (-log M)
EF_{50} = frequency of EFS producing 50% of maximal response to EFS (Hz)
n = number of animals
Values with identical superscript letters are significantly different ($p \leq 0.05$)

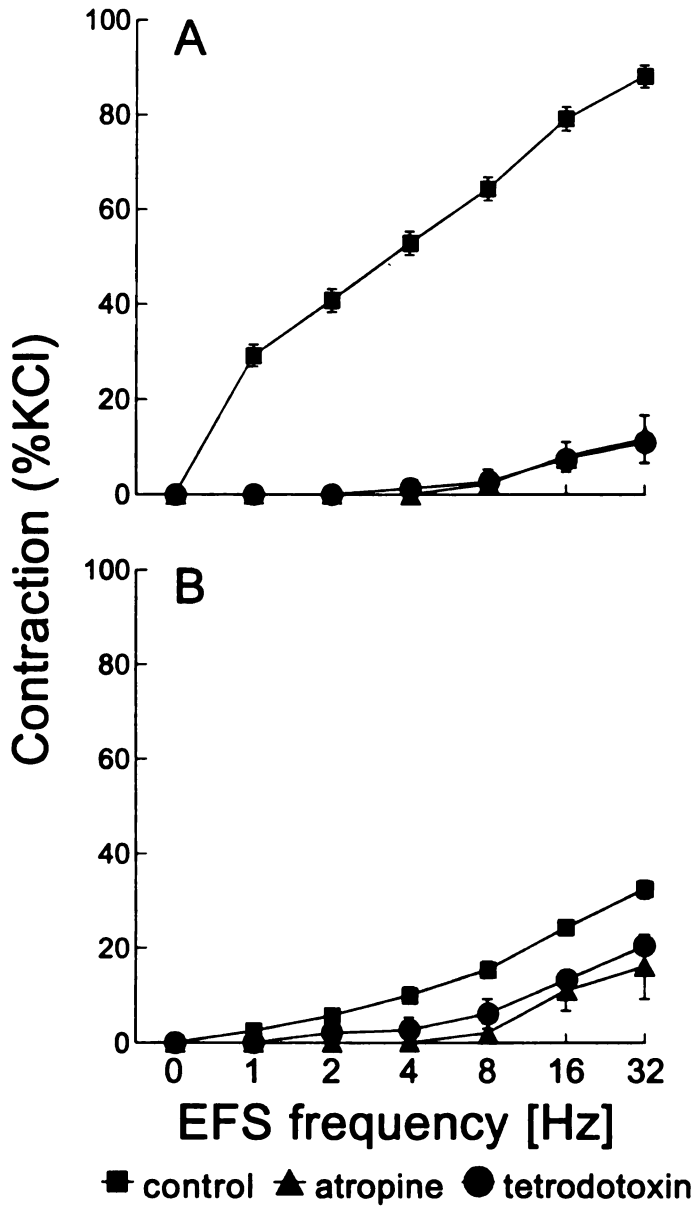

Figure 10. Cumulative frequency-response curves to EFS (20 V, 0.5 ms) of equine SA (A) and equine LP (B). ■, Control; ▲, with 3×10^{-6} M ATR; and ●, with 3×10^{-6} M TTX. Both antagonists significantly altered responses in SA and LP with $p < 0.001$ and $p = 0.017$, respectively, in two-way ANOVA, $n = 12$ animals.

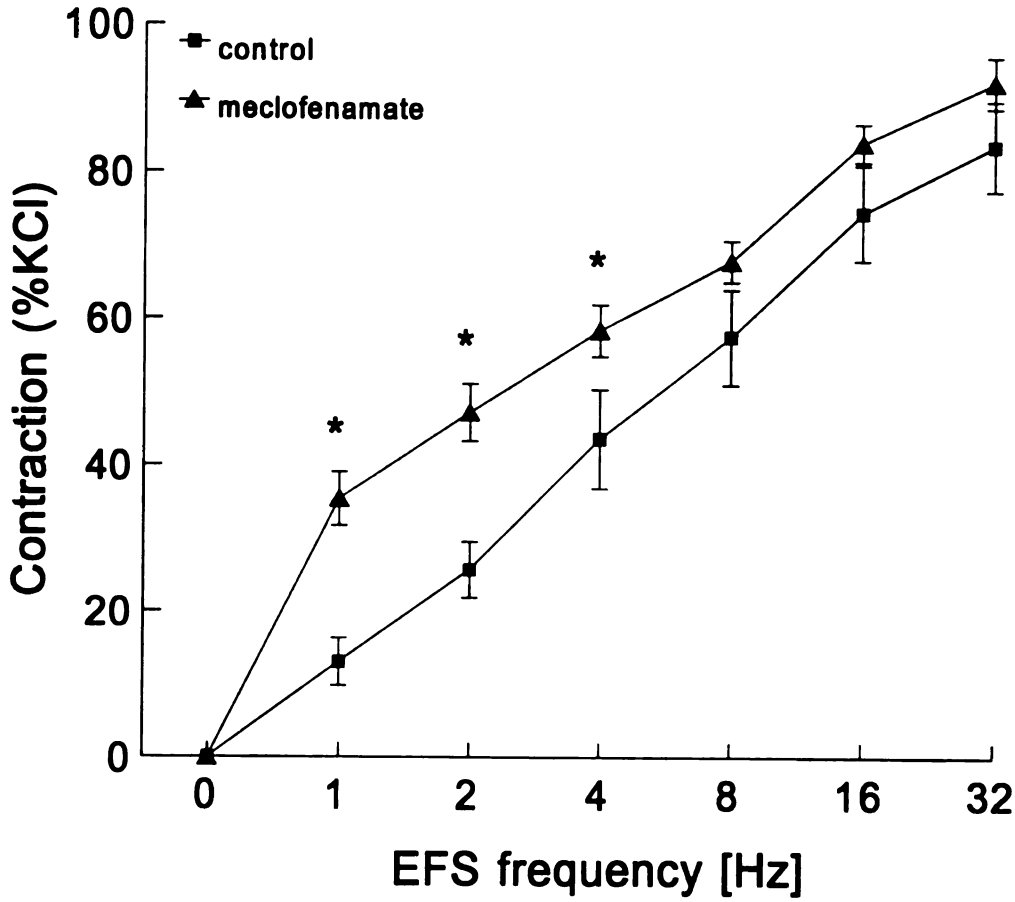

Figure 11. Effect of MEC on SA response to EFS (20 V, 0.5 ms), n = 7; *significantly different from control ($p \leq 0.05$).

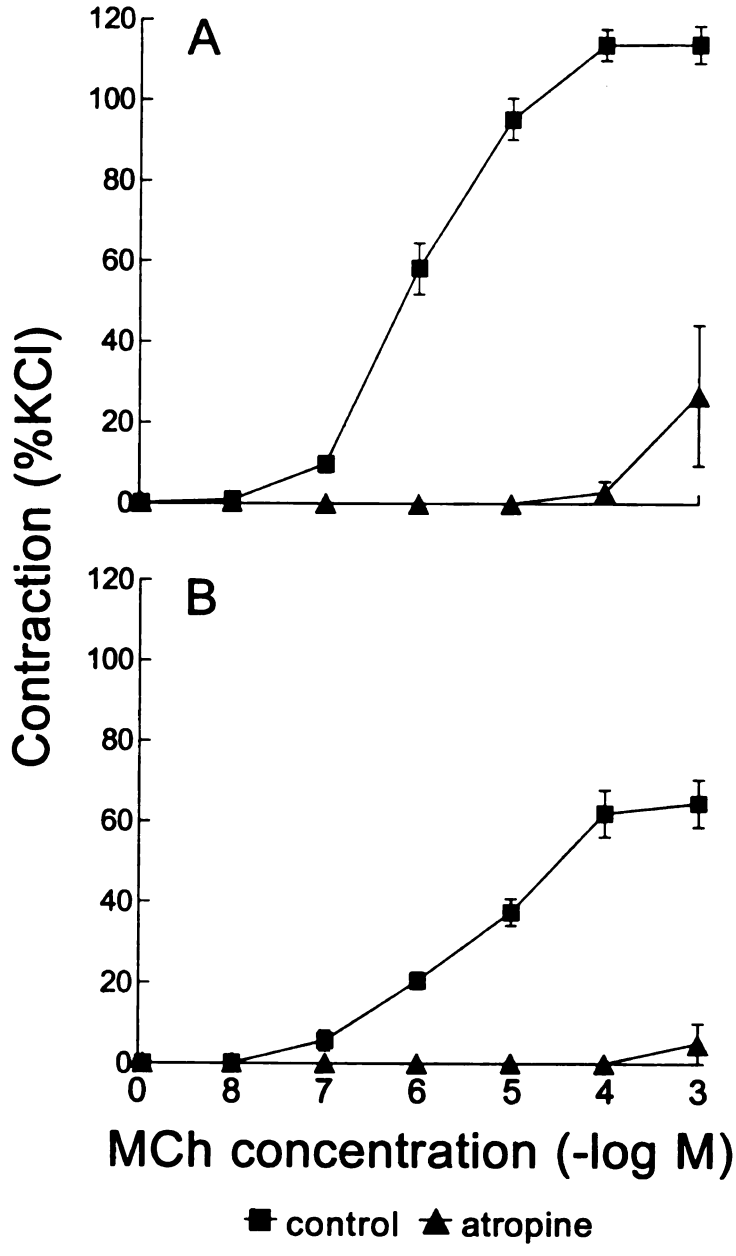

Figure 12. Contractile responses to MCh of equine SA (A) and equine LP (B). ■, Control; and ▲, with 3×10^{-6} M ATR; both tissue responses are significantly inhibited by ATR, $p < 0.001$ in two-way ANOVA, $n = 12$ animals.

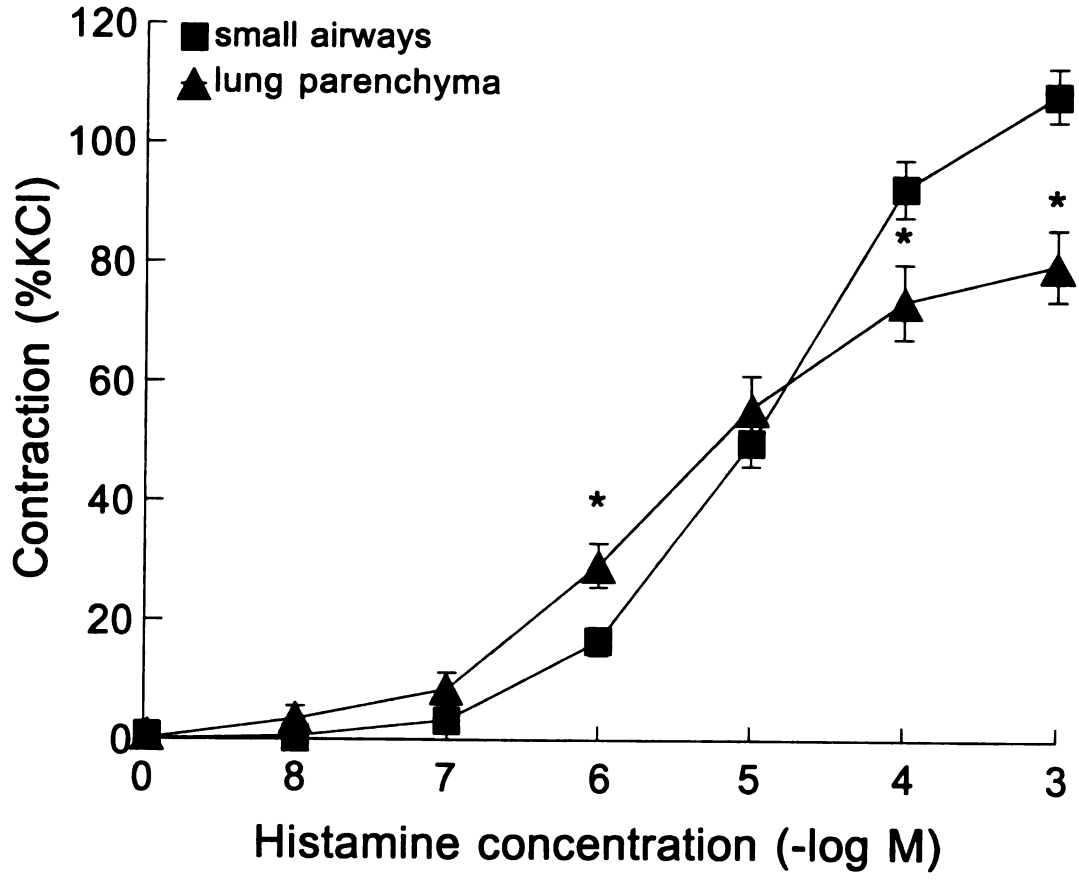

Figure 13. Contractile responses to histamine of equine SA, and LP, n = 9; *indicates significant differences between groups ($p \leq 0.05$).

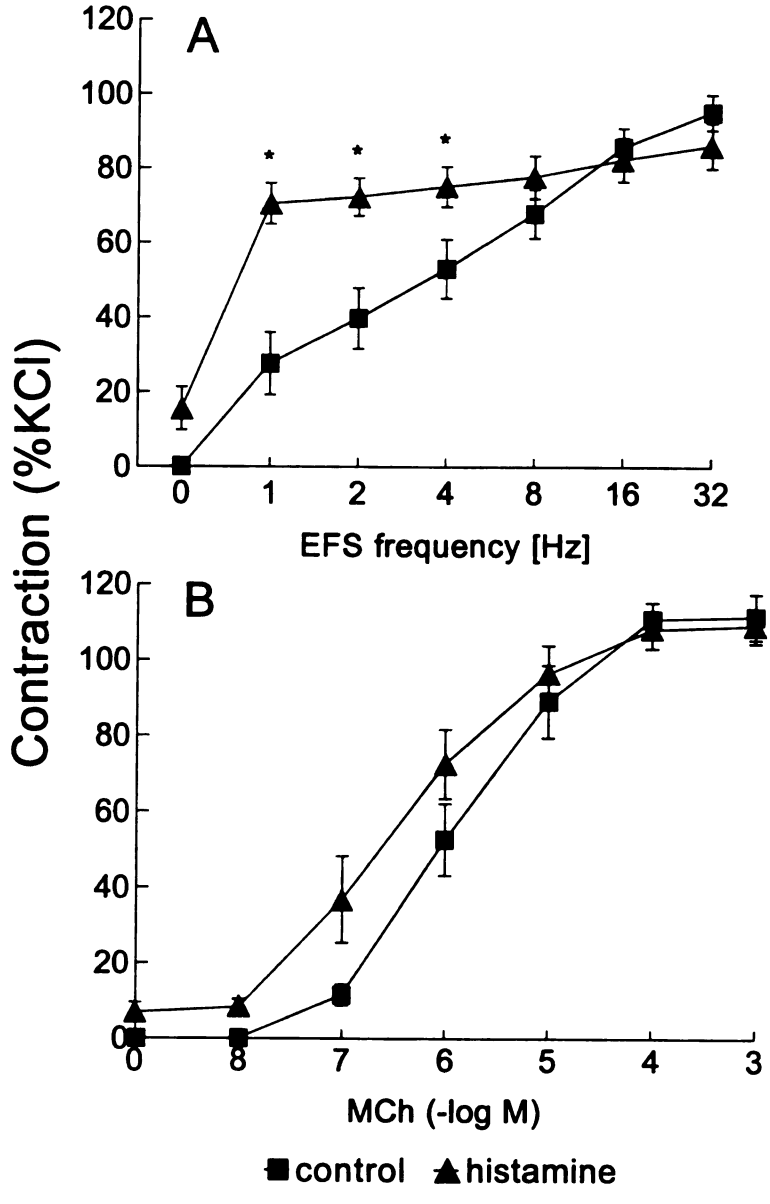

Figure 14. Responses of equine SA in presence of 3×10^{-6} M histamine to (A) EFS (20 V, 0.5 ms) and (B) MCh (10^{-8}–10^{-3} M). In both (A) and (B) the treatment was significantly different from control at $p < 0.001$ and $p = 0.002$, respectively (two-way ANOVA) *Indicates data points different from control ($p \leq 0.05$), $n = 6$ animals.

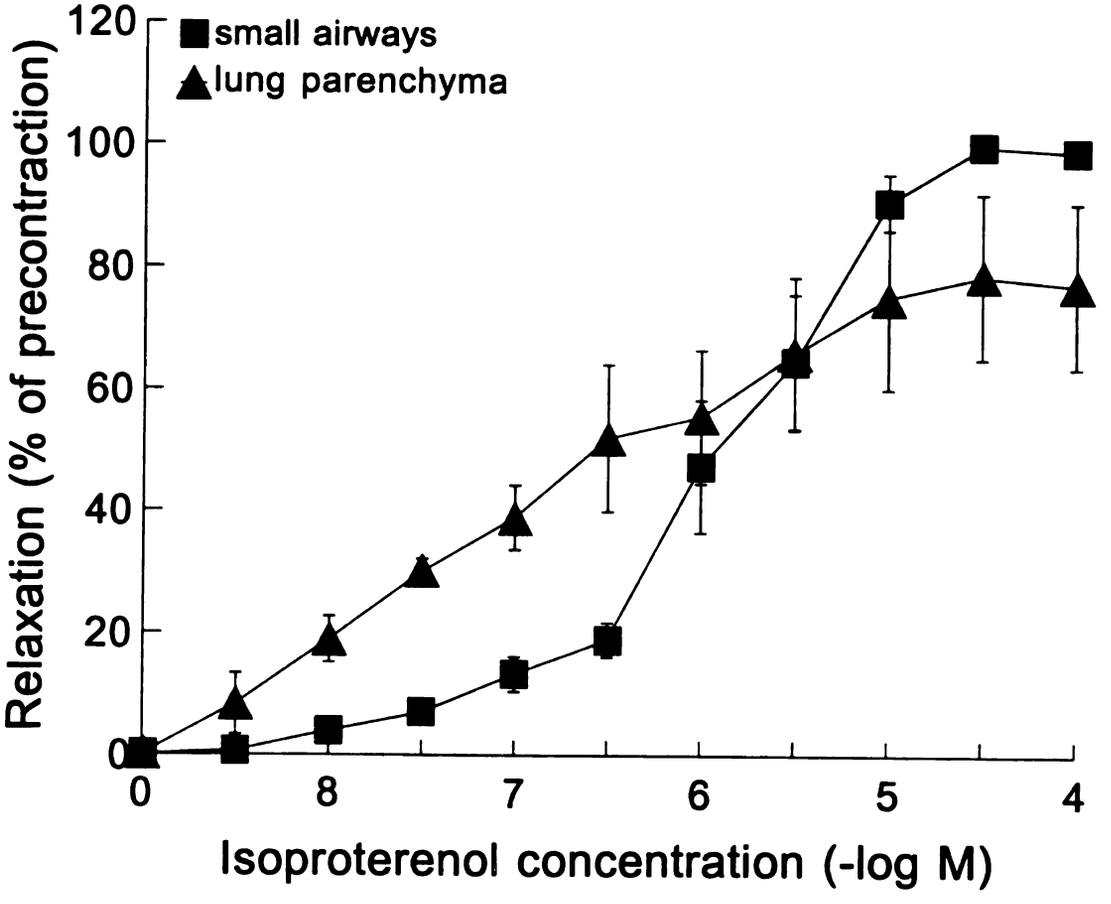

Figure 15. ISO-induced relaxation in trachealis contracted with histamine. Groups are significantly different, two-way ANOVA ($p < 0.001$).

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Chapter 5

RESPONSES OF EQUINE NEUTROPHILS: STUDY OF NEUTROPHIL ACTIVATION IN CONTROL AND RAO HORSES

Introduction

The neutrophil is a major inflammatory cell implicated in the pathogenesis of RAO. In this disease, neutrophil influx into the airway, in response to natural hay and straw, or mold spore challenges, is one of the most consistent observations.[7, 9, 11, 14, 17, 19, 21, 25-27] Thus measurements of neutrophil ratios in BAL fluid is by some considered to be a useful test for diagnosis of chronic pulmonary disease in the horse.[11] Even though neutrophils long have been suggested to be an important factor in the pathogenesis of RAO, the role of neutrophils in this disease remains unclear. Several studies that concurrently measured neutrophil populations in the airways and pulmonary function have indicated that the mere presence of neutrophil in the airways is not a sufficient factor to evoke airway obstruction.[10, 13, 19] However, in these studies, measurements of neutrophil activity were not performed. Thus, lack of a consistent relationship between airway responses and neutrophil recruitment in these studies could possibly arise from variation in neutrophil activity.

Not much information is available about the function of equine neutrophils in contrast to the extensive literature regarding the physiology and metabolism of

neutrophils in other species, particularly in human beings. From what is known, general properties of equine neutrophils are similar to those of other species and the functions of neutrophils, such as migration, phagocytosis, and both oxygen-dependent and -independent antimicrobial mechanisms have been demonstrated in the horse.[3, 12, 15, 22] On the other hand, the response of equine neutrophils to a variety of stimuli is not identical with the responses of other species. For example, fMLP, a formylmethionyl peptide, is a standard chemoattractant for neutrophils of many species, including human. fMLP does not exert chemotactic properties toward equine neutrophils, even though they possess a high-affinity cell surface receptor for this agent.[24] Because of these differences and the paucity of literature about the function of equine neutrophils, it was necessary to determine which compound would be optimal to activate equine neutrophils in vitro. In the present study, I determined respiratory burst activity by means of cytochrome C reduction assay. I have compared the level of activation induced by these compounds between control and heavey horses. To confirm that, in addition to the respiratory burst, other mechanisms are set in motion upon the activation of equine neutrophils, I measured the secretion of myeloperoxidase (MPO), an indicator of neutrophil degranulation. For the purpose of further studies, it was also important to investigate the time course of equine neutrophil responses to different stimuli.

Methods

Animals

For the purpose of this study, blood was collected from two groups of horses. Control horses were clinically healthy animals housed in stables at the MSU Veterinary

Teaching Hospital facilities. Only horses that were clinically normal, based on a daily examination and the temperature charts, were used for blood collection. Horses with a well-documented history of RAO formed the second group. They were brought into the stable a few days before blood collection (usually with their control counterpart) and they were observed for clinical signs of airway obstruction. Clinical score, which highly correlates with lung functions, was used to evaluate the level of airway obstruction.[8] When clinical signs of airway obstruction were obvious, peripheral blood was collected. Blood from both control and RAO horses was collected simultaneously so that both specimens could be tested concurrently during the same experiment, with the exception of experiments in which only control horses blood was used.

Isolation of peripheral blood neutrophils

Blood was collected by jugular venipuncture into sterile Vacuette tubes containing disodium ethylenediamine tetraacetate. A two-step isolation method was used as follows: buffy coat was collected from blood tubes after centrifugation (TJ-6R, Beckman Instruments, Palo Alto, CA) at 1500 rpm for 15 min and gently layered on the surface of a density gradient. A discontinuous density gradient was formed by two layers of 59 and 75% isosmotic, sterile Percoll solutions in 50-ml sterile tubes. After 45 minutes of centrifugation at 3000 rpm and 14°C, neutrophils, separated from other blood cells, accumulated in the form of a cloudy band at the gradient interface. After aspiration, neutrophils were suspended in Ca^{2+}, Mg^{2+}, and phenol-free Hanks' balanced salt solution (HBSS) and spun and washed twice. Isolated cell count and viability were assessed microscopically in a hemacytometer, after a small portion of isolate was diluted (1:100)

in a 0.04% solution of trypan blue. The purity of neutrophils was calculated based on a differential cell count of 200 cells in air-dried smears made from concentrated isolate suspended in a small amount of serum and stained with Hemacolor (Merck).

Measurement of superoxide production

I estimated neutrophil activation by several compounds, measuring $O_2^{-\cdot}$ production with the SOD-inhibitable Cytochrome C reduction assay.[1] Isolated neutrophils were suspended in sterile HBSS without phenol red, and aliquoted into the 4-ml glass tubes (1×10^6 cells/per sample). The tubes were paired, with each pair containing a SOD positive (+) and negative (-) sample. Superoxide dismutase 30 μg/ml was added to the SOD (+) tubes while cytochrome C (1 mg/ml) was added to all tubes immediately before beginning the assay. If the protocol required, the 4 μM cytochalasin B (CB) was added 5 min before the incubation with agonist started. Except for the negative control, all tubes also received neutrophil activators that varied depending on protocol. All of these reagents were added to the tubes with neutrophil suspensions in the volume of 100 μl (10% of final volume). The volume of each tube was adjusted to 1 ml by addition of HBSS if needed, so that the final concentration of neutrophils was 10^6/ml. Each assay was performed in duplicate.

Two types of protocols were performed: the dose-response tests with the standard incubation time of 30 min at 37°C; and time course tests in which one concentration of drug was tested, but the time of incubation varied. When incubation time was completed, tubes were cooled in ice and SOD was added to all SOD (-) samples to prevent further cytochrome C reduction. Next, the volume in each tube was adjusted to

1.85 ml with HBSS and the samples were centrifuged at 3000 rpm for 10 min, at 4°C (TJ-6R, Beckman Instruments, Palo Alto, CA). Supernatant was collected to assign sample absorbances at λ = 550 nm on a Gilford spectrophotometer. Superoxide production was calculated from the differences in absorbance between the SOD (+) and (-) values of given sample, and transformed utilizing molar extinction coefficient of 18.5 and dilution factor.

The response of neutrophils treated with phorbol ester, PMA 10 ng/ml formed the positive control in each experiment. As the negative control, I used neutrophils that were not treated with any stimuli. Neutrophils treated exclusively with CB were the control for the samples in which CB was used, beside the chemotactic ligands to activate neutrophils

Cytochrome C reduction assay protocols

Responses to AA and Ca ionophore (A23187) and PMA

Since AA and A23187 have been reported to activate neutrophils, I measured $O_2^{-}\cdot$ production in response to these compounds. Neutrophils were stimulated with 50 μm or 200 μm AA, 3 μm A23187 or 10 ng/ml PMA. The latter was used as a positive control.

Responses to chemotactic ligands

The dose-response relationship was determined for the chemotactic ligands fMLP (10^{-10}–10^{-6} M) and human recombinant complement fragment C5a (hrC5a, 10^{-10}–10^{-7} M). CB (4 μm) was used to enhance the neutrophil response.

The time course of response was determined in order to observe the dynamics of neutrophil activation by fMLP and hrC5a.

Responses to opsonized zymosan (OZ)

As in protocol 1, the dose-response to OZ (0.003–1 mg/ml) and the time course of the response to 1 mg/ml OZ were determined. Neutrophils exposed to PMA (10 ng/ml) and untreated ones served as positive and negative controls, respectively. The time course of the response to OZ was extended up to 1 hour.

Degranulation (MPO) assay

Secretory activity of neutrophils was measured with the help of the MPO assay. Isolated neutrophils (3.2×10^6/ml) were treated with 4 μM CB for 5 min and incubated with fMLP (10^{-8} or 10^{-7} M), C5a (10^{-9} or 10^{-8} M) or PMA 10 ng/ml. After 15 min incubation at 37°C, tubes were cooled on ice and centrifuged at 3000 rpm. Supernatant (0.2 ml) was collected to determine activity of MPO. A cell lysate was prepared by ultrasonic lysis in the presence of 0.33% Triton X. This lysate was used to determine 100% MPO activity. In addition to concentrated lysate obtained from the neutrophil suspension (3.2 mln/ml), dilutions in HBSS were made to create 50, 33, and 25% lysate solution for further determination of an MPO activity standard curve. From each concentration of lysate, 0.2 ml was used in the MPO activity assay, which was performed according to the procedure by Henson et al. (1979). Briefly, supernatant or lysates were incubated in the presence of H_2O_2 (0.0033%) and o-dianisidine (0.00833%) for 15 min at room temperature, after which 0.1 ml of sodium azide (1%) was added to

stop the reaction. Absorbance was read at λ = 490 nm on the spectrophotometer (Gilford). Absorbance value of supernatant was compared to the standard curve created by linear regression of data from lysate dilutions, and expressed as a % of total MPO activity. The secretory response of equine neutrophils was only studied in a few samples from control horses (n = 1 with fMLP, n = 1 with C5a and n = 2 with PMA).

Compounds used in the study

AA, A-23187, hrC5a fragment, CB, cytochrome C, o-dianisidine, HBSS, H_2O_2, fMLP, Percoll®, PMA, sodium azide, SOD, dismutase, and zymosan A were all obtained from Sigma Chemical Inc. Zymosan was diluted in sterile physiological saline solution (10 mg/ml), vortexed, and incubated at 100°C for 1 hour, cooled, spun, and washed with HBSS. In order to opsonize zymosan, fresh equine serum 1:1-diluted with HBSS without phenol was added to the zymosan pellet so that a final concentration of zymosan was 10 mg/ml. Tubes with serum and zymosan were vortexed vigorously and incubated for 30 min in 37°C. After incubation tubes were spun, supernatant removed, the pellet was resuspended in sterile HBSS, spun, washed twice, and diluted in sterile HBSS without phenol red at 10 mg/ml. Small portions of OZ solution were stored in the freezer and used as needed.

The CB, fMLP, A-23187, and PMA were dissolved in DMSO and frozen in small portions for use during experiments. Arachidonic acid and hrC5a were both diluted in sterile HBSS without phenol red, and stored in small portions at -70°C. Directly before assay, all compounds needed for assay were brought to room temperature and diluted in HBSS to a concentration 10 times greater than their final concentration required in the

assay. Similarly, stock solutions of Cytochrome C and SOD in HBSS without phenol red were prepared directly before assay. Stock solutions of o-dianisidine, H_2O_2, and sodium azide were prepared in deionized water and stored in the refrigerator.

Statistical analysis

Data shown in the text and figures are expressed as ($x \pm SEM$), and n represents the number of horses from which neutrophils were obtained to perform the protocol. Data were calculated and analyzed (Excel 7.0 by Microsoft and SSPS for Windows 7.0 by SSPS Inc. by means of paired or unpaired t-test, one-way ANOVA, or mixed-design factorial ANOVA as appropriate. Post-hoc Dunnet's test was used to compare means between the treatments and the controls. Means were accepted to be significantly different at $P \leq 0.05$.

Results

Neutrophil isolation

Using our procedure we isolated neutrophils with an average purity of 99.1 ± 0.15% and viability of 99.5 ± 0.15%, and numbers more than sufficient to perform all experiments planned on each day.

Superoxide production in neutrophils from control and horses with RAO

Baseline $O_2^{-}\cdot$ production

Neutrophils isolated from control (n = 10) or RAO horses (n = 9) when unstimulated produced only minimal amounts of $O_2^{-}\cdot$, on average 0.55 ± 0.3, and 0.92

± 0.5 nmol of $O_2^{-}\cdot/10^6$ cells, respectively (NS). The CB did not enhance this minimal $O_2^{-}\cdot$ production in unstimulated neutrophils. The average responses to 10 ng/ml PMA (positive control in all tests) were virtually identical in neutrophils from both groups producing over the 30-min period, 54.5 ± 4.9 and 53.7 ± 4.0 nmol of $O_2^{-}\cdot/10^6$ cells in 10 control horses and 9 horses with RAO, respectively.

Superoxide production in response to stimulation via intracellular mechanisms and chemotactic ligands

All agents tested, with the exception of AA, produced a statistically significant increase in $O_2^{-}\cdot$ production by peripheral blood neutrophils (Figure 16 and 17). As indicated on Figure 16, the response to A23187, Ca ionophore was very small (less than 10%) in comparison with PMA response. On average 3 times higher than the response to A23187 was that to chemotactic ligands fMLP and C5a, which both activated respiratory burst to similar degree (Figure 17). Responses to chemotactic ligands were tested in the presence of CB, since, in a pilot study, this agent greatly enhanced the response to fMLP. In the presence of CB, fMLP neutrophils from horses with RAO produced greater response, in paired experiments, and, in the dose-response curve to fMLP, this increase was significant (Figure 17).

Time course of fMLP and C5a and PMA responses

Comparison of the time course response between C5a, fMLP, and PMA demonstrated that C5a and fMLP activate the respiratory burst very rapidly, but only during the first 5 min is $O_2^{-}\cdot$ production observed. Beyond the initial 5 min, the level

of cytochrome C reduction remained constant (Figure 18). PMA-induced activation was long lasting and the relationship between time and $O_2^{-}\cdot$ production was linear over the 30-min period ($r = 0.993$, $p = 0.00007$).

Response to OZ

The $O_2^{-}\cdot$ production in response to OZ is shown in Figure 19. This response was concentration-dependent and, in the 30-min assay, more than two-fold greater than the response to the chemotactic ligands. However, the response to OZ was still only about a half of that induced in neutrophils by PMA. Since OZ at 1 mg/ml appeared to induce submaximal $O_2^{-}\cdot$ production, an additional concentration of 3 mg/ml was used in 2 animals from each group. 3 mg/ml OZ produced only a slightly higher response than 1 mg OZ in the control animal neutrophils, and no further increase in neutrophils from RAO horses, indicating that response had reached a plateau (not shown). The response to OZ in the RAO group was significantly greater than in controls (Figure 19). The increase of $O_2^{-}\cdot$ production in RAO animals was the greatest at OZ concentration of 1 mg/ml.

The time course of $O_2^{-}\cdot$ production in response to 1mg/ml OZ and PMA is plotted in Figure 20. The rate of $O_2^{-}\cdot$ production was slower than that caused by PMA, but the increase of cytochrome C reduction by OZ-activated neutrophils was still observed within 60 min of incubation.

Myeloperoxidase assay

Both fMLP and C5a induced release of MPO from neutrophils. In contrast to the undetectable MPO activity in unstimulated cells, neutrophils treated with 10^{-8} M fMLP (n = 1) and 10^{-8} M C5a (n = 1) released approximately 16% of total MPO activity from horse neutrophils, while 10^{-7} M fMLP released approximately 20% (Figure 21). In both animals, PMA did not induce neutrophil degranulation.

Discussion

In the present study I have determined the activity of equine peripheral blood neutrophils isolated from control and RAO-affected horses to stimulation with several types of agents. The purpose of these experiments was to select the compound which would cause robust activation of neutrophils that could be maintained for a long period of time. This compound would be appropriate for further study of the effects of neutrophils on equine airways provided it did not directly affect the airway smooth muscle response. It was also important to compare the activity of neutrophils isolated from control and RAO animals, because their differential activation could potentially be responsible for differential effects of neutrophils within the airways. Finally, because of insufficiency of literature describing function of equine neutrophils, it was important to study neutrophil functions, to provide additional information about the behavior of equine neutrophils, particularly the time course of neutrophil responses.

I demonstrated that virtually all agents tested, with the exception of AA, induced measurable respiratory burst activity in horse neutrophils. Lack of response to AA is not consistent with an earlier study of equine neutrophils. In 1992, Bochsler et al. using the

cytochrome C reduction assay determined that 50-200 μg AA stimulates $O_2^{-}\cdot$ production of similar magnitude as PMA.[3] However, my experiments, repeated in 5 animals, using several batches of AA in the presence of both negative and positive controls have consistently shown a lack of response to 200 μg AA. It is possible that lack of measurable cytochrome reduction is caused by interference of AA with cytochrome C reduction, despite the respiratory burst induction in neutrophils by AA. Besides this discrepancy in the case of AA, my data were consistent with those of Bochsler, whenever identical compounds were used.[3]

The amounts of $O_2^{-}\cdot$ produced by equine neutrophils in response to other agents varied depending on the type of stimulus (Figure 16, 17, 19). For example, the response to A23187, represents only a minimal fraction of the response to PMA. A similar, relatively low production of $O_2^{-}\cdot$ in response to A23187 was observed in other species. This is consistent with the current understanding of the role of $[Ca^{2+}]_i$ level in the mechanism of neutrophil activation. Even though, $[Ca^{2+}]_i$ is thought to play an important role in the response to neutrophil stimulation, aspects of neutrophil responses other than respiratory burst are predominantly associated with the rise of $[Ca^{2+}]_i$.[2, 6] The concentration-dependent response to chemotactic ligands, fMLP and C5a was identical and on average 3 times higher than to A23187. My study, for the first time, demonstrated that fMLP may produce a significant respiratory burst, approximately equal to 20% of the response to PMA. However, to observe this effect, the presence of CB was required. The CB is a fungal product which interferes with the changes in cytoskeleton that are required for shape change and the cellular motion. It is also thought to prevent the internalization of receptors to which chemotactic ligands are bound, which prevents

rapid desensitization of neutrophils. This process is probably even more important in the horse than in the other species, because the horse possesses less than 1000 fMLP receptors per neutrophil.[4, 24] This very low number of receptors (10-1000 times less then in other species) was suggested to account for lack of the chemotactic response to fMLP, in contrast to hrC5a that induced neutrophil migration.[24] In the same study, equine neutrophils pretreated with CB, released lysosomal enzymes in response to fMLP, reaching maximum activity at 10^{-7} M fMLP. This observation is consistent with my present results, with respect to the maximum of $O_2^{-}\cdot$ production achieved at 10^{-7} M fMLP and enhanced neutrophil respiratory burst response in the presence of CB.[16]

The complement fragment hrC5a is another chemotactic molecule. In contrast to fMLP, C5a induces chemotaxis of equine neutrophils both in vivo and in vitro, and it has been reported to induce relatively high $O_2^{-}\cdot$ production.[3, 5, 23] Therefore I expected at least slightly higher neutrophil response to hrC5a in comparison with that to fMLP. Surprisingly, $O_2^{-}\cdot$ production in response to both hrC5a and fMLP were similar, and the hrC5a potency appeared even lower than that of fMLP. Similar magnitude of effect between these two compounds was also observed in the MPO assay.

Studying $O_2^{-}\cdot$ induction with hrC5a and fMLP, two interesting observations were made. The first regarded the time course of neutrophil activation with these compounds, since both fMLP and C5a induced $O_2^{-}\cdot$ production for approximately 5 min. After the initial 5 min of the respiratory burst activity, no further evidence of $O_2^{-}\cdot$ production could be detected (Figure 18). This observation is consistent with a recent study of respiratory burst activity in equine neutrophils, utilizing lucigenin dependent chemiluminescence. In this study Brazil et al. demonstrated that fMLP stimulates equine

neutrophils in a similar time frame.[4] This transient activation of respiratory burst by both chemotactic ligands explains the relatively weaker response of neutrophils to stimulation with chemotactic ligands in comparison to PMA, which generated $O_2^{-}\cdot$ with a constant rate over the 30-min assay. In contrast, during the 5-min assay, responses induced by hrC5a and PMA were both similar, as in my experiment.

The second important observation was the difference in responses of neutrophils between the control and RAO horses. The respiratory burst response in neutrophils of RAO horses to chemotactic ligands was approximately 30-50% greater than in controls. The number of experiments performed with fMLP allowed me to detect a significant increase in response of neutrophils from RAO horses. Since the control and heavey horses were kept in a similar environment, this phenomenon was not associated with an environmental factor. Furthermore the positive control response to (PMA) between the two groups was similar. Thus, intrinsic respiratory burst capacity of neutrophils in both groups of horses was identical. These data are consistent with observation made by Marr et al. who tested responses of neutrophils in horses utilizing the Cytochrome C assay.[18] These authors observed that peripheral blood neutrophils from both control and RAO horses produce similar responses, but in neutrophils from horses with RAO, after antigen challenge, particularly in the presence of CB, there was a trend of increasing response.[18] Evidence is sufficient to suggest that the circulating neutrophils in RAO horses are possibly exposed to certain endogenous factors (mediators) produced in response to antigen challenge, and therefore become activated more easily than those from control horses. This increased excitability of neutrophils could also explain the increased influx

of neutrophils into the airways of RAO horses in comparison with the control horses challenged with identical antigens.[10, 19, 20]

Another model neutrophil activator tested in this series of experiments was OZ. This particulate stimulus induces phagocytic activity of neutrophils, and, in this natural way, sets in motion both respiratory burst and neutrophil degranulation. In our 30 min. assay, OZ induced a respiratory burst response which was over two-fold greater than that induced by chemotactic ligands. This response is still only about half that induced by PMA. The rate of respiratory burst activation induced by 1mg/ml OZ, in comparison with PMA, was lower. However, in contrast to chemotactic ligands, the response to OZ persisted for 60 min. This long-lasting effect of OZ was responsible for much higher production of $O_2^{-}\cdot$ recorded within the 30 min assay in comparison to fMLP or C5a . On the other hand, the slower rate of $O_2^{-}\cdot$ production induced by OZ versus PMA, resulted in the relatively lower $O_2^{-}\cdot$ production in comparison to PMA response. Similar to the increased response to chemotactic ligands in RAO horses, the response of neutrophils to OZ was significantly greater in the RAO group than in the control.

After these experiments, I selected OZ as the best stimulus for use as an activator of neutrophils in order to study their effects on equine airway responses. The response induced by OZ was relatively large, and the stimulation persists for a long period of time. This method of neutrophil activation appears also to be the most natural, producing the full range of neutrophil activity via the process of phagocytosis. In contrast, activation of neutrophils with chemotactic ligands requires enhancement via CB. Also the PMA-induced activation of neutrophils does not produce a whole spectrum of neutrophil response. It results in rapid and prolonged respiratory burst; however,

without neutrophil degranulation. Moreover, PMA has been reported to induce airway smooth muscle contraction and for that reason it could not be used in my tension study.

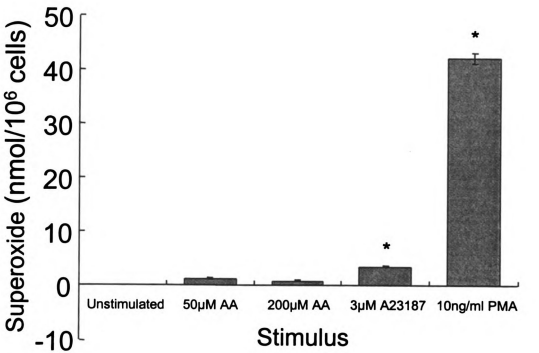

Figure 16. Production of superoxide (nM/10^6 cells) in neutrophils from the control horses treated with 50 μM and 200 μM arachidonic acid (AA), 3 μM calcium ionophore (A23187), and 10 ng/ml phorbol ester (PMA). Baseline superoxide production from unstimulated neutrophils represents the control. Number of animals in each group equals 2, 5, 2, 4, and 5, respectively. * = significantly different from control $p \leq 0.05$.

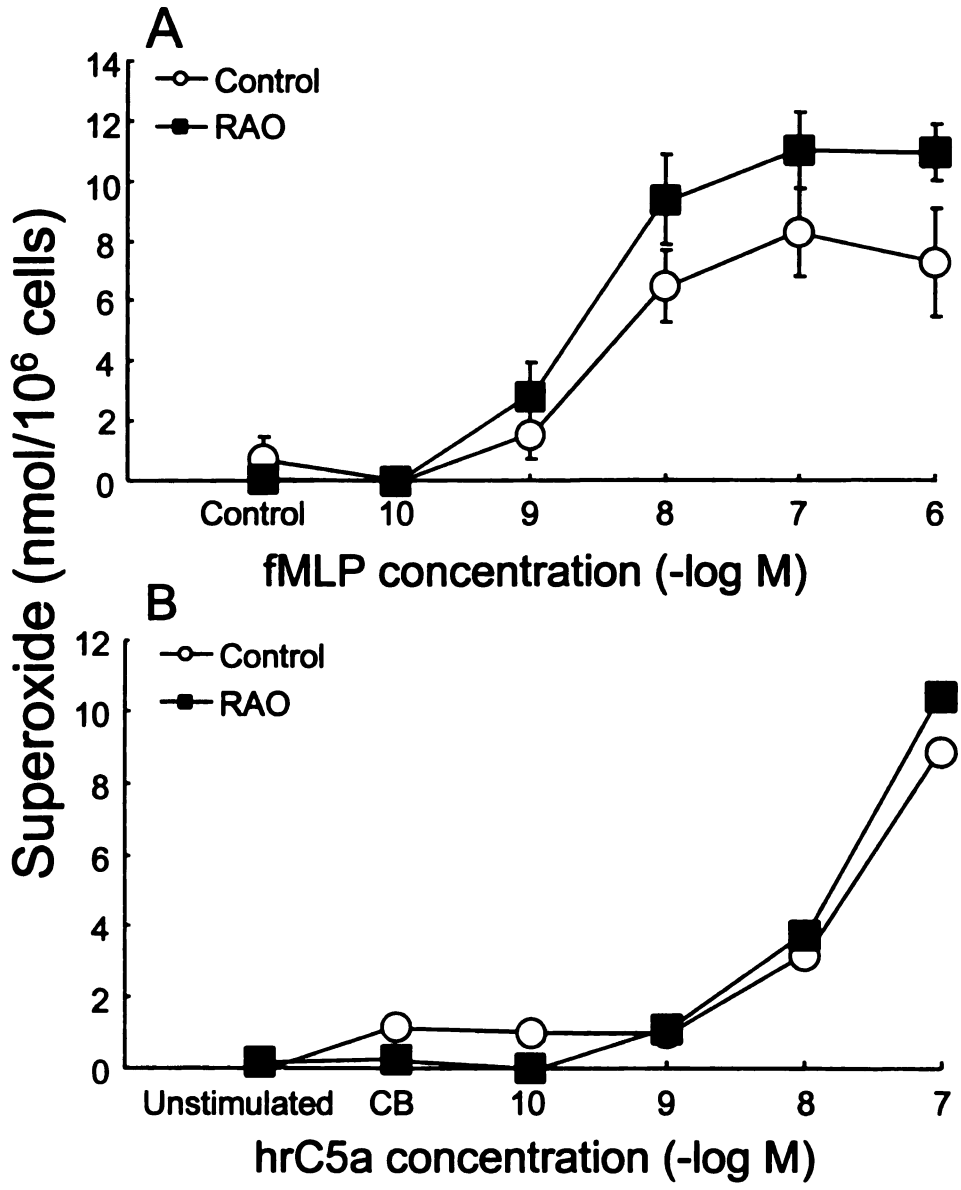

Figure 17. Production of superoxide (nM/10^6 cells) in neutrophils from control and RAO horses treated with 10^{-10}–10^{-6} M formylmethionyl peptide (fMLP, panel A), and 10^{-10}–10^{-7} M human recombinant complement fragment C5a (hrC5a, panel B). Cytochalasin B (CB, 4 μm) was used to enhance the neutrophil responses, and the response to 10 ng/ml phorbol ester (PMA) served as positive control. The response to fMLP was significantly greater in RAO group (ANOVA $p < 0.05$) and significant fMLP-concentration × horse interaction was observed ($p < 0.02$). (In panel A, $n = 6$ and $n = 5$ of control and RAO horses, respectively; in panel B, $n = 2$ for each group of animals.)

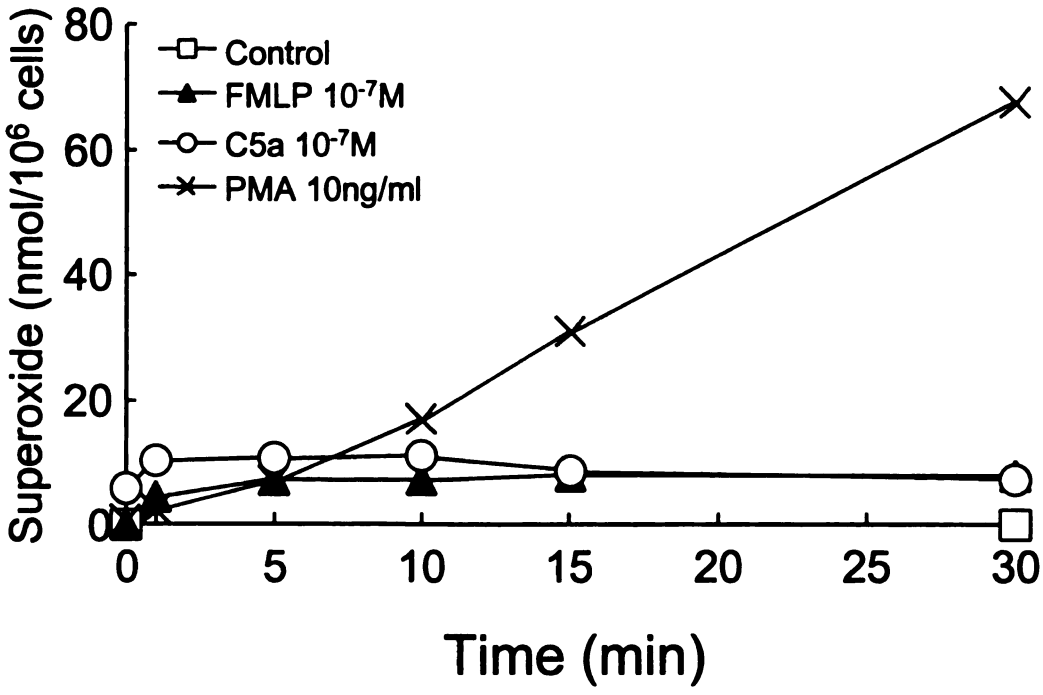

Figure 18. Time course of superoxide (nM/10^6 cells) production in response to 10^{-7} M formylmethionyl peptide (fMLP), 10^{-7} M human recombinant complement fragment C5a (hrC5a), and 10 ng/ml phorbol ester (PMA) in neutrophils from control horses. Cytochalasin B (CB, 4 μm) was used to enhance the neutrophil response to both C5a and fMLP. n = 1 for both chemotactic ligands, and n = 2 for PMA.

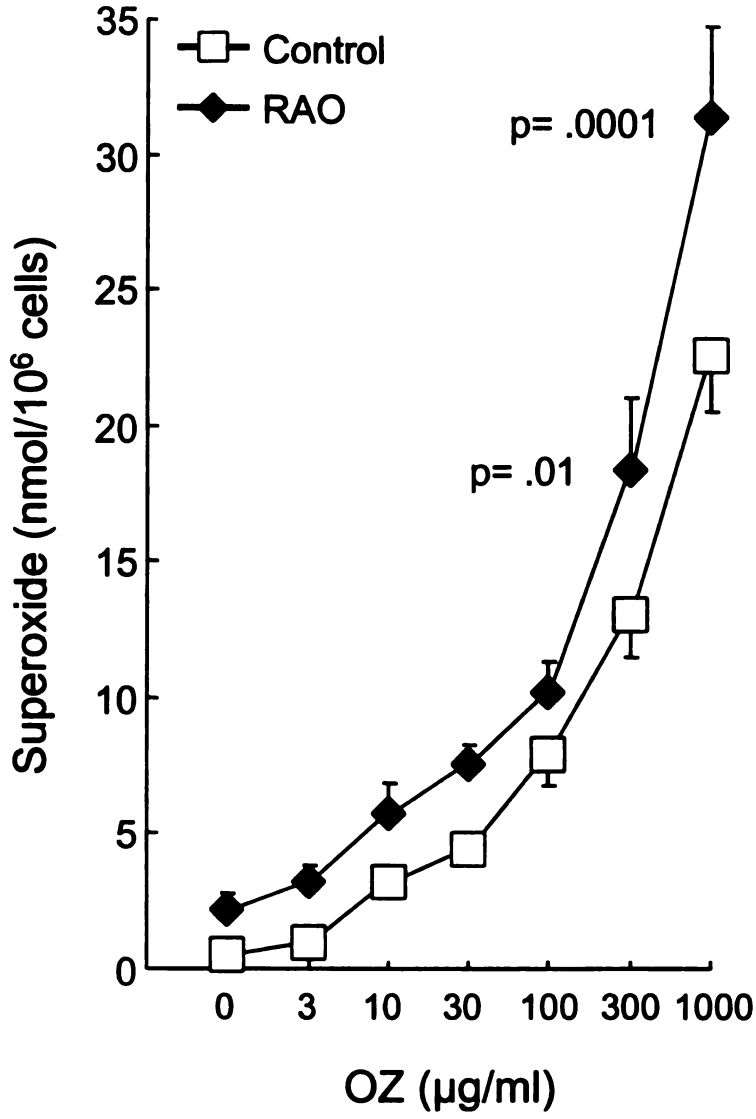

Figure 19. Production of superoxide (nM/10^6 cells) in neutrophils from controls (n = 8) and RAO (n = 5) horses treated with 3-1,000 μg of opsonized zymosan (OZ). Response to OZ was significantly greater in RAO group ($p = 0.01$ and $p < 0.001$ at 300 and 1,000 μg/ml of OZ, respectively).

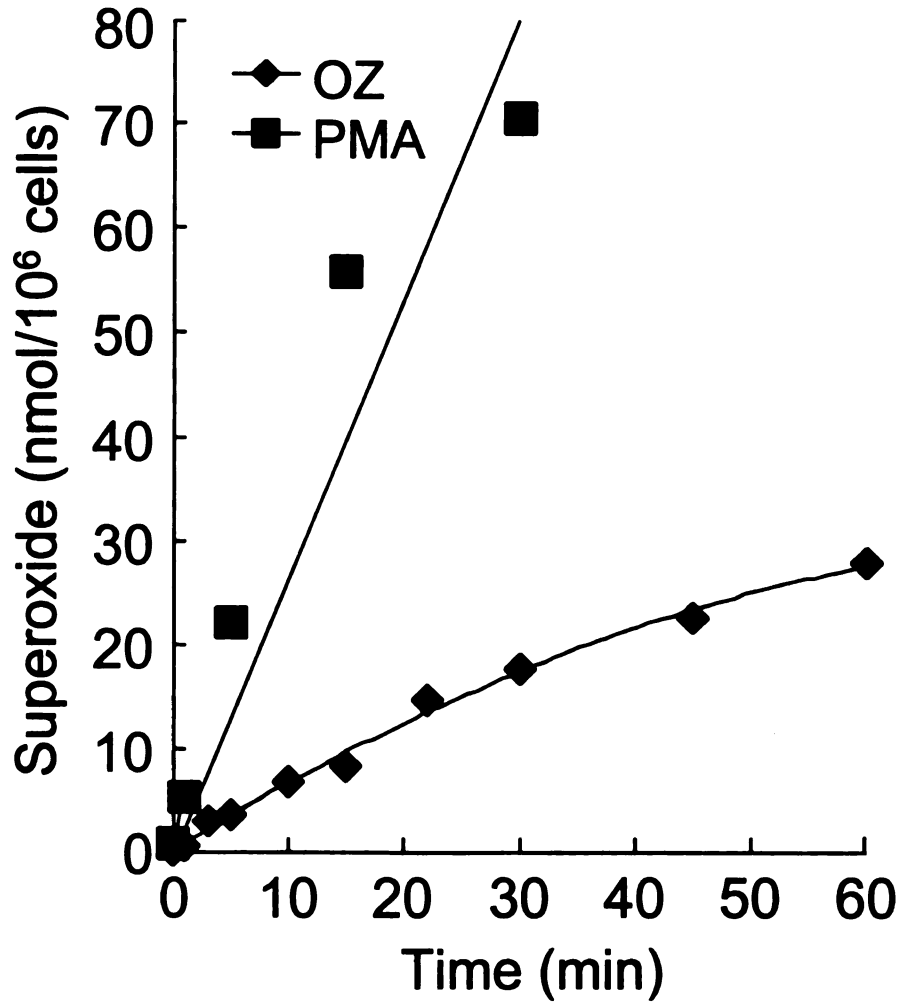

Figure 20. Time course of superoxide (nM/10^6 cells) production response to 1,000 μg/ml of opsonized zymosan (OZ) and 10 ng/ml phorbol ester (PMA) in neutrophils from control horses. Neutrophils were from one control horse.

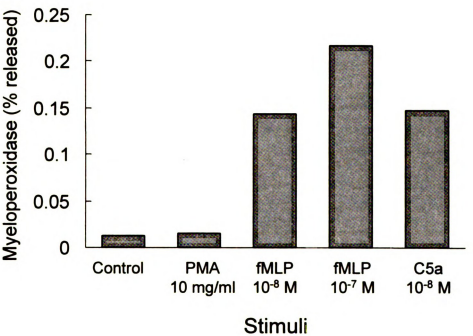

Figure 21. Degranulation of neutrophils, represented as MPO activity in control horse neutrophils treated with fMLP, hrC5a (n = 1), and PMA (n = 2). All cells, including control, were treated with 4 μM cytochalasin B to enhance the degranulation process.

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Chapter 6

INFLAMMATORY MEDIATORS, ACTIVATED NEUTROPHILS, AND BRONCHOSPASM IN EQUINE SMALL AIRWAYS

Abstract

Neutrophilic inflammation in SA, and bronchospasm mediated via M receptors, are features of RAO in horses. Histamine, 5-HT, and LTs are reported to be involved in exacerbation of RAO, and currently histamine has been shown to increase tension response to electrical field simulation (EFS) in equine SA. We have tested the effects of these mediators and the effects of activated neutrophils on the cholinergic responses in SA. Histamine, 5-HT, and LTD_4 had a synergistic effect on EFS responses and only an additive effect on the tension response to exogenous ACh or MCh. Atropine and TTX entirely eliminated the EFS-induced tension response in the presence of all three inflammatory mediators, indicating that augmentation of EFS response applies only to the endogenous cholinergic response. Neutrophils isolated from control and RAO-affected horses were activated by zymosan producing 18.1 $\pm$ 2.3 and 25.0 $\pm$ 2.3 nmol $O_2^{-}\cdot/10^6$ cells/30 min, respectively. However in contrast to the profound effect of mediators, incubation of SA for over 1 hour, in a suspension of zymosan-treated neutrophils up to 30×10^6 cells per ml, did not significantly affect EFS responses of SA isolated from either control or RAO-affected horses. We conclude that in equine SA 1) the endogenous cholinergic responses are subject

to strong facilitation by inflammatory mediators; 2) activated neutrophils do not affect cholinergic responses in SA; and 3) in acute bouts of equine RAO, histamine, LTD_4, and 5-HT (mediators primarily associated with type I allergic reaction), rather than mediators derived from neutrophils, most likely contribute to increased cholinergic airway tone.

Introduction

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

Inhalation of allergens by RAO horses leads to several inflammatory events in the airways, such as neutrophil recruitment and activation,[12, 21] changes in lymphocyte populations, release of histamine from airway mast cells,[19] and activation of the AA cascade in the airway mucosa with a significant shift in the lipid mediator profile.[14] The latter results in a decrease in mucosal PGE_2 production, and an increase of pro-inflammatory lipid mediators such as TX, 15-HETE, cysteinyl LTs, and PAF.[10, 13, 14] We propose that inflammatory mediators released in response to antigen challenge are responsible for altered

cholinergic responses of the airways in RAO. In this context the discrepancy between in vitro tissue behavior and in vivo airway responses could be caused by washout of these mediators from the tissues in vitro, before measurements of tension or ACh release. Additionally, earlier studies were conducted on trachea and bronchi, whereas in RAO the most predominant inflammatory response (retention of mucopurulent secretion and airway wall infiltration) occurs in peripheral airways. Therefore, if inflammation is the source of altered cholinergic tone in the airways, detectable changes in cholinergic responses may be limited to SA.

Some of these issues were addressed in a previous study, in which we confirmed that normal equine SA (20th generation) produce entirely cholinergic contractions in response to nerve stimulation by EFS. These contractions were increased by cyclooxygenase blockade or application of histamine, indicating that inflammatory mediators may have a profound effect on responses to nerve stimulation in equine SA.[22] In the present study, we extended our research to investigate further the effects of inflammation on cholinergic mechanisms in equine SA. Several approaches were used to reach our goal. We have used SA from both control and acutely RAO animals to compare their responses to EFS. Because histamine had quite dramatic effects on the EFS response and virtually no effect on the MCh concentration-response curve, we examined whether cholinergic or other mechanisms are responsible for the effects of histamine on EFS response. We also tested the effect of other inflammatory mediators, reported to be involved in RAO, namely LTD_4 and 5-HT, on cholinergic SA responses. Finally, we were particularly interested in effects of neutrophil-derived mediators, on cholinergic airway responses for many reasons. First in contrast to other inflammatory cells, the number of neutrophils in the airways consistently increases during an exacerbation of RAO.[8, 12, 29] Neutrophil recruitment takes place within a few hours of natural antigen

challenge and in most cases parallels early changes in lung function,[12] neutrophils washed from airways of RAO horses are strongly activated,[21] and, as shown in multiple studies, neutrophil number in bronchoalveolar fluid generally increases with the severity of airway obstruction.[32, 33] Additionally, it has been shown in dog airways that neutrophils are critical to development of both ozone and intravenous-PAF induced hyperresponsiveness.[5] Thus in our final approach we have used isolated and activated neutrophils to test if mediators released acutely from these cells activated in proximity of SA are capable of altering their cholinergic responses.

Methods

Animals

Control horses and horses with RAO were tissue donors for this in vitro study, which was approved by the All-University Committee on Animal Use and Care at Michigan State University. The control group consisted of 17 geldings and mares of various breeds, 7.3 ± 0.8 years old, weighing 901.4 ± 20.2 kg, and free of signs of respiratory disease. At 48 hours before euthanasia, animals were brought from the pasture into the barn and kept on hay and straw.

To induce airway obstruction in horses with a history of RAO, 5 horses (geldings and mares of various breeds, 13.4 ± 4.2 years old, weighing 1148 ± 79.4 kg) were brought from the pasture into the barn and kept on hay and straw until significant clinical signs of airway obstruction had developed (on average 1 week). We assessed the severity of airway obstruction on a daily basis by means of a clinical score system that is tightly correlated with changes in pulmonary functions.[26] As blood donors we have used 2 horses with heaves that

consistently developed severe airway obstruction within 1–2 days of stabling, and 4 control horses stabled in the barn.

Tissue collection

Animals were killed by intravenous injection of pentobarbital sodium, the rib cage was opened, and heart, lung, and trachea excised. Immediately after death the cardiac region of the right lung was collected and suspended in K-H solution (K-H) (composition in mM: 118.4 NaCl, 25.0 $NaHCO_3$, 11.7 dextrose, 4.7 KCl, 2.6 $CaCl_2.2H_2O$, 1.19 $MgSO_4$. 7 H_2O, and 1.16 KH_2PO_4) saturated with 95% O_2 and 5% CO_2. During dissection and experimental protocols, tissues were kept in K-H that was continuously gassed with 95% O_2 and 5% CO_2.

Tissue preparation

Small airway preparations were isolated from the peripheral part of the cardiac region following procedures described previously.[22] In this anatomical location, 1–2 mm OD horse airways represent generation 12–16 and are the smallest airways that still contain some cartilaginous elements. Dissected SA preparations were placed in a 2-ml tissue bath (Radnoti Glass Technology) filled with K-H (38°C), which was replaced every 15 min and bubbled with 95% O_2 and 5% CO_2 during the entire experiment. The lower end of the SA preparations was fixed with surgical silk ties to the glass tissue holder, which secured the tissue at the bottom of the tissue bath. The upper tie was hooked to a force transducer (Model FT 03 Grass Instrument Company, Quincy, MA) installed on a tension manipulator. Isometric force of tissue preparations was recorded on a polygraph (Model 7D or 7E, Grass Instrument Company, Quincy, MA). In this setup, tissues were suspended in the middle of

the tissue bath between two platinum wire electrodes built vertically in the wall of tissue bath. During 2-hour equilibration optimal passive tension was determined by gentle stretching of the tissue and use of 127 mM KCl to induce contraction, followed by 2 applications of EFS (0.5 ms, 1 Hz, 20 V) at 30-min intervals. Optimal tension was in the range of 2–2.2 g. Square wave EFS impulses were generated by a stimulator (Model S88, Grass Instrument Company, Quincy, MA) and delivered to the electrodes via a stimulus power booster (Stimu-Splitter II, Med Lab Instrument, Loveland, CO). After equilibration we treated tissues with KCl for a second time to determine maximal response (100% KCl). The experimental protocols were conducted in 8 muscle baths.

Blood collection and neutrophil isolation

For protocols that required neutrophils, peripheral blood (60 ml from each horse) was collected into EDTA-containing Vacutainer® tubes via jugular venipuncture from an acutely RAO and a control horse. A two-step isolation method was used as follows: buffy coat was collected from blood tubes after centrifugation (TJ-6R, Beckman Instruments, Palo Alto, CA) at 1500 rpm for 15 min and gently layered on the surface of a density gradient made of 59 and 75% isosmotic Percoll solutions. After 45 minutes of centrifugation at 3000 rpm and 14°C, neutrophils accumulated in the form of a cloudy band at the gradient interface. After aspiration, neutrophils were suspended in Ca, Mg, and phenol- free HBSS and spun and washed for 2 times. Cell count, purity, and viability were assessed, and activity of neutrophils stimulated with opsonized zymosan (OZ) was determined by measurement of $O_2^{-}\cdot$ production. For that purpose we used the SOD inhibitable ferricytochrome C reduction assay according

to method developed by Babior et al.[2] and adapted by Tithof et al.,[28] who provided its detailed description in their papers.

Protocols

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

Inflammatory mediators

In the first subset of experiments we tested the effects of inflammatory mediators on SA from control horses. In all protocols, one of the tissues was not treated with any inflammatory mediators and served as a time control. Other tissues, depending on the protocol, were each treated with one concentration of mediator: 3 μM histamine, or LTD_4 (0.3, 1, 3, 10 nMol), or 5-HT (0.01, 0.1, 1, 10 μM). After 15 min of incubation, EFS frequency-response curves were created by application of increasing EFS frequencies (0.05–32 Hz). Frequency was increased when the response to lower frequency had reached a plateau. In order to confirm that mediators affected exclusively the endogenous cholinergic response to EFS (neurally released ACh) two additional tissues were treated: one with a sodium channel blocker TTX (3 μM) and the other with the M receptor antagonist ATR (3 μM). The latter tissues were treated prior to EFS with a concentration of inflammatory mediator, which in a pilot study had the greatest effect on the EFS response.

After the EFS response curve, tissues were washed thoroughly with fresh buffer (with or without inhibitor) and rested for 30 min. The same concentrations of inflammatory

mediator were then added to each tissue bath and after a 15-min incubation period, the concentration-response curves to ACh or MCh (depending on the protocol) were created.

Effects of neutrophils

In a second subset of experiments, we compared responses to EFS in SA of control and horses with ARAO and tested the effects of activated neutrophils on these responses. Because neutrophils of horses with RAO may have different mediator profiles or alternatively the sensitivity of SA to neutrophil-derived mediators in these horses could be different, we applied a crossover design with 4 combinations of neutrophils and tissues isolated from both RAO affected and control horses. In this protocol, untreated control tissue and tissue treated solely with OZ were included in addition to 6 tissue baths in which SA were treated with a mixture of neutrophils and OZ (3 mg/ml). Each time, we used neutrophils isolated from both control and RAO horses. SA were incubated with 3, 10, or 30 $\times$ 10^6 neutrophils/ml. During experiments with neutrophils, the first EFS frequency-response curve was created prior to incubation with neutrophils; the second and third EFS curves were created at 30 and 60 min of incubation in the presence of OZ-activated neutrophils, respectively.

Prior to the series of experiments with SA, we performed pilot studies in which we measured $O_2^{-}\cdot$ production in response to chemotactic ligands (fMLP, hrC5a, LTB_4) and OZ in the neutrophils isolated from peripheral blood of both control and RAO horses. The purpose of these studies was to select the optimal neutrophil activator and to compare responses of neutrophils isolated from control and RAO horses. Additionally, in order to confirm that neutrophils maintained their activity at the time of incubation, activity of neutrophils was tested. We used a fraction of the neutrophils isolated for our tissue

experiment, treated them with OZ and measured $O_2^{-}\cdot$ production by means of the cytochrome C reduction assay.

Agents

On the day of the experiments AA, ATR sulfate, histamine hydrochloride, 5-HT, MCh, and TTX (all from Sigma Chemical Company, St. Louis, MO), were dissolved in deionized water to obtain stock solutions (10 or 100 mM) as needed. The LTD_4 (Calbiochem) was diluted in K-H to 10 μM and frozen in portions that were diluted for use shortly before addition to the tissue baths. Stock solutions of ATR and TTX were directly mixed into K-H, other compounds were serially diluted in K-H, and each concentration was added to the muscle baths in a volume of 1%. The concentrations of all substances are expressed as their final bath concentration. Cytochrome C, fMLP, HBSS salts, Percoll and SOD were all from Sigma. Sterile Percoll, after adjustment of osmolality, and pH by addition of 10X HBSS and 1-n HCl, was diluted to 59% and 75% solutions in HBSS sterile Ca, Mg, and phenol red free and carefully layered in 50 ml tubes as a discontinuous gradient. Superoxide dismutase and cytochrome C were dissolved in sterile HBBS without phenol red. All solutions were prepared directly before use. Zymosan (Sigma) was prepared according to the producer's guidelines, and opsonized in equine serum. Small portions of OZ were frozen and stored at -20°C, and each portion was brought to room temperature directly before use.

Statistics

Tension study data ($\bar{x} \pm$ SEM) shown in the text and figures are expressed as a percentage of response to 127 mM KCl substituted K-H, and n represents the number of horses used in each protocol. To determine drug effects, we applied between-bath comparisons of treated and control tissues. This excluded any effects of time or tachyphylaxis. Data were calculated and analyzed (Excel 7.0 by Microsoft and SSPS for Windows 7.0 by SSPS Inc., on Gateway 2000 P5-133 computer) by means of paired or unpaired t-test, one-way ANOVA, or mixed-design factorial ANOVA as appropriate. Post-hoc Dunnet's test was used to compare means between the treatments and the controls. Means were accepted to be significantly different at $P \leq 0.05$.

Results

Inflammatory mediators

Effect of LTD_4

Leukotriene D_4 contracted the airway in a concentration-dependent manner (Figure 22; however, there was a great deal of variability among individual tissue responses to LTD_4. Response to EFS was augmented by LTD_4 (Figure 23A) and this synergistic effect of LTD_4 in the range of 0.3–3 nM was clearly dose-dependent. Increase of the response to EFS was greatest when tissues were slightly contracted by LTD_4; however, elevation of baseline tension was not absolutely necessary for this augmentation to occur. Both TTX and ATR completely blocked responses to EFS in the presence of LTD_4 (Figure 23B). The response to MCh was additive with the LTD_4-induced contraction but not synergistic (Figure 23C).

Effect of 5-HT

5-HT (0.01–10 μM) contracted only some of SA preparations and the magnitude of 5-HT induced contraction was generally small (Figure 22). Much greater than the effect on the baseline tension was the dose-dependent increase in SA responses to EFS in the presence of 5-HT (Figure 24A). Maximal augmentation was observed at 1 μM 5-HT. In the presence of ATR and TTX, tissues treated with 1 μM 5-HT did not respond to EFS (Figure 24B). 5-HT had no effect on the response to exogenous ACh (Figure 24C).

Effect of histamine

Consistent with our previous data, histamine (3 μM) induced a small contraction of SA and dramatically augmented responses to EFS. In the presence of ATR, responses to EFS in the presence of histamine were abolished (Figure 25), indicating that augmentation of EFS response by histamine was due to an increased cholinergic response and not by activation of other mechanisms.

Neutrophils

For each experiment we were able to isolate consistently a sufficient number of neutrophils (1–4 $\times$ 10^8) with both purity and viability above 98% to perform the tissue bath experiment with neutrophils from both control and RAO horses. We selected OZ as the best neutrophil activator because it does not affect SA responses to EFS, and in contrast to chemotactic ligands that activate neutrophils only for a 5-min period, the OZ-induced respiratory burst lasted over the period of 1 hour (not shown). Neutrophils isolated from

RAO horses stimulated with OZ produce significantly more ($O_2^{-}\cdot$) than those from control horses (Figure 26).

The response to EFS before neutrophils were added was used to compare SA responsiveness from RAO-affected and control horses. Just as in larger airways, SA isolated from horses with RAO, produced weaker responses to EFS than those from control animals (Figure 27). However, responses to KCl were identical.

Even though a sufficient number of neutrophils were isolated and these cells were strongly activated by OZ during each experiment, we did not observe any effect of neutrophils on the EFS response in SA (Figure 28). Coincubation of SA with neutrophils over the period of 1 hour neither increased baseline tension nor significantly affected the response to EFS.

Discussion

In a variety of airway diseases and animal models of airway obstruction, the inflammatory response has been shown to be the crucial event leading to bronchospasm and AHR.[27] In RAO airway inflammation and cholinergically mediated bronchospasm are also associated, but the role of inflammatory response and mechanisms by which it might affect airway tone remain obscure. Additionally, as previously shown in larger airways,[36] and presently in peripheral ones (Figure 27), tissues isolated from horses with RAO are not hyperresponsive. Paradoxically, they produce weaker responses to cholinergic stimulation. This apparent discrepancy between the in vitro and in vivo airway responses provides important information about the mechanism of cholinergic bronchospasm in RAO. Rather than being caused by some chronic changes in nerve terminals or smooth muscle itself, e.g.,

by upregulation of M_3 receptors on ASM, or decreased ACh-esterase activity, the increase in cholinergic airway tone is most likely caused by factors that when present in the airways either facilitate local ACh release or the response of smooth muscle to ACh released by nerves. Because RAO is an inflammatory disease in which both airway cytology and autacoid profile change rapidly in response to antigen challenge, we reasoned that inflammatory mediators may be responsible for altered cholinergic responses of horses with RAO. Several inflammatory mediators are known to cause bronchospasm not only via a direct contractile effect on ASM but also by more complex interactions with mechanisms of airway control. Currently we have shown that histamine has synergistic effects with SA responses to EFS in vitro.[22] To investigate further effects of mediators on cholinergic airway response we applied two experimental models. In the first we recreated the inflammatory micro-environment with the help of inflammatory mediators that are implicated in the pathogenesis of RAO. In the second approach we used activated inflammatory cells.

In response to natural antigen challenge, all three inflammatory mediators, histamine, LTD_4 and 5-HT, were reported to increase in respiratory secretions, urine, or plasma of RAO horses.[10, 11, 19] In our experiments, treatment of SA with any of these mediators caused a quite dramatic leftward shift of the frequency-response curve to EFS. Considering the possibility of interactions of these mediators with the airway responses in vivo, particularly interesting is the large (often many fold) augmentation of SA contraction at the lower range of EFS frequencies. These are the physiological frequencies at which postganglionic parasympathetic nerves are thought to fire periodically in the airways. With regard to the mechanism, we further confirmed that all mediator effects on the EFS responses were exclusively due to modulation of the cholinergic activity since ATR and TTX (blockade of either M receptors

or neuronal fast sodium channels) abolished responses to EFS in the presence of all three inflammatory mediators. This is in contrast to dog airways, in which histamine unmasks otherwise absent α-adrenergic contractions in ATR-treated tissues.[3]

Even though the maximal synergistic effect of mediators was observed when tissues were contracted by mediators up to the level of 10–20% of their maximal response, the effects of mediators on the EFS response are not just related to the contractile status of the tissue. As we observed, particularly with the lower concentrations of mediators, baseline elevation was not necessary to produce a quite impressive increase in tissue response to EFS. We also observed that the effect of inflammatory mediators on EFS response was more long-lasting than their direct effect on tissue tension. While responses to smaller concentrations of mediators started to decrease or even waned after several minutes (compare maximal responses of LTD_4 on Figure 22, with the baseline at Figure 23, representing remaining tension response after 15 min of incubation) the increased response to EFS was present for a long period of time and in the case of histamine persisted for up to 30 min after the washout (author's observation).

The response to exogenous cholinergic stimulation with either ACh or MCh was not subject to similar synergism and was simply additive. This observation indicates that the effects of histamine, LTD_4, and 5-HT are not mediated by an alteration at the level of M-receptors on ASM or by a change of the tissue's mechanical properties due to elevation of the baseline by these mediators. The large effect of mediator on the EFS response and lack of similar effect on exogenous ACh response curve may provide evidence of prejunctional modulation of ACh release from parasympathetic nerve terminals. To make a firm conclusion, measurements of ACh release from SA cholinergic nerves in the presence of

inflammatory mediators is required. We attempted to measure ACh release from SA in the presence and absence of histamine utilizing HPLC coupled with electrochemical detection. Even though this method is very well established in our laboratory for measurement of ACh release in both bronchi and the trachea,[31 37] we could not determine release of ACh from equine SA because the amount of ACh released was very small and below the level of detection.

Regardless of the mechanism by which inflammatory mediators exerted their effect on the EFS response, we have shown that inflammatory autacoids may greatly influence the magnitude of endogenous, cholinergic response in equine terminal airways, and in this respect, several mediators may exert a similar effect. This strong synergism between mediator-induced airway contraction in response to nerve stimulation at the physiological range of frequencies is consistent with the hypothesis that inflammatory mediators released in response to antigen challenge are responsible for increased cholinergic tone of airways in horses with RAO. Noteworthy, all of these mediators could produce effects of similar magnitude. In the clinical course of RAO, where many inflammatory mediators act in concert this may be responsible for the relatively low therapeutic efficacy of compounds that block effects of singular mediators (e.g., antihistamines) in contrast to glucocorticosteroids that blunt all of the inflammatory process.[4, 17]

In our second approach to recreate the tissue inflammatory microenvironment, we used activated neutrophils. Neutrophils are implicated in pathogenesis of RAO because: 1) they are consistently recruited into the airways and their increase in BAL is one of the classic clinical findings in RAO[25]; 2) neutrophils isolated from peripheral blood of RAO horses produced more $O_2^{-}\cdot$ in response to activation with chemotactic ligands and OZ (Figure 26)

and neutrophils in the airways of horses with RAO are strongly activated[21]; and 3) in other species neutrophils or neutrophil-derived inflammatory mediators, such as ROS or TXA_2 have been shown to contract smooth muscle or affect their responses.[7, 15, 35]

Even though indirect evidence supports it, the exact role of the neutrophil in the pathogenesis of RAO is not very clear. On the one hand, antigen challenge-induced recruitment of neutrophils into the airways of RAO susceptible horses is generally concurrent with the first changes in lung functions. This argues for a strong association between these two events. On the other hand, in some exceptional individuals, changes in lung function precede neutrophil recruitment or the recruitment appears earlier than the alterations in pulmonary functions.[12] These last few pieces of evidence indicate that even though the association between neutrophil recruitment and changes in lung function may exist, neutrophils are neither sufficient nor necessary for airway obstruction to occur. Our data clarify this even further. Coincubation of SA with a large number of strongly activated neutrophils over the period of 1 hour did not significantly alter SA responses. In fact there was a tendency to decrease EFS response rather than causing augmentation. Lack of a synergistic effect of neutrophils was not entirely surprising and parallels some other observations. For example, hydrogen peroxide, one of the ROS, produced by activated neutrophils, decreases cholinergic responses in equine trachea,[23] and neutrophil-derived enzyme elastase, in concentrations present in respiratory secretion, decreases tension responses of rabbit trachealis.[7]

Our observation changes our view of the pathogenesis of RAO by suggesting that neutrophils may not be as important in the pathogenesis of this syndrome as it was previously postulated, and that the cholinergic component of airway obstruction in horses is most likely

"neutrophil independent." However the role of the neutrophil cannot be entirely excluded based on our data. Neutrophil products may exert some long-term effects on airways, e.g., promote inflammation, edema formation, and mucus secretion and in this way contribute to airway obstruction by mechanisms not directly related to neuromuscular regulation of airway tone. In contrast to neutrophil-derived mediators, effects of histamine or LTD_4 or 5-HT may explain the mechanisms of increased cholinergic airway tone in RAO. These mediators are associated with a type I allergic reaction (mast cell derived), and in this respect our data support recent reports favoring a Type I reaction as an important mechanism in development of airway obstruction in RAO.[9, 16, 19, 30, 34]

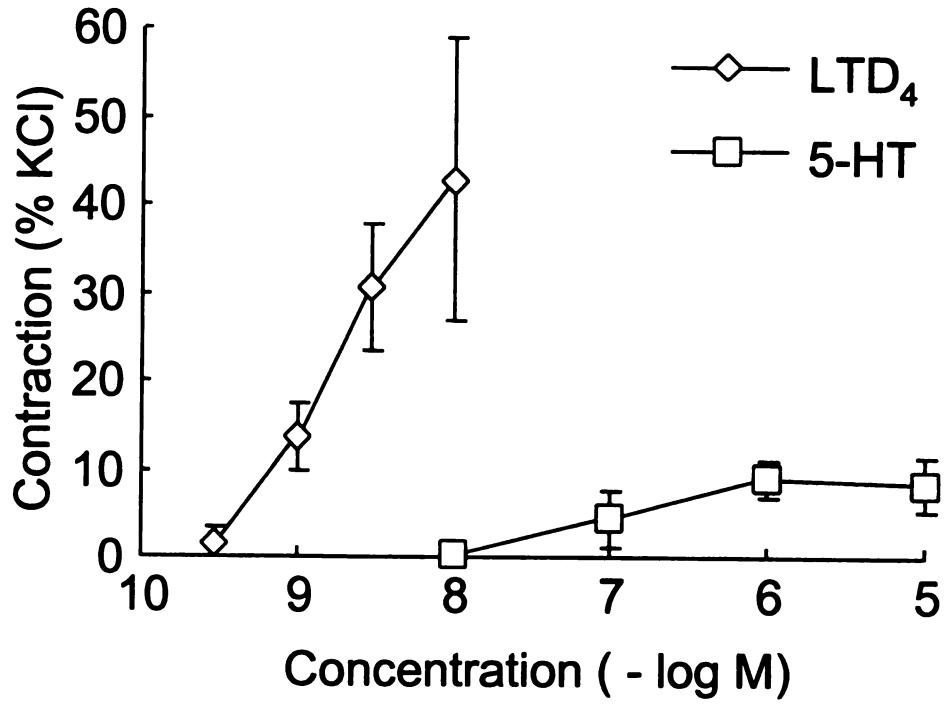

Figure 22. Small airway responses to LTD_4 and 5-HT. Smooth muscle tension is expressed as percentage of tissue contraction evoked by 127 mM KCl.

Figure 23. Effects of LTD_4 on cholinergic SA responses. EFS of tissues treated with LTD_4 was carried out in the presence or absence of ATR or TTX (panel A and B, respectively). The MCh was used as exogenous cholinergic stimulus (panel C). Results with 10 nM LTD_4 are not shown because they overlap with 3 nM (*: significantly different from control, n = 5 animals for each panel).

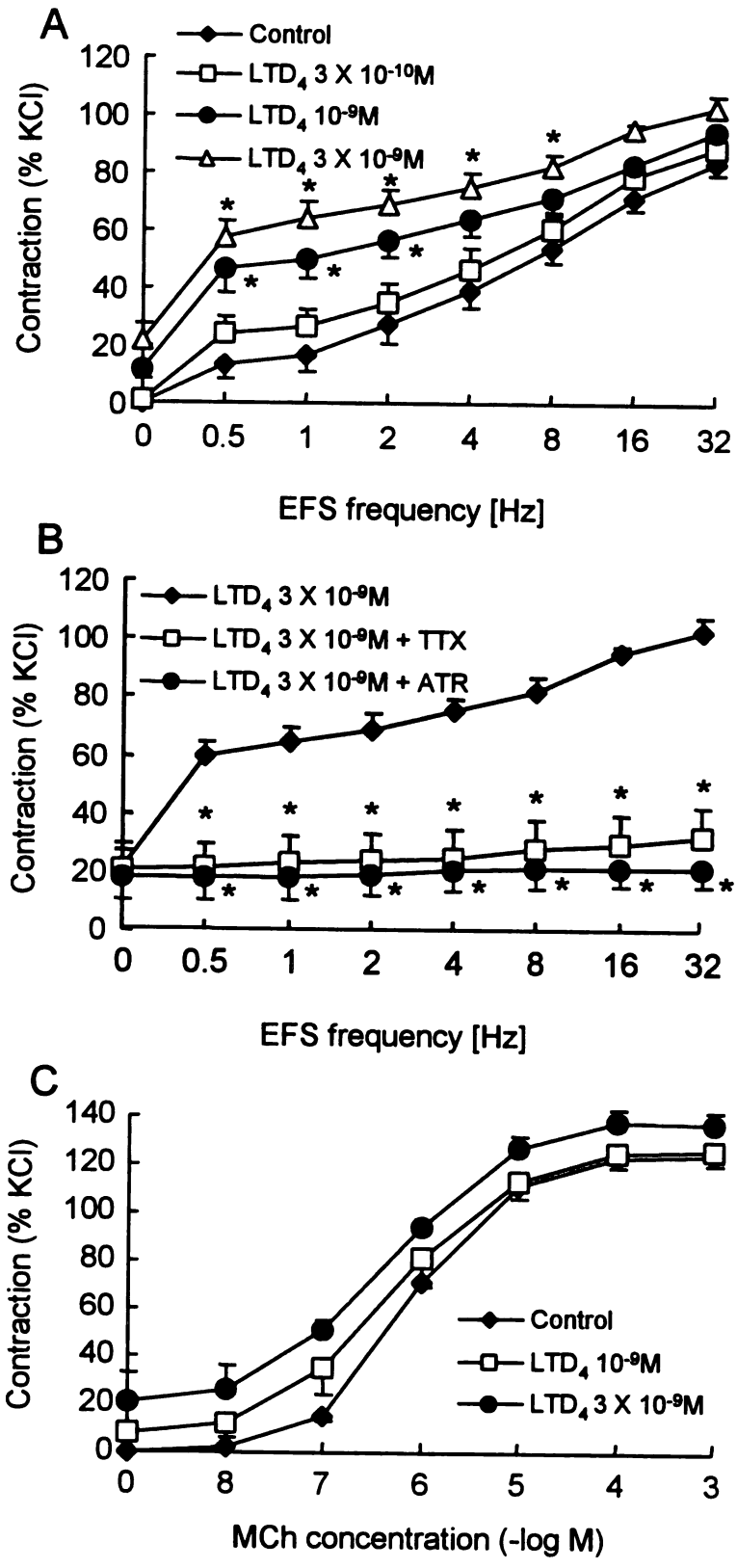
A
Control
LTD4 3 X 10-10M
LTD4 10-9M
LTD4 3 X 10-9M
Contraction (% KCl)
120
100
80
60
40
20
0
0
0.5
1
2
4
8
16
32
EFS frequency [Hz]
B
LTD4 3 X 10-9M
LTD4 3 X 10-9M + TTX
LTD4 3 X 10-9M + ATR
Contraction (% KCl)
EFS frequency [Hz]
C
Control
LTD4 10-9M
LTD4 3 X 10-9M
Contraction (% KCl)
140
120
100
80
60
40
20
0
0
8
7
6
5
4
3
MCh concentration (-log M)

Figure 23

Figure 24. Effects of 5-HT on cholinergic SA responses. EFS of tissues treated with 5-HT was carried out in the presence or absence of ATR or TTX (panel A and B, respectively) and ACh was used as exogenous cholinergic stimulus (panel C). Results with 10 μM 5-HT are not shown because they overlap with 1 μM (*: significantly different from control, n = 5 animals for each panel).

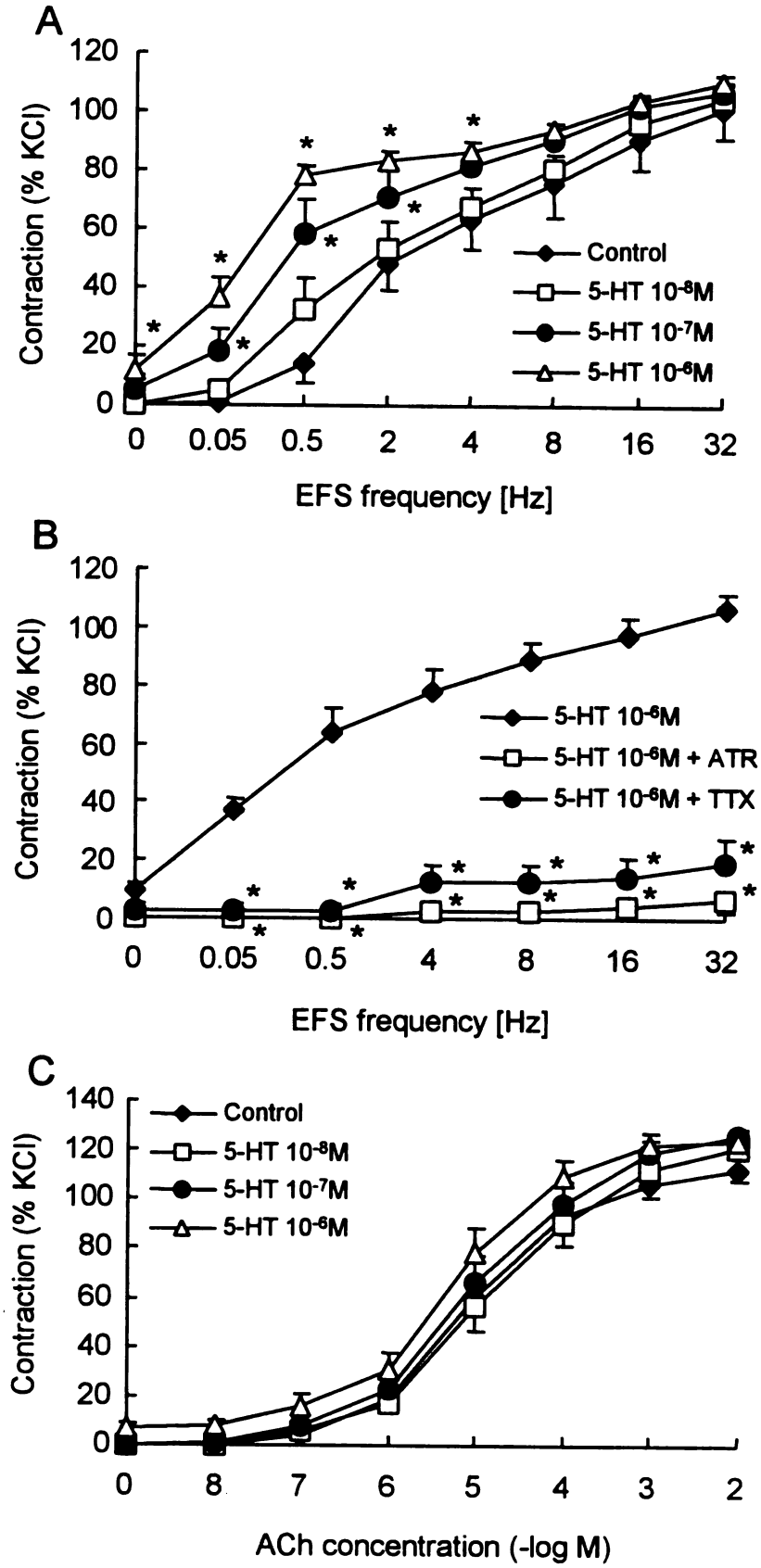
A
Contraction (% KCl)
120
100
80
60
40
20
0
0
0.05
0.5
2
4
8
16
32
EFS frequency [Hz]
Control
5-HT 10-8M
5-HT 10-7M
5-HT 10-6M
B
Contraction (% KCl)
120
100
80
60
40
20
0
0
0.05
0.5
4
8
16
32
EFS frequency [Hz]
5-HT 10-6M
5-HT 10-6M + ATR
5-HT 10-6M + TTX
C
Contraction (% KCl)
140
120
100
80
60
40
20
0
0
8
7
6
5
4
3
2
ACh concentration (-log M)
Control
5-HT 10-8M
5-HT 10-7M
5-HT 10-6M

Figure 24

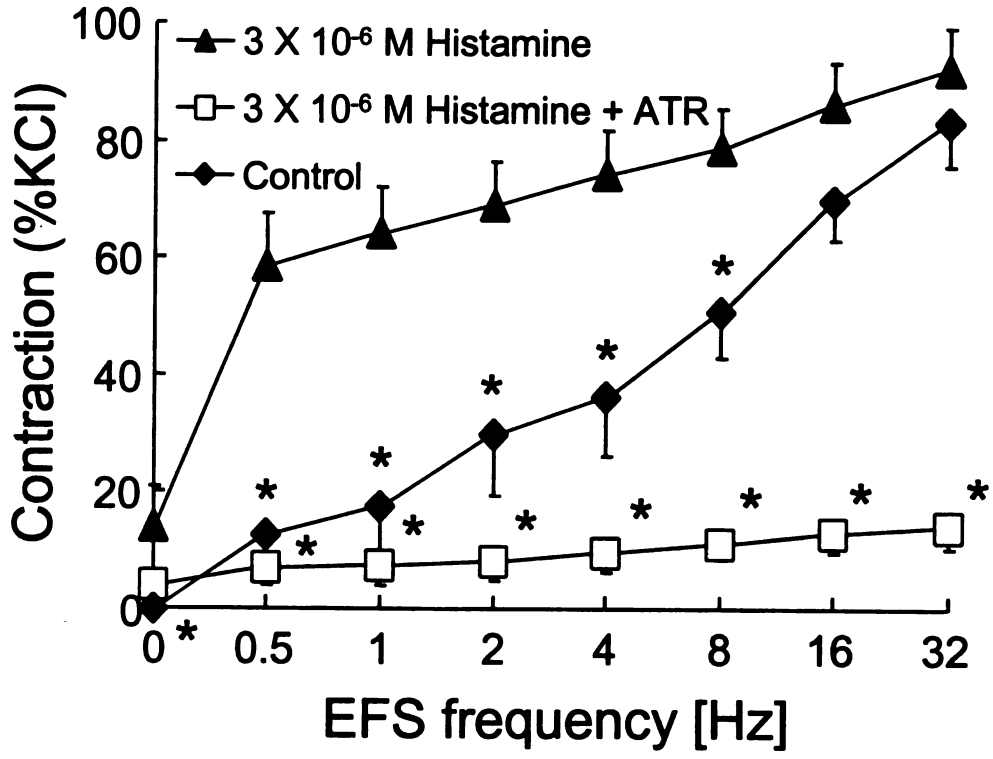

Figure 25. The EFS responses of SA incubated with 3 μM histamine in the presence or absence of ATR (*: significantly different from 3 μM histamine, n = 4).

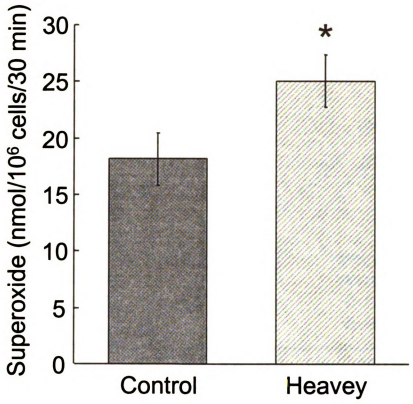

Figure 26. Production of superoxide by peripheral blood neutrophils treated with OZ (1 mg/ml) (*: significantly different from control).

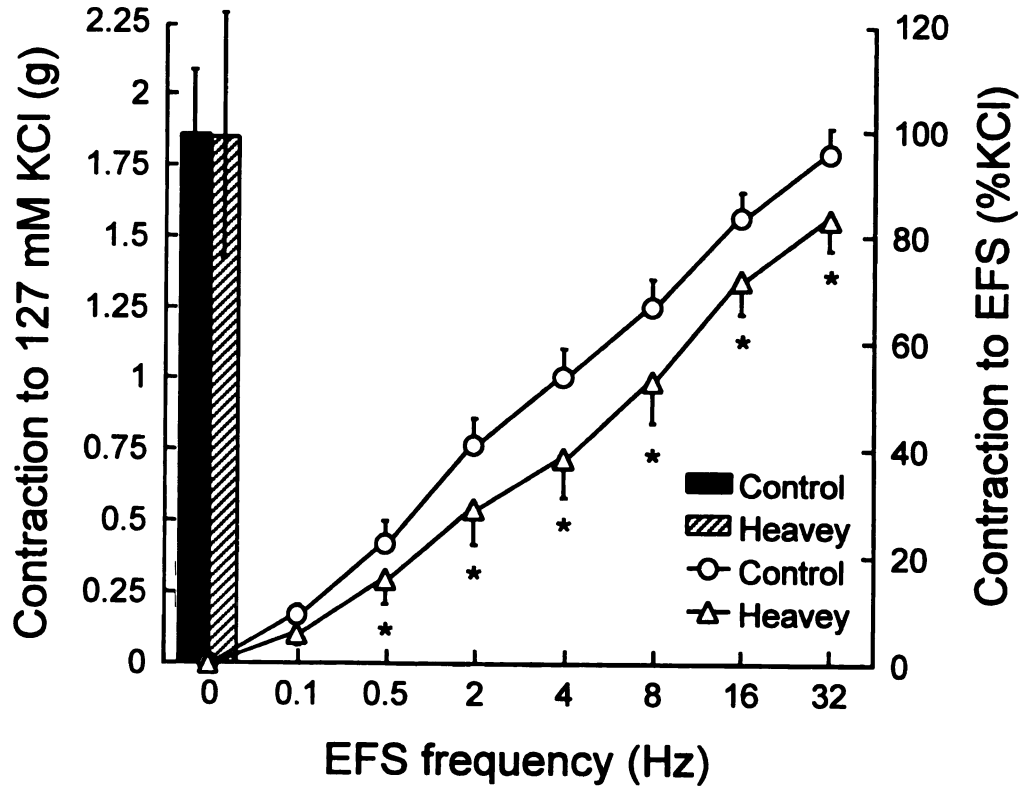

Figure 27. Comparison of EFS-induced tension responses in SA isolated from horses with RAO and controls. Responses to 127 mM KCl and to EFS are expressed in grams and as a percentage of KCl response, respectively (*: significantly different from control, n = 5 per group).

Figure 28. Effect of neutrophils on SA responses to EFS. SA were incubated for 60 min with OZ-activated neutrophils. A: control SA and control neutrophils; B: control SA and neutrophils from RAO horses; C: SA from RAO horses and control neutrophils; D: SA and neutrophils from RAO horses. (In all panels there was no significant effect of neutrophils when compared with tissue treated only with OZ in n = 5 animals.)

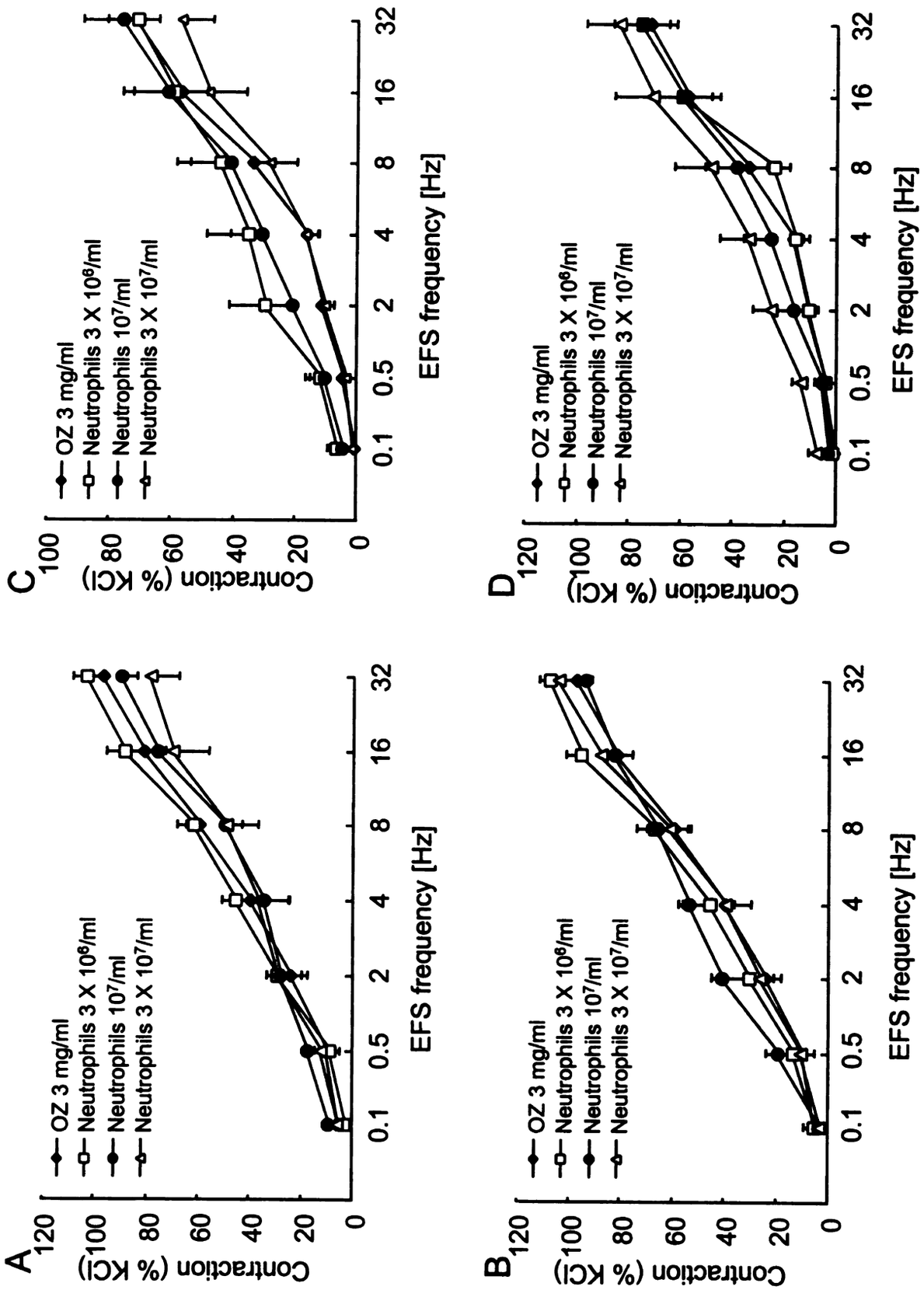
A
B
C
D
Contraction (% KCl)
EFS frequency [Hz]
OZ 3 mg/ml
Neutrophils 3 X 10⁶/ml
Neutrophils 10⁷/ml
Neutrophils 3 X 10⁷/ml
0
20
40
60
80
100
120
0.1
0.5
2
4
8
16
32

Figure 28

REFERENCES FOR CHAPTER 6

REFERENCES FOR CHAPTER 6

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Chapter 7

SUMMARY AND CONCLUSIONS

Beyond any doubt, bronchospasm is a major component inducing airway obstruction in horses with RAO. The mechanism of bronchospasm in RAO is cholinergic, mediated via activation of M receptors on ASM. Although clinical signs of RAO are associated with the exposure to natural hay and straw antigens and airway inflammation, the exact mechanism leading to cholinergic airway obstruction remains unknown. Moreover, there is an obvious discrepancy between the in vivo and in vitro behavior of equine airways. In contrast to in vivo cholinergic bronchoconstriction and AHR to inhaled spasmogens including cholinergic compounds, in vitro hyporesponsiveness to endogenous ACh is observed. I hypothesized that this discrepancy may be caused by studying the airways in vitro, i.e., separated from their inflammatory milieu. Two major components of the inflammatory response are predominantly considered important in the pathogenesis of RAO. On one hand, there is an influx of neutrophils into the airways, with subsequent neutrophil activation. On the other hand, the allergic reaction within the airways is thought to generate mediators of anaphylaxis, such as histamine, 5-HT, and cysteinyl LTs. The experiments described in my dissertation were designed to investigate the mechanisms by which inflammatory response could contribute to development of cholinergic bronchospasm during acute bouts of RAO. In this way

I sought to explain the phenomenon of increased cholinergic response and to determine the role of inflammation on the neuromuscular control of equine airways. For that reason I studied the effects of different inflammatory mediators on cholinergic responses in equine ASM in vitro.

Neutrophil influx and activation in the tissues is associated with oxidative stress due to the release of ROS. It was therefore important to determine the effect of oxidative stress on equine airway responses. In Chapter 3, I report the effects of H_2O_2, a reactive oxygen metabolite, on trachealis muscle isolated from control horses. In this tissue, I studied the effect of H_2O_2 (1 μM-0.1 M) on baseline tension, and on the contractile response to ACh, EFS, and 127 mM KCl.

Beginning at 1 mM, H_2O_2 contracted ASM in a concentration-dependent fashion. This contraction was unaffected by ATR, TTX, or MEC. Above 0.1 mM, H_2O_2 concentration-dependently depressed the responses to ACh, EFS, and KCl. The highest concentration, 0.1 M H_2O_2, decreased maximal response to EFS by 68%, the response to 1 mM ACh by 60%, and the response to KCl by 38%. In the presence of MEC (1 μM), partial but significant protection against 1 and 10 mM of H_2O_2 was observed. Since lack of inhibitory mechanisms may also contribute to increased airway responses, I investigated the effect of H_2O_2 on the relaxant response to ISO. In tracheal strips contracted with 0.3 μM MCh, H_2O_2 had no effect on the ISO concentration-response curve. From this study a few important conclusions become apparent. First, oxidative stress, such as that produced by addition of H_2O_2, is not responsible for cholinergic bronchospasm or cholinergic hyperresponsiveness in horses with RAO. This conclusion is based on the fact that H_2O_2 appears effective only in high concentrations that are in the

upper limits ever reported in biological systems. It is unlikely that such a large number of oxidants would be present throughout the tissues in vivo. Additionally, contraction of airways induced by H_2O_2 was neither a result of neuronal conductance nor mediated via activation of M receptors, and its magnitude was probably too small to become biologically significant. Also the effects of H_2O_2 were opposite to those reported during acute exacerbation of RAO and thus did not confirm my hypothesis. Both in the presence of H_2O_2, or after it was washed from the tissues, there was a decrease, rather than an increase, of tension in response to nerve activation by EFS. A decrease in response to exogenous ACh was also observed in the tissues pretreated with H_2O_2. On the other hand, experiments in vitro have shown predominantly ACh hyporesponsiveness as a feature of ASM from horses with heaves. It is possible that the latter is the result of oxidative stress similar to that caused by incubation of ASM in high concentrations of H_2O_2. This potential role of H_2O_2 fits the paradigm that a variety of mechanisms are part of the pathogenesis of RAO and that no one single factor is responsible for the development of the disease.

The negative result of the latter study convinced me that finding the principal mediator responsible for altered airway function in vivo would not be easy utilizing this model. Therefore, I focused on the development of research models that would better reflect the conditions in vivo. This implicated the use of SA as a segment of airways in which the morphological and pathophysiological changes prevail during exacerbation of RAO, and use of activated inflammatory cells (neutrophils) rather than single mediator.

Chapter 4 of my dissertation describes the development of equine peripheral airway preparations, and the characterization of autonomic control of ASM in these

preparations in vitro. In this study, I compared responses of two groups of terminal airways (1-2.5 mm), SA, and LP in control horses. I was particularly interested in cholinergic responses in these tissues, which represent the periphery of equine respiratory tract. After the contractile response of ASM was normalized in each tissue via contraction with 127 mM KCl, I used EFS to determine the functional innervation in SA and LP preparations. I also measured the exogenous cholinergic responses to the M receptor agonist MCh, in order to test their function in both types of tissues. EFS induced frequency-dependent contractions in SA (T_{max} 88.3 $\pm$ 1.9% KCl) that were virtually abolished by ATR and TTX. In LP, EFS responses were much smaller (T_{max} 32.3 $\pm$ 1.9%) and only partially inhibited by these antagonists. Methacholine contracted SA with a five-fold higher potency than in LP and with a 2-fold greater T_{max} (% KCl).

Analysis of responses in these two groups of tissues indicated that SA in horses possess large number of M receptors and a relatively good innervation with cholinergic nerves. Thus stimulation of nerves in equine SA produced nearly maximal contraction and this response was entirely cholinergic. At the level of LP, the amount of contraction induced by cholinergic stimulation consists of a much smaller fraction of the response to KCl, suggesting a lower density of M receptors. The very small response to EFS, from which only a half is mediated by stimulation of cholinergic nerves, suggests that, in addition to lower density of M receptors, parasympathetic innervation is also very sparse at the level of equine terminal bronchioli and alveolar ducts. EFS-induced relaxation was tested in tissues atropinized and contracted by histamine, to determine the function of inhibitory innervation. In LP or SA, I did not observe a consistent relaxant response to EFS, and therefore the existence of functional inhibitory innervation in SA

or LP is questionable. However, both SA and LP relaxed in response to the β-adrenergic agonist ISO, indicating that these receptors are present throughout the entire respiratory system in horses, in which they provide a universal inhibitory mechanism.

An inhibitory mechanism is also provided by PGE_2 production within the airways. In acute bouts of RAO, mucosal production of PGE_2 decreases, possibly contributing to acute bouts of RAO. Therefore I tested the effect of cyclooxygenase blockade on the responses of SA and LP. In SA but not in LP, a large augmentation of EFS responses at 1-4 Hz was produced by MEC, a cyclooxygenase blocker. These results indicate that the inhibitory mechanisms afforded by prostanoids extends into SA but not LP.

I also tested responses to histamine in the LP and SA, since injected or inhaled histamine precipitates clinical signs of airway obstruction in both control and RAO horses, presumably via peripheral airway contraction. Histamine is also known to increase in the lavage of horses with RAO. In my experiment, histamine contracted SA and LP in a dose-dependent fashion, with nearly five-fold greater potency in the latter.

Much more interesting was the effect of histamine on cholinergic SA responses. Histamine, in a concentration that produced only a small amount of contraction, dramatically augmented the response to EFS, particularly in the lower frequency range. A similar synergistic effect of histamine and EFS response was not observed in the LP preparation. Also, the response to MCh in SA was only slightly shifted to the left by histamine. However, this effect was largely additive and could not explain the dramatic augmentation of EFS response.

In this protocol, for the first time I was able to demonstrate that an inflammatory mediator may increase cholinergic airway response. It is an excellent example of how

my hypothesis about the possible effects of the inflammatory milieu on cholinergic airway response is confirmed. From this study, the following conclusions appeared crucial for my further investigations.

The excitatory input from cholinergic nerves largely determines SA tone, while in LP it has only a small effect. SA represent the size of the airways in which pathological changes can be found in RAO, and they produce a consistent cholinergic response to EFS. Therefore, in contrast to the LP, the SA preparation represented a good model to study the putative factors that may alter cholinergic peripheral airway responses in the course of RAO. Demonstration of the effects of endogenous inhibitory prostanoids and histamine confirmed that indeed the cholinergic ASM response is subject to strong modulation by mediators. This modulation can be both inhibitory or excitatory and applies most of all to endogenous cholinergic response, i.e., ASM contraction arising from cholinergic nerve stimulation. These data are also consistent with the hypothesis that, in horses with RAO, histamine release and an altered prostanoid profile contribute to cholinergically mediated SA obstruction.

One of the main purposes of my study was to resolve the issue about the role of neutrophils in the development of airway obstruction in horses. Having developed a SA preparation, I designed experiments in which I would test whether neutrophils affect the responses of isolated airways in vitro. Before my final question could be addressed, a series of pilot studies had to be performed to obtain a sufficient number of neutrophils and to study the mechanisms of neutrophil activation. These studies are presented in Chapter 5, in which I described techniques to isolate neutrophils from peripheral blood, and to study their activity in response to several agents.

The peripheral blood neutrophils were isolated from blood samples of control and RAO horses by the two-step isolation method utilizing discontinuous Percoll density gradient. This method allowed me to isolate large number of neutrophils with both very high purity and viability. The neutrophils were then activated by several compounds to test their responses to activation. The following agents were used to activate neutrophils: AA, calcium ionophore, PMA, and chemotactic ligands such as fMLP and hrC5a (both in the presence of CB), as well as (OZ). Activation of neutrophils was assessed via cytochrome C reduction assay, which measures respiratory burst activity in these cells. Of all of these agents, only the chemotactic ligands OZ and PMA produced sufficient neutrophil activation to be considered good activators. Also, to my knowledge, my experiment for the first time demonstrated that fMLP may produce significant respiratory burst response in equine neutrophils.

Interestingly, neutrophils isolated from the blood of horses in the acute stage of RAO produced a significantly higher response to chemotactic ligands and OZ, but not to PMA. This indicates that during RAO in horses, the sensitivity of neutrophils to natural stimuli increases, whereas the intrinsic capacity of NADPH oxidase to produce superoxide is not altered. Further study of the time course of neutrophil response narrowed the number of candidate activators to OZ. This was because activation of neutrophils by chemotactic ligands lasted only for the period of 5 min, and PMA could not be used in final experiments with SA because it contracts ASM.

In the final approach, summarized in Chapter 6, I measured responses of SA in the presence of activated neutrophils to determine their effects. To account for the differential response of neutrophil to activation and for differential responses of airways

to the neutrophil-derived products between horses with RAO and the controls, I used a cross-over design that involved all combinations of tissue and neutrophils from healthy and affected animals. I found that activated neutrophils do not directly affect cholinergic responses in SA, in any of the four subsets of tissues from control and RAO animals, even during the incubation period, which exceeded 1 hour. This result excluded the possibility that mediators released from acutely activated neutrophils could significantly modulate cholinergic responses in SA. Therefore, it is unlikely that neutrophils produce factors that directly evoke bronchospasm in acute bouts of RAO. These results are consistent with several studies that questioned the importance of neutrophils in the pathogenesis of RAO. These demonstrated that neutrophils may invade the airway without subsequent airway obstruction; airway obstruction may develop before neutrophil recruitment; and, during remission the alteration of lung function usually persists longer than the increased neutrophil ratio in BAL fluid. The outcome of this experiment is consistent with the observation of H_2O_2 effects in the equine trachea.

In contrast to the lack of effect of neutrophils, mediators of anaphylaxis have demonstrated a strong ability to increase the response to EFS in the equine SA. In addition to histamine, I have tested the effects of 5-HT and LTD_4. Similar to histamine, LTD_4 and 5-HT, in concentrations that only slightly increased baseline tension, dramatically increased the response to cholinergic nerve stimulation. This increase applied particularly to the lower, i.e., physiological range of frequency of parasympathetic nerve stimulation. In the case of all three mediators of anaphylaxis, mechanisms other than a cholinergic one were excluded by treatment with ATR, which entirely blocked the response to EFS but not the direct effect of mediators on ASM tone. The

effect of these mediators on the response to exogenous cholinergic stimulation produced by administration of ACh or MCh was only additive. This observation may suggest a prejunctional effect, i.e., augmentation of ACh release from airway parasympathetic nerves as a mechanism of action of these mediators.

In summary, the experiments presented in my PhD dissertation have shown that the endogenous cholinergic responses of equine airways are subject to strong modulation by inflammatory mediators, and that pro-inflammatory mediators may strongly increase the response to nerve stimulation. Moreover, different types of mediators may produce a similar magnitude of augmentation that is consistent with the multifactorial genesis of the RAO syndrome. These results may explain the mechanisms of increased cholinergic airway tone in heaves via effects of a variety of inflammatory mediators on endogenous cholinergic responses of ASM. I have also found that activated neutrophils do not affect cholinergic responses in SA, and that H_2O_2 decreased rather than increased cholinergic responses, in part due to promotion of inhibitory prostanoid production in isolated trachealis strips. Thus, my data particularly favor an allergic reaction as the source of this mechanism, rather than neutrophilic inflammation, since mediators traditionally associated with mast cells but not activated neutrophils or H_2O_2 contributed to increased cholinergic responses of isolated airways to EFS.