Response of Peripheral Blood Lymphocytes from RAO-affected Horses to β$_2$-Agonist Stimulation

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Recurrent airway obstruction (RAO) affects middle-age horses, inducing bronchoconstriction and airway inflammation. β_2-agonists like salbutamol are used as treatment, promoting airway smooth muscle (ASM) relaxation and bronchodilation. In addition to ASM, inflammatory cells express the β_2-adrenoreceptors (β_2-AR). In other species, β_2-agonists promote peripheral blood lymphocyte (PBL) cytokine expression towards a pro-inflammatory phenotype. RAO horses are a good model for evaluating chronic changes in human asthma. However, little is known about the effect of β_2-agonist stimulation on equine PBL inflammatory response. The aims of this study were to develop an indirect method to evaluate the response of equine PBLs to β_2-agonist stimulation, and to compare it between cells from RAO and non-affected horses. Isolated PBLs were activated with ConA and stimulated with salbutamol. Response to agonist binding was indirectly determined using flow cytometric methodology and verified by Western blot. Activated PBLs from RAO horses demonstrated a significant response to β_2-agonist binding whereas cells from non-affected horses did not. Response of PBLs from RAO horses was attenuated when pre-treated with a β_2-antagonist but unaffected following pre-treatment with a β_1-antagonist, indicating that the response of PBLs from these horses to salbutamol binding was mainly through the β_2-AR. Preliminary investigation of bronchoalveolar lavage (BAL) lymphocytes from RAO horses demonstrated that they also responded to β_2-agonist binding, while cells from non-affected horses did not. These findings represent a novel tool for further investigation of the role of β_2-agonist binding in diseases like asthma and RAO, and support the use of this model for future studies.

DEDICATION

To my father, Bruno Werner Klocker

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ACh: acetylcholine

ANS: autonomic nervous system

APS: ammonium persulfate

ASM: airway smooth muscle

ATP: adenosine triphosphate

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

β-AR: β-adrenoreceptor

β_2-AR: β_2-adrenoreceptor

BCR: B-cell antigen receptor

cAMP: cyclic adenosine monophosphate

CBC: complete blood count

CD: cluster of differentiation

Cdyn: dynamic lung compliance

CGRP: calcitonin gene-related peptide

ChAT: choline acetyltransferase

CLP: common lymphoid progenitor

COLD: chronic obstructive lung disease

ConA: concanavalin A

COPD: chronic obstructive pulmonary disease

DAG: diacylglycerol

ddH$_2$O: distilled water

DPBS: Dulbecco's phosphate buffered saline

EIA: enzyme immunoassay

eNANC: excitatory NANC

EP: prostanoid receptor

FDA: Food and Drug Administration

FITC: fluorescein isothiocynate

GDP: guanosine 5'-diphosphate

GM-CSF: granulocyte macrophage colony-stimulating factor

GPCR: G protein – coupled receptor

GR-α: glucocorticoid receptor-α

GRK: G-protein coupled receptor kinase

GTP: guanosine 5'-triphosphate

IBMX: 3-isobutyl-1-methylxanthine

ICAM: inter-cellular adhesion molecule

IACUC: Institutional Animal Care and Use of Committee

ICYP: -$[^{125}I]$(-)-iodocyanopindolol

IFN-γ: interferon gamma

IL-: interleukin

iNANC: inhibitory NANC

IP_3: inositol triphosphate

ISO: isoproterenol

KDD: kilodaltons

LABA: long acting beta agonist

LTB_4: leukotriene B_4

LTC_4: leukotriene C_4

MHC: major histocompatibility complex

MFI: median fluorescent intensity

nAChRs: nicotinic acetylcholine receptors

NANC: nonadrenergic noncholinergic system

NE: norepinephrine

NK: natural killer

NK-κ B: nuclear factor-κB

NKA: neurokinin A

NKB: neurokinin B

NO: nitric oxide

OCT: organic cation transporter

P_aO_2: arterial oxygen pressure

PBL: peripheral blood lymphocytes

PBMC: peripheral blood mononuclear cell

PDE: phosphodiestrease

PGE_2: prostaglandin E_2

PGD_2: prostaglandin D_2

PKA: protein kinase A

PKC: protein kinase C

pmol: picomol

PNS: peripheral nervous system

ΔPplmax: pleural pressure during tidal breathing

p-VASP-Ser157: phosphorylated VASP at Serine 157

RAO: recurrent airway obstruction

RL: pulmonary resistance

ROS: reactive oxygen species

SABA: short acting beta agonist

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel

SE: standard error

SP: substance P

TCR: T-cell antigen receptor

TEMED: N,N,N',N'-Tetramethylethylenediamine

TGF: transforming growth factor

Th: T helper cell

TNF-α: tumor necrosis factor-α

TTBS buffer: Tris buffer saline and Tween 20.

VASP: vasodilator-stimulated phosphoprotein

VIP: vasoactive intestinal peptide

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Recurrent Airway Obstruction (RAO) is a respiratory disease that affects middle-age horses. Clinically, RAO mainly affects respiratory function but the pathogenesis also involves the nervous and immune systems. Among diseases that affect equine airways, RAO is one of the most common and is most frequently diagnosed in horses that are kept stabled for prolonged times. Currently, there are numerous research groups investigating the pathophysiology, treatment, and prevention in hopes of furthering our understanding of RAO. To accomplish this, it is fundamental to comprehend all the different systems and their individual components that are related to the disease.

In many ways, equine RAO is a good animal model for the study of human asthma because these diseases share characteristics like bronchoconstriction, airway inflammation, airway smooth muscle remodeling, and mucous accumulation. Likewise, there are similarities in the inflammatory response observed in asthma and RAO, especially with regard to the role of lymphocytes and the response of affected horses to corticosteroids and bronchodilator treatments. These similarities further support the idea that examination of tissues from RAO affected horses could provide insight to the cellular and molecular mechanisms that contribute to the development of human asthma. This literature review presents some of the currently available information regarding the most important factors in RAO and asthma in humans. General aspects of the respiratory system will be first presented, and include information regarding clinical aspects of RAO, the inflammatory and bronchoconstrictive aspects of the disease, and the neural control of the airway tone. In addition, a brief review of the β-adrenoreceptor and its role in the pathophysiology of this disease is presented.

1. 2 General Aspects of the Respiratory System

The respiratory system is very important, especially in performance horses. The primary function of this system is to distribute oxygen to all tissues, and to remove carbon dioxide from the blood through breathing. Because of the anatomical characteristics of this system, the horse is an obligate nasal breather. The nasal cavity and turbinate bones are covered by a mucous

membrane which in turn is lined with ciliated epithelium located on the luminal side of the mucosa; the function of cilia is to move in waves and propel mucus and debris to the throat. The horse trachea, which extends from the larynx to the hilus of the lung, is made of 48 to 60 dorsally incomplete hyaline cartilage rings that keep the airways open all the time. It is lined by mucus-secreting cells (called goblet cells) and pseudostratified ciliated columnar epithelium (Robinson and Furlow, 2007, Derksen and Robinson, 2002, Davies, 2005, Shappell, 1999).

The right and left lungs are similar in shape, with a ventral notch at the level of the heart separating the small cranial portion from the larger caudal portion. The right lung is larger than the left because it has an intermediate or accessory lobe. The lung is separated into lobules by fibrous connective tissue which limits the collateral movement of air between different lung regions. After the trachea divides into bronchi (above the heart), it continues dividing into bronchioles, which are made of fibrous and elastic tissue. The tracheobronchial lining is made of columnar, pseudostratified epithelium combined with serous and goblet cells. Bronchioles covered by short ciliated cells and non-ciliated Clara cells divide until they are only a single layer of epithelial cells, called alveoli. Alveoli are dead-ending sacs formed by type I and type II pneumocytes and enclosed by blood capillary vessels. The function of alveoli is gas exchange. Type I pneumocytes cover the majority of the alveoli, and type II pneumocytes contain lamellar cytoplasmic inclusions which function to produce surfactant; type II cells also act as stem cells because they replace type I cells after lung injury. Lymphocytes, macrophages, mast cells, and occasionally eosinophils are found in the lower respiratory tract as well (Robinson and Furlow, 2007, Davies, 2005, Ainsworth, 2004).

1.3 Recurrent Airway Obstruction (RAO)

1.3.1 Overview of the Disease

Recurrent Airway Obstruction (RAO) is a common chronic respiratory disease that most often affects middle-aged horses maintained in confined housing. In affected horses, exposure to aerosolized allergens causes airway inflammation, mucous accumulation, and reversible bronchoconstriction (Robinson, 2001b, Davis and Rush, 2002). Over the years, this disease has had several names, including alveolar emphysema, chronic obstructive pulmonary disease (COPD), chronic obstructive lung disease (COLD), chronic bronchitis, chronic bronchiolitis, small airway disease, broken wind, heaves, and recurrent airway obstruction (Robinson et al.,

1996, Davis and Rush, 2002). These names are based upon the clinical presentation of the disease. In humans, a similar disease called asthma is characterized by airway hyperresponsiveness, reversible airway obstruction, and airway inflammation (Davis and Rush, 2002, Lavoie, 2007, Abraham et al., 2006).

The prevalence of RAO, the most common respiratory disease in mature horses, ranges from 2% to 80% depending on factors like area where the animal lives (in a barn or in pasture), the type of feed and bedding it is provided, the season, and genetics. In a study performed in Britain the authors report a prevalence of 14%, identifying as risk factors increasing age and exposure to urbanized environment, and possibly challenges to the respiratory system early in life. Similarly, a study performed in North America described an increased risk of developing the disease in older horses. In addition, these authors find that Thoroughbred horses were more susceptible than ponies (Léguillette, 2003, Hotchkiss et al., 2007, Couëtil and Ward, 2003).

Results of several studies have identified genetic factors that predispose horses to developing RAO. Studies that have evaluated heritability of the disease have *shown* that foals born of a single RAO affected parent have a 3-fold increased risk of developing the disease and a 5-fold increase if both parents are affected (Gerber et al., 2008). Swinburne et al. report that two chromosome regions in half-sibling families showed a genome-wide significant association with the risk of developing RAO (Swinburne et al., 2009). Several genes located in those regions are related to signaling molecules associated with the immune response. Additionally, Gerber et al. found that in one family line RAO inheritance was autosomal dominant type, while in another family line it was autosomal recessive (Gerber et al., 2009). In a detailed study conducted by Ramseyer et al. these researchers concluded that there was a strong genetic predisposition associated with manifestations of moderate to severe signs of chronic lower airway disease (Ramseyer et al., 2007). These authors also proposed that there exists an RAO heritability factor associated with manifestation of the disease in the sire. Specifically, foals whose sires were RAO affected horses were at significantly greater risk of developing moderate to severe chronic lower airway disease when compared to foals sired by unaffected stallions.

Clinical manifestations of RAO can vary greatly at different stages of the disease. In mild or early disease, affected horses occasionally cough while being fed, groomed, or initiating exercise. As the disease progresses, horses develop reduced exercise tolerance and longer recovery periods. Severely affected horses undergo episodes of dyspnea, tachypnea, nasal discharge, nostril flare, and a marked double expiratory effort (Léguillette, 2003, Davis and

Rush, 2002, Marinkovic et al., 2007, Robinson, 2001b). In more severe and chronic cases, it is also possible to observe weight loss, mainly related to an increase in the amount of energy expended during breathing. In chronic states of the disease, a hypertrophy of the external abdominal oblique muscles ("heave line") can be observed in horses. Affected horses can also manifest alterations in their breathing pattern characterized by a rapid inspiratory phase followed by a forced prolonged exhalation (Davis and Rush, 2002, Léguillette, 2003, Mazan et al., 2004). These signs can be observed in horses housed in a stall where they are exposed to challenges such as barn dust, hay mold, ammonia fumes, fungal spores, and endotoxins (Davis and Rush, 2002).

1.3.2 Diagnostic methods in RAO

A tentative diagnosis of equine RAO can often be made based on the clinical history and an accurate clinical examination of the affected horse. Identification of abnormal lung sounds can be facilitated with the use of a rebreathing bag which stimulates the horse to take deeper breaths and increases pulmonary sounds on auscultation. In horses with mild states of disease, additional diagnostic evaluation is required. Thoracic radiographs are helpful to rule out other lung disease such as neoplasia or silicosis, and an increased interstitial pattern and enhanced peribronchial radio-density are findings that further support the diagnosis of RAO. Endoscopic examination allows visualization of mucoid exudates, tracheal hyperemia, and permits detection of augmented sensitivity to cough. Blood gas analysis provides information about alterations in gas exchange; horses with a moderate state of disease have a PaO_2 under 80 mmHg at rest, while animals presenting arduous breathing can have a PaO_2 of 50 mmHg (Robinson, 2001b, Davis and Rush, 2002, Lavoie, 2007, Léguillette, 2003).

Obtaining a sample of airway fluid and cells by bronchoalveolar lavage (BAL) is a diagnostic technique that permits identification and characterization of inflammation in the airways of RAO affected horses (Davis and Rush, 2002, Hoffman and Viel, 1997, Hoffman, 2008). The cell distribution in bronchoalveolar lavage fluid (BALF) from normal horses is between 5% to 10% neutrophils, 50% to 70% macrophages, 30% to 50% lymphocytes, less than 2% mast cells, and less than 0.1% eosinophils (Robinson, 2001b, Rush and Mair, 2004b). In most cases horses with RAO have an increased percentage of neutrophils (>25%) in their BALF, with >50% neutrophils observed in more severe cases. BAL neutrophil percentage that is between 10% and 25% is less diagnostic of RAO since normal horses housed in a dusty

environment or horses with milder forms of airway inflammation can develop a mild airway neutrophilia (Davis and Rush, 2002, Richard et al., 2010, Robinson, 2001b).

In the past, it was believed that the reversibility of bronchoconstriction associated with RAO could be predicted by assessing the horse's response to a single dose of atropine (the atropine response test) (Robinson, 2001b). This test is no longer considered a reliable method for determining long term response to therapy and prognosis in RAO affected horses, but is considered a way to predict the benefit of treating disease with bronchodilators since atropine acts as a powerful anticholinergic bronchodilator (Rush, 2004). Pulmonary function testing before and after treatment with a β_2-adrenoreceptor agonist can provide some indication of the potential benefit of using bronchodilators in treatment of this disease (Robinson, 2001b).

Determination of lung function in horses with RAO is a sensitive method for detecting mild or subclinical disease. Measurements of parameters like changes in pleural pressure during tidal breathing (ΔPplmax), pulmonary resistance (RL), and dynamic lung compliance (Cdyn) are effective methods for obtaining measure of the severity of the disease and improvement after treatment. Pulmonary function testing is especially useful in research as the results provide an objective and sensitive record of the horse's level of disease and response to treatment (Ainsworth, 2010, Roy and Lavoie, 2003, Davis and Rush, 2002).

Bronchoprovocation is another test that can be used to determine heaves in asymptomatic affected horses. This technique is an adaptation of the test used in humans, and is based on the nebulization of increasing doses of histamine or methacholine, which induce airway hyperresponsiveness. Throughout the test, dynamic compliance (Cdyn) of the lung is monitored as an indirect indicator of the amount of bronchoconstriction the horse develops in response to the amount of either histamine or methacholine to which the horse is exposed. This test examines the position or shift of the dose-response curve, which is a measure of airway sensitivity; the slope of the curve, which is a measure of airway reactivity; and the curve plateau, which is a measure of maximal response. This procedure allows differentiating between normal horses and RAO affected horses with minimal apparent clinical signs (Doucet et al., 1991, Mazan et al., 1999, Klein and Deegen, 1986, Hoffman, 2002, Boulet, 2003, Baroffio et al., 2009).

1.3.3 Pathophysiology of RAO

Recurrent Airway Obstruction affected horses develop airway inflammation, bronchospasm, and mucous hypersecretion when exposed to allergens. Inflammation can cause epithelial cell damage (partial shedding of the epithelium) and destruction of surface cilia followed by submucosal and intercellular edema, and loosening of the basal membrane of epithelial cells. In horses with chronic RAO, the loss of airway epithelium exposes immunologically active tissue to direct contact with aerosolized debris and promotes the development of airway hypersensitivity (Davis and Rush, 2002, Robinson, 2001b, Marinkovic et al., 2007, Puchelle et al., 2006). The mucus then accumulates in the airways due to an increase in production and viscoelasticity. Moreover, changes in mucus glycosylation also reduces its clearability, events that further contribute to airway obstruction (Robinson, 2001b, Léguillette, 2003).

1.3.3.1 Inflammatory response

Clinical RAO has been experimentally induced by exposing affected horses to specific aerosolized antigens such as *Aspergillus fumigatus, Eurotium amsteloidami*, and lipopolysaccharides (Beeler-Marfisi et al., 2010). The specificity of this response suggests that at least part of the airway inflammation associated with RAO is initiated by an acquired immune response. Lymphocytes are the inflammatory cells that mediate the acquired immune response. It is likely that lymphocytes play an important role in the development of RAO by recognizing specific antigens that initiate airway inflammation and the subsequent events that coordinate the inflammatory response (Robinson, 2008, Larché et al., 2003).

In horses, peribronchial lymphocytes act as immunoglobulin-producing B lymphocytes and CD4+ (helper) T lymphocytes. Airway diseases like asthma have been associated with a T helper type 2 (Th2) lymphocyte phenotype based on the cytokine profile of these cells. Among their functions, Th2 cells signal IgE production and neutrophil recruitment into the airways (Davis and Rush, 2002, Léguillette, 2003, Horohov, 2001). Bowles et al. demonstrate that in RAO affected horses the expression of a Th2 cytokine profile in airway lymphocytes was correlated with neutrophil and lymphocyte influx into the airways. Similarly, Lavoie et al. reported an association between Th2 cytokine expression in airway cells and the development of airway neutrophilia (but not eosinophilia) in RAO horses (Lavoie et al., 2001).

1.3.3.1.1 Lymphocytes

Lymphocytes are small (7 – 15 µm in diameter) cells that can be divided into different subpopulations based on their function: **T cells** are related to cell-mediated immunity or acquired immunity, **B cells** play a role in antibody production, and **natural killer** (**NK**) **cells** have a role in innate immunity. Lymphocytes can be further categorized by their capacity to express different surface molecules that facilitate the various functions of the lymphocyte subpopulations. For example, both T cells and B cells express antigen receptors on their surface. However, the T cell antigen receptor (TCR) is a complex structure that recognizes peptide – major histocompatibility complex (MHC) on a presenting cell while B cell antigen receptor (BCR) is simply an antibody expressed on the surface of the cell that directly recognizes and binds antigen. In contrast, NK cells do not express these receptors. Instead, NK cells have the ability to identify and interact with surface molecules that are present in healthy normal cells (self recognition). Tissues with altered surface antigens (such as neoplastic cells) are targeted by NK cells which then signal the immune system of their presence, thus facilitating the elimination of abnormal cells (Tizard, 2009b, LaRosa and Orange, 2007).

Lymphocytes initially develop from hemopoietic stem cells which are derived from the bone marrow and differentiate into a common lymphoid progenitor (CLP). From the CLP, lymphocytes differentiate into either B or T cells. B lymphocytes can proliferate to pre-B cells in the bone marrow after interleukin-7 (IL-7) stimulation. Subsequently, B cells mature in peripheral lymphoid tissues (tonsils, lymph nodes, spleen, bronchial-associated lymphoid tissue, and gut-associated lymphoid tissue) and under the influence of IL-2, IL-4, and IL-5 begin to express antibodies on their surface. The final maturation step of B cells occurs under the effect of IL-6, IL-4, and IL-10 and results in the secretion of antibodies. B cells also serve as antigen presenting cells and can interact with T cells through the B cell receptor (BCR) (Latimer and Prasse, 2003, Jackson, 2007).

In contrast, when CLP migrate to the thymus, they differentiate into T lymphocytes. As maturation and differentiation of T cells progresses, signaling events in the thymus promote the expression of cell surface markers such as cluster of differentiation (CD) and the T cell receptor (TCR). These markers are also used to distinguish various subpopulations of lymphocytes. The final differentiation of lymphocytes into T cytotoxic, helper T, and other T cell subpopulations occurs in peripheral blood, lymph nodes, and spleen under IL-7, IL-1, IL-2, and IL-4 signaling (Latimer and Prasse, 2003, Jackson, 2007, Carrick and Begg, 2008, Cruse et al., 2004).

As mentioned previously, T cells can be divided into subpopulations based on the array of molecules displayed on the surface, the types of signals they generate, and the type of functions they perform. For example, the TCR on T helper cells recognizes an antigen presented by an antigen-presenting cell and this interaction initiates the intracellular signaling events that direct the T cell response. The TCR is a heterodimer. In the vast majority of lymphocytes (95%), the TCR is comprised of α and β peptide chains (TCR α/β), while in the remaining lymphocytes, the TCR is composed of γ and δ peptide chains (TCR γ/δ). On the cell surface, TCR forms a complex structure that includes multiple glycoprotein chains. Two of these chains are paired and form the antigen-binding structure of the TCR (Figure 1), while the rest of the chains help to transmit the signal across the cell membrane after antigen binding. One of these associated structures is the CD3 signal transduction complex (Figure 1). This complex is formed by five protein chains (γ, δ, ε, ζ, and η) arranged in three different dimers γ-ε, δ-ε, and ζ-ζ (or ζ-η). In T helper cells, another protein associated with the TCR is CD4 (Figure 1-1). CD4 allows these cells to recognize exogenous-processed antigen binding to antigen presented in the class II major histocompatibility complex (MHC) on antigen-presenting cells. Conversely, CD8 is also associated with the TCR in cytotoxic T cells and recognizes antigen presented on class I MHC (Figure 1-1). These molecules contribute to the ability of the immune system to recognize and differentiate tissues that are self from non-self. The recognition of non-self or abnormal tissue is achieved in part by the loss or distortion of these markers of histocompatibility (Tizard, 2009b, LaRosa and Orange, 2007, Ceri and Mody, 2004).

CD4+ cells have been shown to contribute to the establishment of the associated inflammatory response both in asthma and RAO. Originally, CD4+ T helper cells were classified as Th1 and Th2 type depending on the cytokines they secreted. Recently, other types of T cells have been identified, including T helper 17 (Th17), regulatory T cells (T reg), and Th9 cells (Figure 1-2). Additionally, another group of T cells named Th0 cells secrete both Th1 and Th2 cytokines. It is believed that Th0 cells are precursors of Th1 or Th2 cells in a transition state between these two populations (Durrant and Metzger, 2010, Carrick and Begg, 2008, Tizard, 2009a, Xing et al., 2011, Afshar et al., 2008, Murphy and Stockinger, 2010).

Th2 cells play an important role in asthma and RAO as they contribute in the initiation and maintenance of these diseases. Signature cytokines secreted by Th2 cells are IL-4, IL-5, and IL-13. The main functions of IL-4 are to stimulate the differentiation of Th2 cells from näive T cells, and promote the production and secretion of IgE from B cells (Lloyd and Hessel, 2010, Larché et al., 2003). IL-13 also signals cells to secrete IgE, and has an effect of increasing

bronchial hyperreactivity, inflammation, and airway smooth muscle (ASM) remodeling (Hamid and Tulic, 2009). In humans, IL-5 promotes the influx of eosinophils into the airways leading to inflammation and tissue damage (Lloyd and Hessel, 2010). In contrast, studies in RAO affected horses have found the expression of IL-5 mRNA in BALF or peripheral blood cells to be too low to stimulate pulmonary eosinophilia (Cordeau et al., 2004, Horohov et al., 2005). In asthma IL-13 induces airway hyperresponsiveness, goblet cell hyperplasia, mucus secretion, and fibrosis. In RAO horses, increased expression of IL-13 has been reported, and is thought to be related to the role that this cytokine plays in regulating IgE production (Kleiber et al., 2005, Finotto, 2008).

In contrast, Th1 cells apparently have a beneficial role in asthma due to their capacity to inhibit development and proliferation of Th2 cells (Durrant and Metzger, 2010, Afshar et al., 2008). The characteristic cytokine secreted by Th1 cells is IFN-γ. In humans, studies have found that IFN-γ prevents that IL-4-activated B cell switch to an IgE-secreting isotype cells, and also inhibits Th2 cell induction. However, in asthmatic patients, upregulation of IFN-γ production has also been associated with the pathogenesis of the disease. In horses with RAO, both increased and decreased expression of IFN-γ have been detected during clinical disease. In addition, CD4+ Th1 type cells from heavey horses have been shown to produce IL-2, leukotriene LTB-4 and integrin ICAM-1. These molecules contribute to recruitment of neutrophils that secrete reactive oxygen species (ROS). These products in turn cause oxidative stress and increased protease activity that cause tissue damage (Lavoie, 2007, Marinkovic et al., 2007, Cordeau et al., 2004). The true role of Th1 and Th2 cells in RAO affected horses remains unclear, and these seemingly conflicting results may be due to differences in the methodology used for measuring this cytokine expression and/or temporal changes that may occur in cytokine expression during the progression of the disease (Finotto, 2008, Hamid and Tulic, 2009).

Th17 cells are known to secrete IL-17, and this cytokine has been associated with the development and severity of asthma in humans (Durrant and Metzger, 2010). Additionally, Th17 cells produce IL-17F, IL-22, TNF, and IL-6; however, a complete understanding of the role of Th17 cells and the role of this cell population in the pathogenesis of asthma is still needed. In horses, the role of IL-17 and RAO has not been extensively studied, and the results of the studies that have been done have not provided conclusive evidence of a role for this cytokine (Vock et al., 2010, Reyner et al., 2009, Barnes, 2008). In humans, regulatory T cells (T reg) reduce airway inflammation and airway hyperresponsiveness through secretion of anti-inflammatory cytokines such as IL-10 and TGF-β. In addition, decreased production of IL-10 has

been proposed as a possible causal mechanism of airway inflammation associated with asthma. However, the suppressive effect of IL-10 is seen only when a chronic antigen exposure occurs. In acute exposure, regardless of the presence of IL-10, airway inflammation occurs in response to exposure to allergens (Vock et al., 2010). In horses, T reg cells have been identified in peripheral blood, but so far their role in RAO is unknown (Robbin et al., 2011, Jäger and Kuchroo, 2010). To date, little is known about Th9 cells, although they are known to synthesize and secrete large amounts of IL-9. Recently it has been reported that these cells may influence the initiation and progression of allergic diseases, especially asthma (Vock et al., 2010, Xing et al., 2011).

1.3.3.1.2 Neutrophils

While it is likely that lymphocytes direct the response, the presence of neutrophils is increased in the airways in RAO horses. In BALF from normal horses, neutrophils represent less than 1% of cells, but in RAO horses this percentage can increase to over 50%. Neutrophils can be found in airways soon after an inhalation challenge in RAO horses, invading and accumulating in the lung within 6 to 8 hours (Robinson, 2001b, Davis and Rush, 2002, Lavoie et al., 2001). This increase in the number of neutrophils in BALF could be the result of an overexpression of neutrophil chemotactic cytokines, especially IL-8 and IL-4 (Robinson, 2001b, Léguillette, 2003, Horohov et al., 2005, Lavoie-Lamoureux et al., 2010). In human asthma, neutrophils are involved in the pathogenesis of severe cases, being aggressive and inducing tissue destruction and airway remodeling. Also, similar to horses, upregulation of IL-8 can contribute to neutrophil accumulation at inflammation sites in people with asthma (Nakagome and Nagata, 2011, Holgate, 2008).

1.3.3.1.3 Mast cells

In RAO affected horses, sensitized mast cells secrete vasoactive molecules, proteases, cyclooxygenase metabolites, cytokines, and chemokines. Histamine secretion is related to initial bronchospasm, vascular leakage, and mucus secretion. Similarly, in humans circulating mast cells have the ability to initiate hypersensitivity reactions by degranulation and secretion of histamine, cysteinyl leukotrienes, and also by producing cytokines (IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, tumor necrosis factor [TNF] and transforming growth factor-β [TGF-β]). Moreover, secretion of chemokines affects smooth muscle contraction, increases microvascular permeability and contributes to the inflammatory response in asthma. In addition, mast cells, in

conjunction with eosinophils, play a role in airway remodeling in humans (Bradding et al., 2006, Davis and Rush, 2002, Léguillette, 2003, Holgate, 2008, Kariyawasam and Robinson, 2007).

1.3.3.1.4 Basophils

Basophils are also present in RAO affected horses, releasing histamine in response to stimulation by exposure to allergens. In humans, basophils increase the type I hypersensitivity response by releasing histamine-containing granules, proteases, cytokines (TNF-α, IL-4, IL-5) and eicosanoids (prostaglandin D_2 [PGD_2], thromboxane A_2). Additionally, an antigen presenting cell-role has been given to these cells (Davis and Rush, 2002, Hamid and Tulic, 2009, Holgate, 2008).

1.3.3.1.5 Macrophages

Alveolar macrophages in RAO affected horses express pro- (TNF-α, IL-1β, and IL-8) and anti-inflammatory (IL-6 and IL-10) cytokines after an aerosolized challenge. Pulmonary macrophages from RAO affected horses are also present in higher numbers than in non-affected horses. The presence of these cells results in signaling an increase in cytotoxic cells and an increased release of pro-inflammatory mediators such as superoxide anion, IL-1, and thromboxane B_2. Macrophages in humans with asthma can release leukotrienes, reactive oxygen and lysosomal enzymes that also contribute to the disease (Laan et al., 2006, Holgate, 2008).

1.3.3.1.6 Eosinophils

Eosinophils are important inflammatory cells found in increased numbers in patients with asthma. However, their precise role is still unclear. They secrete different pro-inflammatory molecules like cytokines and chemokines. Additionally, eosinophils can exert their effects through interactions with mast cells and epithelial cells. In RAO affected horses, the presence of eosinophils is rare, and only occasionally be found in the bronchial tissue and fluids (Holgate, 2008, Venge, 2010, Kariyawasam and Robinson, 2007, Robinson, 2001b, Robinson, 2001a).

1.3.3.2 Bronchoconstrictive response and the neural control in airways

The second key pathophysiologic aspect in RAO and asthma is bronchoconstriction. To better understand this response first it is important to comprehend the mechanisms that regulate the tone of airway smooth muscle.

1.3.3.2.1 Neural control of the Respiratory System and airway smooth muscle tone

The respiratory system is innervated by the autonomic nervous system (ANS) (also called visceral or vegetative), the non-adrenergic-non-cholinergic system, and sensory nerves. The ANS innervation is composed of the afferent and efferent nerves which regulate many functions, including airway smooth muscle tone, microvascular permeability, airway secretion, and recruitment/activation of inflammatory cells. The ANS innervates all tissues and organs in the body except skeletal muscle fibers and forms the major efferent part of the peripheral nervous system (PNS). The ANS is formed by the sympathetic or adrenergic (also called noradrenergic) and the parasympathetic or cholinergic systems. Sensory and parasympathetic nerves reach the airways via the vagus nerve, while sympathetic nerves enter via the spinal cord. The sympathetic nervous system functions to regulate the distribution of resources when the body requires an extra effort such as the "fight or flight" response, when increases in heart rate and blood pressure are needed. The parasympathetic system primarily influences the processes required for the maintenance of the organism ("rest and digest"). Some specific activities of the parasympathetic system include activation of the digestive processes, reduced cardiac activity, regulation of ASM tone, and increased bronchial secretions. The non-adrenergic non-cholinergic (NANC) system is an additional source of innervations that act independently from the autonomic system (Matera et al., 2002, Elenkov et al., 2000, van der Velden and Hulsmann, 1999, Masty, 2008, Verhein et al., 2009, Brodal, 2004).

1.3.3.2.1.1 Sympathetic system

The sympathetic or adrenergic nervous system in horses is composed of preganglionic or presynaptic sympathetic neurons that are located in the lateral side of the thoracic and lumbar segments of the spinal cord. After leaving the spinal cord, the axons of these preganglionic cells synapse with postganglionic or postsynaptic nerves. Based on this arrangement, the sympathetic ganglia are classified into two different groups, the prevertebral and the paravertebral ganglia; there is a third group that is not clearly anatomically organized, and is classified as "other sympathetic ganglia". Preganglionic sympathetic fibers synapse with

the postganglionic neurons near the organ they innervate (van der Velden and Hulsmann, 1999, Matera et al., 2002, Masty, 2008, König et al., 2004).

The principal neurotransmitters of the sympathetic nervous system are norepinephrine (noradrenaline) and epinephrine (adrenaline), both of which bind to adrenergic receptors. Most postganglionic sympathetic neurons release norepinephrine, and they form noradrenergic fibers. In horses, adrenergic fibers are present mainly in larger airways rather than in smaller ones, and the adrenergic response throughout the lung is mediated by the activation of G-protein coupled receptors named α and β adrenergic receptors (Matera et al., 2002, Sonea et al., 1993) (Table 1-1). The major function of the sympathetic system in airways is the regulation of mucosal blood flow; nonetheless, β-adrenergic receptors are broadly distributed in this tissue, being activated by norepinephrine, epinephrine, and other agonist drugs (Rush, 2004, Robinson, 1997a).

1.3.3.2.1.2 Parasympathetic system

The parasympathetic system constitutes the principal neural bronchoconstrictor system in humans and other mammals. In horses, the parasympathetic (cholinergic) system is also called the craniosacral division of the autonomic system, and this name is based on the location of the preganglionic parasympathetic cell bodies (Masty, 2008). The axons of the largest parasympathetic nucleus leave the brain along with the vagus nerve, and from there they are distributed to the organs in the thoracic and abdominal cavity (König et al., 2004). The cholinergic innervation of the airways arrives from the vagus nerve and traverses through local ganglia situated within the alveolar walls. From here, postganglionic fibers diffuse to the ASM and submucosal glands (Matera et al., 2002). In general, this system is the major neural pathway controlling ASM and secretion in all species. In horses, the parasympathetic system also innervates the tracheobronchial tree similar to other species (Verhein et al., 2009, Matera et al., 2002, Baroffio et al., 2009).

The principal neurotransmitter in the parasympathetic system is acetylcholine (ACh) which binds to G protein-coupled muscarinic acetylcholine receptors and nicotinic ligand-gated ion channels. In the airway, ACh acts as a major regulator of airway function. Receptor binding of ACh results in bronchoconstriction, mucus secretion, and airway wall remodeling. Acetylcholine is an acetic ester of choline, being synthesized from choline and acetyl-CoA by choline acetyltransferase (ChAT) (and occasionally by carnitine acetyltransferase).

Acetylcholinesterase degrades ACh to a non-active choline and acetate (Gwilt et al., 2007, van Westerloo, 2010, van der Velden and Hulsmann, 1999, Kummer et al., 2008).

Although ACh is mainly a neuronal product, a non-neuronal release of ACh has also been recently described in the human airways and other tissues. This discovery has changed the paradigm that ACh acts only as a neurotransmitter. Non-neuronal ACh can function as a local signaling molecule regulating basic cell functions like proliferation, differentiation, maturation, migration, and organization of the cytoskeleton. In non-neuronal tissue ChAT activity has been detected throughout the cell, suggesting that the synthesis of ACh takes place throughout the entire cell. In addition, in the neuronal system, ACh is formed and stored in vesicles while in the non-neuronal system, ACh is produced and released using an active transport mechanism mediated by members of the organic cation transporter (OCT) family, which act as bidirectional ACh transporters. In airway epithelium, OCT subtypes 1 and 2 are involved in ACh release (Verhein et al., 2009, Gwilt et al., 2007, Wessler and Kirkpatrick, 2008, Lips et al., 2007, Kawashima and Fujii, 2008).

As previously mentioned, ACh can bind to different type of receptors. Muscarinic receptors can be found in different tissues (cardiac tissue, smooth muscle), at parasympathetic neuroeffector junctions (Table 1-2). Acetylcholine binds to muscarinic receptors differently distributed in the airways. In horses, these receptors are widely present through the airways, mainly localized in smooth muscle and submucosal glands (Törneke et al., 2002, Brenner and Stevens, 2010a).

Acetylcholine also binds to nicotinic ACh receptors (nAChRs). These receptors are ligand gated ion channels formed by 5 subunits (α, β, γ, δ, ε) located on the cell membrane and arranged to form an ion channel. Multiple nicotinic receptor isotypes exist, and differences in activation and inactivation kinetics, and pharmacological characteristics are highly dependent on the specific subunit arrangement. Three groups of nicotinic receptors have been identified: skeletal muscle, autonomic ganglia, and central nervous system. Acetylcholine binding to nicotinic receptors leads to a conformational change that allows sodium ion influx, depolarizing the effector cell. The function of nicotinic receptors in ASM is not clear yet. Studies using isolated and tracheal tissue from dogs, guinea pigs, pigs, and mice have reported both contractile and relaxing function to nicotine and nicotine receptor agonists; contraction is apparently mediated by neuronal ACh and relaxation is mediated, at least partially, by NO (nitric

oxide) secreted by epithelial cells (Gwilt et al., 2007, Brenner and Stevens, 2010a, Racké and Matthiesen, 2004, Tournier and Birembaut, 2011, Racké et al., 2006).

1.3.3.2.1.3 Non adrenergic non cholinergic (NANC) system

In a number of species, including the horse, a third neural system called the non-adrenergic non-cholinergic (NANC) system has been identified. This system is not blocked by either adrenergic or cholinergic antagonists, and can be further divided into inhibitory NANC (iNANC) and excitatory NANC (eNANC) (Jordan, 2001, Kraneveld et al., 2000, Matera et al., 2002).

The iNANC system mediates bronchodilation in the human airways. While present in many species, the distribution of iNANC innervation along the trachea-bronchial tree varies among species. In horses, iNANC nerves innervate the trachea and ASM of central bronchi. The iNANC nerves synthesize nitric oxide (NO) and vasoactive intestinal peptide (VIP). Once produced, NO is liberated by simple diffusion. VIP is known to be a potent relaxant of human airways. Because of their role in the neural bronchodilator pathway, VIP and NO counteract bronchoconstriction, acting as a physiologically inhibitory mechanism to cholinergic nerves (van der Velden and Hulsmann, 1999, Matera et al., 2002, Robinson, 2007, Kraneveld et al., 2000).

In contrast, the eNANC system regulates bronchoconstriction in airways releasing neuropeptides from a subpopulation of nonmyelinated sensory C nerves. Some of these neuropeptides are calcitonin gene-related peptide (CGRP), substance P (SP), neurokinin A (NKA), neurokinin B (NKB), and secretoneurin. The release of SP causes smooth muscle contraction, submucosal gland secretion, vasodilation, cholinergic nerve stimulation, and chemoattraction of inflammatory cells. These effects are mediated by SP binding to the neurokinin receptors (van der Velden and Hulsmann, 1999, Verhein et al., 2009, Matera et al., 2002).

1.3.3.2.2 Bronchoconstrictive response

Normally, an airway bronchoconstrictive response results from a bidirectional communication between the CNS and the airways; however, following repeated exposure to irritants, an increased excitability of airway preganglionic neurons and hyperresponsiveness is observed (Kc and Martin, 2010). The parasympathetic system is the leading portion of the

pulmonary autonomic nervous system, and stimulation of ASM muscarinic receptors results in muscle contraction and bronchoconstriction. Factors such as β-adrenergic blockade, increased vagal tone, and the role of the NANC system can influence airway responsiveness (Boulet, 2003, Canning and Spina, 2009, Rush, 2004).

As previously mentioned, besides the neural secretion of ACh, different cell types can synthesize ACh in the airway. These include epithelial cells, mast cells, macrophages, lymphocytes, monocytes, and neutrophils. Acetylcholine and ChAT are present in human peripheral blood lymphocytes, representing an independent cholinergic system that may also influence the immune response. Studies in the rat and mouse have reported that production of the non-neuronal ACh, especially the one produced in the airway epithelium, is down-regulated in acute allergic inflammation, suggesting a possible role in epithelial shedding and ciliated cell dysfunction (Lips et al., 2007, Kawashima and Fujii, 2004, Wessler and Kirkpatrick, 2008, Neumann et al., 2007, Wessler et al., 2003, Boulet, 2003).

Five subtypes of muscarinic receptors (M1 – M5) have been described. In humans, all of them are found in the lung; M2 and M3 receptors are present in ASM, and contraction is mainly due to activation of M3 receptor. The activation of the M3 receptor can also stimulate an increase in mucus and water secretion from glands and increase ciliary beat frequency of airway epithelium (Verhein et al., 2009). Receptors M1, M3, and M5 activate, through the α-subunit of the $G_{q/11}$-protein, phospholipase C which generates diacylglycerol (DAG) and inositol triphosphate (IP3). These substances then stimulate protein kinase C (PKC) increasing intracellular Ca^{2+}. In contrast, M2 and M4 receptor activation inhibits, via $G_{i\alpha}$- protein, adenylyl cyclase activity and cyclic adenosine monophosphate (cAMP) synthesis, limiting increases in Ca^{2+} concentration (Caulfield, 1993, Felder, 1995, Eglen, 2006, Proskocil and Fryer, 2005). Even though most of the effects related to muscarinic receptors are made through the α subunit of the G protein, there is evidence that the β/γ-subunit also may play a role in the transduction of muscarinic signals (Racké and Matthiesen, 2004).

As mentioned previously, ASM expresses both M2 and M3 receptors, with M3 being in highest number (Racké and Matthiesen, 2004). Abraham et al. reported regional differences in muscarinic receptor density in the equine tracheobronchial tree (Abraham et al., 2007a). The trachea had the highest number of receptors, followed by the bronchus and lung tissue. Additionally, these authors found that the equine lung parenchyma expressed abundantly all three different muscarinic receptor subtypes: M1, M2, and M3. In contrast the bronchial and

tracheal epithelium and the underlying smooth muscle mainly express the M2 receptor and the M3 receptor less frequently. M2 receptors are also named autoreceptors because when activated by ACh, they exert a negative feedback on ACh release. These autoreceptors have been identified in equine tracheal smooth muscle strips, but their activity is not affected by a specific M2 receptor antagonist, suggesting that autoreceptors in horses belong to a different subtype (Wang et al., 1995a, Matera et al., 2002, Scullion, 2007, Wang et al., 1995b).

In has been postulated that abnormal mechanisms in the parasympathetic system could contribute to asthma, RAO or other respiratory diseases. Parasympathetic nerve dysfunction and the related airway hyperresponsiveness may be due to changes in muscarinic receptor activity (number and/or function), findings that have been confirmed in animal models and humans after exposure to virus, ozone and antigen challenge. Another important finding is that airways of horses treated with atropine bronchodilate similarly in RAO-affected and normal animals, suggesting that the function of autoreceptors is not compromised in RAO-affected horses (Belmonte, 2005, Matera et al., 2002, Abraham et al., 2007b). Similarly, Abraham et al. reported that muscarinic receptor number and subtype in RAO-affected horses did not change in response to the disease (Abraham et al., 2007b). In contrast, humans develop an increase in parasympathetic tone associated with inflammatory (or a specific genetic condition), and changes that alter the nitric oxide (NO) in the lung, which then lead to airway hyperresponsiveness and other signs of asthma (Goyal et al., 2010).

While sympathetic system innervation in airways is limited in most species including humans and horses, adrenergic receptors are present in great numbers. In the lung, α, β_1, and β_2 receptors are expressed. Primarily, β_2 receptors mediate ASM relaxation, and β_2 agonists are largely used in humans as well as in horses to generate bronchodilation. In addition, activation of β_1-receptors on parasympathetic nerves inhibits ACh secretion and helps relax smooth muscle (Canning and Spina, 2009, Verhein et al., 2009).

It is well known that airway inflammation alters nerve function; but, it is also important to consider that nerve function can additionally modify the inflammatory response. Adrenergic receptors are expressed in inflammatory cells, and the activation of these receptors can result in either a pro- or anti-inflammatory cytokine release. This interaction is further confirmed by the fact that neurotransmitter release can alter both smooth muscle contraction and inflammatory response (Elenkov et al., 2000, Verhein et al., 2009, Matera et al., 2002, Canning and Fischer, 2001).

The NANC system is present in airways and influences ASM tone as well as the function of blood vessels, glands and epithelium. The main neurotransmitter of iNANC is NO which acts as an intercellular messenger with potent relaxing properties on ASM and vasodilator effects. After stimulation of eNANC nonmyelinated sensory C fibers, neuropeptides are released that act on smooth muscle, mucosal vasculature, and submucosal glands, leading to airway obstruction. In addition, these neuropeptides have a pro-inflammatory effect that promotes inflammatory cell recruitment, adherence and activation. In humans, evidence indicates that a dysfunction of the NANC system is associated with asthma, inducing bronchoconstriction. In RAO-affected horses the function of iNANC is deficient, and the absence of this inhibitory effect results in increased hyperresponsiveness and airway obstruction. Researchers propose that this effect is due to a rise in NO breakdown in affected airways. However, it is still unclear if this effect is due to the inflammatory response, or if it is a response related to the condition in affected horses (Lewis et al., 2006, Matera et al., 2002, Pisi et al., 2009, Yu et al., 1994).

1.3.4 Treatment of RAO

As previously described, horses with RAO and humans with asthma can experience bronchoconstriction and airway inflammation when exposed to specific aerosolized irritants. Thus treatment is aimed at attenuating the inflammatory response and promoting relaxation of ASM. To achieve this treatment, RAO typically requires the use of a combination of different factors that involve the control of pathopysiological components of the disease and minimize exposure to environmental factors. RAO affected horses can be highly sensitive to aerosolized agents like dust, and a brief contact with it can induce disease. Therefore the first recommended approach for treating RAO is to control the environment of the affected horse. It is necessary to eliminate or at least reduce the amount of dust and allergens present in the environment where the horse is located. This, in part, can be achieved by moving stabled horses to a pasture environment. For horses that cannot be maintained on pasture, changes in bedding, feed, and ventilation have been shown to be beneficial. While reducing exposure to aerosolized debris is essential, RAO horses often also require treatment with medications that relieve bronchoconstriction and reduce airway inflammation (Robinson, 2001b, Léguillette, 2003).

Glucocorticoids are the drugs of choice to decrease inflammation in pulmonary diseases. They diffuse across the cell membrane where they bind and activate glucocorticoid receptors-α (GR-α) located in the cytoplasm of target cells. Upon binding, GR dissociates from the complex and translocates to the nucleus where it mediates repression and induction of pro-inflammatory

and anti-inflammatory gene expression. However, the main function of glucocorticoids is to turn off pro-inflammatory genes; these genes are switched on by pro-inflammatory transcription factors like nuclear factor-κB (NF-κB) and are usually activated at sites of inflammation. In addition, GR interact with intracellular molecules to directly attenuate NF-κB activity. In RAO affected horses, both systemic and inhaled forms of corticosteroids have been shown to effectively reduce airway inflammation (Barnes, 2010, Marwick et al., 2010, Léguillette, 2003).

Glucocorticoids have also been shown to improve ASM response to β_2-agonists by modifying the receptor after it has been bound. This prevents β_2-AR downregulation. Studies in human cells demonstrate an inhibitory effect of corticosteroids on Th2 cell accumulation, a response that was not observed when only β_2-agonists were used. Studies in horses reported increases in lymphocyte β_2-AR density after glucocorticoid use (Papich, 2009, Couëtil and Hinchcliff, 2004, Lavoie, 2007, Couëtil et al., 2007, Léguillette, 2003, Loza et al., 2008, Abraham et al., 2003b).

Anticholinergic drugs like atropine promote bronchodilation in RAO-affected horses, but systemic administration of this class of drugs is associated with severe side effects, like tachycardia, mydriasis, and ileus. Administration of aerosolized preparations of anticholinergic bronchodilators (e.g. ipratropium) has been shown to be effective in RAO horses with minimal side effects. Special devices are also required to facilitate effective aerosolized delivery of the medication to the horse. Because of this, anticholinergic drugs are generally not used as much as β_2-agonists (Lavoie, 2007, Couëtil and Hinchcliff, 2004, Papich, 2009, Robinson, 2001b).

Bronchodilators, such as β_2-agonists or anti-cholinergic drugs, are most often used to treat RAO-affected horses (Robinson, 2001b, Davis and Rush, 2002). Even though sympathetic innervation is sparse in the respiratory tract, non-innervated β_2-ARs are widely distributed throughout the equine airways (Rush, 2004). The β_2-agonists most commonly used in horses are salbutamol (also called albuterol) and clenbuterol. These drugs have a rapid but short period of action. In addition, some long-acting β_2-agonists, like salmeterol, are also used in horses, producing bronchodilation for about 8 hours; however, these drugs are used less frequently than short-acting agonists due to a higher cost per animal treated (Kearns and McKeever, 2009, Robinson, 2001b, Lavoie, 2007).

As with human asthma, β_2-agonists play a central role in relieving bronchoconstriction in RAO-affected horses. As mentioned, treatment with β_2-agonists does not reduce inflammation. In fact, human literature suggests increased mortality associated with long-term use of these

drugs (Weatherall et al., 2010, Cockcroft, 2006). β_2-ARs are also found on other tissues, including lymphocytes. Recent studies aimed at examining lymphocyte response to β_2-agonists suggest that drugs like salbutamol may alter cytokine expression in activated T cells in a dose-dependent fashion (Loza et al., 2008, Yamaguchi et al., 2010). Thus, the β_2-AR and the response of differing tissues to β_2-agonist therapy may be a contributing factor that has not been well explained in either asthmatic people or heavey horses (Barnes, 1999). To further explore this relationship it is important to understand how the β_2-AR functions.

1.4 G-protein coupled receptor and the β-adrenoreceptor

The β_2-AR is a member of the G protein – coupled receptor (GPCR) family. GPCR or seven – transmembrane receptors are the most common and largest group of cell-surface receptors. They play an important role in cell signal transduction with more than 800 members in the human genome. GPCRs acquired their name due to their function of linking heterotrimeric G proteins that modulate signal transduction. This vast group of receptors can be activated by a wide variety of ligands including ions, amino acids, fatty acids, neurotransmitters, peptides, and enzymes. Based on their complexity and wide distribution, they are considered an important target for pharmacological intervention and drug development (Wess et al., 2008, Rajagopal et al., 2010, Weis and Kobilka, 2008, Oldham and Hamm, 2008, Millar and Newton, 2010, Massotte and Kieffer, 2005).

1.4.1 Structure and signal transduction

Architecturally, GPCRs consist of seven hydrophobic transmembrane helices connected by three intracellular loops and three extracellular loops, with an extracellular amino (NH_2) terminus, and an intracellular carboxyl (COOH) terminus. G proteins (guanine nucleotide-binding proteins) consist of three units: the α-subunit which contains the guanine-nucleotide binding site, and the β- and γ- subunits which form a dimer or single functional complex. G proteins can be further classified into four groups based on their sequence homology and regulation of effectors ($G\alpha_S$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$) which exist in an inactive form. Ligand binding to the receptor induces a reversible interaction of the intracellular side of the transmembrane receptor with the heterotrimeric G protein (Kobilka, 2007, Bünemann and Hosey, 1999, Weis and Kobilka, 2008, Tesmer, 2010, Miller, 2008, Heilker et al., 2009).

The signaling process in GPCRs is initiated when a ligand binds to the receptor which acts as a dynamic scaffolding protein that interacts with different intracellular signaling molecules or second messengers. In general, GPCRs maintain a basal or constitutive activity which means they can activate G protein without the previous binding of an agonist, increasing or decreasing the receptor activity. This state can be categorized as a low energy state of the receptor. GPCRs become activated when a ligand or agonist binds, leading to a conformational change that catalyzes the exchange of guanosine 5'-diphosphate (GDP) for a guanosine 5'-triphosphate (GTP) on the Gα subunit of the G protein (active state) and the dissociation of the Gβγ subunit. The Gα-GTP active subunit and the freed Gβγ signal to different effectors, including adenylyl cyclase, ion channels, phosphodiesterases and phospholipases, stimulating various second messengers that modulate numerous physiological processes. The signaling process terminates when the Gα subunit hydrolyzes GTP into GDP and rejoins the Gβγ subunit (Brauner-Osborne et al., 2007, Kobilka, 2007, Weis and Kobilka, 2008, Wess et al., 2008, Johnston and Siderovski, 2007, Bünemann and Hosey, 1999, Yao et al., 2009, Oldham and Hamm, 2008, Hwangpo and Iyengar, 2005).

1.4.2 The β-adrenoreceptor (β-AR)

The β-adrenoreceptor (β-AR) family can be divided into three different subtypes, $β_1$, $β_2$, and $β_3$, encoded by three different genes on human chromosomes. Additionally, a fourth subtype of receptor has been pharmacologically identified, the $β_4$-AR (Lymperopoulos and Koch, 2009, Johnson, 1998, Finch et al., 2005).

These receptors are present in cardiac tissue ($β_1$), ASM ($β_2$), and adipose tissue ($β_3$), and to a lesser extent in other tissues. The $β_1$-AR principal ligands are epinephrine and norepinephrine (NE). The $β_2$-AR has greater affinity for epinephrine than for NE. In contrast, the $β_3$-AR type has increased affinity for NE than epinephrine (Benarroch, 2009, Johnson, 1998, Lymperopoulos and Koch, 2009). Radio-ligand competitive binding assays, using the β-adrenergic antagonist –[125I](-)-iodocyanopindolol (ICPY), has been used to determine the presence of the β-AR in healthy horses. Around 200-500 binding sites per lymphocyte are described in horses, a number that is lower than what has been previously reported for other species (1000 binding sites per cell in humans, and 700 binding sites per cell in cattle) (Abraham et al., 2001, LaBranche et al., 2010, Brodde and Wang, 1988, Vermeir et al., 1993). Studies performed using lung lobe samples and bronchial tissue (small bronchi) of RAO affected horses obtained after slaughter showed a decrease in β-AR expression in lung (30% to

40%) and in bronchi (42%) compared to control tissue (Abraham et al., 2006). In agreement with these results, studies using asthmatic tissue have revealed a reduction in β-AR density and function on peripheral lymphocyte surface; however studies in lung tissue are still not conclusive, reporting both decreased and increased receptor density (Connolly et al., 1994, Gao et al., 2001, Qing et al., 1997). In equine lymphocytes, the principal β-AR is the $β_2$-AR subtype. Similarly, in bovine neutrophils, $β_2$-AR is the major receptor subtype present. This receptor distribution is also applicable to the respiratory tract, where the $β_1$- and $β_2$-AR ratio is approximately 20:80 (Abraham et al., 2001, LaBranche et al., 2010).

1.4.2.1 The $β_2$-adrenoreceptor

The $β_2$-adrenoreceptor ($β_2$-AR) shares the same morphological characteristics common to all GPCRs. As previously mentioned, when the α-subunit of the G_S protein binds to the $β_2$-AR, it promotes the exchange of GDP for GTP, activating the receptor. When activation occurs, the Gα-subunit stimulates adenylyl cyclase, an enzyme that catalyzes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), increasing the intracellular concentration of cAMP. cAMP acts as a second messenger, by binding to the regulatory subunit and activating protein kinase A (PKA), which has many intracellular functions including protein phosphorylation (Johnson, 2006, Liggett, 1999, Hwangpo and Iyengar, 2005).

One of the regulatory mechanisms of $β_2$-AR function is desensitization of the receptor. This phenomenon occurs when the receptor is exposed continuously to agonist binding, leading to loss of functionality. This event also has importance for the therapeutic use of agonists, limiting their function (tachyphylaxis). Desensitization involves a three step mechanism (Table 1-3).

1.4.2.1.1 The role of $β_2$-adrenoreceptor in the respiratory system

In horses, $β_2$-AR presence varies depending on the segment of the respiratory tract. Determinations of their population have concluded that the highest number of $β_2$-AR is present in the lung parenchyma, followed by primary and secondary bronchial epithelial cells and in less number in the tracheal epithelium. In contrast, adenylyl cyclase has higher activity in the trachea epithelium, then in brochus and then in the lung (Robinson, 1997a, Abraham et al., 2003a). Törneke, found that the equine tracheal epithelium has higher number of β-AR when compared to tracheal smooth muscle (Törneke, 1999). In humans, $β_2$-ARs are broadly distributed in the lung. ASM (30 – 40,000/cell) and airway epithelial cells show high expression levels, and

submucosal glands, vascular endothelium, type II pneumocytes, eosinophils, lymphocytes, and mast cells are present in detectable levels (Johnson, 2001, Abraham et al., 2003a, Hanania et al., 2010, Badino et al., 2005). Recently, Nguyen et al. used a mouse model to demonstrate pharmacological and genetic evidence that β_2-AR signaling is needed for the development of mucous hyperplasia, airway hyperresponsiveness, and inflammatory cell infiltration into the lung (Nguyen et al., 2009). In ASM, activated β_2-ARs play a key role in bronchodilation (relaxation) related to modifications in cAMP, PKA, and inhibition of Ca^{2+}. In addition, β_2-ARs have been found in rat post-capillary venular endothelial cells, having a role in plasma exudation regulation and fluid balance in alveolar epithelial cells (Sitkauskiene and Sakalauskas, 2005, Broadley, 2006).

1.4.2.1.2 The role of β_2-adrenoreceptor in inflammation and lymphocyte activation

Through their presence in different cell types, β_2-ARs are involved in the inflammatory process by modulating cytokine and inflammatory mediator secretion. Nonetheless, the regulation of the immune response through β_2-AR is poorly understood (Loza et al., 2007). In airway epithelial cells, as already mentioned, β_2-ARs stimulate ciliary beating, and play a role in secretion of IFN-γ (Barnes, 1999, Anderson, 2006). In mast cells, β_2-ARs have been shown to stimulate histamine release. The receptor is also present in lower density in eosinophils where they may exert functions related to the oxidative burst and release of thromboxane and leukotriene C_4 (LTC_4). Peripheral blood monocytes also have functional β_2-AR that may be associated with TNF-α and granulocyte macrophage colony-stimulation factor (GM-CSF) secretion. In contrast, alveolar macrophages do not respond to β_2-agonist stimulation, suggesting that responsiveness could be altered after maturation in the lung. Neutrophils express β_2-ARs, which, when activated, cause increases in cAMP, inhibition of ligand adhesion and accumulation, modification in cytokine release, and induction of apoptosis (Sitkauskiene and Sakalauskas, 2005, Johnson and Rennard, 2001).

Peripheral blood lymphocytes (B and T cells), cells involved in the synthesis and secretion of cytokines like IL-2, GM-CSF, IFN-γ and IL-3 express β_2-ARs. In normal conditions, equilibrium between inhibitory and stimulatory signaling is required. In B cells, activation of β_2-AR modulates the production of antibodies, increasing their secretion. Activation of β_2-AR, with the following increase of cAMP and PKA activation, leads to inhibition of T cell proliferation and modifications in the cytokine profile; however, studies reported that these changes in cytokine synthesis depend on the time when β_2-AR is stimulated in relation to the time when the cell is

activated (Borger et al., 1998, Ceri and Mody, 2004, Torgersen et al., 2002, Wong et al., 2007, Kin and Sanders, 2006). Additionally, it is known that β_2-AR in lymphocytes can rapidly undergo tachyphylaxis when prolonged exposure to agonists occurs (Giembycz and Newton, 2006). In horses, β_2-ARs are present in lymphocytes, having functions similar to human lymphocytes. However, little information about the role of equine lymphocyte β_2-AR is available (Abraham et al., 2001).

There is an increasing belief that an imbalance between Th1 and Th2 cytokines contribute to asthma pathology, favoring the Th2 response. Additionally, it has been proposed that the constant use of short-acting β-agonists promotes airway inflammation through Th2 cell accumulation (Loza et al., 2008, Larché et al., 2003, Hamid and Tulic, 2009). Loza et al. (Loza et al., 2007) demonstrated that exposure to of β_2-agonists led to accumulation of human Th2 cells through increased cell survival. Similarly, the same authors, using cells from asthmatic patients, demonstrated the accumulation effect of β_2-agonists (formoterol and salbutamol) on Th2 cells after IL-2 stimulation. This accumulation was inhibited after corticosteroid treatment (Loza et al., 2008). Goleva et al. reported that the use of β_2-agonists in combination with very low doses of steroids has a suppressive effect on activation and proliferation of T cells from normal patients, but that effect could not be observed in T cells from asthmatic patients; authors suggest that the effect was not observed in cells from asthmatic patients because an increased expression in PDE4 resulted in a greater degradation of cAMP, preventing the signaling cascade (Goleva et al., 2004). Clearly, the effect of chronic β_2-agonist therapy on the inflammatory component of airway disease is not well understood.

1.4.2.1.3 Pharmacology of the β_2-adrenoreceptor

The discovery of "treatments" for asthma started around 1900, when dessicated adrenal glands were demonstrated to be beneficial in the treatment of this disease. Adrenaline was isolated and synthesized in 1901, at the same time its sympathomimetic properties were discovered. The isolation and synthesis of non-selective, selective, short and long acting drugs for the treatment of respiratory diseases started around 1940 and continue to the present day (Tattersfield, 2006).

Adrenoreceptor agonist drugs mimic the effects of sympathetic nervous system stimulation, which is the reason they are called sympathomimetic drugs. The response obtained after stimulation of β_2-AR in the airways is ASM relaxation. The most used and recommended drugs for asthma in humans and RAO in horses are corticosteroids as anti-inflammatory drugs,

and β_2-agonists as bronchodilators. β_2-agonists act through binding and activating β_2-AR, activating adenylyl cyclase, increasing cAMP concentration, and activating PKA. PKA leads to cell relaxation via effect on K^+ channels, Na^+/K^+ ATPases, Ca^{2+} sequestration, Ca^{2+} sensitivity of myosin, and inositol triphosphate (IP_3) formation (Kraft and Leone, 2009, Broadley, 2006, Brenner and Stevens, 2010b).

Important concepts associated with the pharmacology of drugs are potency, efficacy, affinity, efficacy, onset and duration of action. Potency is the dose (concentration) of the agonist that is required to produce an effect of given intensity. Efficacy is the intensity of effect usually measured as the median effective dose, ED50. Affinity can be defined as the ability of the ligand to bind to a receptor. Efficacy is described as the potency of the ligand to induce a response. Partial agonists bind and activate the receptor to variable degrees and are less efficacious compared to full agonists; neutral antagonists have zero efficacy because they cannot change the basal activity of the receptor. Neutral agonists bind and prevent the receptor activation. Inverse agonists can stabilize or suppress the basal state of the receptor without activating it. The onset of action of agonists is determined by the lipophilic properties of the drug and kinetics of binding. Agonists with higher lipophilicity have a slower onset of action. Drugs that are relatively hydrophilic (e.g. salbutamol) have a rapid onset of action because they bind to the receptor in an aqueous phase. Conversely, lipophilic drugs (e.g. salmeterol) have slower onset of action. The duration of action is also influenced by lipophilicity and binding kinetics, and resistance to clearance as well. Drugs like salmeterol and formoterol have longer durations of action than salbutamol due to higher lipophilicity (Panettieri, 2002, Shore and Moore, 2003, Kobilka and Deupi, 2007, Johnson, 2001, Huber et al., 2008, Bhattacharya et al., 2008, Brenner and Stevens, 2010b, Hanania and Cazzola, 2008, Hanania et al., 2010, Anderson, 2006).

1.4.2.1.3.1 β_2-adrenoreceptor agonists

The interaction between a β_2-AR and a β_2-agonist is dependent on the molecular structure and drug size. The activation of a β_2-AR by a β_2-agonist has a therapeutic effect in respiratory diseases like RAO and asthma. Moreover, if given prior to allergen exposure, β_2-agonists can prevent the development of bronchoconstriction. The activation of β_2-AR in the lung promotes a series of beneficial effects like bronchodilation, enhanced mucociliary function in epithelial cells, decreased permeability of the vascular endothelium, decreased mast cell histamine release, and modifications in ANS neurotransmitter release. Selective β_2-agonists have the advantage of not producing serious adverse effects in the cardiovascular system,

when compared with nonselective β_2-agonists. Nonetheless, when high doses of selective β_2-agonists are given they can still activate cardiac β_1-AR, thus increasing the heart rate (Kraft and Leone, 2009, Brenner and Stevens, 2010b, Lin et al., 2008).

β_2-agonists, as previously mentioned, are classified by their onset and duration of action, affinity, potency and efficacy (Papich, 2009). In ASM, salbutamol can reach the active site of the receptor directly from the extracellular aqueous environment. Due to this, it can act rapidly inducing bronchodilation and muscle relaxation, but the duration of action is short and limited (1 to 2 hours duration in horses). This drug was approved by the FDA for use in horses in 2002 in order to treat symptoms such as bronchospasm and bronchoconstriction in RAO affected horses (Robinson, 2001b, Papich, 2009). Clenbuterol is another β_2-agonist that has been used to treat horses with RAO (10 hours duration in horses). The properties of clenbuterol as a bronchodilator are under review because it is a partial β-agonist and has lower intrinsic activity. Negative side effects can be observed in horses treated with clenbuterol. These include sweating, muscle tremor, restlessness, and tachycardia (Kearns and McKeever, 2009, Ainsworth, 2010, Robinson, 2001b, Rush and Mair, 2004a). Salmeterol, a fourth generation molecule that is approximately a thousand times more lipophilic than salbutamol, reaches the active site in a slow process by diffusing laterally from the membrane; this property gives salmeterol a long mode of action compared to other drugs. In horses, it is the bronchodilator with the longest action, with duration between 8 to 10 hours (Furukawa and Lodewick, 2007, Papich, 2009, Robinson, 2001b, Rush and Mair, 2004a, Rush, 2004).

In mast cells, β-agonists and the subsequent receptor activation leads to a decrease in inflammatory mediator release, stabilizing the cells. The use of salmeterol, salbutamol, and formoterol can also inhibit antigen-induced histamine release from human isolated lung cells and mast cells (Papich, 2009, Broadley, 2006). Studies in humans reported that the use of a β_2-agonist in combination with very low doses of steroids can suppress T cell activation and Th1 cytokine production by peripheral blood mononuclear cells in patients with asthma; the authors concluded that although cAMP concentration did increase with the use of β_2-agonist, the expression of phosphodiesterase 4 also did, resulting in cAMP degradation (with no inhibition of Th2 cytokine production) (Goleva et al., 2004). Loza et al. study described, in cells from asthmatic patients, an effect of β_2-agonists (albuterol, formoterol, isoproterenol, and salmeterol) on Th2 cell accumulation; these results could be significant considering that β_2-agonist therapies can increase the Th2 cell population in airways, resulting in hypersensitivity and

intensified frequency and severity of clinical disease following exposure to allergens (Loza et al., 2008).

Adverse effects that can be observed in horses after the administration of high doses of β_2-agonist include increases in heart rate, palpitations, muscle tremors, sweating and excitement. In humans side effects involving the cardiovascular system (tachycardia, hypertension) and increases in blood sugar due to induction of gluconeogenesis in the liver have been observed (Papich, 2009, Broadley, 2006).

Recent studies have shown that long term use of short (SABA)- and long-acting β-agonists (LABA) increase the potential risk of asthma-related death (Ortega and Peters, 2010). The potential mechanisms for this outcome are not clear. Studies in humans have reported increases in mortality rates after LABA use when compared to those in patients using a combination of a β-agonist and inhaled corticosteroids (Cyr et al., 2010, Weatherall et al., 2010, Sears, 2009). The use of LABA has also been associated with significantly increased risk of asthma-related intubations and death with and without the use of inhaled corticosteroids (Salpeter et al., 2010). Studies trying to elucidate the reasons why β-agonists increase asthma mortality have been an important part of asthma research. Since β-agonists are effective as bronchodilators, they could be masking asthma induced inflammation, decreasing the expressed clinical severity of the disease without reducing its underlying cause. Other mechanisms that can contribute to this problem are possible. For example, once tolerance to the drugs is established, the bronchoprotective effect of β_2-agonists is reduced. Additionally, it has been detected that polymorphism or changes in the amino acid sequence of the β_2-receptor (ADRB2) could increase receptor downregulation induced by the use of β-agonists. Also, an increased airway responsiveness to allergens has been detected after prolonged use of β-agonists (Cockcroft, 2006, Hizawa, 2009). In RAO affected horses, the potential negative effects of β-agonists have not been studied yet; nevertheless, it is important to determine if, like in humans, β-agonists affects the β_2-receptor normal function.

1.4.2.1.3.2 β_2-adrenoreceptor antagonists and inverse agonists

Antagonists and inverse agonists have the ability to block a receptor that is normally activated by an agonist, but only the inverse agonist can turn off an empty active receptor. β-antagonists are normally used to treat cardiac problems (hypertension, heart failure, etc), and are contraindicated in patients with concomitant asthma because they increase the risk of bronchoconstriction, especially when non-selective drugs are used. In addition, the

administration of β-blockers (β-antagonists and inverse agonists) is contraindicated in treatment of asthma because their acute administration can cause an increase in airway resistance (Lin et al., 2008, Lipworth and Williamson, 2010). However, recent studies, using mouse models of asthma, have been performed trying to determine the therapeutic effect of inverse agonists (nadolol, cervidolol, bisoprolol, metoprolol). Apparently, the chronic or long term use of these drugs could have beneficial effects by acting on airway epithelial cells and immune cells through inhibition of pro-inflammatory signaling. It is still not fully understood how β_2-inverse agonists play their beneficial role in asthma, but it is thought that they use an intracellular pathway different than β_2-agonists. These results give a new area of research looking for alternative treatments for asthma in humans. Moreover, they give more evidence of the importance of the β_2-AR on the inflammatory response in airway disease, since it is possible that this receptor, depending on the ligand, exerts either a pro- or anti-inflammatory response using different signaling pathways (Dickey et al., 2010, Nguyen et al., 2008, Callaerts-Vegh et al., 2004).

1.5 Research Justification

1.5.1 Justification

In the United States, in 2009, 8.2% of the population (24.6 million people) was affected by asthma, mostly women and children. As previously reviewed, asthma is a common disease that affects humans, causing airway hyperresponsiveness and obstruction, and tissue remodeling. This multifactorial disease involves different systems, and multiple inflammatory cellular components like T cells. The role of activated T cells in asthma has recently received great attention, mostly due to the cytokines they secrete which are implicated in the pathophysiology of this disease. Treatment of asthma requires the use of a combination of therapies, including the use of β_2-agonists as bronchodilators. However, these drugs also exert an effect on inflammatory cells, presumably mediated by PKA. In T cells the activation of the TCR/CD3 complex results in stimulation of different downstream signaling pathways that ends with the activation and translocation of transcription factors, and activation of translation and cell cycle machinery. PKA has the ability to block TCR/CD3-mediated signaling at different downstream points. That is why the use of β_2-agonists and the consequent activation of the β_2-AR could block the TCR-mediated signaling inhibiting the synthesis of cytokines and proliferation of T cells. Conversely, recent studies have reported negative effects of β_2-agonist use, like increased hyperresponsiveness, poor asthma control, and increased mortality in

asthmatic patients. The use of short- and long-acting agonist drugs (e.g. albuterol and salmeterol) increases Th2 type cell accumulation and cytokine production. In addition, the use of agonists in asthma patients with receptor polymorphism (Arg16Gly ADRB2) induces an increased risk of asthma exacerbations (Holgate, 2008, Akinbami et al., 2011, Orihara et al., 2010, Hamid and Tulic, 2009, Loza and Penn, 2010, Loza et al., 2008, Yamaguchi et al., 2010, Basu et al., 2009, Yu and Bukaveckas, 2008). As previously described, equine heaves and human asthma have many characteristics in common. Three key characteristics include: 1) bronchodilation in response to β_2-agonist therapy, 2) a predominantly Th2 cytokine profile associated with the presence of airway inflammation and clinical disease, and 3) chronic disease which results in similar tissue remodeling and which requires long term therapy to minimize clinical signs. Because of these similarities, the RAO affected horse may be an excellent model for examining the effect of β_2-agonists on lymphocyte activation, function, and regulation in both normal and disease affected horses. Yet little is known about the function of the β_2-AR on equine lymphocytes or the effect of β_2-agonist exposure. The goal of this study was to develop a method for evaluating the response of equine peripheral blood lymphocytes (PBLs) to β_2-agonist stimulation. This technique was then used to begin to explore and compare the response in cells from RAO affected horses and non-affected horses. Results, coming from this animal model, could add additional information to the understanding of how T cells respond to the use of agonists, and their role in asthma.

1.5.2 Hypothesis:

H_0: Peripheral blood lymphocytes (PBLs) from RAO-affected and non-affected horses will not differ in their *in vitro* response to stimulation with β_2-agonists

1.5.3 Objectives:

- Develop an *in vitro* method for indirectly measuring the response of equine PBLs to β_2-agonist stimulation
- Measure and compare the response to β_2-agonist stimulation in activated PBLs from RAO-affected and non-affected horses
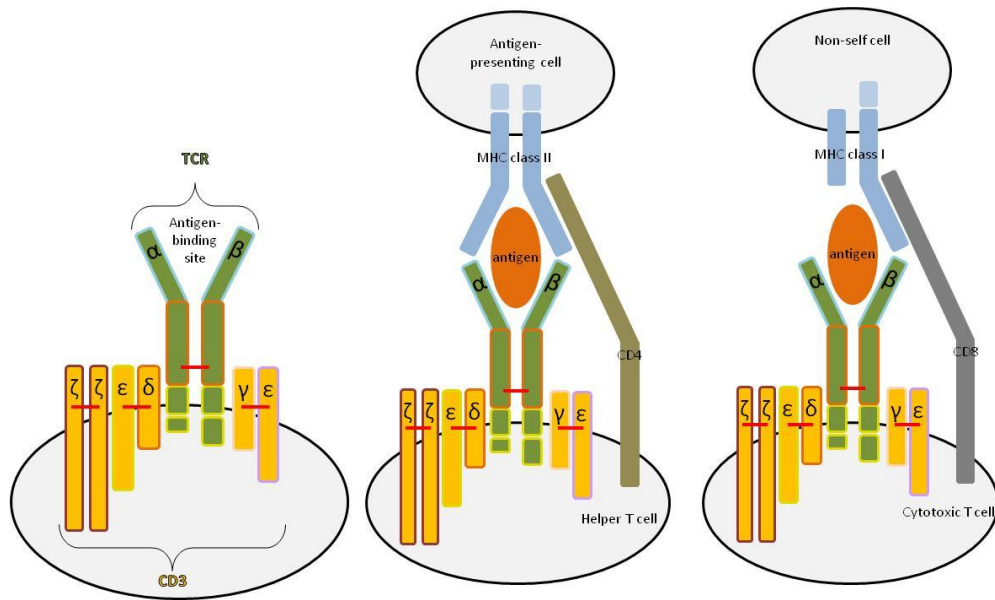
Figure 1-1. Structure of T cell receptor (TCR) (in green) and CD3 structure (in orange). Helper T cell with their associated CD4 protein bind to the class II MHC, and cytotoxic T cells associated with CD8 structure bind to the class I MHC (Adapted from (Tizard, 2009a)).
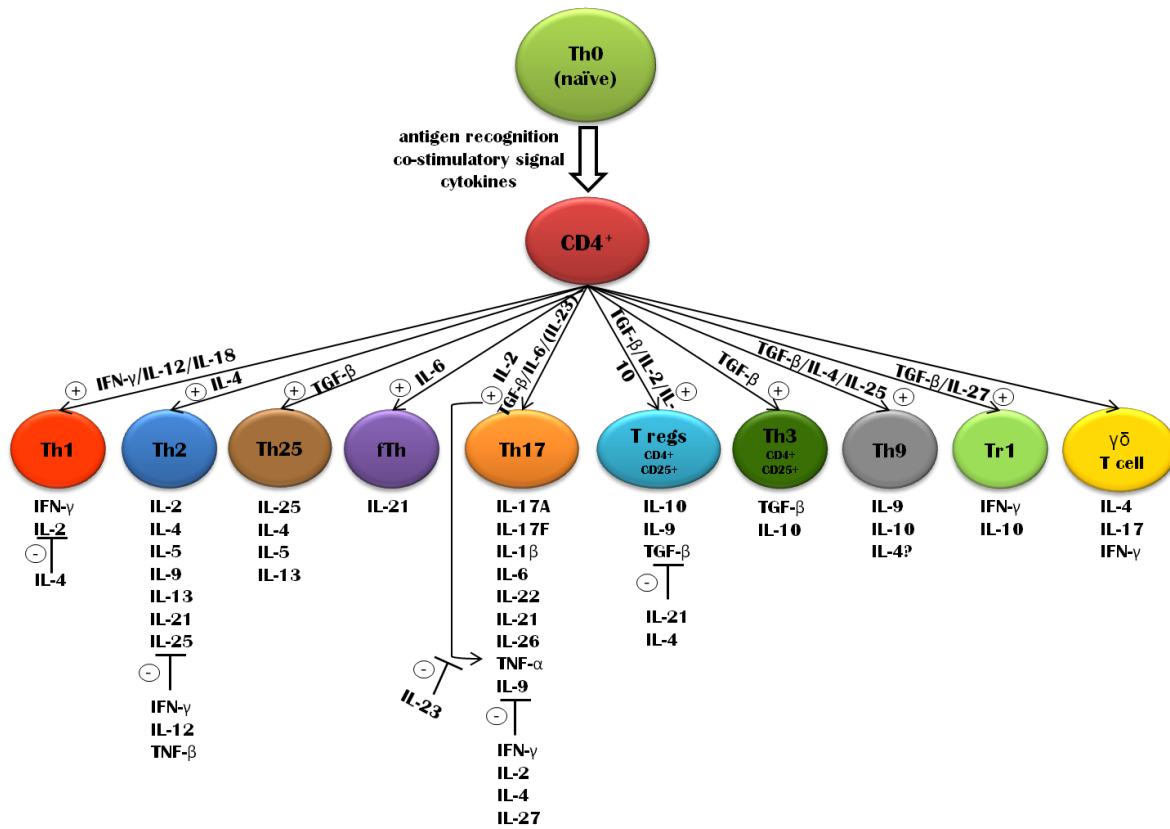
Figure 1-2. Differentiation of Th0 cells (Based on (Finotto, 2008, Murphy and Stockinger, 2010, Paul and Zhu, 2010, Moser and Leo, 2010, Lloyd and Hessel, 2010, Jäger and Kuchroo, 2010, Bonneville et al., 2010, Vock et al., 2010)).

Table 1-1. Adrenergic receptors identified in equine and human airway tissue and inflammatory cells. *Represent presence of the receptor on human tissue only (Based on (Verhein et al., 2009, van der Velden and Hulsmann, 1999, Matera et al., 2002, Kc and Martin, 2010, Lymperopoulos and Koch, 2009)).

Receptor	Location	Function
α_1	ASM*	Contraction
α_2	Cholinergic nerves	$\downarrow$ ACh release
	Prejunctional (autoreceptors)*	Inhibit release of NE and NPY
	Macrophage*	Release inflammatory cytokines
	Neutrophil*	?
β_1	Airways	?
	Submucosal glands*	
	Alveolar walls*	
	Parasympathetic nerves*	Inhibit ACh release (relaxes ASM)
β_2	Cholinergic nerves	$\uparrow$ ACh release
	ASM	Relaxation
	Epithelial cells*	?
	Mast cell*	Inhibit release of histamine
	Eosinophil*	Prevent degranulation

Table 1-2. Muscarinic receptors identified in equine and human airways and inflammatory cells. *Represent presence of the receptor on human tissue only (Based on references (Matera et al., 2002, Gwilt et al., 2007, Törneke et al., 2002, Verhein et al., 2009, Scullion, 2007, Gosens et al., 2006)).

Receptor	Location	Function
M1	Parasympathetic ganglia	Facilitation of neurotransmission and enhance cholinergic bronchoconstriction
	Submucosal glands	↑secretion?
	Alveoli	?
	Mast cell*	Inhibition of histamine release
	T lymphocyte*	↑cytotoxicity
	B lymphocyte*	Proliferation
	Neutrophil*	Chemotaxis
	Eosinophil*	?
	Bronchial epithelium*	Release of monocytes, eosinophil and neutrophil
M2	Postganglionic cholinergic nerves	Inhibit release ACh
	ASM	Inhibit contraction
	T lymphocyte*	↑cytotoxicity
	B lymphocyte*	Proliferation
	Neutrophil*	Chemotaxis
M3	ASM	Contraction
	Submucosal glands	↑mucus secretion
	Epithelial cells	↑ciliar bath?
	Endothelial cells	Vasodilation via NO
	Eosinophil*	Inhibition of activation
	Macrophage*	ACh stimulates release of leukotriene B_4
	Neutrophil*	Chemotaxis
	T lymphocyte*	↑cytotoxicity
	B lymphocyte*	Proliferation
	Bronchial epithelium*	Release of monocytes, eosinophil and neutrophils
M4	Alveoli	?

	Smooth muscle	?
	Postganglionic nerves?	Inhibit release ACh?
	Eosinophil*	Inhibition of activation
	Neutrophil*	?
	T lymphocyte*	↑cytotoxicity
	B lymphocyte*	Proliferation
M5	Eosinophil*	Inhibition of activation
	Neutrophil*	?

Table 1-3. Steps involved in β_2-receptor desensitization (Liggett, 1999, Mutlu and Factor, 2008, Johnson, 2006, McGraw and Liggett, 2005, Shore and Moore, 2003, Finch et al., 2005)

Steps	Events
1. **Phosphorylation**	Protein GPCR kinases 2 and 3 (GRK2 and GRK3) phosphorylate the receptor, leading to a conformational change and a decrease in the interaction between the β_2-AR and the Gα-subunit. PKA can induce a similar response, suggesting that this regulatory process can be initiated by intracellular PKA increases. Receptor phosphorylation results in binding of β-arrestin, a protein that helps reduce coupling of the receptor to the G protein. In a similar manner, β-arrestin induces mobilization of proteins, especially phosphodiesterase IV to the vicinity of the receptor. This protein metabolizes cAMP suppressing the activation of the receptor.
2. **Sequestration**	Internalization occurs when the receptor is exposed to agonists for prolonged periods. Under these conditions, the β_2-AR is internalized into the cell and phosphorylated, thus preventing any signaling process. Internalization occurs in endosomes, which also allows the dephosphorylation of the receptor and fully functional recycling back to the cell membrane. Also, the internalized receptor can be sent to lysosomes for degradation.
3. **Downregulation**	Downregulation does not involve receptor phosphorylation. Instead receptor numbers are reduced through a decrease in transcription (reduced synthesis), messenger RNA (mRNA) degradation, and enhanced receptor protein degradation

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CHAPTER 2

MATERIALS AND METHODS

2.1 Horses

Blood samples were obtained from a total of 19 horses (15 mares and 4 geldings) ranging in age from 6 to 30 years. Control group included 14 horses, and RAO affected horses group included 5 animals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech.

RAO affected horses used in the present study were part of an RAO herd, meeting the diagnostic criteria for RAO as described in the introduction (Chapter 1). In addition, animals from the RAO affected group presented a mild to medium state of the disease (remission). The control group was composed of clinically healthy animals, with no sign of respiratory disease. A complete blood count was performed on all horses at the times blood samples were collected for mononuclear cell isolation.

2.2 Reagents and laboratory materials

All reagents and materials are presented in Table 1 (Appendix A) in the Appendix section of the thesis. The most important are also listed in the section below that describes particular procedures.

2.3 Equine peripheral blood mononuclear (PBM) cell isolation

2.3.1 Reagents:

- Hanks' balanced salt solution, without calcium chloride and magnesium sulphate 1x (Sigma-Aldrich, St. Louis, MO) and 10x (Gibco, Carlsbad, CA)

- Percoll (Sigma-Aldrich) stock

- Dulbecco's Phosphate Buffered Saline (DPBS), without calcium chloride and magnesium chloride (Gibco)

- RPMI 1640 (500mL) (Gibco) complete media with 1.8µl 2-mercaptoethanol (Sigma-Aldrich), 10mL penicillin (10,000UI/mL) & streptomycin (10mg/mL) (Gibco), 10mL L-glutamine in NaCl (Gibco), 5mL sodium-pyruvate (Sigma-Aldrich), 10mL gentamicin (10mg/mL) (Gibco)

2.3.2 Preparation of 59% Percoll:

Iso-osmotic Percoll: to adjust stock Percoll to the same osmolality as 1x Hanks' balanced salt solution, stock Percoll was mixed with 10x Hank's balanced salt solution in the volumes depicted in the table below.

Stock Percoll	300mL	100mL	50mL	40mL	10mL
10X Hanks' balanced salt solution	27.2mL	9.07mL	4.35mL	3.62mL	0.907mL

After iso-osmotic Percoll was prepared, it was mixed with a volume of 1x Hanks' balanced salt solution to produce a 59% gradient solution (see table below). Percoll density of 59% was checked to determine the refractive index using a refractometer. Ideal density was determined to be between 115 and 120. If the density of the solution was not in this range, a small volume of 1x Hanks' solution or iso-osmotic Percoll was added and the density was re-checked until it was within the ideal range.

Reagent	10mL	20mL	40mL	50mL	100mL
Iso-osmotic Percoll	5.9mL	11.8mL	23.6mL	29.5mL	59mL
1X Hanks' balanced salt solution	4.1mL	8.2mL	16.4mL	20.5mL	41mL

2.3.3 Peripheral blood mononuclear (PBM) cells isolation protocol

Peripheral blood mononuclear (PBM) cells were harvested from equine whole blood collected in 10mL tubes with sodium heparin, and isolated using a 59% Percoll density gradient technique.

a. Whole blood was obtained by jugular venipuncture using 10mL (16x100mm) BD (Franklin Lakes, NJ) Vacutainer® Sodium Heparin blood collection tubes and 18Gx 1 ½'' blood collecting needles. Once collected, blood was mixed by manual rotation of the tubes.

b. Tubes were transported to the laboratory and centrifuged for 8 minutes at 600 x g at 27ºC.

c. After centrifugation, plasma from each tube was removed using a plastic transfer pipette and discarded.

d. Preparation of Percoll gradient: 5mL of 59% Percoll was loaded into a 15mL conical tube making sure not to leave residual gradient on the wall of the tube above the level of the gradient.

e. White cell layer (buffy coat) was harvested using a plastic transfer pipette and combined with an equal volume of 1x Hanks' balanced salt solution (without calcium chloride and magnesium sulphate) in a 15mL conical tube. Tubes were mixed using manual rotation.

   The mixture of white cell layer and Hanks' solution was gently layered onto 5mL of 59% Percoll gradient.

f. Transfer was performed carefully to avoid disruption of the gradient-blood interface. After the buffy coat and Hank's balanced salt solution was loaded onto the gradients, the tubes were centrifuged for 40 minutes at 930 x g at 22ºC.

g. After centrifugation, the mononuclear cell layer was identified on the top phase of the 59% Percoll gradient while neutrophils and red cells formed a pellet at the bottom of the tube. This mononuclear layer was gently harvested and transferred to a 50mL conical tube containing 30mL of DPBS (without calcium chloride and magnesium chloride),

using a plastic transfer pipette. These cells were gently mixed with the buffer using a vortex mixer and centrifuged for 8 minutes at 600 x g at 27ºC.

h. The supernatant was aspirated and the pellet was resuspended in 30mL of DPBS (without calcium chloride and magnesium chloride), mixed using a vortex, and centrifuged for 8 minutes at 600 x g at 27ºC.

i. The supernatant was aspirated and the pellet of PBM cells was resuspended in RPMI complete media without fetal calf serum, and mixed carefully.

j. Once the cells were in media, PBM cells were counted using a Cellometer® Auto T4 (Nexcelom Bioscience LLC, Lawrence, MA) automatic cell counter. A total volume of 20µl of the cell suspension was placed on a Nexcelom slide to count cells. PBL concentration was adjusted to obtain a total of 4×10^6 cells per mL. Additional data using the Cellometer® cell counter such as images of counted cells, mean diameter of measured cells, and viability of cells were saved for later reference.

2.4 Stimulation of PBM cells

2.4.1 Justification:

An important component of the treatment in RAO and asthma is the use of bronchodilators like β_2-agonists, acting mainly through β_2-adrenoreceptors on airway smooth muscle. However, it is known that inflammatory cells, like T cells also express this type of receptor. Studies have reported that these drugs can have an effect on inflammatory cells, such as producing an imbalance between Th1 and Th2 cells, favoring the Th2 cytokine production (Yamaguchi et al., 2010, Loza et al., 2007).

To evaluate the response of cells, equine PBM cells were stimulated with different reagents known to increase cAMP intracellular concentration and PKA activity. Reagents that bind to the β_2-adrenergic receptor, salbutamol and isoproterenol (ISO) were used. PGE_2 that binds to prostanoid receptor (EP) receptors and was used as a positive control since it is known to increase cAMP concentration.

2.4.2   Reagent and solution preparation

All reagents used were dissolved following the manufacturer's instructions, and a stock solution of 10mM, based on the molecular weight was prepared as follows:

- Salbutamol: (selective β_2-adrenoreceptor agonist) (Sigma-Aldrich)

  Synonym: Albuterol, α-[(*tert*-butylamino)methyl]-4-hydroxy-*m*-xylene-α,α'-diol

  Molecular Weight: 239.31

  Appearance: Powder

  Solubility (Turbidity): Clear at 50mg/mL in methanol

  For a 10mM stock solution: Based on the molecular weight of salbutamol (239.31), 0.05 grams of salbutamol powder were mixed with 20.89mL of methanol, aliquoted and stored at -20ºC.

- ± - Isoproterenol hydrochloride: (receptor selective β_1 and β_2-adrenoreceptor agonist) (Sigma-Aldrich)

  Molecular Weight: 247.7

  Appearance: White to off-white powder

  Solubility: Clear to very slightly hazy colorless to yellow or faint pink solution at 50mg/mL in water (ddH_2O)

  For a 10mM solution: Based on the molecular weight of isoproterenol (247.7), 12.38 milligrams were mixed with 5mL of ddH_2O, aliquoted and stored at -20ºC.

- Prostaglandin E_2 (dinoprostone): (Cayman Chemical, Ann Arbor, MI)

  Molecular Weight: 352.5

  Appearance: crystalline solid

  Solubility: solvents such as ethanol, DMSO, or dimethyl formamide at 100mg/mL

  For a 10mM solution: Based on the molecular weight of PGE$_2$ (352.5),10 milligrams of PGE$_2$ were mixed with 2.84mL of ethanol 100%, aliquoted and stored at -20ºC.

For reagent solutions concentration calculation: (applied to all reagent concentration calculation)

Stock solution is 10mM

10mM = 10,000µM

10mM = 10,000,000nM

1µM = 1,000nM

So, 1µl of the stock solution was mixed with 1000µl of RPMI complete media obtaining a 10,000nM solution (or 1µl of 10mM=10,000,000nM/1000µl =10,000nM). Based on this solution, different concentrations used for the experiments were calculated and prepared. The same basics were used to calculate other dilutions used for the experiments. In addition, controls were made the same way as above, using matching volumes of the solvent of each the reagent.

2.4.3 PBM cells stimulation protocol

Isolated PBM cells, as described above, were used in a final concentration of $4x10^6$ cells per mL. PBM cells were transferred to 5mL polystyrene round-bottom tubes containing different concentrations of reagents that were prepared from the 10mM stock solution. In addition, the solvent for each reagent was used as its own control. The volume of solvent was the same as the associated volume of stimulant and cell populations otherwise were treated identically (Figure 2-1).

Cells in complete RPMI media 4 x 10^6 cells/ml

30 min. (37^0C;5%CO_2)

Salbutamol (control: methanol)	Isoproterenol (control: ddH$_2$O)	PGE$_2$ (control: ethanol)
250nM, 500nM, 1µM, 2µM, 4µM, 8µM, 16µM	50nM, 200nM, 500nM, 800nM, 1µM, 10µM	50nM, 200nM, 500nM, 800nM, 1µM, 10µM
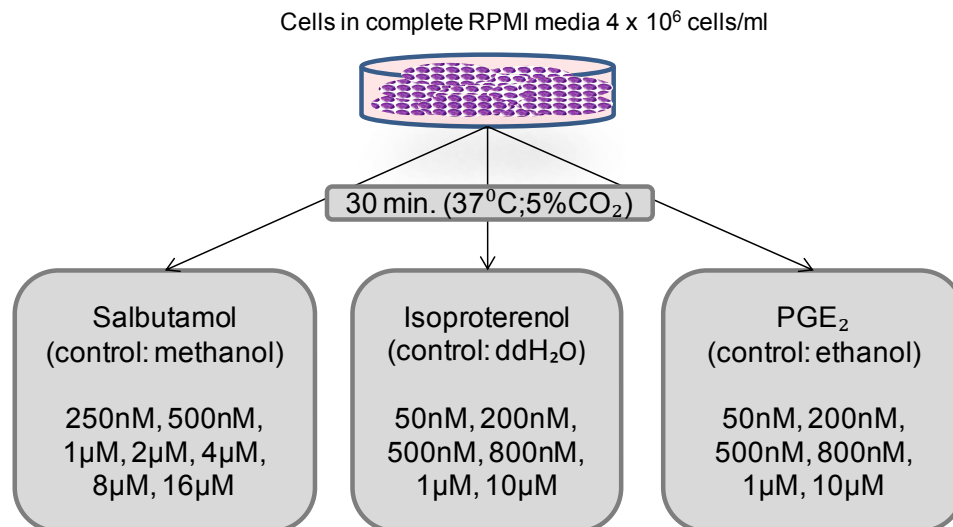
Figure 2-1. Peripheral blood mononuclear cell stimulation after isolation using different reagents known to increase intracellular cAMP.

Tubes containing cell suspension and the reagents were placed in a water jacketed incubator (VWR Scientific, Suwanee, GA) at 37ºC and 5%CO_2 for 30 minutes. After stimulation,

cells were washed twice with DPBS (without calcium chloride and magnesium chloride) for 8 minutes at 600 x g at 27ºC.

2.5 Stimulation of activated PBM cells

2.5.1 Justification:

To determine if the state of activation of the PBM cells influenced their response to a β_2-agonist, cells were pre-incubated and activated with concanavalin A (ConA) (*Canavalia ensiformis*), and after stimulation with salbutamol. ConA is a well known plant lectin that has been used as a cell mitogen, enhancing T cell function and imitating antigen effect (Resch and Ferber, 1988, Hu et al., 2010).

2.5.2 Reagent and solution preparation

- ConA (Sigma-Aldrich) was dissolved in RPMI complete media with 10% fetal calf serum to a final concentration of 5mg/mL.

- RPMI 1640 (500mL) (Gibco) complete media with 1.8µl 2-mercaptoethanol (Sigma-Aldrich), 10mL penicillin (10,000UI/mL) & streptomycin (10mg/mL) (Gibco), 10mL L-glutamine in NaCl (Gibco), 5mL sodium-pyruvate (Sigma-Aldrich), 10mL gentamicin (10mg/mL) (Gibco) with 10% fetal calf serum.

- DPBS, without calcium chloride and magnesium chloride (Gibco)

- Salbutamol (prepared as previously described in section 2.4.2)

2.5.3 PBM cell activation and stimulation protocol

The selected concentration of ConA to activate PBM cells was 2ug/10^6 cells (based on previous reports (Ferrada et al., 2008, Borger et al., 1998)). Isolated PBM cells were resuspended in RPMI complete media with 10% fetal calf serum containing 2ug/10^6 cells of ConA, and cultured in a water jacketed incubator (VWR Scientific) at 37ºC and 5%CO_2 for 24 and 48 hours.

After ConA activation, cells were washed with DPBS (without calcium chloride and magnesium chloride), and stimulated with different concentrations of salbutamol, using as a control its solvent following the same protocol as the previous experiment (Figure 2-2).

Cells in complete RPMI media 4 x 10^6 cells/ml
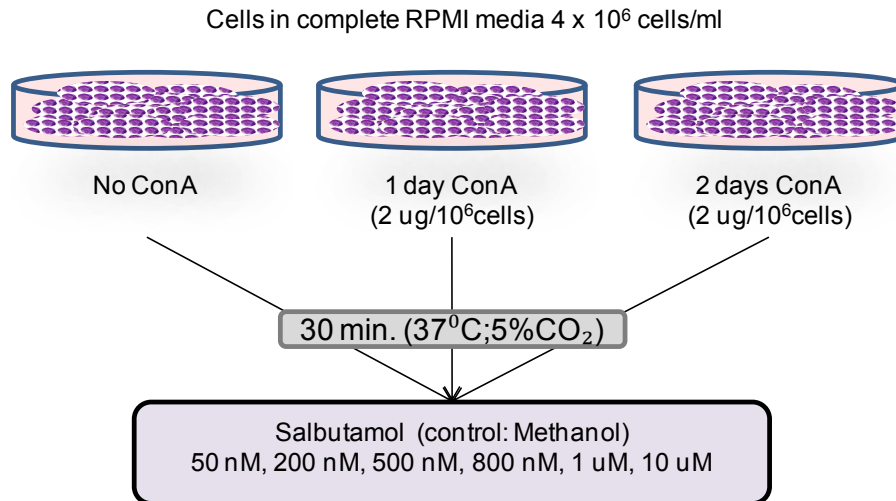
Figure 2-2. Peripheral blood mononuclear cell activation with ConA for different periods of time and stimulation with different concentrations of salbutamol.

2.6 Pre-culture with antagonist and stimulation of activated PBM cells
2.6.1 Justification:

As previously mentioned, it is known that PBM cells express β_2-adrenergic receptors on their surface, and that binding of reagents like β_2-agonists can have an effect on these cells.

To determine if the cell stimulation was made through the binding of a β_2-agonist like salbutamol to the β_2-adrenergic receptor activated PBM cells were pre-incubated with antagonists.

2.6.2 Reagent and solution preparation

The selected β-antagonists were ICI 118,551 and atenolol. Similar to the reagents for PBM cell stimulation, a 10mM stock solution of the antagonists was prepared as follows.

- ICI 118,551 hydrochloride (selective β_2-adrenergic receptor antagonist) (Sigma-Aldrich)
  Molecular Weight: 313.86
  Appearance: White powder

Solubility: Soluble at 12mg/mL in water with warming to 60^0C

For a 10mM solution: Based on the molecular weight of ICI (313.86), 5 milligrams of ICI were mixed with 1.593mL of ddH$_2$O, and placed in a 60^0C water bath until dissolved. Aliquoted and stored at -20^0C after dissolved.

- S(-)-Atenolol (selective β_1-adrenergic receptor antagonist) (Sigma-Aldrich)

  Molecular Weight: 266.3

  Appearance: White powder

  Solubility: Ethanol

  For a 10mM solution: Based on the molecular weight (266.3), 10 milligrams of atenolol were mixed with 3.75mL of ethanol 100%, aliquoted and stored at -20^0C.

- ConA (Sigma-Aldrich, St. Louis, MO) was dissolved with RPMI complete media with 10% fetal calf serum to a final concentration of 5mg/mL.

- RPMI 1640 (500mL) (Gibco) complete media with 1.8µl 2-mercaptoethanol (Sigma-Aldrich), 10mL penicillin [10,000UI/mL] & streptomycin (10mg/mL) (Gibco), 10mL L-glutamine in NaCl (Gibco), 5mL sodium-pyruvate (Sigma-Aldrich), 10mL gentamicin (10mg/mL) (Gibco) with 10% fetal calf serum

- DPBS, without calcium chloride and magnesium chloride (Gibco, Carlsbad, CA)

- Salbutamol (prepared as previously described in section 2.4.2)

2.6.3 PBM cell pre-culture with antagonists and salbutamol stimulation protocol

After ConA activation for 24 hours (same protocol as above), cells were washed with DPBS (without calcium chloride and magnesium chloride), and pre-cultured for 15 minutes with ICI 118,551, atenolol, or both antagonists together in a water jacketed incubator (VWR Scientific) at 37ºC and 5%CO$_2$. Solvents for each antagonist were used as their own control.

Subsequently, cells were washed with DPBS (without calcium chloride and magnesium chloride), and stimulated with one concentration of salbutamol (500nM) (Figure 2-3). Activation

time and salbutamol concentration for stimulation were selected based on the results from the previous experiment.

Cells in complete RPMI media 4×10^6 cells/ml
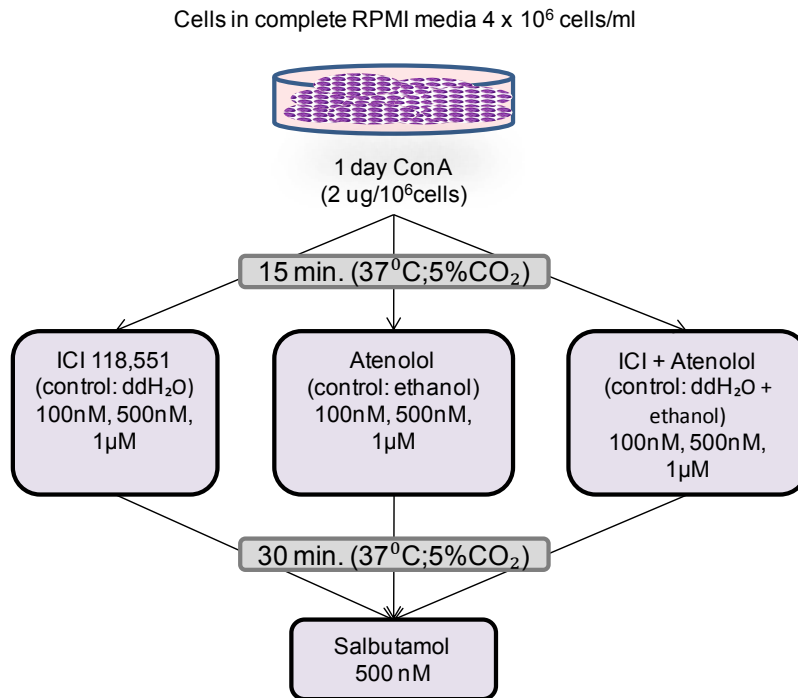
Figure 2-3. Peripheral blood mononuclear cells activated for one day with ConA, pre-cultured with β-adrenoreceptor antagonists, and stimulated with salbutamol.

2.7   Measurement of vasodilator-stimulated phosphoprotein (VASP) phosphorylation
2.7.1   Justification:

For an indirect determination of protein kinase A (PKA) activity, VASP phosphorylation was measured using flow cytometry technique.

VASP is a phosphoprotein found in many different cells, and as part of the ENA/VASP family of proteins, has a central role as regulator of actin assembly and cell motility. In addition, it is known that mammalian VASP proteins are substrates of cAMP- and cGMP-dependent serine and threonine kinases, PKA and PKG, respectively. PKA is composed of two catalytic subunits maintained in an inactive conformation when it is bound to a regulatory subunit dimer; when two molecules of cAMP bind to the regulatory subunit, it induces a conformational change that leads to the release of the catalytic subunit, able to phosphorylate substrates. VASP has three phosphorylation sites, Ser157, Ser239, and Thr278; however, the PKA preferred

phosphorylation site is Ser157 (Krause et al., 2003, Mosenden and Taskén, 2011). Therefore, after β₂-adrenergic receptor activation, increased cAMP concentrations raise PKA activity, leading to VASP Ser157 phosphorylation (Figure 2-4). The primary antibody used in the present study recognizes only the amino acid Serine 157 of VASP when is phosphorylated by PKA. Hence, antibody binding can be used to detect variations in phosphorylated VASP and this is considered an indirect measure of PKA activation.
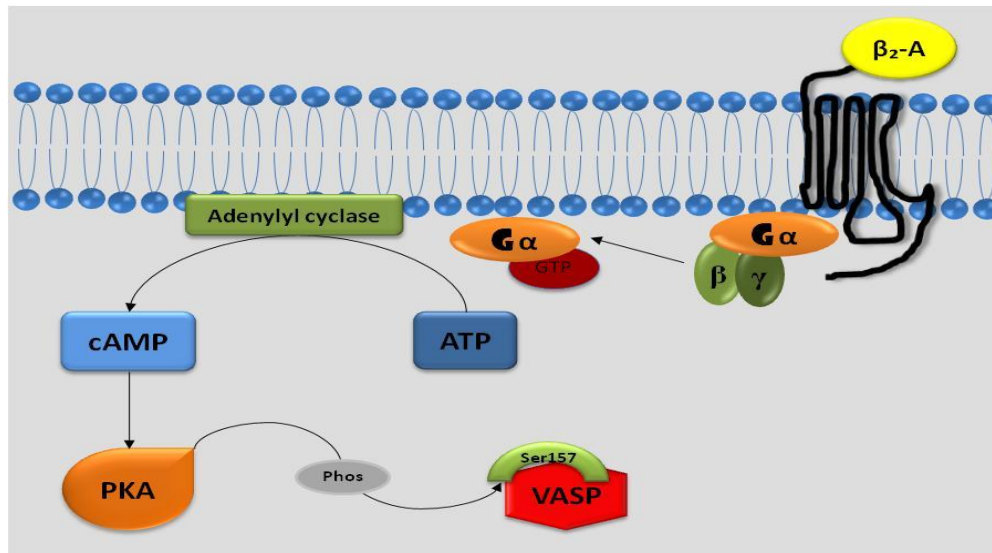
Figure 2-4. Signaling pathway after a β₂-agonist binds to a β₂-adrenoreceptor. β₂-A: β₂-adrenoreceptor; Gα,β,γ: G-protein; GTP: guanosine 5'-triphosphate; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; Phos: phosphorylation; Ser157: Serine 157; VASP: vasodilator stimulated phosphoprotein.

2.7.2   Reagent and solution preparation

- 10% formalin (VWR Scientific)

- DPBS, without calcium chloride and magnesium chloride (Gibco)

- Permeabilization solution: 500mL DPBS (without calcium chloride and magnesium chloride), 0.5g gelatin, 2.5g saponin, 5mg NaN_3, and 25µl Tween 20

- DPBS gel: 500mL DPBS (without calcium chloride and magnesium chloride), 0.5g gelatin, 5mg NaN_3

2.7.3   Antibody preparation

2.7.3.1 Primary antibody

Primary mouse monoclonal IgG_1 anti-phospho-VASP (pSer 157) antibody (NanoTools, Germany; 0085-100/VASP-5C6; 100ug/mL) was used. Primary antibody was diluted to a concentration of 1:500 w/v in permeabilization solution.

2.7.3.2 Secondary antibody

Secondary FITC Alexa Fluor®488 goat anti-mouse IgG (H+L) 2 mg/mL (Invitrogen Molecular Probes, Eugene, OR) antibody was used. Secondary antibody was diluted at a concentration of 1:1000 w/v in permeabilization solution.

2.7.4 Phosphorylated VASP detection protocol

a.   After PBM cells were stimulated as mentioned above, cells were fixed with formalin 10% ($100\mu l/10^6$ cells) for 10 minutes in the refrigerator (vortex while adding to avoid clumps). After the fixation process, cells were washed with DBPS (without calcium chloride and magnesium chloride) for 8 minutes at 600 x G at 27ºC.

b.   Cell pellet was permeabilized with permeabilization solution ($100\mu l/10^6$ cells) for a period of 1 hour in the refrigerator.

c.   After permeabilization, 100µl of cell suspension per well were distributed in each well of a 96 well V-plate.

d.   20µl of primary antibody was added to each well. 96 well V-plate was placed on ice (in the dark) for 30 minutes on a rocker.

After 30 minutes, cells were washed twice with 100µl of DPBS gel and centrifuged at 600 x g for 3 minutes at 20ºC.

e.   100µl of secondary FITC antibody was added per well. The 96 well V-plate was placed on ice (in the dark) for 30 minutes in the rocker; after that, cells were washed twice with

100µl of DPBS (without calcium chloride and magnesium chloride), and centrifuged at 600 x g for 3 minutes at 20ºC.

f. Cells were resuspended in 100µl of DPBS (without calcium chloride and magnesium chloride) and transferred to 1.2mL microtiter tubes. Further PBLs were gated, and phosphorylated VASP fluorescence using a Flow Cytometer BD® FACSAria I was detected. Emission wavelength was determined with a 533nM filter and excitation wavelength with a 488nM filter.

g. Data were analyzed using the FlowJo 7.2 software for Windows, and saved as medians and means of phosphorylated VASP fluorescence.

2.8   Western blot
2.8.1   Justification

As previously mentioned, VASP is a substrate for PKA phosphorylation, having a molecular weight of 46KDD before phosphorylation. Therefore, to confirm that the anti-phospho-VASP primary antibody that was used for flow cytometry was binding to VASP, immunoblotting technique was performed.

2.8.2   Reagent and solution preparation

- Lysis buffer: 150mM NaCl, 10mM trizma base, 0.5% triton X-100, 0.5% w/v deoxycholate

- Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL)

- Laemmli buffer (Bio-Rad, Hercules,CA)

- ProSieve® color protein markers (Lonza, Allendale, NJ)

- Running buffer (Bio-Rad, Hercules, CA)

- Transfer buffer: 2.9g/1L Tris base, 14.5g/L glycine, 200mL methanol, ddH$_2$O for 1L final volume

- TTBS buffer: 6.1g/L Tris base, 8g/L sodium chloride, 0.29g/L potassium chloride, ddH$_2$O to 1L final volume, adjust to pH7.6, add 0.5mL/L Tween-20

- Blocking buffer: TTBS buffer and 5% non-fat dry milk

2.8.3 SDS-PAGE gel preparation:

Gels were made using a casting frame system (Bio-Rad, Hercules, CA). Both glasses (spacer and short plates) were placed in the casting frame with the short plate facing the front of the frame, and the pressure cams were closed to secure the glasses in the frame. The gel cassette assembly was placed on the grey casting stand gasket, and secured with the spring loaded lever on the casting strand.

Resolving gel (5.1mL ddH$_2$O, 2.3mL 30% acrylamide mix, 2.5mL 1.5M Tris [pH8.8], 0.1mL 10% sodium dodecyl sulfate [SDS], 0.1mL 10% ammonium persulfate [APS], 0.004mL TEMED [N,N,N',N'-Tetramethylethylenediamine]) was prepared in a conical tube, mixed and loaded in the glass cassette, using a glass pipette to fill the cassette. ddH$_2$O was put over the resolving gel to assure a straight edge was formed. The gel was allowed to polymerize for 40 – 45 minutes. After polymerization, the gel was rinsed with ddH$_2$O.

Stacking gel (4.2mL ddH$_2$O, 1.0mL 30% acrylamide mix, 0.76mL 0.5M Tris [pH6.8], 0.06mL 10%SDS, 0.06mL 10%APS, 0.006mL TEMED) was placed over the polymerized resolving gel using a glass pipette, filled until the top of the short glass. Comb was placed between both glasses. Gel was allowed to polymerize for 40 – 45 minutes. After polymerization, the comb was removed and the wells were rinsed with ddH$_2$O.

2.8.4 Immunoblotting protocol

a. After PBM cells were stimulated with either salbutamol or PGE$_2$, cells were washed with cold DPBS (without calcium chloride and magnesium chloride) for 8 minutes at 600 x g at 27ºC.

b. 200µl of lysis buffer were added to the cell pellet, allowing cells to lyse for 25 minutes in a shaker. Lysed cells were transferred to microcentrifuge tubes, and centrifuged for 8 minutes at 14,000 rpm. Supernatant was transferred to new microcentrifuge tubes and stored at -80ºC.

c. For protein quantification, a Pierce® BCA Protein Assay Kit was used following the user's manual instruction.

d. After protein was quantified, 5µg (detection of p-VASP-Ser157) or 8µg (quantification of VASP and p-VASP-Ser157 [Appendix II]) of protein per sample were mixed 1:1 with Laemmli buffer. Tubes were placed for 8 minutes in a hot bath (85 – 90ºC).

e. For the electrophoresis, samples and color protein markers (ProSieve®) were loaded in duplicates in a 7% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and run at 120V for 90 minutes in running buffer.

f. For the electroblotting or membrane transfer, SDS-PAGE gel was assembled in a transfer module, using a nitrocellulose membrane (Trans-Blot Transfer Membrane® 0.45µm, Bio-Rad), filter paper (Mini-Trans-Blot® filter paper, Bio-Rad), and fiber pads (Bio-Rad, Hercules, CA) as showed in Figure 2-5. Filter paper and nitrocellulose membranes were soaked 30 minutes before transfer in transfer buffer. Nitrocellulose membrane was placed between the gel and the positive side (red) of the module (negative proteins transferred to the membrane located in the positive side of the module). Transfer was made at 100V for 1 hour in transfer buffer.
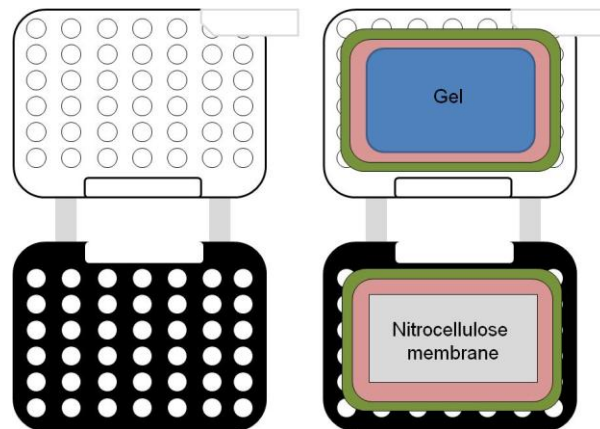
Figure 2-5. Transfer module arrangement, fiber pads (green), filter paper (pink), gel (blue), and nitrocellulose membrane (grey).

g. After the transfer step, the membrane was blocked for 1 hour in a rocker, using 10mL of blocking buffer. The membrane was washed for 15 minutes with TTBS buffer in the rocker.

h. For antibody staining, membranes were probed with a primary mouse monoclonal IgG1 antibody (anti-phospho-VASP pSer 157) (Nanotools, Germany) or a primary mouse monoclonal IgG$_{2a}$ antibody (VASP [A-11]) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies were diluted to a 1:2000 w/v concentration (0.5µg/ml) in TTBS buffer. The membrane was left in a rotamixer overnight at 4ºC.

i. Next, the membrane was washed twice with TTBS buffer, for 20 minutes in a rocker. Then the membrane was stained using a fluorescent secondary antibody IRDye800® conjugated affinity purified sheep anti-mouse IgG (H&L) (Rockland, Gilbertsville, PA). Antibody solution was made at a 1:5000 w/v concentration. The membrane was placed, covered on a rocker for 1 hour at room temperature. Then the membrane was washed three times with TTBS buffer for 20 minutes in a rocker.

j. Analysis of the membrane was performed in a Near-Infrared (NIR) fluorescence detection method, using an Odyssey® Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

k. Digital images were saved as TIFF files to further analyze them with ImageJ software (National Institute of Health, Bethesda, MD) for Windows.

2.9 Statistical analysis

Normal probability plots were generated to verify that data followed an approximate normal distribution. Subsequently, the effects of days of ConA, dose of salbutamol (or other compound as appropriate), and RAO status on response to stimulation were assessed using mixed model analysis of covariance with baseline and solvent associated measurements as covariates. Horse was included in the model as a random blocking factor. Model adequacy (that the errors followed a normal distribution with constant variance) was verified by inspecting the residual plots. Statistical significance was set at $p < 0.05$. All analyses were performed SAS version 9.2 (Cary, NC, USA).

2.10 Materials and Methods for Additional Data (Pilot Studies)

2.10.1 Determination of p-VASP-Ser157 in bronchoalveolar lavage cells

2.10.1.1 Justification

Bronchoalveolar lavage (BAL) is a technique developed in order to recover fluid from peripheral airways and alveoli. Cytologic analysis of BAL fluid (BALF) is used as a measure of lung inflammation and the diagnosis of RAO (Hoffman, 2008, Robinson, 2001). The purpose of this experiment was to gather preliminary information about how the response of lymphocytes from the airways of RAO and non-affected horses compared to the response of ConA stimulated PBLs.

2.10.1.2 Reagents

- Sterile saline solution

- RPMI 1640 (500mL) (Gibco) complete media with 1.8µl 2-mercaptoethanol (Sigma-Aldrich), 10mL penicillin [10,000UI/mL] & streptomycin (10mg/mL) (Gibco), 10mL L-glutamine in NaCl (Gibco), 5mL sodium-pyruvate (Sigma-Aldrich), 10mL gentamicin (10mg/mL) (Gibco)

- DPBS, without calcium chloride and magnesium chloride (Gibco)

- Salbutamol (prepared as previously described in section 2.4.2)

- PGE_2 (prepared as previously described in section 2.4.2)

2.10.1.3 BALF cells collection and stimulation protocol
  a. BAL fluid collection tube was passed through the nose and trachea into the lower airways in both RAO- and non-affected horses after sedation (detomidine 0.01mg/Kg and butorphanol 0.01mg/Kg intravenously [IV]).
  b. A sterile saline solution (300mL) was instilled in 100mL aliquots and BALF was recovered using 60mL syringes.
  c. Each sample was placed in a sterile collection cup and placed on ice until processed (within 30 minutes).
  d. BALF was mixed by manual rotation, transferred to 50mL tubes and centrifuged for 8 minutes at 500 x g at 20ºC.

e. After centrifugation, supernatants were aspirated and discarded. Each cell pellet was washed with DPBS without calcium chloride and magnesium chloride for 8 minutes at 500 x g at 20ºC.

f. Each cell pellet was resuspended in RPMI complete media without fetal calf serum.

g. Cells were counted using a Cellometer® Auto T4 automatic cell counter (Nexcelom Bioscience LLC, Lawrence, MA). Cells were adjusted to a final concentration of 2×10^6 cells per mL.

h. BALF cells were stimulated with salbutamol (500nM) and PGE_2 (500nM) for 30 minutes (37ºC; 5%CO_2); solvent for each stimulant was used as its own control (Figure 2-6). The salbutamol concentration was selected based on our previous experiments (dose response curve) where the 500nM concentration induced significant differences between both groups.

i. After stimulation, cells were fixed, permeabilized and stained following the same protocol used to detect p-VASP-Ser157 in PBLs cells using flow cytometry (Chapter 2, section 2.7).

BALF cells in complete RPMI media 2×10^6 cells/ml

30 min. (37^{0}C;5%CO_2)

Salbutamol
(solvent: methanol)
500nM

PGE_2
(solvent: ethanol)
500nM
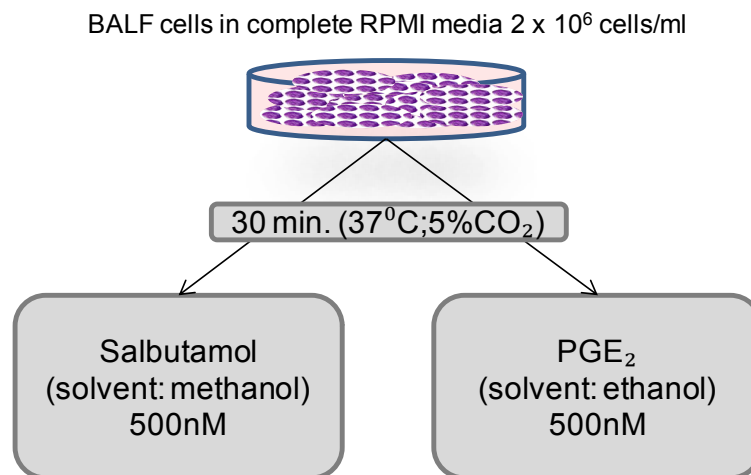
Figure 2-6. Isolated BALF cells from RAO and non-affected horses stimulated with salbutamol (500nM) and PGE_2 (500nM).

2.10.2 Cyclic AMP concentration measurement

2.10.2.1 Justification

As previously mentioned, stimulation of the β_2-AR induces the activation of adenylyl cyclase through the Gα subunit of the G-protein. This enzyme catalyzes the conversion of ATP into cAMP, which in turn activates PKA. Measuring the cAMP concentration can be used as a complementary or confirmatory endpoint to the determination of p-VASP-Ser157 by flow cytometry after cell stimulation.

2.10.2.2 Reagents

- Salbutamol (prepared as previously described in section 2.4.2)

- PGE$_2$ (prepared as previously described in section 2.4.2)

- IBMX (3-isobutyl-1-methylxanthine)

- EIA assay (Cayman Chem.)

2.10.2.3 cAMP concentration measurement protocol

a. Isolated PBMC were activated for 48 hours with ConA (2ug/10^6 cells) (37ºC; 5%CO$_2$).

b. After activation, cells were stimulated with salbutamol (800nM) and PGE$_2$ (500nM). In addition, IBMX (500µM) was added based on previously described methods (LaBranche et al., 2010) (Figure 2-7).

PBM cells in complete RPMI media 2×10^6 cells/ml

2 days ConA
($2 \, ug/10^6$ cells)

30 min. (37^0C; 5%CO_2)

| Cells + IBMX (500µM) | Cells + Salbutamol (800nM) | Cells + Salbutamol/IBMX (800nM/500µM) | Cells + IBMX (500µM) | Cells + PGE$_2$ (500nM) | Cells + PGE$_2$/IBMX (500nM/500µM) |
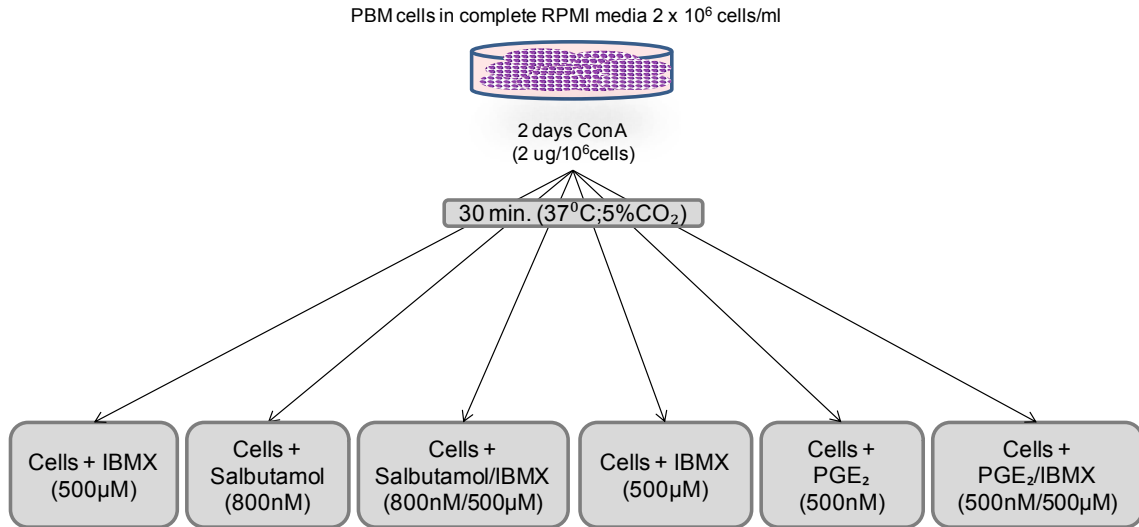
Figure 2-7. PBMC isolated from RAO and non-affected horses, activated with ConA for 48 hours and stimulated with salbutamol (800nM), PGE$_2$ (500nM), and IBMX (500µM).

c. After stimulation, cells were centrifuged for 8 minutes at 600 x g at 4ºC. After centrifugation, tubes were decanted and blotted on paper tissue.

d. Each cell pellet was resuspended in 125µl of HCl (0.1M) and incubated for 20 minutes at room temperature. Cells were centrifuged for 5 minutes at 600 x g at 25ºC.

e. The supernatant for each sample was used to perform the assay following the instructions manual provided by the company.

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CHAPTER 3

RESPONSE OF PERIPHERAL BLOOD LYMPHOCYTES FROM RAO HORSES TO β_2-AGONIST STIMULATION USING AN INDIRECT METHOD

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Abstract

Recurrent airway obstruction (RAO) is a common respiratory disease that affects middle-age horses, leading to bronchoconstriction and airway inflammation. β_2-agonists like salbutamol (albuterol) are frequently used as a treatment for RAO because these medications promote airway smooth muscle (ASM) relaxation and bronchodilation. In addition to ASM, other tissues including inflammatory cells express β_2-adrenoreceptors (β_2-AR). In other species, β_2-agonists promote peripheral blood lymphocyte (PBL) cytokine expression towards a pro-inflammatory phenotype in a dose-dependent fashion. The ability of β_2-agonists to promote a chronic, pro-inflammatory response could explain the association between prolonged use of short-acting β_2-agonists and increased mortality in human asthmatics. In many ways, the RAO horse in a good model for evaluating chronic changes associated with disease that cause persistent non-septic airway inflammation (like human asthma), since this is one of the few examples of a naturally occurring asthma-like-syndrome in a non-human species. However, little is known about the effect of β_2-agonist stimulation on equine PBL inflammatory response and few tools are available to assist in the exploration of this relationship. The aims of this study are to develop an indirect method to evaluate the response of equine PBLs to β_2-agonist stimulation, and to compare this response of cells from RAO and non-affected horses. Equine PBLs were isolated from blood, activated with ConA and stimulated with salbutamol. The response to agonist binding was indirectly determined using flow cytometric methodology and verified by Western blot analysis. Activated PBLs from RAO horses demonstrated a significant response to β_2-agonist binding whereas cells from non-affected horses did not. The response of RAO horses was attenuated when PBLs were pre-treated with a β_2-antagonist but were unaffected when pre-treated with a β_1-antagonist, indicating that the response of PBLs from RAO horses to β_2-agonist binding was mainly, if not exclusively, mediated by the β_2-AR. Preliminary investigation of the response of airway lymphocytes, retrieved by bronchoalveolar lavage from RAO horses and non-affected horses also indicated that RAO horses responded to β_2-agonist, while cells from non-affected horses did not. The results of this study indicate that the β_2-AR on PBLs from RAO horses have a distinct response to agonist binding, and the response *in vitro* appears to mimic the *in vivo* response, at least within the limits of the methods applied in these experiments. These findings represent a novel tool for further investigation of the role of β_2-agonist binding in the long term persistent airway inflammation in diseases like asthma and RAO, and support the use of this model for future studies aimed at better defining this effect.

Keywords

3.1 Introduction

Recurrent Airway Obstruction (RAO) is a common chronic, inflammatory respiratory disease that affects middle-aged horses (Robinson 2001; Léguillette 2003). In general, RAO is characterized by clinical signs that include cough, nasal discharge, nostril flare, respiratory distress, and reduced exercise tolerance that can vary depending on the severity of the disease (Davis and Rush 2002; Lavoie 2007). RAO horses develop airway inflammation, bronchoconstriction and mucous hypersecretion in response to exposure to aerosolized irritants present in their environment (Robinson 2001). Clinical disease is exacerbated in susceptible horses exposed to inhaled irritants like fungi and molds, suggesting that RAO is an allergic reaction (Cordeau, Joubert et al. 2004) mediated by lymphocyte antigen recognition and subsequent inflammatory response (Larché, Robinson et al. 2003; Robinson 2008). In horses, peribronchial lymphocytes act as immunoglobulin-producing B lymphocytes and as CD4+ (helper) T cells (Horohov 2001; Léguillette 2003) and it is known that helper T cells type 2 (Th2) and their secreted cytokines have an important role in RAO, contributing to cell differentiation, bronchial hyperreactivity, inflammation, and airway smooth muscle (ASM) remodeling (Cordeau, Joubert et al. 2004).

In addition to being a common disease of horses, equine RAO mimics many characteristics of human asthma including chronic inflammation of the respiratory system, airway smooth muscle (ASM) remodeling, airway hyperreactivity, and mucous accumulation (Hamid and Tulic 2009). Treatment for both diseases is also similar; corticosteroids have been shown to effectively reduce airway inflammation, while bronchodilators induce ASM relaxation (Davis and Rush 2002). β_2-agonists are largely used to relieve ASM contraction (Léguillette 2003) in RAO horses and human asthmatics. However, recent studies have reported that long term use of short- and long-acting β-agonists increase the potential risk of asthma-related death (Ortega and Peters 2010). One proposed reason for this association is that these medications may mask asthma induced inflammation, decreasing the clinical severity of the disease without attenuating long term effects of persistent inflammation (Cockcroft 2006).

As in ASM, inflammatory cells also express β_2-adrenoreceptors (β_2-AR) (Kin and Sanders 2006; Wong, Murthy et al. 2007). These receptors have been shown to play a role in the inflammatory

process by modulating cytokine and inflammatory mediator secretion (Loza, Peters et al. 2007). The β_2-AR is a member of the G protein coupled receptor (GCPR) family. β_2-agonist binding results in activation of adenylyl cyclase, through signaling by the Gα subunit of the G protein. Adenylyl cyclase subsequently catalyzes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) increasing the intracellular concentration. cAMP acts as a second messenger that binds to and activates the regulatory subunit of protein kinase A (PKA) (Liggett 1999; Hwangpo and Iyengar 2005; Johnson 2006).

Studies from human asthmatics suggest that asthma results from a disparity in the production and secretion of Th1 and Th2 cytokines by airway lymphocytes. In asthmatics, the predominance of Th2 cytokine production and secretion is associated with the development and persistence of clinical disease (Larché, Robinson et al. 2003; Akan and Lemanske 2007; Nakagome and Nagata 2011). More recent reports indicate that the intensity of the Th2 response may be highly variable among individual asthmatics (Holgate 2010) and in some cases both Th1 and Th2 cytokines are increased in cells retrieved from asthma patients (Loza, Foster et al. 2010). As with asthma, there is some evidence that a shift towards Th2 cytokine expression predominates in airway lymphocytes retrieved from the airways of RAO horses (Lavoie, Maghni et al. 2001; Bowles, Beadle et al. 2002; Cordeau, Joubert et al. 2004). However, this also has not been a consistent finding among all investigators who have explored the cytokine phenotype expressed from airway lymphocytes retrieved from RAO horses (Horohov, Beadle et al. 2005; Kleiber, McGorum et al. 2005). With a few exceptions, the potential influence of medications other than corticosteroids on lymphocyte cytokine expression has not been explored.

Even though inflammatory cells express the β_2-receptor, the effect of β_2-agonist binding on inflammatory cell function remains unclear. Studies of human monocytes indicate that salbutamol (also known as albuterol) significantly inhibits the release of tumor necrosis factor (TNF)-α (Ezeamuzie and Shihab 2010); in mast cells, β_2-agonists have an inhibitory effect on the release of histamine and cytokines like TNF-α (Bissonnette and Befus 1997). Airway neutrophils retrieved from humans treated with salmeterol exhibit a significant decrease in IL-8 release (Reid, Ward et al. 2003). The use of long acting β_2-agonists contributes to the inhibition of eosinophil activation (Johnson 2002), and in basophils it has been reported that β_2-agonist exposure inhibits IL-4, IL-13 and IgE-mediated histamine release (Kleine-Tebbe, Frank et al. 1994; Gibbs, Vollrath et al. 1998). In T cells, the use of β_2-agonists (salbutamol, isoproterenol, salmeterol) promotes IL-2-stimulated accumulation of Th2 cells (Loza, Peters et al. 2007). In

contrast, (R)-salbutamol has an anti-inflammatory influence on T cells, including inhibition of cell proliferation and cytokine production (Baramki, Koester et al. 2002; Ferrada, Gordon et al. 2008). β_2-ARs have been identified on equine PBLs (Abraham, Brodde et al. 2001) although the effect of β_2-agonist binding has not been explored. The aims of the present study are to develop an indirect method for measuring the response of activated equine PBLs to β_2-agonist stimulation, and to subsequently apply this technique to compare the effect of salbutamol (albuterol) stimulation on PBLs from RAO and non-affected horses. The results of this study indicate that distinct differences exist between the response of PBLs from RAO and non-affected horses to β_2-agonist binding. Further exploration of these differences could provide new insights to the pathophysiology of allergic airway disease and the role of β_2-agonist in chronic inflammation tissue changes associated with these diseases.

3.2 Materials and methods

3.2.1 Animals

Blood samples were obtained from fifteen adult horses (13 mares and 2 geldings of mixed breeds), five RAO horses and ten non-affected horses. The average age of the RAO horses was 21.4 years (range 18-30 years), and 14.8 years (range 8-23 years) for the non-affected horses. RAO horses were part of an RAO herd known to develop clinical signs when exposed to conditions such as dusty hay or housing in a barn. At the time of sampling, RAO horses had not received any medications for at least four weeks. No efforts were made to characterize their RAO status beyond casual observation, and horses did not display overt signs of significant airway disease. Non-affected horses were clinically healthy, with no signs of respiratory disease, including nasal or ocular discharge or cough. A complete blood count (CBC) at the time of blood sample collection was performed in all horses (RAO- and non-affected horses), and no abnormalities were detected. All procedures were approved by the Institutional Animal Care and Use of Committee (IACUC) at Virginia Tech.

3.2.2 Equine peripheral blood mononuclear cell (PBMC) isolation

Equine peripheral blood mononuclear cells (PBMC) were harvested from whole blood collected in tubes with sodium heparin, and isolated using a 59% Percoll density gradient technique. Briefly: whole blood was obtained by jugular venipuncture and centrifuged for 8 minutes at 600 x g at 27ºC. After centrifugation, plasma from each tube was removed, the white cell layer (buffy coat) was harvested and mixed (1:1) with Hanks' Balanced Salt Solution Modified without

calcium chloride and magnesium sulfate (Sigma-Aldrich, St. Louis, MO). Tubes were mixed using manual rotation, and the mixture was gently pipetted over 5mL of 59% Percoll solution. The 59% Percoll solution was made with 5.9mL iso-osmotic Percoll (10mL stock Percoll and 0.907mL 10x Hanks' solution) mixed with 4.1mL 1x Hanks' balanced salt solution without calcium chloride and magnesium sulfate; refractive index was checked using a refractometer, and the gradient composition was adjusted to achieve a density between 115-120. Tubes were carefully balanced to minimize vibration and centrifuged for 40 minutes at 930 x g at 22ºC with slow acceleration and minimal breaking at the end of centrifugation. After centrifugation, a white cell layer was visible above the 59% Percoll gradient. Cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) without calcium chloride and magnesium chloride, and resuspended in RPMI complete media (RPMI 1640, 2-mercaptoethanol, penicillin [10,000UI/mL], streptomycin [10mg/mL], L-glutamine in NaCl [200mM], sodium-pyruvate [100mM], gentamicin [10mg/mL]) without fetal bovine serum, and counted using a Cellometer® Auto T4 (Nexcelom Bioscience LLC, Lawrence, MA) automatic cell counter. Cells were brought to a final concentration of 4×10^6 cells/mL.

3.2.3 PBMC stimulation

To evaluate PBL response to β_2-agonist binding, after isolation PBMCs were stimulated for 30 minutes at 37ºC in a humidified atmosphere with 5% CO_2, using different concentrations of salbutamol (Sigma-Aldrich, St. Louis, MO) (50nM, 200nM, 500nM, 800nM, 1µM, 4µM, 10µM) and prostaglandin E_2 (PGE_2, Cayman Chemical, Ann Arbor, MI) (200nM, 800nM). Stimulants were dissolved in methanol and ethanol respectively, and used as their own controls, using same concentrations.

3.2.4 Phosphorylated vasodilator-stimulated phosphoprotein (p-VASP-Ser157) detection

In PBLs to indirectly measure the effect of β_2-agonist binding, determination of phosphorylated VASP was performed. VASP is a well conserved phosphoprotein which is selectively phosphorylated by protein kinase A (PKA) at the amino acid serine 157. Therefore, after a β_2-agonist binds to the β_2-AR, an increase in cAMP concentration leads to activation of PKA and phosphorylation of VASP. Measuring p-VASP-Ser157 could be used as an indirect method to determine activation of the β_2-AR and increases in PKA activity (Krause, Dent et al. 2003; Mosenden and Taskén 2011).

3.2.4.1 Western blot

To detect and confirm that the primary antibody (Ab) we used was binding to p-VASP-Ser157 in equine PBMCs, immunoblotting was performed. Briefly: isolated and stimulated PBMCs with salbutamol (800nM) and PGE_2 (800nM) were washed and lysis buffer (150mM NaCl, 10mM Tris, 0.5% Triton X-100, and 0.5% deoxycholate) was added to the cell pellet, allowing cells to lyse for 20 minutes in a shaker. Once lysed, samples were centrifuged at 14,000 x g for 8 minutes, and the supernatant was kept. Protein was quantified using a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), and a total of 4µg of protein was used per sample. Protein sample was mixed 1:1 with Laemmli buffer (Bio-Rad, Hercules, CA) and placed in a hot bath (95ºC) for 5 minutes. Samples and color protein markers ProSieve® (Lonza, Allendale, NJ) were loaded in a 7% discontinuous sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) to perform electrophoresis. Gels were run at 120V for 1.5 hours, and then transferred to a nitrocellulose membrane (Trans-Blot® Transfer Membrane 0.45µm, Bio-Rad, Hercules, CA) at 100V for 1 hour. After transferring, the membrane was placed in blocking buffer (TTBS buffer [6.1g/L Tris base, 8g/L sodium chloride, 0.29g/L potassium chloride, ddH_2O to 1L, adjust pH to 7.6, 0.5mL/L Tween 20] and 5% non-fat dry milk) for 1 hour in a rocker. The membrane was stained overnight at 4ºC, using the same primary Ab for p-VASP-Ser157 as used for flow cytometry, at a concentration of 1:2,000 w/v prepared in TTBS buffer. After washing with TTBS buffer, the membrane was stained with a fluorescent secondary IRDye800 labeled sheep anti-mouse IgG Ab (Rockland, Gilbertsville, PA), at a concentration of 1:10,000 w/v prepared in TTBS buffer. Secondary Ab was left for 1 hour at room temperature in a rocker, and further washed with TTBS buffer. The membranes were developed using an Odyssey® Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE), and digital images were processed using ImageJ software (National Institute of Health, Bethesda, MD).

3.2.5 Activation of PBMCs

To determine if the state of activation of PBMC influenced their response to β_2-agonist stimulation, cells were pre-incubated and activated. To activate cells, PBMCs were placed in RPMI complete media with10% fetal calf serum and concanavalin A (ConA) (2ug/10^6 cells) (Sigma-Aldrich, St. Louis, MO) for one or two days and incubated with humidification at 37ºC in air with 5%CO_2.

3.2.6 Evaluation of the effect of pre-culture with antagonists

Isolated PBMCs were cultured for 24 hours in ConA (as previously described) then pre-cultured for 15 minutes with β-antagonists (37ºC; 5%CO_2). For these experiments, ICI 188,551 (selective β$_2$-antagonist) and atenolol (selective β$_1$-antagonist) (Sigma-Aldrich) were used at 100nM, 500nM, and 1μM concentrations. Antagonists were dissolved in ddH$_2$O and ethanol respectively and used as their own control. After antagonist pre-culture, cells were stimulated for 30 minutes with salbutamol (500nM; 37ºC; 5%CO_2).

3.2.7 Measurement of vasodilator-stimulated phosphoprotein phosphorylation (p-VASP-Ser157)

Phosphorylation of VASP at Serine 157 (p-VASP-Ser157) was measured as an indirect method to detect increases in intracellular activity of protein kinase A (PKA). Briefly, after activation and stimulation, PBMC were fixed in 10% formalin for 10 minutes at 4ºC, and permeabilized for 1 hour at 4ºC using a permeabilization solution (DPBS without calcium chloride and magnesium chloride, 0.5% saponin, 0.1% gelatin, 5mg NaN$_3$, 0.005%Tween 20). After cells were permeabilized, cells were transferred to a 96-well V-plate to be stained and analyzed using flow cytometry. Staining was performed using a primary mouse monoclonal antibody (Ab) that specifically recognized p-VASP-Ser157 at Serine 157 (NanoTools, Teningen, Germany) at a 1:500 w/v concentration diluted in permeabilization solution, for 1 hour on ice in a rocker. Cells were washed with DPBS gel (DPBS without calcium chloride and magnesium chloride, 0.1% gelatin, 5mg NaN$_3$) and labelled using a secondary goat anti-mouse IgG fluorescein isothiocyanate (FITC) labeled Alexa Fluor® 488 Ab (Invitrogen, Carlsbad, CA) at a concentration of 1:1000 w/v for 1 hour in a rocker. Stained cells were analyzed for p-VASP-Ser157 fluorescence using a BD FACS Aria® flow cytometer, exciting at a wavelength of 488 nm and detecting at 533 nm. Peripheral blood lymphocytes (PBLs) were gated based on their forward and side scatter characteristics (size, granularity and internal complexity), and data were analyzed using FlowJo 7.2 for Windows software.

3.2.8 Statistical analysis

Normal probability plots were generated to verify that the data followed an approximate normal distribution. Subsequently, the effects of treatments and RAO status on response to stimulation were assessed using mixed model analysis of covariance with baseline and reagent vehicle associated measurements as covariates. Horses were included in the model as a random blocking factor. Model adequacy was verified by inspecting the residual plots. Statistical

significance was set at p<0.05. All analyses were performed using SAS 9.2 software (Cary, NC, USA).

3.3 Results

3.3.1 Detection of phosphorylated VASP (p-VASP-Ser157)

Western blot analysis of protein derived from stimulated PBMCs from RAO- and non-affected horses demonstrated binding of the primary anti-pVASP-Ser 157 antibody to a 50 kilodalton (KDD) protein (Figure 3-1), which corresponds to the reported MW of p-VASP-Ser157 in many species.

The response of PBLs to salbutamol stimulation was also detected by measuring an increase in median fluorescent intensity (MFI) in ConA stimulated PBLs with or without salbutamol and PGE_2 stimulation using previously described methods (section 2.7 of methods) in which fluorescence labeled anti-p-VASP-Ser157 is used to indirectly indentify an increase in p-VASP-Ser-157 (Loza, Foster et al. 2006; Loza, Peters et al. 2007). PBLs stimulated with both salbutamol and PGE_2 showed increased median fluorescent intensity (MFI) when compared to cells without stimulation. Increased p-VASP-Ser157 was detected as a shift of the curve in the flow cytometry histogram to the right (Figure 3-2).

3.3.2 Peripheral blood lymphocyte response to β_2-agonist

In the absence of ConA activation, PBLs from RAO and non-affected horses did not respond to salbutamol stimulation regardless of the concentration tested (Figure 3-3 A). After one day of ConA activation, cells from RAO and non-affected horses showed a significant (p=0.04) increase in MFI values between cells from both groups, and compared to unstimulated cells at 500nM (p=0.002) and 10µM (p=0.01) respectively (Figure 3-3 B). Activation of PBLs from RAO horses for two days with ConA resulted in a significant (p=0.04) increase in MFI values from baseline to 800nM salbutamol concentration (Figure 3-3 C). In contrast, cells from non-affected horses did not increase MFI values with different salbutamol concentrations (Figure 3-3 C). Significant differences were detected between cells from both groups at 500nM (p=0.04), 800nM (p=0.01), and 10uM (p=0.04) salbutamol concentrations, where cells from RAO horses showed the higher MFI values.

PBLs from RAO horses pre-cultured with ICI 188,551 and stimulated with salbutamol showed a non-significant tendency to decrease MFI values indicative of p-VASP-Ser157 when compared

to cells stimulated only with salbutamol (Figure 3-4). In addition, PBLs from RAO horses showed a non-significant tendency to decrease MFI values to similar values obtained in cells without stimulation with increasing ICI 118,551 concentrations (1μM). Conversely, no antagonist effect was observed in PBLs pre-cultured with atenolol in both RAO and non-affected horses (Figure 3-5). When cells were pre-cultured concurrently with both antagonists, the MFI tended to be reduced as compared to cells stimulated with salbutamol alone. This reduction was not complete and only differed significantly from the response of cells stimulated with salbutamol alone at the lowest concentration of antagonists (data not shown).

3.4 Discussion

The aims of this study were to develop an indirect method to be used for determination of agonist stimulation of the β_2-AR in equine activated PBLs, and to compare this response in cells from RAO and non-affected horses. The indirect method developed in the present study was modified from a previously reported method in which an increase in p-VASP-Ser157 was used as a measure of response to β_2-agonist stimulation in human PBLs. In this study, the presence of VASP in freshly isolated equine PBMC was first determined and confirmed using Western blot analysis. Our immunoblotting analysis showed that the molecular weight for VASP is 46 KDD, shifting to 50 KDD when the protein is phosphorylated by PKA, molecular weight also noted by others (Halbrügge and Walter 1989; Krause, Dent et al. 2003; Pula and Krause 2008). VASP proteins are members of a family of structurally well conserved proteins found in vertebrates and invertebrates (Kwiatkowski, Gertler et al. 2003). Mammalian VASP proteins are known substrates of cAMP-dependent serine kinase PKA. Phosphorylation of VASP at serine 157 has been previously reported has a good indicator of PKA activity in PBLs, since the phosphorylation is mediated selectively by PKA (Halbrügge, Eigenthaler et al. 1992; Loza, Foster et al. 2006; Loza, Peters et al. 2007). Several studies demonstrated an association between an increase the phosphorylated state of VASP and activation of PKA in a variety of tissue types including human platelets (Halbrügge and Walter 1989; Butt, Abel et al. 1994), human endothelial cells (Nolte, Eigenthaler et al. 1991), human airway smooth muscle cells (Roscioni, Kistemaker et al. 2009), murine cardiac myocytes (Sartoretto, Jin et al. 2009), and human neutrophils (Eckert and Jones 2007). Also, previous studies reported the presence of VASP and p-VASP-Ser157 in human T and B cell lymphocytes (Halbrügge, Eigenthaler et al. 1992). Similarly, Loza et al. reported a molecular shift in VASP molecular weight after T cells were stimulated with PGE_2 and isoproterenol (Loza, Foster et al. 2006). In the present study, a protein band located at 50 KDD was observed in PBMC from RAO- and non-affected horses.

This serves as evidence that the anti-human p-VASP-Ser157 antibody used in this study is likely recognizing equine VASP in a specific fashion (Loza, Foster et al. 2006). This antibody was then used to detect changes in FITC-labeled anti-p-VASP-Ser157 using flow cytometric analysis. A positive response was indicated by an increase in the MFI of the lymphocyte population. Stimulation of PBLs from RAO and non-affected horses with salbutamol and PGE_2 induced an increase in MFI. These results are similar to previous studies performed in stimulated human T cells (Loza, Foster et al. 2006; Loza, Peters et al. 2007) and provide indirect evidence that the methods described by Loza and coworkers can be used to assess the response of equine PBLs to 2-agonist stimulation.

T cells express β_2-AR in a number of species including humans and horses (Abraham, Brodde et al. 2001; Wahle, Stachetzki et al. 2001). The stimulation of this receptor can lead to a variety of responses, and the cAMP-dependent pathway has been widely studied. Although cAMP can activate numerous molecules, in T cells the majority of its effects are mediated through PKA (Taskén and Stokka 2006). In this study, detection of p-VASP-Ser157 was used as a measure of response to agonist binding to the β_2-AR in activated equine PBLs (Loza, Foster et al. 2006; Loza, Peters et al. 2007). Salbutamol is a common short-acting β_2-agonist bronchodilator that binds to the β_2-AR increasing cAMP concentration and PKA activity. In the present study, PBLs from non-affected horses tended to have higher baseline MFI values compared to cells from RAO horses but the results were not statistically significant. When stimulated with salbutamol, non-activated PBLs from RAO and non-affected horses showed minimal response. However, when PBLs were pre-cultured with ConA, cells from RAO horses demonstrated greater baseline MFI and had a greater response to salbutamol stimulation as compared to cells from non-affected horses. Hastie et al. reported that epithelial cells from asthmatic patients expressed significantly lower baseline p-VASP-Ser157 values as compared to non-asthmatic patients. However, when epithelial cells were stimulated with salbutamol, the VASP ratio (phosphorylated 50 KDD to 46 KDD VASP) was significantly higher in cells from asthmatic patients as compared to non-stimulated cells (Hastie, Wu et al. 2006). A decrease in the percentage of β_2-AR has been reported in respiratory airway tissue (peripheral lung and bronchi) from RAO affected horses; in addition, the number of high affinity state receptors, functionally bound to the G protein were decreased resulting in a reducing of baseline adenylyl cyclase activity (Abraham, Kottke et al. 2006). These authors concluded that tissue from RAO horses showed not only a decrease in receptor number and coupling capacity of the receptor to the G-protein, but also a decrease intracellular cAMP accumulation as a consequence of attenuation on constitutive

adenylyl cylclase activity. In the present study, it is possible that prior to ConA activation, PBLs had a similarly diminished expression of receptors on the cell surface, and/or a reduction in baseline adenylyl cyclase activity, leading to decreased intracellular concentrations of cAMP, activated PKA and p-VASP-Ser157 in the cell.

Activation with mitogens like ConA (plant lectin) has long been used to enhance T cell function and to mimic antigen binding effect (Resch and Ferber 1988; Borger, Hoekstra et al. 1998; Hu, Li et al. 2010). Additionally, thymocytes and splenocytes incubated with ConA display an increased number of β_2-AR (Radojcic, Baird et al. 1991). In the present study, PBLs were activated for one or two days using ConA, and stimulated with salbutamol. After one day activation, cells from RAO and non-affected horses showed a significant increase in MFI values at 500nM and 10µM compared to baseline, respectively. This suggests that activation of cells could have an effect on receptor expression, responding to stimulation with salbutamol and increasing MFI. Similar results were reported by Ferrada et al., where only ConA and phorbol 12-myristate 13-acetate (PMA) activated T cells decreased cytokine secretion after (R)-salbutamol stimulation as compared to non-activated cells (Ferrada, Gordon et al. 2008).

After two days of ConA activation, PBLs from RAO horses showed a significant increase in MFI values after salbutamol (800nM) stimulation when compared to baseline. In contrast, cells from non-affected horses did not show any increase in MFI after salbutamol stimulation. Similarly, Borger et al. detected increased concentrations of cAMP in ConA activated T cells stimulated with increasing concentrations of isoproterenol (β-AR agonist) (Borger, Hoekstra et al. 1998). Reports by Hastie et al. using epithelial cells from asthmatic patients indicate that significant increases in p-VASP-Ser157 occur when leukocytes from bronchoalveolar lavage fluid (BALF) were co-cultured with epithelial cells. This increase was not observed in cells from non-asthmatic subjects. The authors concluded that a soluble signal from BAL leukocytes may serve to activate epithelial cells from asthmatic patients leading to increased p-VASP-Ser157. These authors also found increased levels of p-VASP-Ser157 in epithelial cells *in vivo* after regular inhalation of salbutamol (Hastie, Wu et al. 2006). Results of the present study showed that only ConA activated cells from RAO horses responded to salbutamol stimulation *in vitro* as compared to cells without activation or to cells from non-affected horses, and the difference between response of salbutamol stimulated PBLs from RAO and non-affected horses was statistically significant. Moreover, preliminary results (data not shown) using cells from BAL fluid harvested from RAO and non-affected horses indicate that only airway lymphocytes from RAO horses responded to salbutamol. The discovery that lymphocytes in culture respond similarly to

lymphocytes retrieved from the airway of horses suggest that the response of lymphocytes to β_2-agonist binding is due to a receptor-associated□characteristic or mechanism that is unique to the RAO horses examined in this study, and is consistent in these cells regardless of whether they are retrieved from the airway or peripheral blood. Alternatively, it is possible that the inflammatory response in the airways of RAO horses is not contained only in that system, but has more of a generalized effect on cells like circulating lymphocytes, leading to changes such as increasing the expression and/or reactivity of the β_2-AR on these cells. These preliminary results also support the prediction that the *in vitro* response of activated PBLs to β_2-agonist stimulation could be an acceptable model for further exploration of the effect of these drugs on the function of airway lymphocytes in RAO horses. Minimally, these similarities between the response of PBLs and BALF lymphocytes warrant further investigation.

Salbutamol (called albuterol in the U.S.) is a selective β_2-agonist and a hydrophilic molecule that binds to the receptor extracellularly. It is characterized by a rapid onset of action but a short duration of action, mainly due to its hydrophilic characteristic. When administered at high doses, it can also bind and activate the β_1-AR (Brenner and Stevens 2010). In the present study, cells were also pre-cultured with β_1- and β_2-antagonists to confirm that salbutamol response was exerted through the β_2-AR. Cells from RAO horses, pre-cultured with ICI 118,551 (selective β_2-antagonist) and stimulated with salbutamol, showed a non-significant decrease in MFI values compared to cells stimulated only with salbutamol. Moreover, a significant increase in MFI values was observed in cells from RAO horses stimulated with salbutamol when compared to cells without stimulation, and this increase was not observed when the antagonist was added before the stimulation. These results indicate that salbutamol stimulation in PBLs from RAO horses was through by β_2-AR binding. Similarly, Borger et al. reported that ICI 118,551 was able to antagonize the effect of isoproterenol in human activated T lymphocytes (Borger, Hoekstra et al. 1998). Also, Loza et al. reported that in human T cells ICI 118,551 induces an inhibitory effect when added to T cell culture previous to isoproterenol stimulation (Loza, Peters et al. 2007).

When atenolol (selective β_1-antagonist) was used in pre-culture cells from RAO horses before salbutamol stimulation no antagonist effect was observed. In addition, a significant increase in MFI values was detected when PBLs were stimulated with salbutamol and atenolol, suggesting that the reagent did not have an antagonist effect on salbutamol binding. Similarly, Scott et al. reported that atenolol did not antagonize the bronchodilator effect of isoproterenol in horses; these authors concluded that the agonist exerts its effect principally through the β_2-AR (Scott,

Berney et al. 1991).   Similarly, Borger et al. reported that atenolol did not antagonize isoproterenol stimulation in ConA-activated human T lymphocytes, indicating that the β_1-adrenoreceptor was not involved in this stimulation (Borger, Hoekstra et al. 1998). These results and those previously described results suggest that salbutamol stimulation activated the β_2-AR in activated PBLs from RAO horses, and that the β_1-AR is not involved cell stimulation.

3.5 Conclusions

Results from the present study indicate that response induced by agonist stimulation of the β_2-AR can be indirectly detected by measuring p-VASP-Ser157 in equine PBLs. In addition, only activated PBLs from RAO and non-affected horses responded to salbutamol stimulation. However, only activated cells from RAO horses significantly increased their MFI compared to cells from non-affected horses. Furthermore, agonist stimulation was mediated by activation of the β_2-AR in PBLs from RAO horses.   Results from this study indicate that flow cytometric analysis of p-VASP-Ser157 can be a useful tool for the study of β_2-AR stimulation with commonly used drugs for the treatment of RAO and asthma.

Acknowledgements
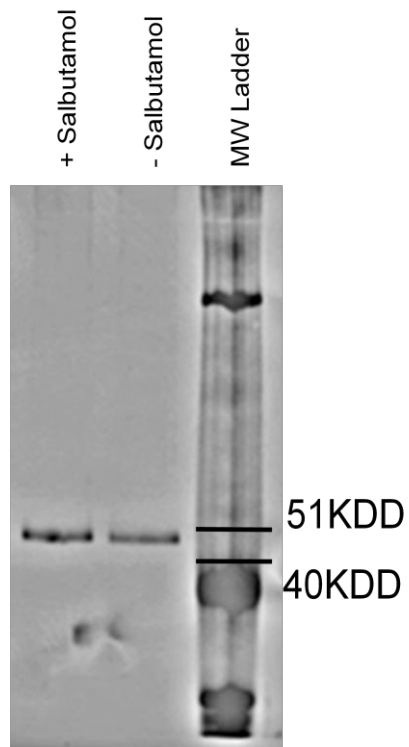
Figure 3-1. Western blot analysis for p-VASP-Ser157 in equine activated PBMC stimulated with salbutamol (800nM) and PGE_2 (800nM). Protein extracts were separated by electrophoresis using a 7% SDS-PAGE gel.
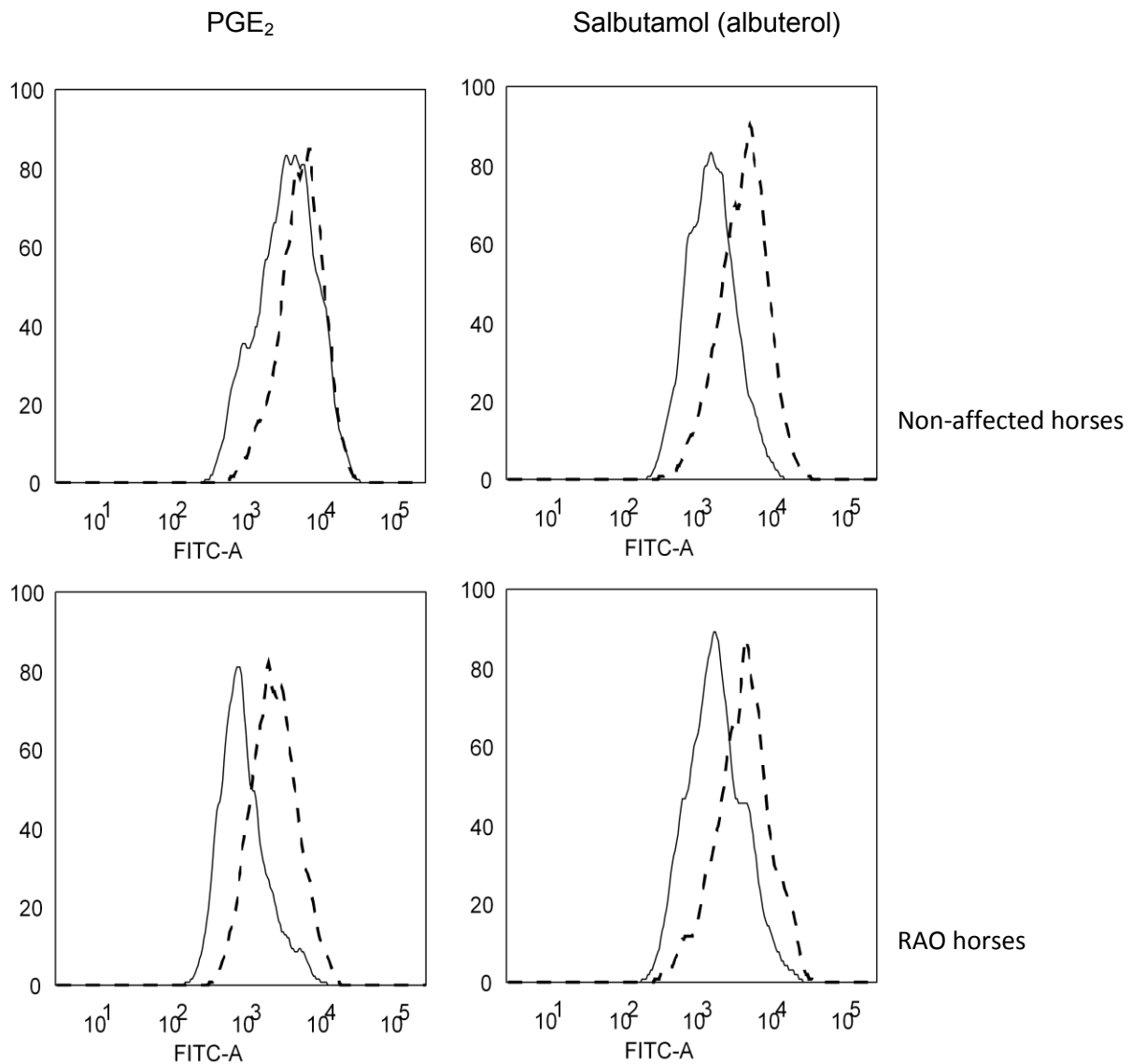
Figure 3-2. Histograms represent phosphorylated VASP fluorescence (x-axis) of PBLs from RAO horses and non-affected horses. Freshly isolated cells were stimulated for 30 minutes with both albuterol (salbutamol) (4μM) and PGE$_2$ (200nM) (dashed line). Continuous line represents cells without stimulation. After stimulation, cells were analyzed for VASP-Ser157 using flow cytometry.
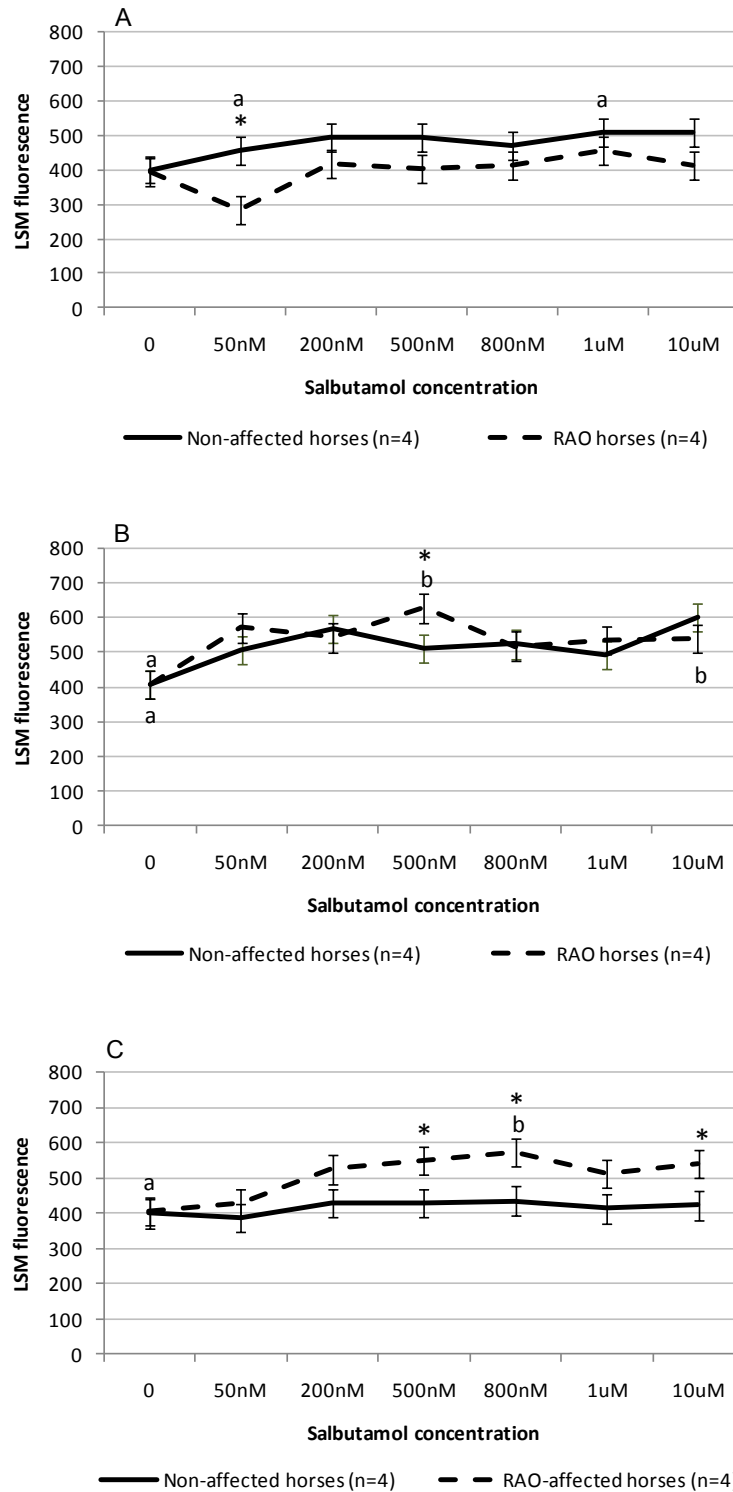
Figure 3-3. Least square mean and standard error of the median fluorescent intensity for phosphorylated VASP-Ser157 in equine PBLs stained with an anti-phospho-VASP antibody, using flow cytometry. A. Freshly isolated PBLs stimulated with salbutamol (albuterol) for 30

minutes. B. Isolated PBLs activated for one day with ConA (2ug/10^6 cells) and then stimulated with different concentrations of salbutamol for 30 minutes. C. Isolated PBLs activated for two days with ConA (2ug/10^6 cells) and then stimulated with different concentrations of salbutamol. *Indicate that the response of PBLs from the RAO horses differed significantly (p<0.05) from the response of PBLs from the non-affected horses at that concentration of salbutamol.

Different letters indicate a significant difference (p<0.05) in the response to different concentrations of salbutamol in cells from the same group of horses (RAO and non-affected). Letters above the graph lines correspond to RAO horses and letter below the graph line correspond to non-affected horses.
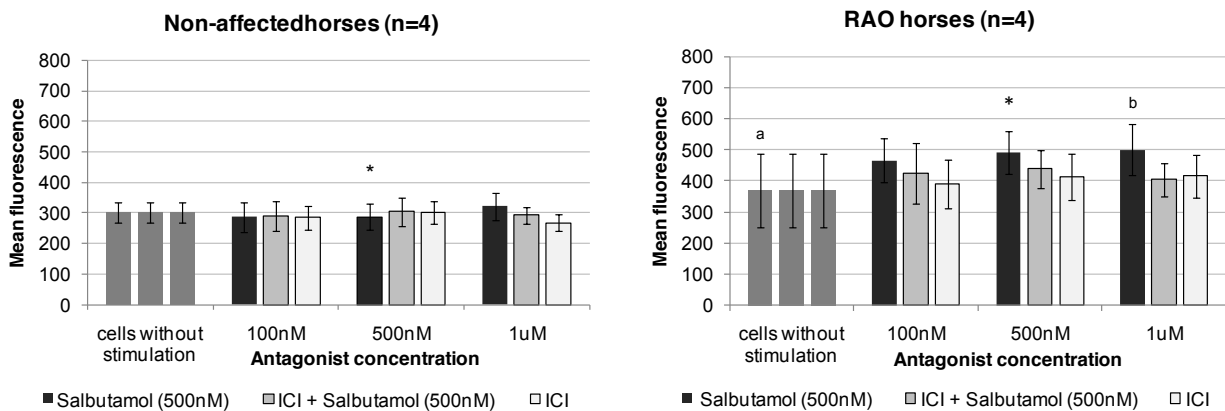
Figure 3-4. Mean and standard error of the mean for the median fluorescent intensity for phosphorylated VASP-Ser157 detected in PBLs from RAO and non-affected horses using flow cytometry. All groups of cells were activated for 24 hours with ConA (2ug/10^6 cells) before some were pre-cultured with ICI 188,551 (β_2-antagonist) for 15 minutes. Following this, designated groups of cells were stimulated with salbutamol (albuterol) (500nM) for 30 minutes.
* Indicates a significant difference (p<0.05) in the response of PBLs from RAO and non-affected horses to salbutamol stimulation.
Different letters indicate a significant difference (p<0.05) in PBLs from RAO horses.

Non-affected horses (n=4)

Mean fluorescence / Antagonist concentration

cells without stimulation, 100nM, 500nM, 1uM

■ Salbutamol (500nM) ▫ Atenolol + Salbutamol (500nM) ▫ Atenolol

RAO horses (n=4)

Mean fluorescence / Antagonist concentration

cells without stimulation, 100nM, 500nM, 1uM

■ Salbutamol (500nM) ▫ Atenolol + Salbutamol (500nM) ▫ Atenolol
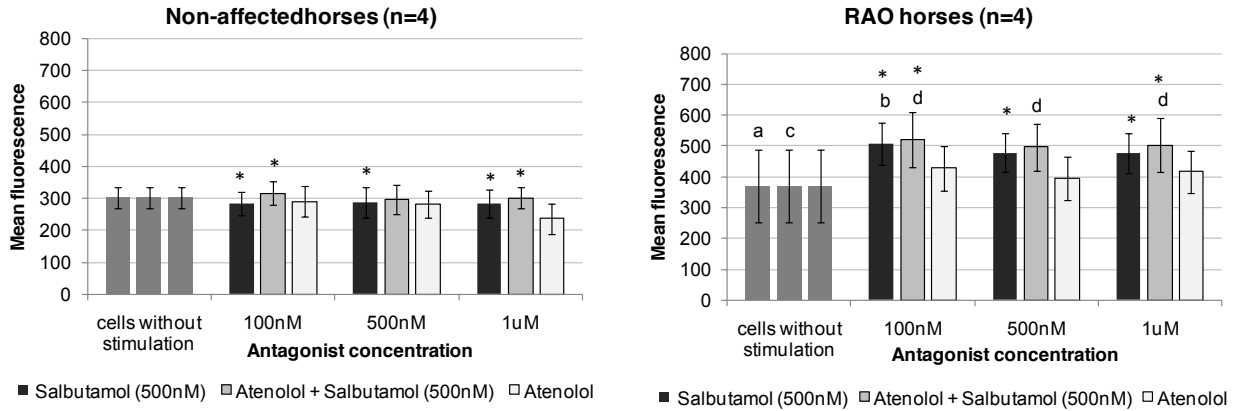
Figure 3-5. Mean and standard error of the mean for the median fluorescent intensity for phosphorylated VASP-Ser157 detected in PBLs from RAO and non-affected horses using flow cytometry. All groups of cells were activated for 24 hours with ConA (2ug/10^6 cells) before some were pre-cultured with atenolol (β_1-antagonist) for 15 minutes. Following this, designated groups of cells were stimulated with salbutamol (albuterol) (500nM) for 30 minutes.

* Indicates a significant difference (p<0.05) in the response of PBLs from RAO and non-affected horses to salbutamol stimulation and to salbutamol stimulation with the correspondent antagonist concentration.

Different letters indicate a significant difference (p<0.05) in PBLs from RAO horses between baseline (cells no stimulation) and the correspondent antagonist concentration.

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CHAPTER 4

SUMMATIVE INTERPRETATION AND FUTURE STUDIES

Summative Interpretation

1. **The developed method appears to be a valid technique to detect variations in phosphorylated VASP at the amino acid Serine 157 (p-VASP-Ser157) after salbutamol stimulation in activated equine peripheral blood lymphocytes (PBLs).**

Results obtained using Western blot analysis (Figure 3-1 in Chapter 3), confirmed that the antibody used to detect p-VASP-Ser157 recognized a single protein with the same molecular weight (50 KDD) as reported for other species (Krause et al., 2003, Pula and Krause, 2008). This finding is consistent with previous reports that indicate VASP is a well conserved protein across a number of species (human, monkey, rat, mouse, guinea pig. Anti-p-VASP-Ser157 antibody was then FITC labeled, and flow cytometric methodology was used to detect increases in intracellular p-VASP-Ser157 following stimulation with reagents known to activate PKA in other species (Figure 3-2 in Chapter 3). The results of the Western blot analysis coupled with flow cytometry provide reasonable evidence that this method of detecting a response to β_2-agonist binding is both specific for this response and adequately sensitive to detect moderate changes in intracellular phosphorylated VASP. Results of repeated flow experiments were generally consistent and provided similar results as those described in previous studies (Loza et al., 2006). These findings support the conclusion that the available anti-p-VASP-Ser157 antibody is capable of specifically detecting changes in equine intracellular phosphorylated VASP and that this methodology, coupled with flow cytometry, is a valid method for measuring the response of equine PBLs to β_2-agonist binding (Loza et al., 2006).

While the results of these experiments provide strong circumstantial evidence that the anti-p-VASP-SER157 binds specifically to equine phosphorylated VASP, this conclusion could be strengthened by isolating the antibody-protein complex from the Western blot, removing the antibody, and determining the precise sequence of amino acids in the protein that the antibody recognizes. This sequence could then be compared to those previously published on HomoloGene web page (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=homologene&dopt=AlignmentScores&list_uids=7592) to confirm that the sequence is similar to that of VASP from other

species. This information could also be used to determine how well VASP is conserved in horses as compared to humans, dogs, cattle, and other species.

In addition, this method is only an indirect measure of changes in cAMP (and subsequent activation of protein kinase A [PKA]). Including a second, different method for measuring cellular response to β_2-agonist would also serve to validate the flow cytometry methodology developed in this study. Preliminary effort to measure cAMP in these cells was attempted and is reported in Appendix B, section 5.

2. **Based on the results described in Chapter 3, only activated PBLs from RAO horses responded to β_2-agonist stimulation while cells from non-affected horses did not.**

Salbutamol stimulation did not induce any response in PBLs from either RAO or non-affected horses unless they were first activated for 24 or 48 hours with ConA. When PBLs from RAO horses (but not non-affected horses) were activated with ConA and then stimulated with salbutamol, an increase in p-VASP-Ser157 was detected. Results of previous studies demonstrated that activation of lymphocytes with mitogens enhanced their function and increased the expression of the β_2-AR on their surface (Borger et al., 1998, Hu et al., 2010, Radojcic et al., 1991). In addition, other researchers have shown that only cells from asthmatic patients demonstrated an increase in p-VASP-Ser157 after treatment with salbutamol (Hastie et al., 2006). These findings suggest that the expression and/or reactivity of the β_2-AR in PBLs from RAO and non-affected horses differ, especially when cells are in an activated state. However, the data presented in this study are only suggestive and conformation of this difference requires quantification of receptor expression and characterization of the receptor response in PBLs from both RAO and non-affected horses.

Another explanation for the different response to salbutamol stimulation among cells from RAO and non-affected horses could be variations in some of the components of the cAMP signaling pathway. For example, the structure of PKA could be modified in RAO horses such that it is more reactive to small increases in cAMP as compared to PKA from non-affected horses. In the present study, the evaluated outcome was p-VASP-Ser157, but direct measurement of the response of other molecules involved in the signaling pathway could further define differences that exist between the RAO and non-affected horse.

3. **Antagonist experiments showed that salbutamol mainly exerted its influence through the β_2-AR in cells from RAO horses.**

To determine if PBL response to salbutamol stimulation was predominantly mediated by binding to the β_2-AR, activated PBLs were pre-cultured with selective β_1- and β_2- antagonists before stimulation with salbutamol. Cells from RAO horses that were pre-cultured with ICI 118,551 (a selective β_2-antagonist) tended to demonstrate less or no response to salbutamol as compared to cells cultured with salbutamol alone (Figure 3-4, Chapter 3). In contrast, the addition of atenolol (β_2-antagonist) had minimal effect on the response of PBLs from RAO horses to salbutamol stimulation (Figure 3-5, Chapter 3)

While these results suggest that the effect of salbutamol is mediated mainly through the β_2-AR, the data are not statistically significant. However, considering the small number of samples, the consistent trend of the data is persuasive evidence and the inclusion of only a few more horses in each group may be adequate to achieve statistical significance.

In addition, while salbutamol is thought to mediate its effects through the β_2-AR, it is possible that binding to an array of other receptors occurs in horse tissues. If additional studies do not confirm that the effect of salbutamol is mediated through the β_2-AR, then further investigation of the possible binding to receptors (especially G-protein coupled receptors) outside of β-family should be pursued.

4. **Airway lymphocytes retrieved by bronchoalveolar lavage fluid (BALF) tended to respond in a similar fashion to salbutamol stimulation as activated PBLs.**

As a pilot study, airway lymphocytes were retrieved by BAL from 2 RAO and 2 non-affected horses. When cultured *in vitro* with salbutamol the airway cells tended to respond in a similar manner as the pattern observed in activated PBLs. Specifically, airway lymphocytes from RAO horses responded to salbutamol stimulation while cells from non-affected horses did not. Airway lymphocytes from both horse groups responded to stimulation with PGE_2. Even though BALF cells experiments were performed using a small number of horses the results are promising and warrant further investigation.

To detect a response after salbutamol stimulation, airway lymphocytes from RAO horses did not require pre-activation with ConA, while the PBLs did. This difference could be due to a difference in the functional state of PBLs and airway lymphocytes in RAO horses. ConA activation mimics antigen binding (Resch and Ferber, 1988, Borger et al., 1998, Hu et al., 2010), so activation of PBLs with ConA could mimic events that occur when airway lymphocytes encounter inhaled antigens that elicit the RAO response. However, the process must be related to more than only the activation state of the lymphocyte, since the response of PBLs from non-affected horses to salbutamol stimulation is not improved by ConA activation. Perhaps the difference is related to the "level" of activation of cells from RAO verses non-affected horses. In any chronic disease state, the organs and the related inflammatory response do not function as entities separate from the entire body. Thus, the effects of inflammation in the lung (like cytokine release) are likely not restricted to the lung. It is possible that the PBLs in horses with RAO experience some exposure to inflammatory cytokines such that they are in an "inflammation ready" state when they are retrieved from the horse's blood. In the absence of further activation, they do not respond to agonist stimulation. However, when exposed to ConA, the cells are already primed to respond and alter their function in a way that allows them to mount a measureable response to salbutamol. In contrast, the fully quiescent PBLs from non-affected horses do not receive enough "signaling" from ConA to take them to a fully activated state in a 48 hour period. To better understand the effect of lung inflammation on PBL response to salbutamol, measuring circulating cytokines at the time of blood collection might be beneficial. In addition, examining markers of activation independent of and in association with VASP phosphorylation on PBLs might provide evidence of a different, pre-existing state of activation in PBLs from RAO horses as compared to cells from normal horses. The possibility that lung inflammation might have a systemic effect on the status of the circulating lymphocytes has not be discussed previously in research associated with equine RAO or human asthma, and could be a contributing factor to the increased mortality in asthmatic patients that use short-acting β_2-agonist. Again, the horse may prove to be a good model (based on similarities of the disease and ease of repeated sampling) for additional evaluation of this relationship.

Future Studies

The information generated by this study provides the foundation for a multitude of future projects, both in basic and clinical science. However, the next steps should focus on experiments that further verify the findings presented in this document. In addition, studies that begin to define the effect of β_2-agonist binding on the inflammatory response should be considered. Some specific studies include:

1. **Evaluate if PBLs response to β_2-agonist stimulation differs depending on the RAO status of the horse (remission/exacerbation).**

   It is important to consider the effect of disease status on cell response since only cells from RAO horses respond to agonist stimulation. This disease is incurable, and it is likely that RAO horses experience subclinical airway inflammation that is difficult to detect without extensive testing and examination. In this study, no attempt was made to define the clinical status of the RAO horses from which blood was collected. While none of the horses demonstrated significant signs of disease, it is possible that they experienced varying chronic low levels of airway inflammation, and it is not known if disease state affects the response of airway or PB lymphocytes to β_2-agonist stimulation. To explore the possible relationship between horse disease status and cellular response, the experiments described in this study could be repeated but include an assessment of the horse's status at the time of sample collection. In addition, both blood and airway lymphocytes could be collected from the same horse at the same time and evaluated simultaneously. These results could be used to assess the relationship between airway and PB lymphocyte response.

2. **Determine if different levels of PKA activation can influence cytokine expression profiles in PBLs, and determine if these changes contribute to cell differentiation (Th1 vs. Th2 cell profile) and airway inflammation in RAO horses.**

   In humans, studies have shown that activation and stimulation of the β_2-AR leads to increases in cAMP concentration and activation of PKA. One of the outcomes of PKA activation is modification of the expression of pro- and anti-inflammatory cytokines by T cells (Taskén and Stokka, 2006, Hamid and Tulic, 2009, Loza and Penn, 2010). Additional reports based on response of cells from humans with asthma have showed that β_2-agonist stimulation can influence lymphocyte differentiation towards a Th2 cell response, leading to the secretion of cytokines that contribute to the progression of the disease (Loza and Penn,

2010, Yamaguchi et al., 2010). Equine RAO and human asthma share many characteristics and among these is the array of cytokines that are present in the airways of affected humans and horses. Consequently, using cells from RAO horses may permit the evaluation of the effect of agonist stimulation on T cell differentiation and provide information that is useful in the treatment of both RAO and asthma.

3. **Further establish similarities between the *in vivo* response and the *in vitro* model.**

The preliminary results presented in Appendix B indicate that activated PBLs and airway lymphocytes from RAO horses tend to behave in a similar manner when stimulated *in vitro* with salbutamol. Future studies which include evaluation of airway and blood lymphocyte response before and following administration of a β_2-agonist could serve to effectively "translate" our current bench top findings to the actual barn side response of the whole animal. Establishing a correlation between bench top and barn side information could permit the development of a powerful tool for better understanding the effect of β_2-agonist (and other medications) on horses with RAO and humans with asthma.

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APPENDIX A

Table 1. Reagents and laboratory materials used in the experiments of the present study.

Reagents:

Product name	Laboratory	Catalog number	Additional information
Percoll (stock)	Sigma	P1644	pH 8.5 – 9.5 (25°C), 500 ml.
Hanks Balanced Solution 1x	Sigma	H6648	With N_aHCO_3, without phenol red, Calcium chloride and magnesium sulphate.
Hanks Balanced Solution 10x	Gibco	14185-052	500 ml. With N_aHCO_3, without phenol red, Calcium chloride and magnesium sulphate.
DPBS w/o Ca-Mg	Gibco	14190	Without Calcium chloride and Magnesium chloride.
RPMI 1640	Gibco	22400	(+)L-Glutamine, (+) 25mM HEPES, N_a pyra, gent, penn/strep.
Salbutamol (Albuterol)	Sigma	S8260	100 MG
Isoproterenol	Sigma	I5627	5 G
ICI 118,551	Sigma	I127	5 MG
Prostaglandin E_2 (PGE_2)	Cayman Chemical	14010	10 MG
Concanavalin A	Sigma	C2272	Biotin conjugate, type IV, lyophilized powder
DMSO	Sigma	D8418	50 ml.
Formalin 10%	VWR	VW3239-1	Contains: Formaldehyde, Water, Sodium phosphate monobasic, Sodium phosphate dibasic, Methanol.
1° Antibody	Nanotools	0085-100/VASP 5C6	Mouse monoclonal antibody to VASP (phosphor-Ser 157) clone 5C6. IgG
2° Antibody FITC	Invitrogen	A-11001	Alexa Fluor® 488 goat anti-mouse IgG (H+L) 2mg/mL
2° Antibody for Western blot	Rockland		IRDye800 sheep anti-mouse IgG
Permeabilization solution			In 500 ml. DPBS (w/o Ca and Mg): 0.5% saponin, 0.1% gelatin, 0.01%NaN_3, 0.005% Tween.
DPBS with gel			In 500 ml. DPBS: 0.5 g gelatin, 5 mg NaN_3.
Gelatin from porcine skin Type A	Sigma	G2500	500 G
Tween 20	Aldrich	274348	500 ml.
Sodium azide (NaN_3)	Sigma	S-8032	
Saponin (from	Sigma	S7900	25 G

Quillaja Bark)			
Albumin Bovine 30%	MP Biomedicals	0215027050	50 ml.
2-Mercaptoethanol	Sigma	M-7522	
Penicillin	Gibco	15140-122	10,000UI/ml
L-Glutamine	Gibco	25030-081	200mM (100X)
Na-Pyruvate	Sigma	S8638	100mM
Gentamicin	Gibco	15710-072	10mg/ml
Lysis buffer			NaCl, Tris, Triton X-100, Deoxycholate
Laemmli buffer	Bio-Rad	161-0737	
Running buffer	Bio-Rad	161-0772	10x stock
ProSieve Color protein markers	Lonza	50550	10-190kDa
Nitrocellulose membrane	Bio-Rad	162-0115	Trans-blot transfer membrane 0.45µm
Filter paper	Bio-Rad	170-3932	
Transfer buffer			pH8.3 Tris base, Glycine, Methanol, ddH$_2$O
TTBS			Tris base, Sodium chloride, Potassium chloride, ddH$_2$O adjust pH7.6, Tween-20
Sodium Dodecyl sulfate (SDS) 10%	Bio-Rad	161-0301	for 10% solution:1gr in 10mL ddH$_2$O
Ammonium persulfate (APS)	Bio-Rad	161-0700	for 10% solution: 1gr in 10mL ddH$_2$O
TEMED	Sigma	T9281	N,N,N',N'-Tetramethylethylenediamine

Materials:

Product name	Company	Order number	Additional information
15 ml. conical tubes	Corning	430791	
45 ml. conical tubes	Corning	430829	
Plastic transfer pipettes	SAMCO (Corning)	202-20S	
10 ml. plastic pipettes	Fisherbrand	13-678-11E	
25 ml. plastic pipettes	Fisherbrand	13-678-11	
2 ml. plastic pipettes	Fisherbrand	13-678-11C	
Tips for pipettes	USA Scientific	1111-2021	1000 µl blue Pack 1000
		1111-1006	200 µl yellow Pack 1000
		1111-4000	0.5 – 10 µl Pack 1000
Glass tips for vacuum	Fischerbrand	13-678-20B	Case of 1440
96 well plates V-	Corning, Costar	3896	

bottom			
12x75mm 5 ml tubes	USA Scientific	1450-2810	20 bags of 25
Polypropylene Microtiter Tubes	Fisherbrand	02-681-376 (Bagged)	Plain tubes, Nonsterile, Bagged
Cytofunnel-single	Shandon	5991040	
Microcentrifuge tubes 2.0mL	USA Scientific	1620-2700	2.0 mL Graduated tube with flat cap. Package of 500/bag
Microcentrifuge tubes 1.5mL	USA Scientific	1615-5500	Natural, 500/bag
Microcentrifuge tubes 0.5mL	USA Scientific	1605-0000	Natural 5 bags of 200
Cover Glass	Fischerbrand	12-548-B	1 pack 1oz.
Cytoslide for cytospin	Shandon (Fischerbrand)	5991051	Case of 100
Cytofunnel Single	Thermo Shandon	A78710003	Box of 40
Blood collection tubes Sodium Heparin	BD Vacutainer	366480	10mL 16x100mm
Needles	Air-tite Products Co., Inc.		
Cell counting chambers (slides)	Nexcelom	SD100	
Cell culture flask	Corning	430641	With vent cap, 75cm^2 cell growth area

APPENDIX B

Additional Experiments and Results not included in Chapter 3

This Appendix includes additional pilot experiments that were performed to obtain preliminary data that will help in future experiments. These additional data include:

1. Dose response curves performed in freshly isolated PBLs from RAO and non-affected horses stimulated with salbutamol, isoproterenol and PGE_2

2. Western blot analysis performed in activated PBLs from RAO and non-affected horses, to detect differences between VASP (46 KDD) and p-VASP-Ser157 (50 KDD) after stimulation with salbutamol and PGE_2

3. Pre-culture of activated PBLs with both β_1- and β_2-antagonists together and then stimulated with salbutamol from RAO and non-affected horses to determine a possible role of the β_1-receptor in salbutamol stimulation.

4. Determination of p-VASP-Ser157 in bronchoalveolar lavage fluid lymphocytes after salbutamol and PGE_2 stimulation.

5. Measurement of cAMP concentration in activated PBMC from both RAO and non-affected horses after stimulation with salbutamol and PGE_2.

1. Dose response curves

1.1 Background/Justification

As described in Chapter 1, it is well known that equine peripheral blood lymphocytes (PBLs) express β_2-adrenoreceptors (β_2-AR) but the response to β_2-agonist binding has not been yet explored. In other species including humans, the response of the β_2-AR to agonist binding has been indirectly quantified by measuring changes in the phosphorylation status of the vasodilator-stimulated phosphoprotein (VASP) (Loza et al., 2006). The β_2-AR is a G-protein coupled receptor, and downstream events that follow agonist binding include an increase in intracellular cyclic adenosine monophosphate (cAMP) concentration, activation of protein kinase A (PKA), and subsequent phosphorylation of VASP. VASP is a protein that is well conserved across species, and is selectively phosphorylated at the amino acid serine 157 by activated PKA. Studies which have examined the ability of specific β_2-agonists to initiate β_2-AR intracellular signaling events have compared pre and post-binding VASP phosphorylation (p-

VASP-Ser157) as an indirect measure of this response. The downstream signaling events and the amino acid composition of VASP are well conserved across species so it was predicted that this methodology could be modified for use in horse tissue.

1.2 Specific Goals

1. Determine if the *in vitro* stimulation of equine PBLs with increasing concentrations of salbutamol, isoproterenol (ISO), or prostaglandin E_2 (PGE_2) results in an increase in p-VASP-Ser157 that is detected using previously described flow cytometric. techniques and measuring changes in median fluorescent intensity (MFI).
2. Identify the concentration(s) of each reagent (salbutamol, ISO, PGE_2) that induces a measurable response in equine PBLs and determine if that concentration(s) is the same for cells from RAO horses as compared to cells from non-affected horses.

1.3 Methods and Design

Isolated equine peripheral blood mononuclear cells (PBMC) were stimulated with salbutamol (selective β_2-adrenoreceptor agonist), ISO (receptor selective β_1 and β_2-adrenoreceptor agonist), and PGE_2 used as a positive control due to its capacity to increase cAMP.

As previously mentioned, PBLs express β_2-AR, and to evaluate the response of equine PBMC to agonist stimulation, different concentrations of agonists were selected to perform a dose response curve. The dose range used was selected based on previous studies (Hastie et al., 2006, Loza et al., 2007, Loza et al., 2006, Borger et al., 1998, Ferrada et al., 2008, Abraham et al., 2006). In order to assess non-specific changes in p-VASP-Ser157 that might occur after incubation with the agonist solvent, MFI of cells incubated for the same time in equal volume of solvent was measured and this value was used as a covariance in the final statistical analysis (as described below).

The following concentrations were evaluated for each of the agonists tested, and PBLs were prepared as described in Chapter 2.

- Salbutamol (solvent: methanol): 250nM, 500nM, 1µM, 2µM, 4µM, 8µM, 16µM
- ISO (solvent: distilled water): 50nM, 200nM, 500nM, 800nM, 1µM, 10µM
- PGE_2 (solvent: ethanol): 50nM, 200nM, 500nM, 800nM, 1µM, 10µM

Isolation, stimulation, and antibody staining of PBLs for p-VASP-Ser157 were performed using the protocol previously described in Chapter 2 (sections 2.3, 2.4, and 2.7).

Results for dose response curves were presented as least square means and standard error (SE). MFI obtained for each sample stained with only secondary antibody (considered as nonspecific binding of the antibody) was subtracted to the correspondent value of MFI in cell populations stained with primary and secondary antibody. As previously mentioned, MFI obtained in samples cultured with each solvent were used as a covariance in the statistical analysis, which means that the analysis corrected for any response due to the addition of the agonists solvent alone (rather than the agonist).

1.4 Results

1.4.1 Response to salbutamol

Incubation with salbutamol did not induce an increase in MFI compared with unstimulated cells at any dose tested. This response was similar in cells from both RAO and non-affected horses (Figure B-1).
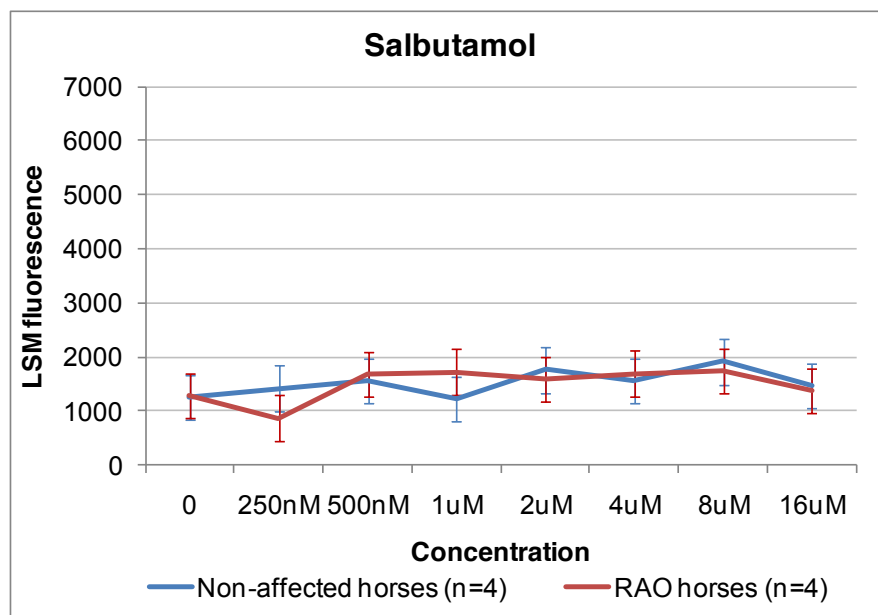

Figure B-1. Least square mean ± SE of MFI for p-VASP-Ser157 in equine PBLs from RAO horses (n=4) and non-affected horses (n=4) stimulated with different concentrations of salbutamol.

1.4.2 Response to isoproterenol (ISO)

MFI did not increase in PBLs from either horse group regardless of the concentration of ISO as compared to baseline. At 500nM and 800nM concentrations the MFI of cells from non-affected horses was significantly greater than RAO horses (p=0.04, p=0.01, respectively) (Figure B-2).

Figure B-2. Least square mean ± SE of MFI for p-VASP-Ser157 in equine PBLs from RAO horses (n=3) and non-affected horses (n=6) stimulated with different concentrations of isoproterenol.

* Indicates that the response of PBLs from RAO-horses differed significantly (p<0.05) from the response of PBLs from non-affected horses at that concentration of ISO.

1.4.3 Response to prostaglandin E_2 (PGE_2)

Equine PBLs stimulated with PGE_2 showed a concentration dependent increase in MFI. Cells from both RAO and non-affected horses demonstrated significant (p<0.05) increases from the baseline value. In addition, MFI was significantly greater in cells from non-affected horses as compared to cells from RAO horses at 500nM (p=0.03) (Figure B-3).
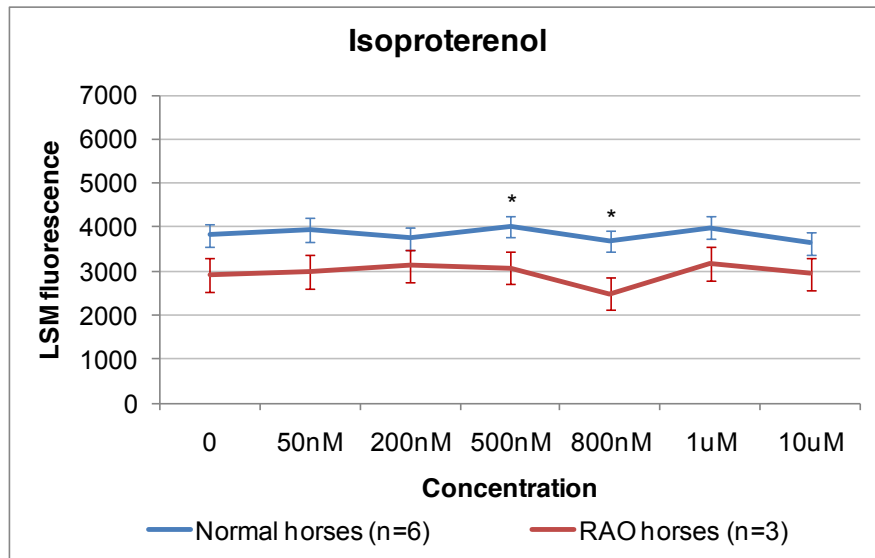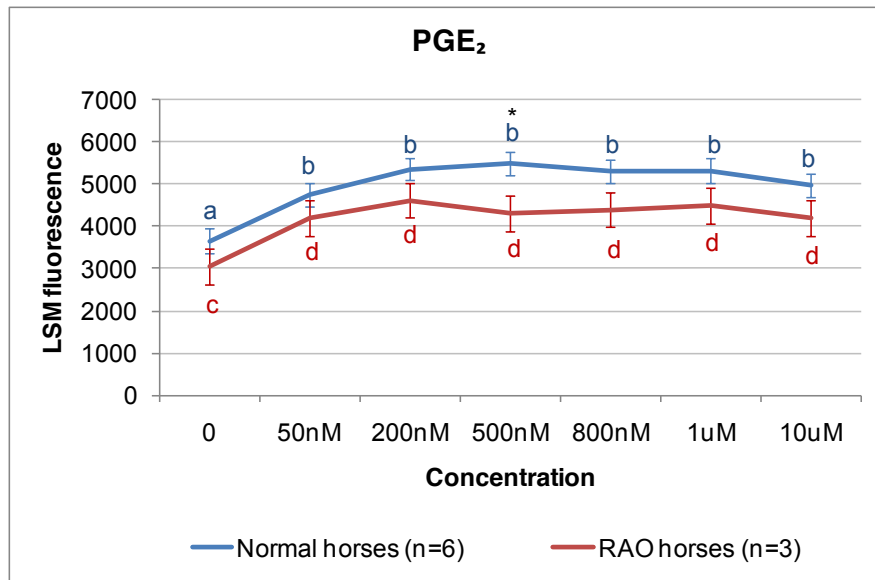
Figure B-3. Least square mean ± SE of MFI for p-VASP-Ser157 in equine PBLs from RAO horses (n=3) and non-affected horses (n=6) stimulated with different concentration of PGE_2.

* Indicates that the response of PBLs from RAO horses differed significantly ($p<0.05$) from the response of PBLs from non-affected horses at that concentration of PGE_2.

Lower case letters indicate that the response to two different concentration of PGE_2 differed significantly ($p<0.05$) within the same group of cells.

1.5 Interpretation/Discussion

The specific goals of this experiment were:

1. Determine if *in vitro* stimulation of equine PBLs with increasing concentrations of salbutamol, isoproterenol (ISO), or prostaglandin E_2 (PGE_2) results in increased concentrations of p-VASP-Ser157 that are detectable using previously described flow cytometric techniques.

2. Identify the concentration(s) of each reagent (salbutamol, ISO, PGE_2) that induces a measurable response in equine PBLs and determine if that concentration(s) is the same for cells from RAO horses as compared to cells from non-affected horses.

Goal 1: to determine if *in vivo* stimulation of equine PBLs with increasing concentrations of salbutamol (albuterol), isoproterenol (ISO), or prostaglandin E_2 (PGE_2) results in increased concentrations of p-VASP-Ser157 that are detectable using previously described flow cytometric techniques.

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No significant response was observed using salbutamol or ISO as stimulants. An absence of detectable response indicates that PBLs from horses do not respond to this stimulation or that the method tested in the experiment is not valid for use in horse tissue.

In horses, it is known that PBLs express the β_2-AR (Abraham et al., 2001). Based on this, a response to β_2-agonist stimulation was expected. It is possible that the method that was chosen to measure this response was not applicable to equine cells. However, the results obtained after PGE_2 stimulation provided some evidence that changes in p-VASP-Ser-157 could be detected using this methodology. A significant increase in MFI from baseline was observed in cells from both RAO and non-affected horses after stimulation with PGE_2 mimicking the response described in PBLs from other species, including humans (Loza et al., 2006).

PGE_2 binds to at least four prostanoid receptor (EP) subtypes which belong to the G-protein coupled receptor family. Stimulation of EP2 and EP4 increases intracellular concentrations of cAMP, and consequently activate PKA (Chung, 2005, Regan, 2003). In human PBMC, increases in p-VASP-Ser157 after stimulation with different concentrations of PGE_1 and PGE_2 were reported (Halbrügge et al., 1992). In human T cells increased p-VASP-Ser157 was observed after stimulation with ISO and PGE_2, with PGE_2 being more effective than ISO in eliciting a response (Loza et al., 2006). A possible explanation for this finding is that EP receptors, specifically subtypes 2 and 4, are involved in the activation of the cAMP pathway in equine tissues. The EP4 can undergo rapid desensitization while EP2 does not undergo agonist-induced internalization (Hata and Breyer, 2004). These findings suggest that the constitutive expression of EP receptors is higher and adequate to stimulate a response in PBMCs regardless of their activation state.

An additional characteristic of the β_2-AR is that the receptor can rapidly become desensitized after agonist stimulation through mechanisms previously described in Chapter 1 (Abraham et al., 2006, Oostendorp et al., 2005). This response could result in minimal response to β_2-agonists. In this study, cells stimulated with the highest concentration of β_2-agonists tended to have the lowest level of p-VASP-Ser15. While the reduction was not significant relative to baseline values, it could suggest that receptor desensitization contributes to the lack of response to β_2-agonists stimulation (Abraham et al., 2006, Oostendorp et al., 2005).

The lack of response to β_2-agonist stimulation may also be due to an alteration or defect in the signaling events that result from agonist binding. Heijink *et al.* provided evidence that the difference between responses in T cells from asthmatic patients after stimulation with a β_2-

agonist (fenoterol) and PGE$_2$ was due to a defect in the β$_2$-adrenergic system; specifically a decreased capacity of the β$_2$-AR to bind to the G-protein (Heijink et al., 2004).

It could also be possible that freshly isolated cells do not express an adequate number of β$_2$-AR to initiate a detectable increase in MFI in response to agonist stimulation. Gao *et al.* also demonstrated that the number and function of the β$_2$-ARs on human lymphocytes depends on whether an asthmatic is in remission or experiencing clinical disease (Gao et al., 2001). Other researchers have shown that activation of thymocytes and splenocytes with Concanavalin A (ConA) (plant mitogen) increases the surface expression of the β$_2$-AR (Radojcic et al., 1991). The PBLs used in this study were not activated prior to addition of the agonist which could explain the absence of response.

Since the response was minimal with salbutamol or ISO, additional studies were designed to determine if activating PBLs with ConA prior to agonist stimulation resulted in a measurable increase in p-VASP Ser-157. ConA activation did increase the response in cells from RAO horses but not from the unaffected horses and the results of these studies are described in detail in Chapter 3.

Goal 2: identify the concentration(s) of each agonist (salbutamol, ISO, PGE$_2$) that induces a measurable response in equine PBLs and determine if that concentration(s) is the same for cells from RAO horses as compared to cells from non-affected horses.

The optimum concentration of PGE2 for stimulating both populations of cells (RAO and non-affected horses) appears to be 200nM based on MFI. However, if the goal of future experiments is to examine mechanistic differences between the response of cells from RAO horses and those of non-affected horses, the 500nM concentration could also be considered since that was the only concentration in which there was a significant difference between the two cell populations.

In contrast, stimulation with salbutamol did not produce differences in MFI between cells from each group. Likewise, neither population of cells demonstrated a significant response at any concentration of ISO, although there was a significant difference in response between cells from RAO and non-affected horses at 500nM and the 800nM ISO concentration. Reasons for this lack of response are described in detail with regard to the first goal of this study. Based on previously published studies, further experiments were performed aimed at determining if the

state of activation affected the response of equine PBL to β_2-agonist binding and results are described in detail in Chapter 3.

1.6 Additional/Future Studies

Within groups of horses, response to the three agonists varied from horse to horse regardless of which group (RAO or unaffected) the horse was in. Increasing the sample size would reduce the effect of individual variation. Also, clinical evaluation of RAO horses was based on assessment of physical parameters only and more sensitive tests of horse status (pulmonary function testing or evaluation of bronchoalveolar lavage cytology) were not performed. Variation in the horse's clinical status could affect the "activation" state of lymphocytes retrieved from these horses. Further grouping of RAO horses based on clinical status could minimize some of the horse to horse variation observed in this study and permit better characterization of the relationship between disease status and PBL response to β_2-agonist stimulation.

2. Western blot
2.1 Background/Justification

The flow cytologic methods that were used in the previous study utilized reagents (antibodies) that were designed to specifically recognize p-VASP ser-157 in human and mouse cells. While the response observed in equine tissues was similar to that reported in mouse and human cells, the increase in MFI could also have been due to nonspecific binding of the antibody reagents that were used in the study. To provide more evidence that the anti p-VASP ser-157 antibody was binding to this protein exclusively to horse tissue, Western blot analysis was performed and the results of this study were described in Chapter 3. The aim of this experiment was to use Western blot technique to measure relative amounts of phosphorylated and un-phosphorylated VASP in PBMC from RAO and non-affected horses before and after stimulation with salbutamol and PGE_2. Halbrügge et al. describe a change in the molecular weight of un-phosphorylated VASP (46 KDD) to 50 KDD after phosphorylation in human lymphocytes (T and B cells) (Halbrügge et al., 1992). The goal of this study was to examine the relative amount of un-phosphorylated VASP to p-VASP ser-157 in ConA activated cells from RAO and non-affected horses before and after stimulation with PGE_2 or salbutamol and to compare the results using Western blot analysis to those obtained by flow cytometry.

2.2 Specific Goals

1. Verify that reagents used in other species to identify un-phosphorylated VASP recognized as a single protein from equine tissues that was 46 KDD molecular weight using Western blot analysis.

2. Use an alternative method to verify that changes in p-VASP-Ser157 detected by flow cytometric technique correspond to those found using Western blot analysis.

2.3 Methods and Design

Western blot analysis was performed on samples derived from PBMC that were activated with ConA for 48 hours then stimulated with salbutamol (800nM) or PGE_2 (800nM). Cells cultured in RPMI complete media without salbutamol or PGE_2 were used as a "control" population and were included to provide a baseline for comparison. Selection of activation period and concentration of both reagents were made based on the results of previous experiments described in Chapter 3. The 800nM concentration was specifically selected because it was the only concentration in which there was a significant difference in response between both groups. After activation and stimulation, cells were lysed and protein was quantified following the same protocol described in Chapter 2 (section 2.8). Gels were loaded as showed in Figure B-4.

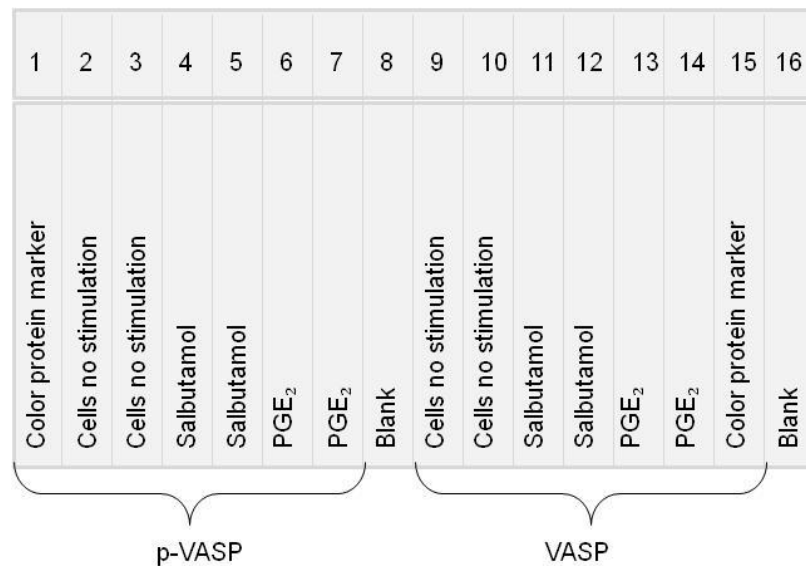| 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Color protein marker	Cells no stimulation	Cells no stimulation	Salbutamol	Salbutamol	PGE_2	PGE_2	Blank	Cells no stimulation	Cells no stimulation	Salbutamol	Salbutamol	PGE_2	PGE_2	Color protein marker	Blank

<center>p-VASP   VASP</center>

Figure B-4. Distribution of samples and color protein marker in a 7% SDS-PAGE gel for electrophoresis and detection of VASP and p-VASP-Ser157 in ConA activated and stimulated with salbutamol (800nM) or PGE_2 (800nM) PBMC from RAO and non-affected horses.

Protein levels are expressed as percentage relative to the control (cells without stimulation) identified as band relative intensity. Results are presented in a descriptive form, and no statistical analysis was performed.

2.4 Results

Statistical analysis was not performed on these data due to the small sample size (n=3). However, based on subjective evaluation, cells from non-affected horses did not appear to increase the relative amount of p-VASP-Ser157 as compared to un-phosphorylated VASP in response to stimulation with salbutamol. However, a trend towards a shift in favor of p-VASP-Ser157 was seen with PGE_2 stimulation (Figure B-5 A).

PBMCs from RAO horses did not demonstrate an increase in p-VASPSer-157 relative to un-phosphorylated VASP when stimulated with salbutamol or PGE_2 (Figure B-5 B).
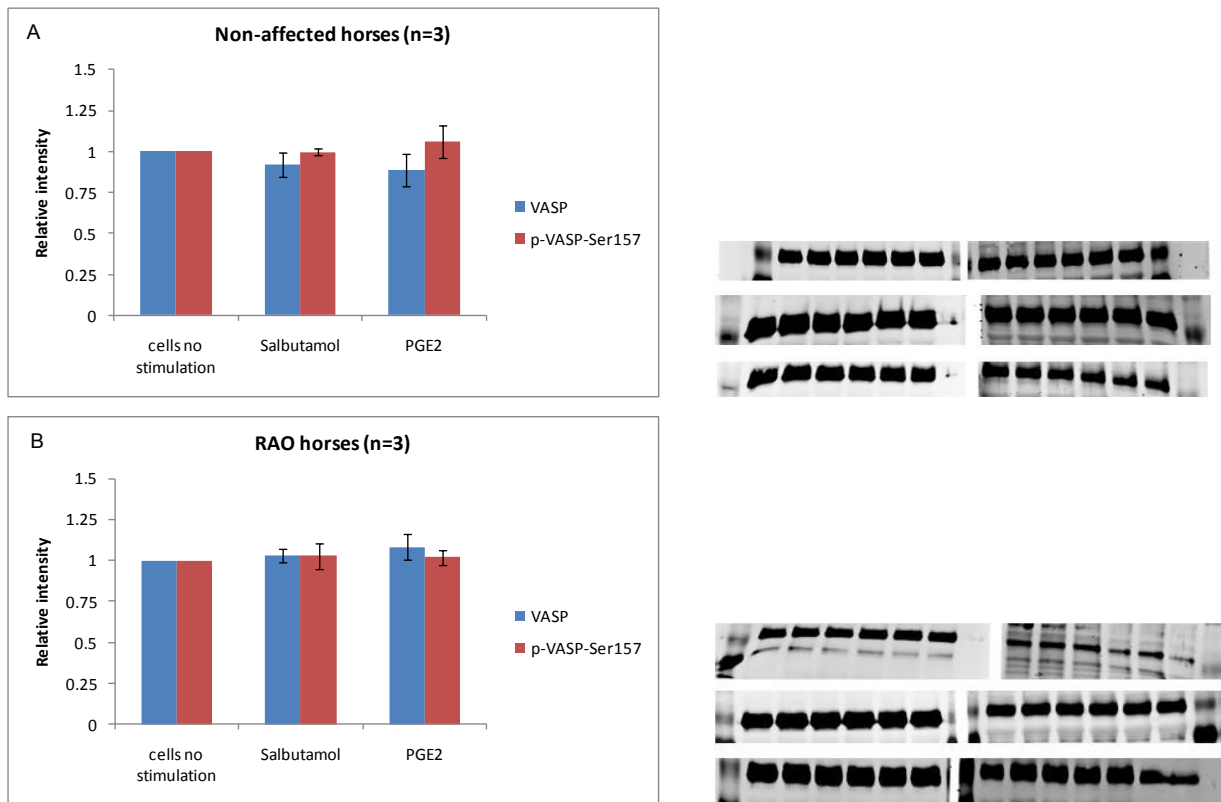

Figure B-5. Western blot analysis for VASP and p-VASP-Ser157 in equine PBMC expressed as relative intensity to the control (unstimulated cells) and standard error of the mean. A. Cells from non-affected horses group (n=3). B. Cells from RAO horses (n=3).

2.5 Interpretation/Discussion

The specific goals of this experiment were:

1. Verify that reagents used in other species to identify un-phosphorylated VASP recognized as a single protein from equine tissues that was 46 KDD molecular weight, using Western blot analysis.
2. Use an alternative method to verify that changes in p-VASP-Ser157 detected by flow cytometric technique correspond to those found using Western blot analysis.

Goal 1: VASP and p-VASP-Ser157 antibodies detected a protein band located at 46 KDD and 50 KDD respectively. As previously mentioned, phosphorylation of the amino acid Ser157 of VASP by PKA induces a shift in the molecular weight of the protein from 46KDD to 50KDD (Krause et al., 2003, Pula and Krause, 2008). Several studies have described the presence of VASP and p-VASP-Ser157 in human platelets (Butt et al., 1994, Halbrügge and Walter, 1989, Iyú et al., 2011), human endothelial cells (Nolte et al., 1991), human airway smooth muscle cells (Roscioni et al., 2009), mice cardiac myocytes (Sartoretto et al., 2009), human neutrophils (Eckert and Jones, 2007), and human lymphocytes (Halbrügge et al., 1992, Loza et al., 2006) and in each of these studies, the molecular weight of the two proteins has been the same. In the present study, a 46 KDD protein band was recognized by the anti-un-phosphorylated antibody and a 50KDD molecular weight protein band was identified by the anti-p-VASP-Ser157 antibody. While these findings are not absolute, they provide persuasive evidence that these reagents were binding specifically to the two forms of VASP.

Goal 2: No difference was observed between VASP and p-VASP-Ser157 in activated cells from RAO horses stimulated with salbutamol or PGE_2 and this finding differed from what was consistently observed using the flow cytometric technique. Cells from non-affected horses showed a tendency to increase the relative intensity of p-VASP-Ser157 in cells stimulated with PGE_2 which was similar to the results obtained using flow cytometry. This increase in p-VASP-Ser157 corresponded with an apparent decrease in unphosphorylated VASP. The absence of response observed in cells from RAO horses could be explained by:

- Potential need to perfect the technique and design of the experiment. This was a pilot study in which cells from only three horses per group were evaluated and only a single Western blot was performed. In addition, only one concentration of each agonist was used. To have confidence in the results obtained using this methodology, the experiment should be repeated several times until the outcome is repeatable and consistent. In addition,

lymphocytes were not separated from monocytes so the cell population used for the study differed slightly from that examined by flow cytometry (where gating on specific cell types is possible). Finally, the cells used in this study were not simultaneously evaluated using flow cytometric methods. Therefore, it is possible that changes in clinical status of the RAO horses may have influenced the cell responses to the agonist.

2.6 Additional/Future Studies

Additional studies aimed at perfecting the Western blot technique are required before the results can be considered meaningful. Increasing the number of horses from which cells are obtained, examining different protein loading amounts, titering of the antibodies, and using standards for densitometry measurements are just some examples of aspects of the techniques that should be evaluated. In addition, running side-by-side experiments in which both Western blot analysis and flow cytometric evaluation is performed on the same population of cells would provide a more valid data set for comparison, and better characterizing the clinical status of the RAO horses could provide more interpretable and comparable results.

3 Effect of Pre-culture with Antagonists
3.1 Background/Justification

To evaluate if the response to agonist stimulation in PBLs was due to specific β_2-AR binding, cells were pre-cultured with either a β_1- or β_2-antagonists. These results are described in detail in Chapter 3 and generally showed that the response of PBLs from RAO horses to salbutamol appeared to be antagonized exclusively by pre-culture with a β_2-antagonist. In this study, the effect of concurrent pre-culture with both a β_1- and β_2-antagonists on equine PBLs response to salbutamol was explored.

3.2 Specific Goal

1. Evaluate the response of equine PBL from RAO and non-affected horses to salbutamol stimulation following pre-culture with both ICI 188,551 (selective β_2-adrenergic receptor antagonist) and atenolol (selective β_1-adrenergic receptor antagonist).

3.3 Methods and Design

Equine PBM cells were activated, pre-cultured with ICI 188,551 and atenolol and then stimulated with salbutamol (500nM). After stimulation, cells were fixed, permeabilized, and

stained for p-VASP-Ser157 antibodies following the same protocol previously described in Chapter 2 (section 2.6.3) to be analyzed using flow cytometry. Results are presented as means and standard error (SE). MFI obtained by staining with secondary antibody only was considered as measure of non-specific binding of this antibody and this value was subtracted from MFI obtained by staining with primary and secondary antibody.

3.4 Results

In cells from non-affected horses, salbutamol did not increase MFI at any concentration when compared to unstimulated cells. In addition, no antagonist effect was observed. These findings were consistent with those discussed in Chapter 3.

The cells from RAO horses expressed an increase MFI in response to salbutamol alone, which was a statistically significant increase from baseline in all of the experiments in this study. The addition of the antagonists prior to addition of salbutamol tended to decrease the MFI values as compared to cells stimulated with salbutamol alone. However, cells pre-cultured with both antagonist and stimulated with salbutamol, demonstrate a significant increase in MFI when compared to unstimulated cells.

The populations of cells from RAO horses that were pre-cultured with the antagonists, but not stimulated with salbutamol also demonstrated a significant increase in MFI as compared to unstimulated cells at the lowest concentration of antagonists. This effect was not observed at the higher concentrations of antagonists (Figure B-6).
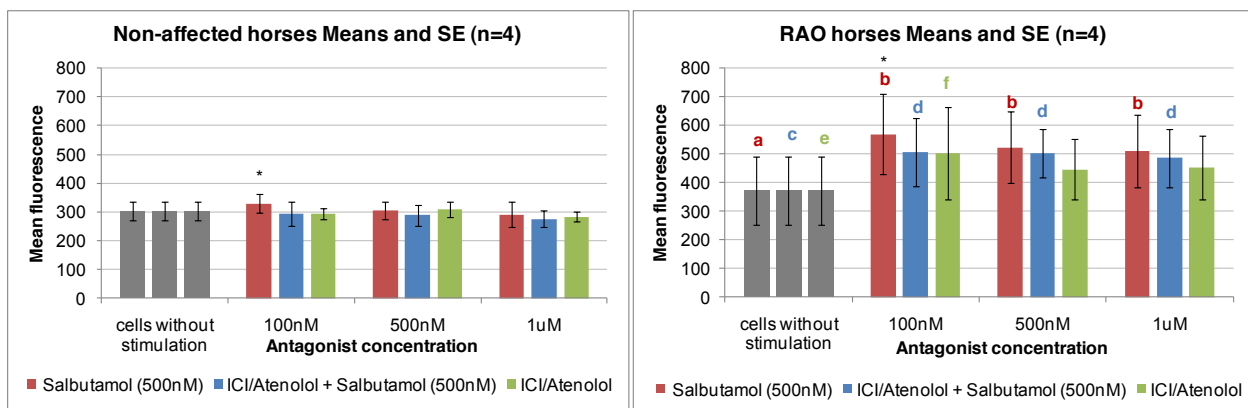

Figure B-6. Mean ± SE of MFI for p-VASP-Ser157 in equine PBLs from RAO and non-affected horses. All groups of cells were activated for 24 hours with ConA, before some were pre-cultured with ICI 188,551 (β_2-agonist) and atenolol (β_1-antagonist) together for 15 minutes.

Following this, designated groups of cells were stimulated with salbutamol (500nM) for 30 minutes.

* Indicates that the response of PBLs from RAO horses differed significantly ($p<0.05$) from the response of PBLs from non-affected horses at that concentration of both antagonists.

Different letters indicate a significant difference ($p<0.05$) in PBLs from RAO horses between baseline (cells no stimulation) and the correspondent antagonist concentration.

3.5 Interpretation/Discussion

The specific goal of this experiment was:

1. Evaluate the response of equine PBL from RAO and non-affected horses to salbutamol stimulation following pre-culture with both ICI 188,551 (selective β_2-adrenergic receptor antagonist) and atenolol (selective β_1-adrenergic receptor antagonist).

Stimulation with salbutamol did not induce a significant increase in the MFI in cells from non-affected horses when compared to unstimulated cells. Consequently, it was not surprising to find that addition of the antagonists had no effect on these cell populations. This finding corresponds with the response previously described in Chapter 3.

In contrast, the response of cells from RAO horses demonstrated a significant increase in MFI in response to stimulation with salbutamol, and this result was also similar to that observed in previous studies described in Chapter 3. The antagonists tended to reduce the response to salbutamol at the lowest antagonists concentrations, however MFI values were significantly increased when compared to unstimulated cells. This result differed from those obtained in previous experiments (Chapter 3) where cells were pre-cultured with either ICI or atenolol. When present alone, ICI appeared to have a greater antagonistic effect on salbutamol stimulation than when combined with atenolol. The selectivity of the drug is based on the structure of the drug that fits in the active site of the receptor (Hill, 2010). Therefore, a combination of drugs in appropriate concentrations could induce an interaction or conformational changes that may limit the efficacy of the drug.

The non-significant decrease in MFI in cells from RAO horses stimulated with salbutamol and both reagents (100nM) could be the result of an antagonist effect exerted by ICI 188,551 (β_2-antagonist) on the β_2-ARs present in activated PBLs. Considering that the β_2-AR is mainly, if not exclusively present in PBLs, atenolol should not have an effect on the receptor stimulation.

However, this response was not observed with higher antagonist concentrations, which could indicate that nonspecific response results when of both antagonists were used at higher concentrations. Drugs interact with receptors binding to specific sites of the receptor inducing a conformational change which triggers a response. The selectivity of the drug is based on the structure of the drug that fits in the active site of the receptor (Hill, 2010). Therefore, a combination of drugs in high concentrations could induce an interaction or conformational changes that may limit the efficacy of the drug.

3.6 Additional/Future Studies

Additional studies in which the concentration of one antagonist is held constant while the concentration of the second antagonist is varied may provide more information about how these antagonists interact. In this study, both antagonists were added at the same time. However, if some sort of competition for receptor binding is responsible for the partial agonistic effect, the order in which the antagonist is added may also affect outcome and this too should be explored. For example, if atenolol acts by blocking ICI binding, pre-incubating with ICI followed by atenolol may result in a different outcome as compared with cells which are pre-cultured with atenolol first.

4 Determination of p-VASP-Ser157 in Bronchoalveolar Lavage Cells
4.1 Background/Justification

Bronchoalveolar lavage (BAL) is a technique developed in order to recover fluid from peripheral airways and alveoli. Cytologic analysis of BAL fluid (BALF) is used as a measure of lung inflammation and the diagnosis of RAO (Hoffman, 2008, Robinson, 2001). Using an *in vitro* system, the results of experiments described in Chapter 3 demonstrated that activated PBLs from RAO horses responded to salbutamol stimulation while cells from non-affected horses had no response. While these results were interesting, it is hard to predict how well results obtained *in vitro* correlate with what happens *in vivo*. The purpose of this experiment was to gather preliminary information about how the response of lymphocytes from the airways of RAO and non-affected horses compared to the response of ConA activated PBLs. The outcome of this experiment could give information regarding the possible use of our *in vitro* model for the purpose of better understanding the effect of β_2-agonist therapy on the inflammatory response in the lung.

4.2 Specific Goal:

1. Determine if BALF cells from RAO and non-affected horses stimulated with salbutamol mimicked the *in vitro* response obtained in ConA activated PBLs.

4.3 Methods and Design:

Cells from the airways of horses with clinical RAO and non-affected horses were harvested using a previously reported method (Hoffman, 2008) and treated following the method described in Chapter 2 (section 2.10.1). Results presented here are only descriptive and are showed as MFI and range. No statistical analysis was performed.

4.4 Results

An increase in MFI in BALF lymphocytes harvested from non-affected horses and stimulated with PGE_2 was observed as compared to unstimulated cells and salbutamol stimulated cells. No response was observed with salbutamol stimulation when compared to unstimulated cells (Figure B-7 A).

In cells from RAO-affected horses, a slight increase in MFI was detected in BALF lymphocytes stimulated with salbutamol and PGE_2 as compared to the MFI of unstimulated cells (Figure B-7 B).
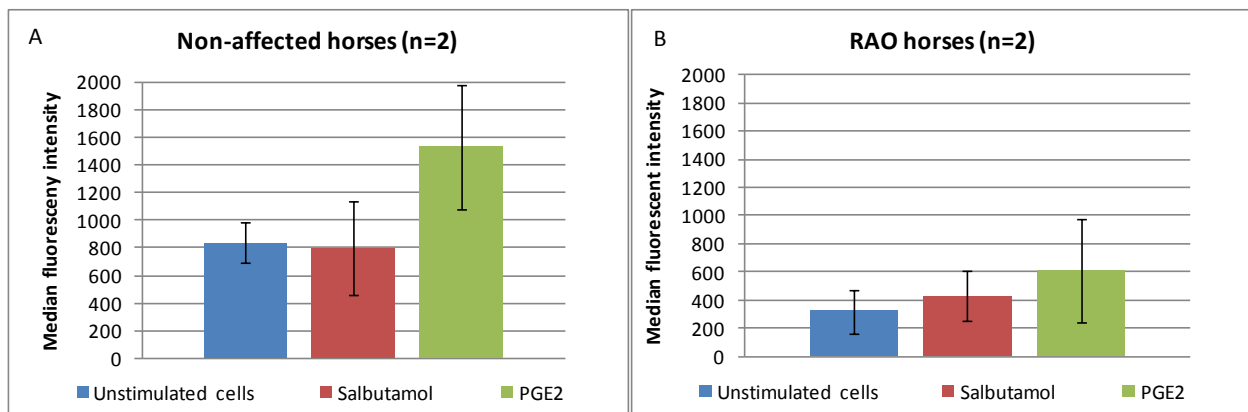

Figure B-7. A. Median fluorescent intensity and range of p-VASP-Ser157 detected in BALF lymphocytes from non-affected horses (n=2) stimulated with salbutamol (500nM) and PGE_2 (500nM). B. Median fluorescent intensity and range of p-VASP-Ser157 detected in BALF lymphocytes recovered from RAO horses (n=2) stimulated with salbutamol (500nM) and PGE_2 (500nM).

4.5 Interpretation/Discussion

The specific goal of this experiment was:

1. Determine if BALF cells from RAO and non-affected horses stimulated with salbutamol mimicked the *in vitro* response obtained in ConA activated PBLs.

In this experiment, airway lymphocytes from non-affected and RAO horses demonstrated a similar pattern as that observed when PBLs from these horses were activated with Con A and stimulated with the same agonists. These findings suggest that lymphocytes retrieved from RAO horses are likely in a state of activation which allows them to respond to salbutamol. This response is similar to that induced by ConA activation at least with respect to activation of PKA. But the downstream effect on the inflammatory response needs to be further examined. Of interest, airway cells from non-affected horses do not express a response to salbutamol, similar to the effect seen in PBLs even after they are stimulated with ConA. As previously mentioned, mitogen activation is commonly used to enhance T cell function by imitating the antigen binding effect (Resch and Ferber, 1988, Hu et al., 2010) and causing an increased expression of the β_2-AR (Radojcic et al., 1991). It is therefore likely that the addition of ConA to PBLs from non-affected horses activates those cells. Yet, they do not respond to salbutamol stimulation. These findings suggest that activation alone is not adequate to induce a response to salbutamol. Both the PBLs and airway lymphocytes from RAO horses must differ in their expression of the "activated" state in such a way as to increase response to the β_2-AR. This could be related to the stage of activation of lymphocytes. This could explain the difference observed in airway lymphocyte populations from RAO and non-affected horses. However, it is harder to apply this reasoning to the differences in responses observed between ConA activated cells from RAO and non-affected horses since the "activation state" of these cells should be similar. These findings more likely suggest that a difference in the mechanisms that regulate β_2-AR expression and or sensitivity exists between tissue from RAO and non-affected horses. This difference does not seem to affect the constitutive expression and/or function of the β_2-AR. However, regulation of the inducible form/function of the β_2-AR may be of significant importance in this and related diseases.

Stimulation with PGE_2 also induced higher MFI values in BALF lymphocytes from both RAO and non-affected horses as compared to unstimulated cells or cells stimulated with salbutamol. In section 4.1.4.3 it is described that PBLs stimulated with 500nM PGE_2 showed a significant increase in MFI when compared to baseline both in cells from RAO and non-affected horses.

These results demonstrate a similarity in the response of PBLs and airway lymphocytes to β_2-AR stimulation, and serve as further evidence that our in vitro system may be a good model for predicting in vivo responses.

4.6 Additional/Future Studies

1. While the results of this study suggest that there is a similarity in the response of ConA activated PBLs and BALF lymphocytes, evaluating a larger number of non-affected and RAO horses is indicated to further validate the similarity in the *in vivo* and *in vitro* responses. To better understand how closely these responses correlate, cellular characterization of the BALF could assist in determining if horses are experiencing airway inflammation at the time they are sampled.

2. Collecting blood and BALF lymphocytes at the same time from the same horse, and analyzing PBLs and BALF lymphocytes in a side-by-side fashion could provide a more accurate picture of whether the *in vitro* models reflect what is happening in the horse. Performing these experiments during different disease states in the RAO horses could also provide information about the effect of disease status on lymphocyte activation/response and further validate the use of the *in vitro* model to explore what is happening in the horse.

3. Measuring other downstream components in the cAMP pathway (cAMP concentration, gene expression), could help to identify additional similarities between PBLs and BALF cells, and provide further confirmation that the *in vitro* model is a useful tool for evaluating the *in vivo* response.

5 Cyclic AMP Concentration Measurements

5.1 Background/Justification

As previously mentioned, stimulation of the β_2-AR induces the activation of adenylyl cyclase through the Gα subunit of the G-protein. This enzyme catalyzes the conversion of ATP into cAMP, which in turn activates PKA. Measuring the cAMP concentration can be used as a complimentary or confimatory endpoint to the determination of p-VASP-Ser157 by flow cytometry after cell stimulation.

5.2 Specific Goals:

1. To measure the concentration of cAMP in unstimulated and stimulated cells from RAO and non-affected horses using a commercial cAMP competitive enzyme immunoassay (EIA) kit.

2. Employ an alternative method to verify the previously described response of PBLs to salbutamol stimulation using flow cytometric methodology.

5.3 Methods and Design

Isolated PBMC ($2x10^6$ cells/mL), as previously described in Chapter 2 (section 2.3), from RAO horses (2 horses) and non-affected horses (2 horses) were used. Cells were stimulated with salbutamol (800nM) and PGE_2 (500nM). Stimulant concentrations were selected based on previously described results (Chapter 3 and section 4.1.4.3, respectively) where significant differences were obtained.

IBMX (3-isobutyl-1-methylxanthine) is a known inhibitor of cAMP phosphodiesterase (PDE). Phosphodiesterases are a family formed by up to 11 isoenzymes (PDE1-PDE11). Type 4 cAMP- specific PDE (PDE4) catalyzes the hydrolysis of cAMP into AMP decreasing its concentration (Jin et al., 2010, Torphy, 1998, Spina, 2008). To prevent cAMP breakdown during the incubation period, IBMX was added to cell culture.

The technique was performed using the described methods in Chapter 2 (section 2.10.2) and following the manufacturer recommendations (Cayman Chem).

Results were obtained after data were analyzed using a program provided by Cayman Chemicals (Ann Arbor). Results are presented as means of each repetition and expressed as picomoles per milliliter (pmol/mL). No statistical analysis was performed.

5.4 Results

The addition of IBMX tended to increase cAMP concentration in unstimulated cells (Figure B-8 A and B).

cAMP concentration did not increase in cells from non-affected horses after salbutamol stimulation, and the addition of IBMX did not affect this result (Figure B-8 A).

In cells from RAO horses, cAMP tended to increase after salbutamol stimulation, and that effect was enhanced by the addition of IBMX (Figure B-8 A).

Cells from both RAO and non-affected horses showed increased cAMP concentration after stimulation with PGE_2. This increase was enhanced by the addition of IBMX. In addition, this increase appeared to be relatively larger in non-affected horses as compared to RAO horses (Figure B-8 B).
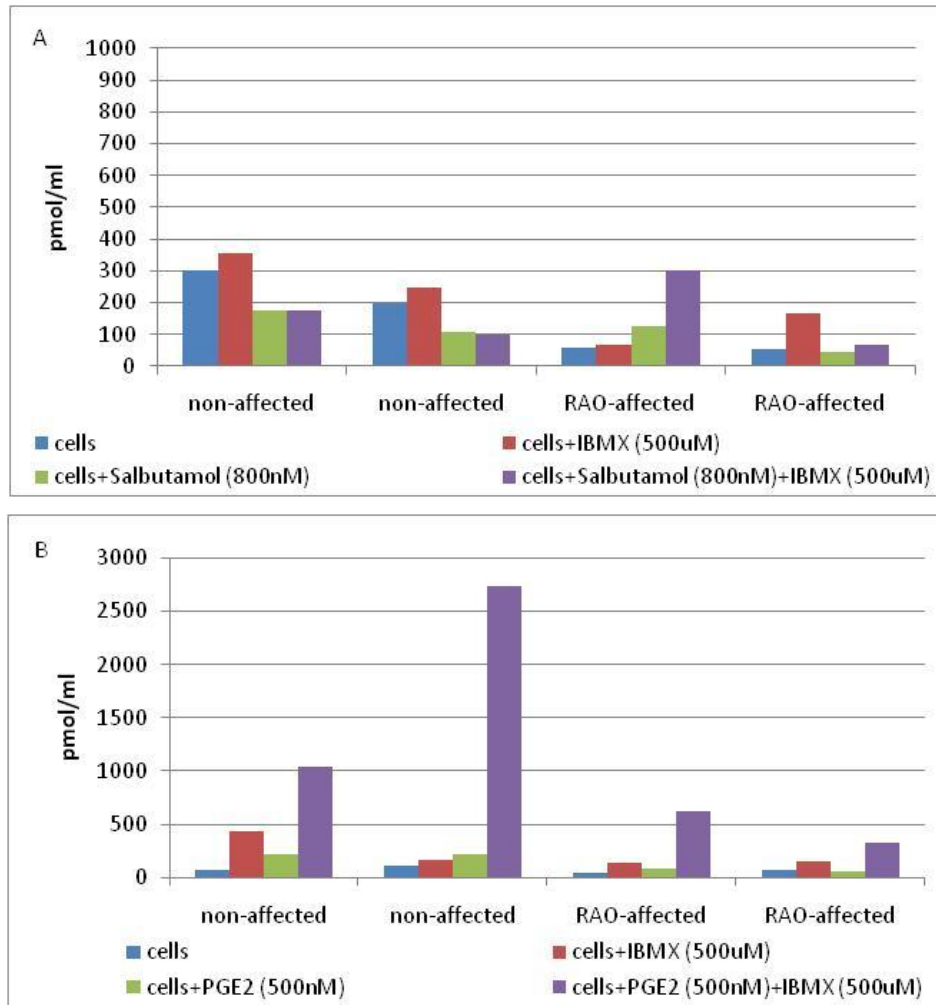

Figure B-8. Determination of cAMP concentration using an EIA commercial kit. Isolated PBMC from RAO horses (n=2) and non-affected horses (n=2) were activated with ConA for 48 hours and stimulated with: A. salbutamol (800nM) and/or IBMX (500µM), and B. PGE_2 (500nM) and/or IBMX (500µM).

5.5 Interpretation/Discussion

The specific goals for this experiment were:

1. To measure the concentration of cAMP in unstimulated and stimulated cells from RAO and non-affected horses using a commercial cAMP competitive enzyme immunoassay (EIA) kit.
2. Employ an alternative method to verify the previously described response of PBLs to salbutamol stimulation using flow cytometric methodology.

Goal 1: cAMP concentrations in PBMC with and without stimulation were measured using a commercial EIA kit. The addition of IBMX tended to induce an increase in cAMP concentration in cells from both groups stimulated with salbutamol and PGE_2. This response was expected based on previous reports in which the use of IBMX significantly increased cAMP concentration after agonist stimulation (LaBranche et al., 2010, Bloemen et al., 1997) by reducing the conversion of cAMP to ATP.

Concentration of cAMP in PBMC from non-affected horses did not increase after salbutamol stimulation, even when IBMX was used. This response is similar to findings detected using flow cytometric techniques. Previously discussed causes for these results include a lower expression of the β_2-AR in horses not affected with the disease. Also, modifications downstream in the cAMP pathway could contribute to this outcome, including lower function of adenylyl cyclase or less efficient binding of the receptor to the G-protein. When IBMX was added to the cell culture, an increase in cAMP was expected but not observed. These findings suggest that the absence of increase of cAMP in response to β_2-AR binding is more likely due to a lack of increase in production rather than increased degradation of cAMP.

In contrast, cells from RAO horses tended to increase in cAMP concentration in cells in response to salbutamol and this response was further increased by the addition of IBMX. These results are similar to those described previously using flow cytometric techniques (Chapter 3), where activated PBLs from RAO horses significantly responded to salbutamol stimulation. The observation that the addition of IBMX further increases intracellular cAMP concentration indicates that the effect of increased cAMP may be amplified by decreasing degradation of this molecule. These findings provide direction for development of novel therapeutic approaches to the use of bronchodilators, and studies currently are underway in which the potential use of PDE inhibitors as a treatment of human asthma is being explored.

Goal 2: Employ an alternative method to verify the previously described response of PBLs to salbutamol stimulation using flow cytometric methodology.

As observed with the flow cytometric techniques (Chapter 3), both RAO and non-affected horses tended to demonstrate an increase in intracellular cAMP in response to PGE_2 stimulation, and this response was amplified by the addition of IBMX. The amplitude of these responses varied from horse to horse, and in this experiment, non affected horses tended to have a greater baseline and increase in cAMP. However, as with other studies, no attempt was made to closely characterize the RAO horses' clinical condition and horses were also not age matched. However, changes in cAMP concentration mimicked those observed when measuring changes in p-VASP-Ser157 by flow cytometry. These findings suggest that binding of β_2-agonist to the β_2-AR results in activation of PKA through increased intercellular cAMP concentrations. These findings provide evidence that the signaling mechanism initiated by β_2-AR agonist binding is similar in horses as in other species. The observation that the changes in cAMP concentrations that occur in response to salbutamol and PGE_2 binding followed a similar pattern as changes in p-VASP-Ser157 serve to validate the results of both experiments.

5.6 Additional/Future Studies

1. Results obtained measuring cAMP concentration are promising, however a larger number of horses is needed in order to confirm these preliminary findings. It would be interesting to correlate EIA with flow cytometric findings in order to establish a true relationship between both techniques.

2. Measure cAMP concentration in stimulated equine PBLs after incubation with β_2-antagonist. This will add information and help to confirm that stimulation is exerted mainly through the β_2-AR.

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