EFFECTS OF AEROBIC EXERCISE ON THE ASTHMATIC LUNG

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ABSTRACT

Asthma is identified by the clinical symptoms of wheezing, chest tightness, dyspnea and cough, and by the presence of airway hyperresponsiveness (AHR) to inhaled stimuli. We have reported previously that repeated bouts of moderate intensity aerobic exercise attenuate airway inflammatory responses in a mouse model of atopic asthma. Because the effects of exercise on physiological responses are dependent upon several variables, including total exercise duration, the hypothesis of the current work was that differing amounts of moderate intensity aerobic exercise exert dissimilar effects on AHR and airway inflammation. The aims to test this hypothesis included: 1) determine the effect of multiple bouts of moderate intensity aerobic exercise on AHR; and 2) determine the effect of a single bout of moderate intensity aerobic exercise on AHR and airway inflammation. These aims were completed in two independent studies. In the first study, we determined the effects of moderate intensity aerobic exercise at repeated bouts on AHR in mice sensitized and challenged with ovalbumin (OVA). Results show that repeated bouts of exercise attenuate AHR in OVA-treated mice as compared with controls; these effects were blocked in the presence of a β2-adrenergic receptor (AR) antagonist. Repeated bouts of exercise also decreased ASM thickness and PGE2 production significantly in OVA-treated mice. Together, these data indicate that repeated bouts of moderate intensity aerobic exercise attenuate AHR via a mechanism that involves β2-AR.
In the second study, we delineated the effects of a single bout of moderate intensity aerobic exercise on AHR and airway inflammation in OVA-treated mice. Results show that, within the lungs of OVA-treated mice, exercise attenuated inflammatory mediator production, leukocyte infiltration, and NF-κB activation as compared with controls. In contrast, a single bout of exercise had no effect on AHR and airway remodeling in OVA-treated mice. These findings suggest that a single bout of aerobic exercise at a moderate intensity attenuates airway inflammation but not AHR or airway remodeling in OVA-treated mice.

From these studies, we hypothesize that moderate intensity aerobic exercise attenuates AHR and airway inflammation sequentially within the asthmatic lung; future studies to test this hypothesis are discussed.
DEDICATION

This dissertation is dedicated to my parents Michael and Elaine Hewitt, who have given me guidance and advice throughout my life and taught me the fundamentals of personal and professional success. I thank them for all of their support through the years, and hope that I have made them proud.
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CHAPTER 1

ASTHMA

Asthma Overview

Allergic or atopic asthma, the most common form of asthma, is identified by the presence of characteristic clinical symptoms of wheezing, chest tightness, dyspnea, cough, and by the presence of reversible airway narrowing and/or airway hyperresponsiveness (AHR) to a variety of inhaled bronchoconstrictor stimuli. Asthma is classically characterized as an inappropriate immune response to common allergens. For many patients, the disease has its roots in infancy, and both genetic factors (atopy) and environmental factors (viruses, allergens, and occupational exposures) contribute to its inception and evolution.

Figure 1. Pathogenesis of Asthma. Asthma pathogenesis involves complex interactions with both environmental factors and also genetics. Long acting beta-agonists (LABA) and Corticosteroid treatments for asthma are shown along with their detrimental side effects that are seen after prolonged use.
Asthma Statistics

The incidence of asthma has increased in industrialized countries at an alarming rate. In these countries the incidence of asthma has doubled since 1980 with 1 out of every 10 people in the United States (US) affected, translating into approximately 33 million people with asthma. Since asthma does not cause death except in severe cases and asthmatics require a means to control symptoms, healthcare costs related to asthma treatment are rising. According to a Trends in Asthma Morbidity and Mortality article, in 2007 asthma costs the US an estimated $19.7 billion a year in healthcare costs. Asthma was responsible for an estimated 27.3 million lost work and school days in 2005.

Inflammatory Cascade in the Airways: Early and Late Phase Responses

The first part of this process called the early phase is caused by an immediate reaction that is triggered by the allergen contact. The result is the production of IgE and the degranulation of mast cells which releases preformed and synthesized inflammatory mediators such as histamine, proteases, and enzymes. These mediators promote microvascular permeability, high levels of mucus secretion, and smooth muscle contractions in the vascular smooth muscle. The second stage, or late-phase is characterized by a delayed and sustained inflammatory response which involves the release of cysteinyl leukotrienes, prostaglandins, cytokines (i.e. IL-4, IL-5, IL-13), chemokines (i.e. IL-8, MCP-1, RANTES), eosinophil chemoattractant factors, adhesion molecules (i.e. ICAM-1, VCAM-1), and matrix metalloproteinases. The release of these factors coincides with the recruitment of macrophages and leukocytes such as helper T-cells, lymphocytes, eosinophils, and neutrophils promoting the inflammatory cascade.
Airway epithelial cells and airway smooth muscle (ASM) also contribute to this inflammatory response during this phase and the sequelae from this phase cause further tissue damage and airway remodeling through epithelial sloughing, airway smooth muscle proliferation, and basement membrane thickening.\(^5\)

Role of Genetics in the Pathogenesis of Asthma

Most genes implicated in asthma and allergy susceptibility have been identified through studies that sought to identify an association between variants such as single nucleotide polymorphisms (SNPs). These genes are located in the main pathways that influence allergic inflammation and asthma or asthma-related (intermediate) phenotypes. Asthma susceptibility genes fall into four main groups: i) genes associated with innate immunity and immunoregulation; ii) genes associated with T helper 2 (Th2)-cell differentiation and effector functions; iii) genes associated with epithelial biology and mucosal immunity; and iv) genes associated with lung function, airway remodeling and disease severity. There isn’t a clear understanding of how these genetic abnormalities play into this complex disease. However, many of the genes that have been associated with asthma share the same regions of their respective chromosome. Because of this, there exists the possibility that if one gene is affected it would have a ripple effect on the other genes of the same region. One example is interleukin 4 (IL-4), IL-13, CD14, and the β2-adrenergic receptor (ADRB2), and the serine peptidase inhibitor, Kazal type 5 (SPINK5) all sharing the q31 region of chromosome 5. The aforementioned genes have all been implicated in asthma. If one of these genes was altered with an addition or deletion it would affect the genes in the same region resulting in the limiting or ablation of function in multiple genes. These factors taken together may pre-dispose an
individual to become atopic; or having the ability to produce IgE in the presence of common environmental proteins such as pollen or house dustmite\textsuperscript{7}.

**Inflammatory Transcription**

After the early phase, the late phase is characterized by the release of cytokines, chemokines, and many other pro-inflammatory mediators that were mentioned above. Release of these mediators is tightly regulated through the activation of pro-inflammatory transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1\textsuperscript{8}. The transcription factor of most interest in asthma is NF-κB for two reasons; i) the inflammatory genes that NF-κB transcribes are genes classically associated with asthma (IL-4, IL-5, MCP-1, RANTES, VCAM-1, etc) and ii) NF-κB is potently inhibited by glucocorticoids, the most effective treatment of asthma\textsuperscript{9}. In addition, Poynter and colleagues demonstrated that NF-κB activity is increased in the airways of an OVA-driven murine model of asthma\textsuperscript{10}. Another study by Yang and co-workers demonstrated that the NF-κB subunit p50 is required for the induction of eosinophilia in an OVA-driven murine model of asthma\textsuperscript{11}. When cells in the lung receive an inflammatory stimulus, it activates the inhibitor of I-κB kinase (IKK)-2 which in turn releases the active form of the transcription factor NF-κB\textsuperscript{8}. A dimer of the p50 and p65 subunits translocate into the nucleus and bind specific κB recognition sites and also to co-activators such as cAMP-reponse-element-binding-protein-binding protein (CBP) or p300/CBP-associated factor (pCAF), which has intrinsic histone acetyltransferase (HAT) activity\textsuperscript{12}. This results in the acetylation of core histone H4, resulting in increased expression of genes encoding multiple inflammatory proteins\textsuperscript{12}. 
Inflammatory Cytokines and Chemokines in the Lung

Asthma is a disease associated with the activation of \( T_{H2} \) (CD4+) cells expressing interleukins IL-4, IL-5, and IL-13\(^5\). These cytokines coincide with IgE-mediated eosinophilic inflammation in the lungs. When IL-4 and IL-13 are activated they promote Ig switching from IgG to IgE in the immune system which stimulates the recruitment of eosinophils to the site of inflammation in the lung\(^5\). IL-5 has been implicated in eosinophil maturation, recruitment, and enhancing the survival of eosinophils\(^5\). Another cytokine that has been linked to asthma recently is IL-12p40\(^13\). This cytokine is interesting in that it can differentially dimerize to perform different functions in the immune system. The dimers more relevant to asthma are IL-12p70, IL-12p40, and IL-12p80\(^13\). The form most produced is IL-12p40 which has been shown to be produced by activated inflammatory cells including macrophages, neutrophils, microglia, and dendritic cells; along with keratinocytes, and airway epithelial cells\(^13\). IL-12p70 consists of an IL-12p40 subunit and an IL-12p35 subunit while IL-12p80 is a homodimer of two IL-12p40 subunits. The different dimers and the pathways they activate are shown in Figure 2.
Figure 2. IL-12p40/80 Signaling. The IL-12p40 dependent cytokines, IL-12p70, IL-23, and IL-12p80 along with their respective receptors and the pathways they activate. Adapted from “IL-12p40: an inherently agonistic cytokine” by A.M. Cooper and S.A. Khader, 2007, Trends in Immunology, 28, p. 33. Copyright 2007 by Elsevier LTD. Adapted with permission.

It has been established with studies in vitro that IL-12p80 has the ability to antagonize IL-12 function, specifically the function of IL-12p70. The role of IL-12p80 as an antagonist of IL-12p70 function was first proposed by Gately and colleagues and supported by the fact that endogenous rmIL-12p80 protects mice from IL-12-dependent but not TNF-dependent shock. This cytokine also blocks in vivo interferon (IFN)-γ production and inhibits IFN-γ synthesis from splenocytes treated with IL-12p70. IL-12p40 and IL-12p80 have also been implicated in promoting the pathogenesis of asthma through promoting Th2 responses and dampening any Th1 response. This association has been seen with IL-12p40/80 acting with potentially damaging inflammatory responses within the lung. Specifically, IL-12p40/80 as identified using western analysis, are seen at high levels in the BAL fluid of asthma patients and are associated with macrophage accumulation. IL-12p40/80 has also been implicated as an inducer
of dendritic cell migration from the lung to the lymph node after a challenge by a pathogen or allergen\textsuperscript{17}. This may help to initiate the inflammatory response and bias it towards the T\textsubscript{H}2 lineage through the expansion of particular T\textsubscript{H}2 T-cell populations.

Chemokines represent a large family of proteins secreted by many immune and non-immune cells with an essential role in inflammatory and immune reactions\textsuperscript{18}. Chemokines are divided into two major subfamilies on the basis of arrangement of the two N-terminal cysteine residues, CXC and CC, depending on whether the first two cysteine residues have an amino acid between them (CXC) or are adjacent (CC)\textsuperscript{19}. A generally recognized function of chemokines is the control of leukocyte migration and recruitment under normal (homeostatic) conditions and, more importantly, during inflammatory settings\textsuperscript{18}. In asthma, CC chemokines are activated including eotaxin, monocyte chemoattractant protein (MCP) – 1, and regulated upon activation normal T-cell expressed and secreted (RANTES). Eotaxin is produced mainly by epithelial cells of lung and gut and mediates preferential attraction of eosinophils to a site of inflammation\textsuperscript{20}. MCP-1 has also been implicated in inflammatory disorders of the lung including asthma\textsuperscript{21}. Mechanisms by which MCP-1 may be acting include recruitment of regulatory and effector leukocytes; stimulation of histamine or leukotriene release from mast cells or basophils; induction of fibroblast production of transforming growth factor-beta (TGF-beta) and procollagen; and enhancement of T\textsubscript{H}2 polarization\textsuperscript{21}. RANTES is expressed on many cell types including eosinophils, basophils, and mast cells\textsuperscript{22}. It has been found to play an important role in allergic lung inflammation causing lung leukocyte infiltration, bronchial hyperresponsiveness, and the recruitment of eosinophils to airways in asthma\textsuperscript{22}. 
Airway Remodeling and Asthma

Airway remodeling can be defined as changes in composition, organization and quantity of cells and extracellular constituents of the airway\textsuperscript{23}. Some components are reversible, either spontaneously or through pharmacologic interventions. However, the more prominent abnormalities, such as globlet cell hyperplasia seen in the epithelium, airway smooth muscle (ASM) thickening, increased vessel vasculature, and increased deposition of extracellular matrix, are generally thought of as more permanent and refractory to therapy\textsuperscript{24}. There are multiple cells types that are affected by airway remodeling but the ASM that lines the airways has become a centerpiece of research in recent years. The thickness and reactivity of the ASM surrounding the airways can have a direct impact on the caliber of the airways. Given this an increase in ASM mass, caused by hyperplasia, hypertrophy, and/or increased extracellular matrix deposition, is an important component of the remodeled wall in asthmatic airways\textsuperscript{25}. In asthmatics, the ASM proliferates farther out of the central airways and into the peripheral airways\textsuperscript{25}. This is accompanied by decreased ASM apoptosis than it would normally in non-asthmatic individuals\textsuperscript{25}. This hypertrophy and hyperplasia of the ASM is thought to induce stronger contractions from the ASM resulting in airway narrowing and closure. In chronic asthma the airways become thickened, not only due to an increase in ASM, but also as a consequence of the laying down of new matrix protein including collagen fibers, increased proliferation of microvessels along with vascular leakage and deposition of proteoglycans, with the ability to sequestrate water\textsuperscript{25}.

Asthmatic airways can be characterized by the increased influx of immune cells including, eosinophils, macrophages, leukocytes, and to a lesser extent neutrophils. These cells are accompanied by increased inflammatory mediator expression, as described above, in the airways. Accompanying the influx of these cells and mediators
is the remodeling of the airways. In childhood asthma airway remodeling is seen preceding classical asthma symptoms indicating that the airway remodeling in asthma is independent of inflammation\textsuperscript{24}. We and others have published data reinforcing this idea of the separation of remodeling and inflammation\textsuperscript{26}; our data will be presented in detail within Chapter 3. Data has shown that when eosinophils are knocked out through genetic manipulation no airway remodeling is observed, however airway inflammation and airway hyperresponsiveness (AHR) persists\textsuperscript{26}.

A tissue in the airways that is affected by the laying down of addition extracellular matrix is the lamina reticularis, or the basement membrane in the airways\textsuperscript{27}. This membrane is mainly used for adhesion of cells and connects the basolateral side of epithelial cells with other tissues including ASM that are further away from the surface of the airways. In asthma the laying down of addition extracellular matrix proteins such as collagen and elastin can cause the thickening of the basement membrane and contribute to the stiffness of the airways along with airway narrowing and closure\textsuperscript{27}.

The role of epithelial and goblet cells in the remodeling of airway in asthma are much more pronounced than changes in ASM or the surrounding vasculature. In asthma epithelial cells become abnormal and have shown to be so both \textit{in vitro} and \textit{in vivo}\textsuperscript{28,29}. The expression profile of cadherins and also epidermal growth factor (EGF) is abnormal in asthmatic epithelium\textsuperscript{28,29}. The proliferation rate of asthmatic epithelium is increased in atopic asthmatics and even more so in severe asthmatics\textsuperscript{30}. Asthmatic airways produced excess mucus, which is unable to be removed by normal ciliary movement\textsuperscript{23}. Goblet cells are a primary source of mucus within the airway and have been shown to be hyperplastic in asthmatic airways\textsuperscript{23}. A recent study showed the functional link between airway inflammation and increases in goblet cell number\textsuperscript{31}. In this study, both T\textsubscript{H}2 cytokines and dust mite allergy (DMA) induced the expression of SAM pointed domain-containing ETS transcription factor (SPDEF) in Clara cells, which
resulted in their differentiation into globlet cells\textsuperscript{31}. This process was dependent on STAT-6, suggesting a role for interleukin (IL)-14 or IL-4\textsuperscript{31}.

An often neglected aspect of airway remodeling is the role of the vasculature in the asthmatic lung. In both asthmatic children and adults, it has been reported that there is an increase in the number of blood vessels per square millimeter compared to non-asthmatics\textsuperscript{23}. This increase in blood vessel number has also been shown to be true in a rat model\textsuperscript{32}. The mechanism by which this is thought to occur is through increased expression of vascular endothelial growth factor (VEGF) which promotes the growth of new blood vessels\textsuperscript{33}. This growth of new blood vessels causes increased vascular permeability or “leak” from the vessels into the lung making it easier for immune cells to migrate out of the bloodstream and into the lung\textsuperscript{33}. It is thought the reason for increased vascularization through blood vessel growth serves to help better oxygenate the tissues\textsuperscript{33}. However under chronic inflammation conditions such as the ones in asthma this compensatory mechanism may be detrimental to lung function.

Lung Function and Asthma

The primary function of the lungs is to arterialize mixed venous blood through gas exchange. Gas exchange in the lungs can be divided into three parts. The first is ventilation or the process of distributing air throughout the lungs\textsuperscript{34}. Second is gas exchange of O\textsubscript{2} into the bloodstream and CO\textsubscript{2} out of the bloodstream\textsuperscript{34}. The last part is to distribute the O\textsubscript{2} in the bloodstream to the different tissues in the body, while at the same time disposing of the CO\textsubscript{2} through exhalation\textsuperscript{34}. In order to understand the relationship between asthma and lung function, there are a few terms that must be defined. Total lung capacity (TLC) is the maximum amount of air that an individual’s lungs allow them to inhale\textsuperscript{36}. Inspiratory capacity (IC) represents the maximal volume of
gas that can be inspired; whereas vital capacity (VC) is the maximal volume of air that can be expelled from the lungs forcefully\textsuperscript{35}. An individual’s expiratory reserve volume (ERV) shows maximal amount of air that be expired from the end-expiratory level\textsuperscript{35}. Meanwhile, residual volume (RV) is the volume of air remaining in the lungs at the end of a maximal expiration\textsuperscript{35}. In the Figure 3 below, these lung mechanics are illustrated in the form of a spirometry test. The lung mechanics of a normal individual performing a spirometry test is shown in Figure 3. An asthmatic with narrowed airway performing the same test is also shown in Figure 3. The predicted TLC of each person is the same. However in the asthmatic individual there is a significantly higher RV than in the normal individual indicating the asthmatic subject cannot move air in and out the lungs as efficiently as the normal individual. This becomes more evident when the individual undergoes the Maximal Breathing Capacity portion of the test where they take multiple breaths repeatedly. In the non-asthmatic individual the inspiration and expiration volumes remain constant whereas the asthmatic’s inspiration and expiration volumes decrease thereby increasing the RV and decreasing the amount of air moving in and out of the lungs. Along with this phenomenon IC, ERV, and VC are all observed as being significantly decreased in the asthmatic individuals.
Airway Smooth Muscle Physiology

Surrounding the airways is a layer of smooth muscle that is under control of the nervous system and is responsible for regulating airway caliber. This airway smooth muscle (ASM) responds to stimuli from the nervous system and also stimulus signals from the epithelium which causes this muscle to either relax or contract. In the case of asthma, the ASM can become over-reactive to stimuli that asthmatics become atopic to. For reasons not well understood, the ASM in the airways proliferates\(^24\). This proliferation is thought to induce stronger contractions when stimulated causing further airway narrowing and closure. The signaling cascades that control contraction and relaxation in
the ASM are very complex and poorly defined. There are 2 groups of receptors mediating contraction and relaxation in the ASM. The muscarinic receptors are mainly responsible for contraction of the airways while the β-adrenergic receptors are responsible for relaxation.

Muscarinic Receptors and Asthma

The muscarinic receptor family is comprised of five receptors. Of these five, three have been implicated in allergic airways diseases such as asthma; M1, M2, and M3. The M1 receptor is expressed solely on the nerves innervating the ASM and has been implicated as being a receptor responsible for ASM relaxation by limiting the release of acetylcholine (Ach) which promotes ASM contraction\textsuperscript{36,37}. Using knock-out mice, when the M1 receptor was mutated and rendered inactive an increase in airway resistance was observed in mice when they are given a bronchoconstriction challenge\textsuperscript{37}. The M2 receptor has been shown to be expressed on both ASM and also the nerves that innervate the ASM\textsuperscript{36,37}. The M2 receptor seems to have a dual role in ASM contraction/relaxation; if the receptor is expressed on the ASM it is contractile\textsuperscript{36}. The M2 receptors expressed on the innervating nerves are thought to be responsible for limiting the release of acetylcholine and limiting ASM contraction\textsuperscript{36}. Using mice with the M2 receptor knocked-out it was found that the M2 receptor is only responsible for approximately 20-25\% of the ASM contraction\textsuperscript{37}. However, the M2 receptor has a lower activation threshold than the receptor that is responsible for the majority of the ASM contraction, the M3 receptor. Therefore the M2 receptor is activated first when in the presence of Ach or a contractile agonist. The M3 receptor is found only on the ASM\textsuperscript{36,37} in the airways and is responsible for approximately 80\% of the ASM contraction which again was illustrated using knock-out mice\textsuperscript{37}. The M2 and M3 receptors signaling
cascade, which is shown in Figure 4, promotes ASM contraction by causing the release of $\text{Ca}^{2+}$ from the sarcoplasmic reticulum and inhibit adenylate cyclase which produces cyclic adenosine monophosphate (cAMP) within ASM cells\textsuperscript{38}. The release of $\text{Ca}^{2+}$ leads to the activation of calmodulin which then binds myosin light chain kinase (MLCK)\textsuperscript{38}. Once bound MLCK attaches phosphates to Myosin Light Chain (MLC) which causes the myosin head to contract\textsuperscript{38}. The M2 receptor follows a similar cascade of events to cause contraction but does not differ in that it promotes the uptake and release of $\text{Ca}^{2+}$ and inhibits cAMP production\textsuperscript{38}.

Figure 4. M2 and M3 signaling cascades. M2 and M3 signaling cascades that result in airway smooth muscle (ASM) contraction. Note: Adapted from “Signal-transduction pathways that regulate visceral smooth muscle function. III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles” by W.T. Gerthoffer, 2005, American Journal of Physiology and Gastrointestinal and Liver Physiology, 288, p. G849. Copyright 2005 by The American Physiological Society. Adapted with permission\textsuperscript{38}. 

![Diagram of M2 and M3 signaling cascades](image-url)
β₂ - Adrenergic Receptor and Asthma

The role of the β₂ – adrenergic receptors (β₂ – AR) in the airways is to promote ASM relaxation. It has been the basis for most bronchodilator treatments in asthma or other chronic obstructive pulmonary diseases (COPDs). The receptor can be found on the surface of multiple cells types in the airways including epithelial cells, ASM, and immune cells such as T-cells all express β₂ – AR. The traditional concept of agonist activation of the β₂ – AR was akin to a lock and key: the agonist fit into the receptor and caused it to adopt a conformation that was favorable for coupling to G₃⁹. However, it is now clear that the receptor is continuously "toggling" between various conformations in the absence of agonist⁹. This is readily appreciated by measuring basal adenylyl cyclase activity in membranes from transfected cells expressing various levels of the β₂ – AR⁹. As expression increases, so does basal (nonagonist) adenylyl cyclase activity⁹. Once the receptor is activated it signals through the G₃ class of G – proteins.
Figure 5. $\beta_2$ – Adrenergic Receptor Activation and Signaling. Signaling events associated with $\beta_2$-adrenergic receptor mediated relaxation of airway smooth muscle, Adapted from “Heterotrimeric G protein signaling: role in asthma and allergic inflammation” by E.N. Johnson and K.M. Druey, 2002, Journal of Allergy and Clinical Immunology, 109, p. 592. Copyright 2002 by Mosby Inc. Adapted with permission.

Adenylate cyclase is activated causing increased cAMP production by the hydrolyzation of ATP. cAMP inhibits the release of $Ca^{2+}$ and therefore inhibiting contraction and promoting relaxation of the ASM. It maintains this relaxation through a variety of ways including reducing the amount of $Ca^{2+}$ that is allowed to enter the cell, inhibiting $Ca^{2+}$ release from the sarcoplasmic reticulum, and sequestrating what $Ca^{2+}$ is in the cell to the sarcoplasmic reticulum. Without $Ca^{2+}$ calmodulin cannot be activated to cause contraction.
β2 - Adrenergic Receptor Regulation in Asthma

Associated with β2 – adrenergic receptor (β2 – AR) activation is the autoregulatory process of receptor desensitization41. This process operates as a precaution to prevent overstimulation of receptors in the face of excessive β2 – agonist exposure. The mechanisms by which desensitization can occur consist of three main processes: (1) uncoupling of the receptors from adenylate cyclase, (2) internalization of uncoupled receptors, and (3) phosphorylation of internalized receptors41. The principal mechanism of homologous, short-term, β2 – agonist – promoted desensitization of the ADRB2 is phosphorylation of the receptor by PKA or a G protein – coupled receptor kinase (GRK), usually GRK – 241. This phosphorylation ultimately leads to the formation of a binding site for β – arrestin which inhibits β2 – AR function by preventing the association with the Gs protein42. This simple form of desensitization is a transient process and might be reversed within minutes of removal of the agonist. After prolonged periods of agonist exposure, an internalization of receptors occurs that results in some loss from the cell via clathrin – coated pits surface41;43. This process, sequestration, might play a role in short – term regulation of the receptor, because while sequestered, dephosphorylation of the receptor occurs41. Internalization of the receptors takes longer than uncoupling, but full reversal normally occurs within hours41.
Airway Hyperresponsiveness and Asthma

Airway hyperresponsiveness (AHR) is a hallmark clinical symptom of asthma\(^{44}\). At least two components of AHR have been identified: i) baseline AHR, which is persistent and presumably caused by airway remodeling due to chronic recurrent airway inflammation; and ii) acute and variable AHR, which is associated with an episodic increase in airway inflammation due to environmental factors such as allergen exposure\(^{44}\). In asthma, abnormalities of airway smooth muscle (ASM), airway remodeling, and airway inflammation have all been implicated as contributing factors to AHR\(^{45}\).
In addition to its central role in limiting airflow and regulating variable AHR, it is increasingly evident that chronic structural and phenotypic alterations of the ASM exaggerate ASM contraction\textsuperscript{46}. Studies have shown that ASM surrounding the airways of asthmatics is considerably thicker\textsuperscript{47,48}. Knowing that, detailed analyses have been performed on ASM bundles obtained from asthmatics and indicate that increases in both ASM cell number (hyperplasia) and cell size (hypertrophy) are present and contribute to the increased reactivity of the ASM\textsuperscript{49,50}. Ebina and colleagues proposed two different asthma phenotypes for ASM in the asthmatic lung. The first being primarily characterized by ASM hyperplasia in the central airways, while the other is primarily characterized by ASM hypertrophy throughout the bronchial tree\textsuperscript{49}. Recent studies performed \textit{in vitro} suggesting asthmatic ASM cells exhibit an intrinsic functional change that facilitates cell proliferation\textsuperscript{51}. This change is partially explained by changes in extracellular matrix (ECM) protein deposition by asthmatic ASM. In this study, Johnson and colleagues cultured ASM cells from asthmatics. These cells were lifted from the plate but the ECM laid down by the asthmatic ASM cells was left. When normal non-asthmatic ASM cells were cultured on the plate containing asthmatic ECM, the normal cells showed increased proliferation comparable to the asthmatic ASM cells\textsuperscript{52}. Although studies to date have strongly suggest that ASM play a large role in promoting AHR through the mediation of airway caliber, the exact contribution is not yet clear.

Airway remodeling is characterized by persistent structural changes to the airway wall\textsuperscript{44}. It is generally believed that both airway remodeling and chronic AHR maybe induced by chronic or prolonged airway inflammation\textsuperscript{53}. However, the role of airway remodeling in promoting AHR has recent come under heavy scrutiny. Recently, there have been studies suggesting that airway remodeling is a protective mechanism and by causing airways to become stiffer, they would allow less airway narrowing and closure as a result of ASM contraction\textsuperscript{54,55}. This has led to the concept that airway inflammation
and airway remodeling happen in a parallel manner and not sequentially\textsuperscript{44}. This fits with the hypothesis that asthmatics exhibit an intrinsic inability to appropriately repair epithelial cell injury in response to environmental agents leading to the secretion of a variety of growth factors, mediators and cytokines by the activated airway epithelium, which drives the airway remodeling and promotes persistent airway inflammation contributing to AHR\textsuperscript{56}.

Numerous studies have demonstrated a direct association between allergen-induced changes in AHR and T\textsubscript{H}2 cell driven eosinophilic airway inflammation\textsuperscript{53,57}. There has been evidence that AHR and the characteristic eosinophilic inflammation observed in asthma are dissociated. In these studies, eosinophils lineages were either knocked out in mice or anti-IL-5 therapy was administered to humans with no change in AHR observed\textsuperscript{26,58}. Humbles and colleagues performed their studies in mice where they knocked out the eosinophil lineage. Their results indicated even in the absence of eosinophils both airway remodeling and AHR persisted\textsuperscript{26}. Similar results were observed in Leckie and colleagues where human asthmatics were administered anti-IL-5 therapy intravenously to observe its effect on the late asthmatic response (LAR). Their results showed a significant decrease in eosinophils found in the blood\textsuperscript{58}. However, AHR was remained unchanged in these patients indicating the anti-IL-5 therapy had no affect\textsuperscript{58}. Although these studies were important in illustrating the differential effects immune cell populations can have on the airways, it is important to note that there are other cell types in the airways that can also promote AHR. Mast cells which are an important source of inflammatory mediators such as histamine, prostaglandins, and leukotrienes are found in increasing numbers in asthmatic airways and their presence within the ASM correlates with AHR\textsuperscript{59}. 
Prostanoids and Asthma

Mediators derived from the arachidonic acid pathway are generated through the lipoxygenase and cyclooxygenase (COX) pathways, resulting in two major classes of lipid mediators produced in asthma – the leukotrienes and the prostaglandins. In the cyclooxygenase pathway, however, the situation is more complex because of the production of a wider array of lipid mediators synthesized by the inducible cyclooxygenase-2 (COX-2) enzyme during inflammation. The properties of these COX-2-derived mediators are diverse, and they have both anti-inflammatory or pro-inflammatory properties and bronchoconstrictor or bronchodilator properties. Attention has been directed towards individual products of the cyclooxygenase pathways, particularly PGE2. Important sources of PGE2 in the asthmatic lung include mast cells, dendritic cells, epithelial cells, and airway smooth muscle (ASM) cells. PGE2 has both anti and pro-inflammatory effects which can be divided into two groups, i) effects on airway smooth muscle and ii) effects on epithelium. In ASM cells PGE2 can have a brochodilatory effect if inhaled. However, PGE2 also has bronchoconstrictor properties as well. PGE2 has the ability to activate four separate E-Prostanoid (EP) receptor subtypes, which complicates the interpretations of PGE2’s effects. Kunikata et al recently showed in an allergic model of inflammation that PGE2 itself is not active in suppressing inflammation. Another study examined, in vitro, the crosstalk between different EP receptors PGE2 activates and the β2-adrenergic receptor (ADRB2) that are both expressed on ASM cells. McGraw and colleagues tagged each receptor with a fluorescent marker and performed fluorescence resonance energy transfer (FRET), a technique used in live tissues to help determine whether two proteins are close enough to interact. This study found that when specific EP receptor subtypes were activated...
(in this case EP1) these receptors would uncouple the ADRB2 from its G-protein rendering it insensitive to agonists. This finding was significant because PGE2 has a short half life meaning cells in the airways must secrete high concentrations of PGE2 to produce an effect. It has been reported that in asthmatic airways PGE2 levels do significantly increase. This data suggests that while PGE2 may have anti-inflammatory effects, the pro-inflammatory effects may be significantly more potent.

A recent study has shown that PGE2 has a pro-inflammatory role in promoting TH2 responses in allergic airways diseases such as asthma. This study performed in vitro showed that PGE2 has the ability to almost completely inhibit the Stat pathways which are known to promote TH1 response when stimulated. It has been well established that asthma is a TH2 mediated disease and this study suggests PGE2 may function to repress any TH1 responses that would hinder a TH2 response.

Murine Models of Atopic Asthma

The usefulness of animal model for studying human asthma has been heavily debated. Investigators who believe mouse models are useful in studying human asthma have shown there are important similarities between experimental mouse allergic airway disease and human asthma. These similarities include, TH2 cytokine production, memory T-cell responses, globlet cell hyperplasia, IgE production, and development of increased airway resistance (AHR).

The allergens used in murine models of asthma vary, the four that are most commonly used in studies are OVA-albumin, house dust mite extract, cockroach allergen, and ragweed extract. OVA-albumin is the allergen most commonly used and is an extract isolated from chicken egg whites. The other three are isolated from allergens commonly found in the environment. In the case of house dust mites and
cockroaches, these are insects living in homes and the population is exposed to them on a daily basis. Ragweed is pollen that is released from grass in the spring while blooming and is part of the reproductive cycle. When these allergens are given to mice that have been primed against the particular allergen they exhibit similar reactions creating a murine model of human asthma.

Murine models of asthma are usually classified into one of two groups, acute and chronic. The difference between the two models is the number of time exposed to allergen and also the concentration of the allergen. Murine models involving exposure to allergens in short-term and high-levels through either injection of inhalation are considered acute. Whereas animals subjected to low concentrations of antigen through aerosolization for extended periods of time up to 6-8 weeks are considered chronic. Both models have their respective uses, with acute models used to induce acute inflammation in lung tissue particularly in the peribronchial and perivascular compartments of the lung, which is much different from a chronic reaction. Contrast this with chronic models, which exhibit remodeling within the lung including smooth muscle hyperplasia and subepithelial fibrosis. Human asthma and some mouse models are suppressed to some degree by leukotriene antagonists, mast cell depletion, IgE antagonists, and corticosteroids.

Beyond the similarities mentioned above, mouse models provide several potential advantages for studying allergic airways disease. These include using mice as a first model for testing experimental agents for efficacy and in vivo toxicity. The great expense of human trials relative to animal studies makes it advantageous to screen potential agents in mice before testing them in humans. Mice are plentiful, relatively inexpensive, and easy to breed and house. There are fewer regulatory concerns for in vivo studies of mice than for in vivo studies with most other vertebrate species. A recent advancement in research on asthma has been the introduction of mice that are
transgenic or overexpressing genes implicated in human asthma are now available and producible.

Another advantage has been the development and availability of inbred mice that exhibit defined immunologic and airway physiologic properties, including differences in susceptibility to allergic airway disease. These features of allergic airway disease susceptibility are strain dependent. Different mouse strains exhibit varying levels of susceptibility to allergens that are commonly used to induce an asthmatic model. Shinagawa and co-workers recently showed there are significant histological differences to allergens. The mouse strains Shinagawa used were A/J, Balb/cj, C57BL/6 and C3H/HeJ. This study showed that A/J mice revealed features typical of airway remodeling, i.e., airway wall thickening and increased collagen depositions were observed after 12 weeks antigen exposure. Persistent airway hyperresponsiveness (AHR) was observed in chronically antigen-exposed A/J mice. Eosinophilic inflammation, collagen deposition, airway wall thickening, and AHR were all less marked in BALB/c mice than in A/J mice. In C57BL/6 and C3H/HeJ mice, eosinophilic inflammation, airway wall thickening, and AHR were not observed at all, although slightly increased collagen deposition was observed. This difference in susceptibility based on mouse strain illustrates the importance of choosing the correct mouse strain for murine models of human asthma.

Finally, there exist some important differences between human asthma and mouse models of allergic airway disease. The differences that most separate murine models of asthma from human asthma are: i) asthma does not spontaneously develop in mice; ii) unlike human airways, most mouse airways beyond first-generation bronchi lack smooth muscle bundles; iii) eosinophils are distributed differently in mice vs human subjects and iv) not all effects of TH2 cytokines are similar in mice and human subjects. However, a mouse model that is optimized for observing an asthma
phenotype can produce useful information. This information can help researchers to screen agents for potential pitfalls and problems. While mouse models of allergic airway disease are far from exact, their similarities and cost-effectiveness make them a viable alternative to human studies.

Current Pharmacologic Treatments

Current treatments of asthma have focused on treatment of the inflammatory and airway reactivity aspects of asthma. Steroids such as, fluticasone, beclomethasone, and budesonide to name a few; are prescribed as an inhaler to help control inflammation within the lung while short acting β-agonists (SABAs) and long acting β-agonists (LABAs) are prescribed to treat airway narrowing. These steroids work by binding the glucocorticoid receptor in the lung. Once bound the activated receptors can directly or indirectly influence gene transcription. These receptors prevent the activation of the inflammatory transcription factors NF-κB and AP-1 by either binding them directly or through histone modification limiting access to the DNA. The β-agonists work by binding the β2-adrenergic receptor (β2 – AR). The β2 – AR is the main receptor found in the airways responsible for airway relaxation and is expressed on both airway epithelium and airway smooth muscle (ASM). After β2 – AR is bound, it activates adenylate cyclase to produce cyclic adenylate monophosphate (cAMP) to promote relaxation.

Other therapies that have been suggested for use in treating asthma have been deep inspirations and anti-leukotriene therapy. Leukotrienes are products of the arachidonic pathway shown to be significantly increased in asthmatics. They have been implicated in promoting bronchoconstriction, vascular leak, immune cell recruitment, and mucus production. Leukotrienes have been shown to be synthesized by different cells including eosinophils, neutrophils, basophils, lymphocytes,
macrophages, and mast cells. Specifically, mast cells, macrophages, and eosinophils are capable of forming leukotrienes directly from arachidonic acid. Anti-leukotriene therapies are currently based on preventing the binding of the leukotriene receptors (Zyflo) responsible for promoting inflammation and also to block leukotriene synthesis (Singulair). Studies have hypothesized that the majority of airway narrowing is through increased airway smooth muscle (ASM) contraction. From this data it has been hypothesized that if an asthmatic individual performs deep breathing exercises it would help to disassociate the ASM to prevent airway closure.

Steroids, SABAs, and LABAs have been extremely effective at treating asthma with little or no short-term side effects. However, after prolonged periods of use, there can be side effects that detrimental to an asthmatic’s health and are unrelated to their asthma symptoms. For example prolonged use of steroids can lead to skeletal muscle wasting, osteoporosis, and glaucoma. Prolonged use of β-agonists has been shown to induce cardiac arrhythmias, reflex tachycardia, and tremors. These side effects from prolonged use prove to be as bad at the disease they are treating. In light of this an alternative or adjunct therapy is needed to help lessen their dependence on their pharmacological treatments.

**EXERCISE**

**Exercise Benefits**

Exercise has long been known to be beneficial for humans and other animals. In the case of humans, exercise has been shown to reduce the risk factors for many debilitating and fatal diseases such as diabetes, heart disease, strokes, and even improves immune function. The effects are mainly determined by the duration, intensity, and frequency of the exercise. In Figure 7 below is the J-Curve which illustrates the relationship between a person’s exercise lifestyle and the incidence of
upper respiratory infections (URI) and the percent morbidity and mortality\textsuperscript{80}. From the graphs it is apparent that if an individual lives a moderate exercise lifestyle they have the lowest incidence of URIs and the lowest percent morbidity and mortality. A moderate exercise lifestyle would involve an individual exercising 3 to 4 times a week for 30-45 minutes per bout at a target heart rate of 65-70\% of their maximum heart rate\textsuperscript{81}. The exhaustive exercise lifestyle is shown to be detrimental in graph in Figure 7A; this represents your marathon runner. When exercising for long periods of time there can actually be a negative effect on the immune system causing the individual to become immunosuppressed. Therefore moderate exercise has been adopted by many hospitals and societies such as the American Thoracic Society (ATS) and the American College for Sports Medicine (ACSM) for use in patients with asthma and other COPD diseases to perform pulmonary rehabilitation and improve the overall quality of life for these patients. ATS and ACSM went so far as to recommend aerobic exercise for asthmatics in 1999 and 2000 respectively.

![Figure 7. Beneficial Effects of Moderate Exercise Lifestyle. Curves showing the relationship between the intensity of exercise lifestyle with the percent incidence of A) Upper Respiratory Infections (URI); and B) percent Morbidity and Mortality of people. Adapted from “Exercise, infection, and immunity” by D.C. Nieman, 1994, International Journal of Sports Medicine, 15, p. S131. Copyright 1994 by International Journal of Sports Medicine. Adapted with permission\textsuperscript{80}.](image-url)
Exercise, the Immune System, and Lung Function

Exercise has been shown in several studies to have beneficial effects in both healthy and asthmatic individuals. These studies use exercise type, intensity, duration, and frequency to achieve these beneficial results. Exercise causes the redistribution of lymphocyte populations from the periphery circulation into the vascular compartments while the overall proportion of memory to naïve T-cell lymphocytes in the periphery increased. These results suggest the memory lymphocytes are redistributed to the periphery circulation in response to exercise. Another study conducted by Ceddia and Woods showed exercise enhanced macrophage phagocytosis, antitumor activity, and chemotaxis yet showed reduced macrophage major histocompatibility complex II (MHC II) expression and antigen-presentation ability. Acute exercise has also been shown to have an effect on the β2-adrenergic receptor (β2 – AR) and its different signaling and regulatory elements. In this study rats were acutely exercised and the protein expression levels of β2 – AR increased. The protein expression levels of GRK-2 and β-arrestin, which are responsible for β2 – AR regulation and internalization, declined after exercise. The molecule, ubiquitine, which targets a peptide for degradation was also reduced in rats that underwent acute exercise.

EXERCISE AND ASTHMA

Link between Exercise and Asthma

Several clinical studies have shown that aerobic exercise training as part of a pulmonary rehabilitation program improves the overall physical fitness and health of
respiratory disease patients. These studies followed the guidelines of ACCP/AACVPR for pulmonary rehabilitation programs that recommend the implementation of low to moderate intensity aerobic exercise for respiratory disease patients. Most of these studies to date have focused on COPD patients as opposed to asthmatic patients.

Emtner and colleagues performed a clinical study with mild atopic asthmatics where the patients study exhibited increased lung function and decreased exercised induced bronchospasm (EIB) and asthma-related symptoms\textsuperscript{81}. Long term follow-up of the patients involved in the study indicated that those who maintained exercise regiment decreased number of ER visits\textsuperscript{81}. Henriksen and colleagues along with Basaran et al have both performed similar studies on asthmatics which focus on children that have the disease\textsuperscript{85,86}. Their main results mirrored Emtner’s results with improved cardiovascular function, less pharmacological dependence, and an improved quality of life\textsuperscript{85,86}.

In our previous work, this lab has shown that exercise can also cause decreased inflammation in a murine mouse model of atopic asthma\textsuperscript{79}. Specifically, repeated bouts of moderate intensity aerobic exercise lessened the following pro-inflammatory parameters in mice that were sensitized and challenged with OVA: i) mucus production and epithelial cell hypertrophy/hyperplasia in lung tissue; ii) cellular infiltrate and total protein concentration in the airway lumen; iii) secretion of the pro-inflammatory mediators KC, IL-4, and IL-5 into the airway lumen; iv) expression of the adhesion molecule VCAM-1 in intact lung tissue; and v) production of OVA-specific IgE in serum. Data also demonstrated that exercise decreased NF-\(\kappa B\) nuclear translocation and \(\text{I} \kappa \text{B} \alpha\) phosphorylation in the lungs of OVA-sensitized mice as compared with OVA sensitized sedentary controls.

Vieira and colleagues performed a study similar to our previous work which helped to validate our model and previous data. Although there were some significant differences related to the sensitization and exercise protocols they showed similar
Inflammatory mediators, IL-4 and IL-5 were both shown to be significantly decreased when compared to sedentary mice that were sensitized with OVA. Vieira et al also presented data indicating that exercise decreased not only immune infiltrate found in the bronchial lavage fluid but also immune cell within the lung tissue. With regards to airway remodeling, in Vieira’s murine model of asthma, they observed a significant decrease in collagen and elastic fibers in the exercised mice. This was accompanied by decreased thickness of the airway smooth muscle (ASM) in mice that were subjected to repeated exercise bouts. In this study IL - 10 release was shown to significantly increase with exercise; from that data the authors concluded that this increased IL - 10 release was in response to exercise and represents the mechanism by which exercise exerts its effects.

Exercise Mediated Attenuation Mechanisms

Exercise stimulates the hypothalamic–pituitary–adrenal (HPA) axis to upregulate its release of neuroendocrine-related factors, including the endogenous glucocorticoid (GC) corticosterone (cortisol in humans), into the circulation. Likewise, exercise induces the release of catecholamines (Cchs), including epinephrine from the adrenal medulla. Each of these neuroendocrine factors can modulate immune-related events, such as cytokine production, surface molecule expression, and lymphocyte proliferation. Other neuroendocrine factors, such as growth hormone and prolactin, as well as neuropeptides, including substance P and neuropeptide Y, are also released during exercise and may play a role in exercise-induced changes in immune responses.

GCs have been shown to be potently anti-inflammatory and, therefore, have been a main-stay of asthma therapy for over 30 years. GCs have been shown to inhibit
the synthesis of a wide variety of inflammatory mediators, including cytokines (IL-1β, TNFα, IL-6, GM-CSF), chemokines (IL-8, MCP-1, RANTES), adhesion molecules (ICAM-1, VCAM-1), and inflammatory enzymes (COX-2, iNOS)⁸⁹. However, after prolonged periods of use exogenous steroids can trigger deleterious side effects such as skeletal muscle wasting, glaucoma and osteoporosis, which diminish their overall effectiveness⁸⁸. GCs exert their anti-inflammatory effects via binding to intracellular glucocorticoid receptors; like β₂-adrenergic receptors, glucocorticoid receptors are widely expressed in cells and tissues relevant to the pathogenesis of asthma. GC receptors are considered ligand-activated transcription factors that act, either directly or indirectly, to regulate gene transcription. Activated glucocorticoid receptors can either bind directly to DNA (transcriptional trans-activation) or to other transcription factors (transcriptional trans-repression) in order to modulate gene transcription¹². In particular, GC receptors can bind the pro-inflammatory transcription factors AP-1 and NF-κB (p65 subunit) physically to inhibit their transcriptional activation of inflammatory genes¹². Importantly, activated GC receptors can also inhibit NF-κB-mediated gene expression through the induced gene expression of the NF-κB inhibitor, IκB⁹¹. Emerging data now suggest that GC may also modulate gene expression via regulation of chromatin structure and initiation complex accessibility to genes¹².

Pastva and colleagues have reported on the roles of GCs in a murine model of atopic asthma. Mice were sensitized to induce atopy, a subset were implanted with a pellet made of the GC receptor antagonist, RU486 and exercised⁸⁸. Exercise significantly reduced all inflammatory parameters in mice that were sham implanted⁸⁸. However, mice implanted with the GC antagonist exhibited an inflammatory phenotype that was significantly different from the exercised mice implanted with the sham pellet. The GC antagonist had completely reversed or ablated the beneficial effects of
These data support that GCs are indeed potently anti-inflammatory asthmatic phenotype.

Pastva and co-workers also examined the mechanism by which GCs attenuated the inflammatory response by observing the level of NF-κB activation from nuclear extracts of lung homogenate. These were probed for the p65 subunit of NF-κB using an electrophoretic mobility shift assay (EMSA). In exercised mice there was no detectable p65 from the EMSA as compared with sedentary controls, which showed a strong p65 band from the nuclear extract. When exercised mice implanted with the GC antagonist were probed for the p65 subunit, they also exhibited a significant increase in p65 found in the nuclear extract. These data strongly suggest GCs prevented the translocation of NF-κB subunits to the nucleus. Figure 3 shows a representation of how GCs may inhibit the transcription of these inflammatory genes. Low doses of GCs work best because the HPA axis has the ability to become tolerized to GCs which would require the adrenal glands to release increased levels of GCs to obtain the same effect. This is a possible explanation of why some asthmatics become steroid insensitive after periods of repeated use.
Figure 8. Glucocorticoid Mediated Attenuation of Inflammatory Gene Transcription. Representation of a possible mechanism that Glucocorticoids (GCs) may inhibit the transcription factor function of NF-κB thereby attenuating the asthmatic phenotype. Adapted from “Corticosteroid effects on cell signaling” by P.J. Barnes, 2006, The European Respiratory Journal, 27, p. 413. Copyright 2006 by ERS Journals LTD. Adapted with permission.

There is a large body of evidence indicating that, in addition to being crucial neurotransmitters and hormones, catecholamines (Cchs) are important immunomodulators during health and disease. It has been shown that Cchs, once released from the adrenal glands, can bind and activate the β2 – adrenergic receptor (β2 – AR). Early studies that addressed immune cell activity regulation by Cchs and β2 – AR stimulation used unfractionated populations of CD4+ T – cells. β2 – AR stimulation on an unfractionated population of CD4+ T cells inhibited T cell proliferation by decreasing IL-2 expression and secretion as well as IL-2 receptor (IL-2R) expression via a cAMP-
dependent mechanism\textsuperscript{93}. In human T cells, $\beta_2$ – AR stimulation inhibited nuclear factor (NF)-kB activation via a mechanism that involved the stabilization of its inhibitor protein, I\textkB$\alpha$\textsuperscript{93}. Activation of the inflammatory response by pathogens leads to the rapid release of innate cytokines (IL-1 and TNF) that, in turn, stimulate the sympathetic nervous system to release Cch\textasciitilde{}s from sympathetic nerve endings in immune organs\textsuperscript{95}. This inhibits natural killer (NK) cells and macrophage pro-inflammatory cytokine production, providing negative-feedback inhibition to dampen the inflammatory response and restore homeostasis\textsuperscript{95}.

**Dissertation Objectives**

Rates of asthma in children and adults have continued to rise at alarming rates in developed countries such as the United States. These individuals have classically been treated with bronchodilators such as steroids and $\beta$ – agonists. Steroids are the most effective way to treat the inflammatory aspect of asthma while $\beta$ – agonists are used to open airways. With prolonged usage of these pharmacologic treatments come many adverse side effects. Numerous studies in the last 30 years have shown the beneficial effects of exercise in mice with mild to moderate atopic asthma. Although the clinical symptoms of asthma have been well characterized, the underlying molecular and immunological mechanisms of the asthmatic response remain ill-defined. Addressing these mechanisms, recent studies have shown exercise in atopic asthmatic models had a profound effect on the immune system. The overarching hypothesis of these studies is that differing amounts of moderate intensity aerobic exercise exert dissimilar effects on AHR in an OVA-driven mouse model of atopic asthma.
Two specific aims were developed to test this hypothesis. The first aim was to determine the effect of multiple bouts of moderate intensity aerobic exercise on AHR in an OVA-driven mouse model of atopic asthma. The hypothesis of this aim was that multiple bouts of moderate intensity aerobic exercise would attenuate AHR in OVA-treated mice via a mechanism that involves increased $\beta_2$-adrenergic receptor responses and decreased inflammation. For these studies, female Balb/cj mice were used to engineer an allergic asthmatic model by subjecting them to a sensitization protocol using the chronic allergen ova albumin (OVA). This chicken egg protein is commonly used to induce an allergic asthmatic model. Following the initial sensitization, mice were exercised at a moderate intensity via repetitive running on a motorized
treadmill. During the exercise protocol, mice were challenged with OVA via aerosolization (Figure 10).

**A. OVA-Sensitization**

OVA-sensitization  
Dy0  Dy14  
i.p. w/50ug OVA  
i.p. w/50ug OVA

**B. OVA-Challenge & Exercise**

OVA-challenge / exercise  
Dy21-25  Wks 4 - 7  
neb w/5mg/ml OVA for 30m/dy for 5X/wk  
neb w/5mg/ml OVA for 10m/dy for 3X/wk

**C. Analysis**

Analysis  
24 – 48h post  
sacrifice  
exer 3X/wk at 13.5m/min for 45m

Figure 10. Experimental Design for Aim #1.

Upon completion of the sensitization/exercise protocol, the mice were sacrificed. 24 hours post the last exercise bout, the lungs were lavaged, excised, and examined for changes in leukocyte infiltration, airway smooth muscle (ASM) thickness, β2 – adrenergic receptor expression, β2 – AR signaling molecules, and PGE2 production. Pulmonary function was measured 48 hours post the last exercise bout using a FlexiVent system and the resistance (R) was measured. These results were prepared and submitted to Nature Medicine for publication and are presented in Chapter 2.

The second aim was to determine the effect of a single bout of moderate intensity aerobic exercise on AHR in an OVA-driven mouse model of atopic asthma. The hypothesis of this aim was that a single bout of moderate intensity aerobic exercise
attenuates airway inflammation but no AHR in OVA-treated mice. For these studies, female Balb/cj mice were used to engineer an allergic asthmatic model by subjecting them to a sensitization protocol using the chronic allergen ova albumin (OVA). Following the initial sensitization as shown in the previous figure, mice were exercised for a single exercise bout at a moderate intensity on a motorized treadmill. During the exercise protocol, mice were challenged with OVA via aerosolization (Figure 11).

Figure 11. Experimental design for Aim #2.

Upon completion of the sensitization/exercise protocol, the mice were sacrificed. 24 hours post the last exercise bout, the lungs were lavaged, excised, and examined for leukocyte infiltration and NF - κB activation. The inflammatory mediators KC, RANTES, MCP-1, IL-12 p40/80, IL-5, IL-13, and PGE₂ were measured. Airway resistance was also measured in these mice using a FlexiVent system and pictures were taken of the lung morphology after a single bout of moderate aerobic exercise. These results were prepared and accepted to the American Journal of Respiratory Cellular and Molecular Biology (AJRCMB) for publication and are presented in Chapter 3.

Data presented in Chapters 2 and 3 will be further critiqued in the discussion and conclusion section (Chapter 4).
MODERATE INTENSITY AEROBIC EXERCISE REDUCES AIRWAY HYPERRESPONSIVENESS IN A MURINE MODEL OF ASTHMA

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Format adapted for thesis
ABSTRACT

We have reported previously that repeated bouts of aerobic exercise at a moderate intensity attenuate asthma-related airway inflammation in mice sensitized and challenged with ovalbumin (OVA). The current study determined the effects of moderate intensity aerobic exercise training on asthma-related airway hyperresponsiveness (AHR) in these mice. Mice were sensitized/challenged with OVA or saline and exercised at a moderate intensity 3X/wk for 4 wks. 48h post protocol completion, mice were analyzed for changes in AHR via methacholine challenge and subsequent mechanical ventilation. Results show that repeated bouts of exercise decrease total lung resistance approximately 60% in OVA-treated mice as compared with controls. Because β₂-adrenergic receptors (AR) play a role in bronchodilatation, the role of β₂-AR in exercise-mediated improvements in AHR was examined. Application of the β₂-AR antagonist butoxamine HCl blocked the effects of exercise on total lung resistance in OVA-treated mice completely. In parallel, airway smooth muscle (ASM) cells were harvested from OVA-treated and exercised mice and examined for changes in the protein expression of β₂-AR and G-protein receptor kinase-2 (GRK-2); GRK-2 promotes β₂-AR desensitization. Surprisingly, exercise had no affect β₂-AR expression in ASM cells of OVA-treated mice; however, exercise decreased GRK-2 expression by 50% in these cells as compared with controls. Repeated bouts of exercise also decreased ASM thickness and PGE₂ production significantly in OVA-treated mice as compared with controls; both of these parameters have been implicated in AHR. Together, these data indicate that moderate intensity aerobic exercise attenuates AHR via a mechanism that involves β₂-AR.
INTRODUCTION

Allergic or atopic asthma, the most common form of asthma, is identified by the presence of characteristic clinical symptoms of wheezing, chest tightness, dyspnea and cough, and by the presence of reversible airway narrowing and/or airway hyperresponsiveness (AHR) to a variety of inhaled bronchoconstrictor stimuli. For the treatment of asthma-associated AHR, patients are typically administered inhaled, long acting β₂-agonists. β₂-agonists bind to and activate β₂-adrenergic receptors (β₂-AR), which are expressed on a variety of cell types, including airway smooth muscle (ASM) cells, airway epithelial cells and mast cells.

Upon binding β₂-agonist, the β₂-AR couples with the stimulatory G protein (Gs) to stimulate adenylate cyclase, cAMP production and subsequent protein kinase A (PKA) activation. Activated PKA stimulates myosin light chain kinase and myosin light chain phosphatase; these events lead to decreased myosin light chain activity and smooth muscle tone. Desensitization occurs upon G-protein receptor kinase-2 (GRK-2)-mediated phosphorylation of β₂-AR, which uncouples the receptor from the Gs resulting in reduced cAMP levels and PKA inactivation. GRK-2-mediated phosphorylation of β₂-AR also facilitates the binding of the receptor to β-arrestin that promotes receptor endocytosis. The prostaglandin PGE₂ may also regulate β₂-AR densitization via activation of the E prostanoid receptor-1, which is expressed on ASM cells. Activation of the EP1 receptor uncouples the β₂-AR from its G-protein complex resulting in its desensitization.

Several clinical studies suggest that continued aerobic exercise training improves the overall physical fitness and health of asthmatics and reduces their disease-related hospital admissions; these studies also observed improvements in exercise-induced
bronchoconstriction following physical training. We have reported previously that moderate intensity aerobic exercise training reduces lung inflammatory responses in an ovalbumin (OVA) - driven mouse model of pulmonary inflammation \(^7,8\). To extend this work, we hypothesized that moderate intensity aerobic exercise would also attenuate asthma-associated AHR. To test this hypothesis, mice were sensitized and challenged with OVA or control saline and exercised on a motorized treadmill at a moderate intensity as reported previously \(^7,8\). At the conclusion of the protocol, changes in AHR were monitored upon exposure to increasing doses of methacholine, a bronchoconstrictor stimulus. In parallel, changes in \(\beta_2\)-AR expression, G-protein receptor kinase-2 (GRK-2) expression, ASM thickness, and PGE\(_2\) content were measured. Collectively, results presented herein indicate that moderate intensity aerobic exercise attenuates AHR via a mechanism that involves \(\beta_2\)-AR. The implications of these findings for the treatment of mild to moderate asthma are discussed.
MATERIALS AND METHODS

Animals

Female BALB/cJ mice (3-5 wk old; The Jackson Laboratory, Bar Harbor, ME), a strain susceptible to OVA-induced IgE responses\(^9,10\) were utilized. Mice were housed in a pathogen-free containment facility and maintained in autoclaved Microisolator cages (Lab Products, Maywood, NJ). Mice were provided with food (Teklad, Madison, WI) and water ad libitum. Mice were allowed to acclimate to housing condition for 1 wk before experimental manipulation. All animal treatments were approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health recommended guidelines.

OVA-Sensitization and Exercise Protocol

OVA-sensitization and moderate-intensity aerobic exercise training were performed as described previously\(^7,8\). We have shown previously that the utilization of this OVA sensitization scheme in BALB/cJ mice initiates an airway inflammatory response and immunoglobulin profile that is consistent with those observed in asthma\(^7\). Exercised mice underwent bouts of moderately-intense aerobic exercise on a motorized treadmill (Exer 6M; Columbus Instruments, Columbus, OH). Exercise bouts were performed three times per wk for a total of 10 bouts; mice exercised for 45 min at 13.5 m/min (0% grade). As noted in our earlier study, previous reports have defined moderate intensity aerobic exercise as brief (15 – 60 min) bouts of treadmill running at 50 - 75% maximum \(O_2\) consumption or \(\sim15-22\) m/min\(^7,11\). All exercise bouts included brief warm-up and cool-down periods so that the total treadmill time was \(\sim60\) min.
Mechanical Ventilation

48 hours post the last sensitization/exercise bout, mice were mechanically ventilated and challenged with increasing concentrations of methacholine as described previously \(^{12}\). Briefly, mice were anesthetized with diazepam (17.5 mg/kg) and ketamine (450 mg/kg) and a tracheotomy tube (18G) was inserted and connected to the inspiratory and expiratory ports of a ventilator (Flexivent, SCIREQ, Montreal, Quebec, Canada). Mice were ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2 ml with a positive end-expiratory pressure of 2–4 cm H\(_2\)O. Increasing concentrations of methacholine (0-50 mg/mL) were administered via aerosolization. From 20 s up to 3 min after each aerosol challenge, resistance (R) was recorded continuously; an average value of R was taken in order to express changes in murine airway function. For studies involving the \(\beta_2\)-AR antagonist butoxamine HCL (Sigma), mice received a nebulization of either butoxamine HCL (200mg/ml) or saline prior to each methacholine challenge as just described; R was recorded continuously.

ASM Isolation and Western Blot Analysis

Tracheas were removed and the trachealis muscle was isolated, centrifuged, and resuspended in a digestion buffer containing collagenase, soybean trypsin inhibitor, and elastase as described previously \(^{13}\). The resulting cell suspension was filtered to remove debris and resuspended in Ham’s F-12 medium (supplemented with 10% FBS). Isolated \textit{ex vivo} ASM cells were immediately lysed and prepared for Western blot analysis as described previously \(^{14}\). Equivalent amounts of protein lysates (25 \(\mu\)g/lane) were electrophoresed and transferred to PVDF membrane blots. Blots were blocked with 5% BSA-containing buffer and then stained with either a rabbit anti-mouse anti-\(\beta_2\)-AR
antibody (Abcam) or anti-GRK-2 antibody (Santa Cruz) followed by a goat-anti-rabbit IgG antibody conjugated to horseradish-peroxidase (HRP). Immunoblots were developed using chemiluminescence and imaged using an Chemidoc XRS Imaging Station (BioRAD).

To verify ASM phenotype and to account for differences in lane loading, blots were stripped and re-probed with HRP-conjugated antibodies directed against the ASM marker \( \alpha \)-smooth muscle actin (Santa Cruz). Re-probed blots were developed via chemiluminescence and analyzed as just described. Results were quantitated via densitometry.

Lung Fixation and ASM Thickness Measurements

Lungs were extracted and prepared for analysis as described previously \(^7\)\(^8\). Briefly, mice were injected with a lethal dose of ketamine/xylazine. The mice were treacheaotomized using an 18G catheter and the lungs were filled with 10% paraformyldehyde. Once the lungs were filled the trachea was tied off to prevent leakage of the paraformaldehyde using suture silk and lungs were removed from the body cavity. All excess tissue was clipped off and the lungs were suspended in a tube of 10% paraformaldehyde, embedded in paraffin, and then was sliced into 10 \( \mu \)m sections for staining. Paraffin tissue sections were rehydrated as described \(^7\)\(^8\). Rehydrated tissue was stained with a biotinylated primary antibody against \( \alpha \)-smooth muscle actin (Dako) and subsequently probed with steptavidin – peroxidase. Staining was completed with incubation with 3,3′ – diaminobenzadine (DAB) + substrate resulting in a brown – colored precipitate at the antigen site. Slides were then visualized under a light field
microscope. Airways with a longitudinal diameter between 150-200 μm were identified and ASM thickness was measured using the imaging program Metamorph.

Lavage Cell and Protein Analysis

Mice were lavaged as described previously. Briefly, mice were lavaged with 1.5 mL of 0.9% saline flushed in and out of the lungs 3 times. Cell viability was determined via trypan blue exclusion and cell types were differentiated on cytospin preps using Diff-Quik stain. Cell differentials were determined from at least 500 leukocytes using standard hematological criteria. BALF supernatant samples were analyzed for differences in PGE₂ content via ELISA (R&D Systems).

Statistical Analysis

Data were analyzed using SPSS Version 11.0. Results are reported as group means ±SD. A One-way ANOVA determined differences among the group means and the Bonferroni post-hoc analysis determined which group means differed significantly (at a level of p≤0.05).
RESULTS

Aerobic Exercise at a Moderate Intensity Decreases Airway Resistance in OVA-Treated Mice

To determine the effect of a moderate intensity aerobic exercise training on AHR in OVA-treated mice, mice were mechanically ventilated and challenged with increasing concentrations of methacholine (0-50 mg/mL); changes in resistance (R) were recorded continuously. Results shown in Figure 1 demonstrate that, at the highest methacholine dose, OVA-treated, sedentary mice exhibited a total lung resistance that was approximately 4-fold greater than saline-treated (S, E) controls. In contrast, mice that were OVA-treated and exercised displayed a 65% reduction in total lung resistance as compared with OVA-treated, sedentary mice; this level of resistance was equivalent to that observed in control mice (S, E). Significant decreases in overall lung resistance were also observed in OVA-treated and exercised mice at intermediate methacholine doses (20mg/ml, 40mg/ml) as compared with sedentary controls (Fig. 1).

Exercise-Mediated Improvement of AHR in OVA-Treated Mice is Dependent Upon β₂-AR

Because β₂-AR has been implicated in bronchodilatation, its role in exercise-mediated attenuation of AHR in OVA-treated mice was examined. Mice were ventilated and challenged with methacholine at increasing concentrations as described above. Prior to each methacholine challenge, however, mice received a nebulization of either saline or the specific β₂-AR antagonist butoxamine HCL. At the highest methacholine dose (50 mg/ml), treatment with the β₂-AR antagonist increased total lung resistance significantly in mice that were OVA-treated and exercised as compared with controls (Fig. 2A); significant increases were also observed at methacholine concentrations...
30mg/ml and 40mg/ml. In OVA-treated (Fig. 2A) and saline-treated (Fig. 2B) sedentary mice, however, treatment with the β₂-AR antagonist had no significant effect on total lung resistance. Interestingly, saline-treated and exercised mice that were nebulized with the β₂-AR antagonist exhibited increased total lung resistance as compared with controls (Fig. 2B).

Moderate Intensity Aerobic Exercise Alters GRK-2, but not β₂-AR, Protein Expression in the Lungs of OVA-Treated Mice

β₂-AR are expressed on a variety of cell types, including ASM cells, and have been shown to facilitate bronchodilatation ¹. To determine the effect of moderate intensity aerobic exercise training on β₂-AR protein expression, ASM cells were harvested and prepared for Western blot analysis. Data presented in Figure 3A suggests that moderate intensity aerobic exercise training does not alter β₂-AR protein expression on ASM cells. OVA-treatment also did not affect β₂-AR expression in these cells (Fig. 3A).

In parallel with analyses of β₂-AR expression, studies also determined the effect of moderate intensity aerobic exercise training on GRK-2 protein expression. GRK-2 phosphorylates β₂-AR, which promotes desensitization of the receptor and, thereby, reduces its bronchodilatory actions ¹². Results shown in Figure 3B indicate that OVA-treatment of sedentary mice increased GRK-2 expression approximately 4-fold above saline-treated controls. In contrast, mice that were OVA-treated and exercised exhibited a 50% reduction in GRK-2 expression as compared with OVA-treated, sedentary mice; this level of GRK-2 expression was equivalent to that observed in saline-treated controls (S, E).
Aerobic Exercise at a Moderate Intensity Decreases ASM Thickness in OVA-Treated Mice

Increases in airway wall thickness have been implicated as an underlying mechanism in the initiation and/or exacerbation of AHR. Because increases in ASM thickness contribute most to the increased airway wall thickness in asthmatics, the effect of moderate intensity aerobic exercise on ASM thickness in OVA-treated mice was determined. To this end, lung tissues were harvested and stained with a biotinylated α-smooth muscle actin antibody and subsequently probed with steptavidin – peroxidase; tissues were then analyzed for changes in ASM thickness via Metamorph analysis. Data presented in Figure 4 demonstrate that OVA-treatment of sedentary mice increased ASM thickness 2-fold as compared with saline-treated controls (Fig. 4A, 4B, 4E). Mice that were OVA-treated and exercised, however, displayed a 35% reduction in ASM thickness as compared with sedentary controls (Fig. 4C, 4D, 4E); this level of thickness was equivalent to that observed in saline-treated control mice (Fig. 4A, 4C, 4E).

Moderate intensity aerobic exercise attenuated PGE2 levels in the lungs of OVA-Treated Mice

Increases in PGE2 levels within the lung have been implicated in the initiation and/or exacerbation of AHR. To determine the effect of moderate intensity aerobic exercise on PGE2 levels within the lungs of OVA-treated mice, mice were bronchioalveolar lavaged and the resulting lavagates were analyzed for changes in PGE2 content. Results presented in Figure 5 demonstrate that OVA-treatment of sedentary mice increased PGE2 levels approximately 250-fold as compared with saline-
treated controls (S, E). In contrast, mice that were OVA-treated and exercised displayed an 80% reduction in PGE$_2$ levels as compared with OVA-treated, sedentary mice (Fig. 5); however, these levels were significantly greater than those observed in saline-treated controls (S, E).
DISCUSSION

In this study, repeated bouts of aerobic exercise at a moderate intensity decreased total lung resistance, GRK-2 protein expression in ASM cells, ASM thickness, and PGE\textsubscript{2} production as compared with sedentary controls. Treatment with a $\beta_2$-AR antagonist blocked the effects of exercise on total lung resistance in OVA-treated mice. Together, these results suggest that repeated bouts of exercise improve AHR in OVA-treated mice via a mechanism that involves $\beta_2$-AR.

We have reported previously that repeated bouts of aerobic exercise at a moderate intensity attenuate airway inflammation and remodeling in OVA-treated mice\textsuperscript{7}; our current findings extend and complement this original study. In a separate study, we demonstrated that a single bout of moderate intensity aerobic exercise decreased airway inflammatory responses, but had no effect on airway remodeling or AHR\textsuperscript{12}. These seemingly contrasting results between our collective studies may be explained, in part, by the differences in the length of the exercise protocols and exposure to OVA. The effects of exercise on physiological responses are dependent upon several variables, including the total duration of the exercise protocol\textsuperscript{18-23}. The mechanism that underlies such a disparity likely involves changes in the levels of circulating hormones (e.g. glucocorticoids, catecholamines) that are released from the hypothalamic – pituitary – adrenal axis (HPA) upon exercise. It is possible that repeated exercise bouts permit a sustained increase in endogenous glucocorticoids (GCs) that, in turn, attenuate airway inflammatory responses, remodeling, and AHR. We have reported previously that repeated bouts of exercise at a moderate intensity increase serum levels of corticosterone in OVA-treated mice as compared with sedentary controls\textsuperscript{8}. GCs are potent anti-inflammatory agents and have been shown to decrease airway remodeling
and AHR in both animal models and asthmatic patients\textsuperscript{24,25}. In addition, increased exposure to OVA treatments during repeated exercise bouts may induce a tolerizing effect that dampens the overall asthmatic response and renders it more susceptible to the attenuating effects of HPA-derived circulating hormones. To this end, several studies have demonstrated that prolonged allergen exposure results in decreased inflammatory responses\textsuperscript{26-30}.

Data presented herein demonstrate that, in both saline- and OVA-treated mice, exposure to the specific $\beta_2$-AR antagonist butoxamine HCl blocked the effects of exercise on total lung resistance in response to methacholine-challenge; this observation argues for a role of $\beta_2$-AR in exercise-mediated attenuation of AHR. Upon activation, $\beta_2$-AR facilitates bronchodilatation through coupling to the Gs-protein complex and subsequent decreases in myosin light chain activity and smooth muscle tone\textsuperscript{1}. Associated with $\beta_2$-AR activation is the autoregulatory process of receptor desensitization that involves phosphorylation of $\beta_2$-AR via GRK-2 and its subsequent uncoupling from the Gs-protein complex. GRK-mediated phosphorylation of $\beta_2$-AR also facilitates the binding of $\beta_2$-AR to $\beta$-arrestins, which promote its endocytosis and subsequent desensitization\textsuperscript{2}. Interestingly, repeated bouts of exercise had no effect on $\beta_2$-AR protein expression but did decrease GRK-2 protein expression in ASM. Barnes and co-workers have indicated that decreasing the expression of GRK-2 within the airways may lessen $\beta_2$-AR desensitization\textsuperscript{31}. Together, these results indicate that repeated bouts of moderate intensity aerobic exercise may attenuate AHR by decreasing GRK-2-mediated densitization of $\beta_2$-AR.
Thickening of the airway wall is an indicator of asthma severity. Factors that contribute to increased airway wall thickness in the asthmatic lung include epithelial thickening, reticular basement membrane thickening, increased extracellular matrix, and fibrosis; however, ASM hypertrophy and/or hyperplasia contributes most to overall airway wall thickening. Data presented herein demonstrate that, in OVA-treated mice, repeated bouts of aerobic exercise at a moderate intensity decreased ASM thickness to control levels; however, it is unclear whether exercise lessened ASM swelling and/or proliferation. Within the OVA-treated lung, exercise-mediated decreases in ASM thickness correlated positively with exercise-mediated attenuation of AHR. The relationship between increased airway wall/ASM thickness and AHR has been explored in both clinical and animal model studies. Several studies suggest that increases in total wall thickness are associated with increased AHR. For example, Boulet and co-workers reported a positive correlation between airway wall thickening and hyperresponsiveness to methacholine challenge in asthmatic patients with airway obstruction. The findings presented in the current study support this observation. In contrast, Niimi and co-workers reported that there exists a negative correlation between airway wall thickness and AHR. These authors suggested that increased airway stiffness due to increased airway thickening may prevent ASM shortening and subsequent narrowing of the airway lumen.

Within the asthmatic lung and airways, PGE₂ is considered protective against allergen-induced AHR (reviewed in). In contrast, results presented herein suggest that increased PGE₂ levels in the lungs of sedentary, OVA-treated mice do not protect against AHR. Specifically, results presented in Figure 5 show that exercise-mediated decreases in PGE₂ levels correlate with decreased total lung resistance in response to methacholine challenge. These findings support previous observations that PGE₂ induces AHR via a signaling pathway that is dependent upon Rho/ROCK activation and
Ca$^{2+}$ release$^{34}$. Such conflicting results may be explained by the differential expression and utilization of E prostanoid receptors (EP1 – EP4). EP receptors, which are expressed on multiple cell types such as ASM cells, mediate the effects of PGE$_2$ within the lung and airways. Because multiple EP receptor subtypes may be expressed simultaneously in a single cell, there exists the possibility that an on-going inflammatory response would produce opposing PGE$_2$-mediated actions within the lung and airways. For example, Liggett and co-workers have reported that activation of the EP1 receptor on ASM uncouples the $\beta_2$-AR from its G-protein complex resulting in $\beta_2$-AR-desensitization$^{35}$. Penn and colleagues have also shown that the EP2 receptor regulates cytokine-mediated desensitization of the $\beta_2$-adrenergic receptor ($\beta_2$-AR) on ASM$^{36}$. Activation of the EP1 and EP3 receptors elevate intracellular calcium levels$^{17}$, which has been implicated in AHR$^{34}$. In addition, activation of the EP2 and EP4 receptors increases intracellular cAMP concentrations$^{17}$, which promote bronchodilatation.

Throughout the moderate intensity aerobic exercise protocol, none of the OVA-treated mice exhibited labored or difficult breathing, which could have indicated exercise-induced bronchoconstriction. Interestingly, previous studies have observed improvements in exercise-induced bronchoconstriction following physical training$^{37-39}$. Emtner and colleagues have reported that adults with mild-moderate asthma who underwent a physical training program for 10 weeks exhibited increased ventilatory capacity and decreased exercise-induced bronchospasm$^{40}$. Likewise, Henriksen and co-workers demonstrated a beneficial effect of endurance training on exercise-induced bronchoconstriction and working capacity$^{38}$. Additional studies have reported similar findings with regard to exercise-mediated improvement in ventilatory capacity$^{39,41}$ and
bronchial hyperresponsiveness \(^42\) of asthmatic patients. As such, there may exist an inverse dose response for the efficacy of exercise in the treatment of asthma-related hyperresponsiveness. For example, training at a moderate intensity may provide beneficial effects as the data presented herein suggests; however, strenuous training may exacerbate disease parameters \(^43\).

As noted above, the results of the current study demonstrate that repeated bouts of aerobic exercise at a moderate intensity attenuate asthma-associated AHR via a mechanism that involves \(\beta_2\)-AR. Exercise-mediated decreases in GRK-2 protein expression, ASM thickness, and PGE\(_2\) levels within the OVA-treated lung support this conclusion. Because our previous work shows that exercise lessens inflammatory responses within the lungs of OVA-treated mice via an endogenous GC-dependent mechanism, future studies will determine whether endogenous GCs also drive the effects of exercise on \(\beta_2\)-AR responses and subsequent hyperresponsiveness. This approach is further supported by the observation that inhaled corticosteroids enhance \(\beta_2\)-AR responses \(^31\).
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FIGURE LEGENDS

Fig. 1. Moderate intensity aerobic exercise attenuates AHR in OVA-treated mice. Mice were OVA-treated and exercised as described in Methods. At the conclusion of the protocol, mice were assessed for changes in AHR via mechanical ventilation with methacholine challenge at increasing concentrations. Results are presented as total lung resistance (cm H$_2$O/ml/s; *p<0.02, as compared with sedentary, OVA-treated mice; n = 6 - 8 mice per group).

Fig. 2. β$_2$-AR antagonist blocks the effect of moderate intensity aerobic exercise on AHR in OVA-treated mice. Mice were OVA-treated and exercised as described in Methods. At the conclusion of the protocol, mice were assessed for changes in AHR upon challenge with methacholine at increasing concentrations. Prior to each methacholine challenge, mice received a nebulization of either butoxamine HCL or saline. Results for (A) saline-treated and (B) OVA-treated mice are presented as total lung resistance (cm H$_2$O/ml/s; ‡p<0.02 as compared with exercised, saline-treated; §p<0.02 as compared with EO, saline-treated; n = 6 - 8 mice per group).

Fig. 3. Moderate intensity aerobic exercise decreases protein expression of GRK-2, but not β$_2$-AR, in the ASM of OVA-treated mice. Mice were OVA-treated and exercised as described in Methods. At the conclusion of the protocol, ASM cells were harvested and prepared for Western blot analyses of (A) β$_2$-AR (65kD) and GRK-2 (80kD) protein expression. (B) Densitometric analyses were performed; differences in lane loading were normalized via measurement of α-smooth muscle actin levels. Results are reported as fold-difference in protein expression (†p<0.02, as compared with sedentary, saline-
treated controls; *p<0.02, as compared with sedentary, OVA-treated mice; n = 4 - 7 mice per group).

Fig. 4. Moderate intensity aerobic exercise decreases ASM thickness in OVA-treated mice. Mice were OVA-treated and exercised as described in Methods. At the conclusion of the protocol, whole lung tissues were harvested and prepared for peroxidase staining and ASM thickness measurements with MetaMorph software. (A-D) Representative images of lung tissue stained for α-smooth muscle actin and probed with peroxidase (40X magnification). Arrows indicate ASM layer; asterisks indicate airway lumen. (E) Results of Metamorph analysis are presented as ASM thickness in microns (*p<0.02, as compared with sedentary, OVA-treated mice; n = 4 - 8 mice per group).

Fig. 5. Moderate intensity aerobic exercise attenuates PGE2 levels in the lungs of OVA-treated mice. Mice were OVA-treated and exercised as described in Methods. At the conclusion of the protocol, mice were bronchioalveolar lavaged; resulting lavagates were analyzed for changes in PGE2 content via PGE2-specific ELISA (†p<0.01 as compared with sedentary, saline-treated controls; *p<0.01 as compared with sedentary, OVA-treated; n = 6 - 8 mice per group).
Fig. 1
**Fig. 3A**

![Fig. 3A](image1)

**Fig. 3B**

![Fig. 3B](image2)
Fig. 4

Sedentary Exercised
Saline

OVA

Fig. 4E

ASM Thickness (microns)

w/saline w/OVA

Sedentary Exercised

†
Fig. 5

![Bar graph showing PGE2 Concentration (pg/mL) with saline and OVA for Sedentary and Exercised groups.](image-url)

- **Sedentary**
  - w/saline
  - w/OVA

- **Exercised**
  - w/saline
  - w/OVA

*Significant difference compared to sedentary group.
†Significant difference compared to saline group.
ACUTE EXERCISE DECREASES AIRWAY INFLAMMATION, BUT NOT RESPONSIVENESS, IN AN ALLERGIC ASTHMA MODEL

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ABSTRACT

Previous studies have suggested that the asthmatic responses of airway inflammation, remodeling, and hyperresponsiveness (AHR) are interrelated; in this study, we utilized exercise to examine the nature of this interrelationship. Mice were sensitized and challenged with ovalbumin (OVA); mice were then exercised via running on a motorized treadmill at a moderate intensity. Data indicate that, within the lungs of OVA-treated mice, exercise attenuated the production of inflammatory mediators, including chemokines KC, RANTES, and MCP-1 and IL-12p40/p80. Coordinately, OVA-treated and exercised mice displayed decreases in leukocyte infiltration, including eosinophils, as compared with sedentary controls. Results also show that a single bout of exercise significantly decreased phosphorylation of the NFκB p65 subunit, which regulates the gene expression of a wide variety of inflammatory mediators. In addition, OVA-treated and exercised mice exhibited decreases in the levels of Th2-derived cytokines IL-5 and IL-13 and the prostaglandin PGE2, as compared with sedentary controls. In contrast, results show that a single bout of exercise had no effect on AHR in OVA-treated mice challenged with increasing doses of aerosolized methacholine (0 – 50 mg/ml) as compared with sedentary mice. Exercise also had no effect on epithelial cell hypertrophy, mucus production, or airway wall thickening in OVA-treated mice as compared with sedentary controls. These findings suggest that a single bout of aerobic exercise at a moderate intensity attenuates airway inflammation but not AHR or airway remodeling in OVA-treated mice. The implication of these findings for the interrelationship between airway inflammation, airway remodeling and AHR is discussed.
Keywords: asthma, aerobic exercise, airway inflammation, remodeling, hyperresponsiveness
INTRODUCTION

Asthma is characterized by the clinical symptoms of wheezing, chest tightness, dyspnea and cough, and by the presence of reversible airway narrowing and/or airway hyperresponsiveness (AHR) to a variety of inhaled bronchoconstrictor stimuli (1). Although multifactorial in origin, asthma is considered an inflammatory process that is the result of an inappropriate and sustained immune response to innocuous antigens. This response involves increased levels of chemokines, cytokines and prostaglandins that perpetuate tissue damage and airway remodeling through the recruitment and activation of leukocytes, including Th2 cells and eosinophils.

The interplay between the mechanisms that drive AHR, airway remodeling and airway inflammation has been examined extensively. Numerous studies have suggested a causal relationship between increases in cellular inflammation of the airways and AHR (2). Likewise, increases in airway remodeling, including goblet cell hyperplasia and airway wall thickening, have been implicated in the pathogenesis of AHR (2). Because anti-inflammatory therapy does not completely resolve AHR in chronic asthmatics, the role of inflammation in the initiation and exacerbation of AHR is in question.

Previous reports have demonstrated that acute aerobic exercise at a moderate intensity affects both immune responses and lung function in healthy subjects (3,4); therefore, we utilized acute exercise as a tool to further examine the relationship between airway inflammation, airway remodeling and AHR. Because lung function is compromised in asthmatics, we hypothesized that acute aerobic exercise at moderate intensity would attenuate airway inflammatory responses but not airway remodeling or AHR in an ovalbumin (OVA)-driven mouse model of allergic asthma. For this study, mice were sensitized and challenged with OVA or control saline and exercised for one bout at
a moderate intensity on a motorized treadmill. At the conclusion of the protocol, changes in leukocyte infiltration, mediator production, airway remodeling and AHR were monitored. Results presented herein indicate that a single bout of moderate intensity aerobic exercise attenuated airway inflammation, but not airway remodeling or AHR.
MATERIALS AND METHODS

Animals

Female BALB/cJ mice (3-5 wk old; The Jackson Laboratory, Bar Harbor, ME), a strain susceptible to OVA-induced IgE responses (5;6), were utilized. Mice were housed in a pathogen-free containment facility and maintained in autoclaved Microisolator cages (Lab Products, Maywood, NJ). Mice were provided with food (Teklad, Madison, WI) and water ad libitum. Mice were allowed to acclimate to housing condition for 1 wk before experimental manipulation. All animal treatments were approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health recommended guidelines.

OVA-Treatment and Aerobic Exercise Protocol

At the start of the protocol, mice were assigned randomly into sedentary and exercised groups. Sensitization (dys 0, 14 via i.p.) and challenge (dys 21 – 25, dy 28 via aerosolization) with OVA (Sigma Chemical, St. Louis, MO) or saline was performed as described previously (7;8). Exercised mice underwent a single bout of moderately-intense aerobic exercise on a motorized treadmill (Exer 6M; Columbus Instruments, Columbus, OH) for 45 min at 13.5 m/min (0% grade) on Day 28 of the protocol. As noted in our earlier study (7), previous reports have defined moderate intensity aerobic exercise as brief (15 – 60 min) bouts of treadmill running at 50 - 75% maximum O₂ consumption or ~15-22 m/min (9;10;11;12). The exercise bout included brief warm-up and cool-down periods so that the total treadmill time was ~60 min.
Lavage Cell and Protein Analyses

24 h after completion of the OVA-treatment / exercise protocol, mice were euthanized via i.p. injection of ketamine (8.7 mg/kg) / xylazine (1.3 mg/kg) and prepared for bronchoalveolar lavage (BAL) as described previously (7). Cell viability was determined via trypan blue exclusion and cell types were differentiated on cytospin preps using Wright-Giemsa stain. Cell differentials were determined from at least 500 leukocytes using standard hematological criteria. BAL samples were analyzed for mediator expression via Mouse Multiplex Assay (Bio-Rad Inc., Hercules, CA). PGE$_2$ was analyzed via enzyme linked immunosorbent assay (ELISA; Invitrogen, Carlsbad, CA).

NFkB p65 Phosphorylation Analysis

24 h after completion of the OVA-treatment / exercise protocol, lungs were harvested and protein extracts were prepared as described previously (8). Equivalent amounts of protein from each sample were analyzed for differences in total and phosphorylated NFkB p65 via ELISA according to the manufacturer’s instructions (Millipore, Billerica, MA).

Airway Hyperresponsiveness Analysis

Mice were mechanically ventilated and challenged with increasing concentrations of aerosolized methacholine as described previously 48 hours post the last OVA-treatment/exercise bout (13;14). This time-point was chosen to be consistent with the current literature. Briefly, mice were anesthetized with diazepam (17.5 mg/kg) and ketamine (450 mg/kg) and a tracheotomy tube (18G) was inserted and connected to the
inspiratory and expiratory ports of a ventilator (Flexivent, SCIREQ, Montreal, Quebec, Canada). Mice were ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2ml with a positive end-expiratory pressure of 2–4 cm H₂O. Increasing concentrations of methacholine (0-50 mg/mL) were administered via aerosolization. From 20 s up to 3 min after each aerosol challenge, resistance (R) was recorded continuously; an average value of R was taken in order to express changes in airway function.

Airway Remodeling Analysis

Lungs fixed in 70% alcoholic formalin and paraffin-embedded were stained with alcian blue-periodic acid-Schiff hematoxylin (PASH) as described previously (7). After random coding, the degree of epithelial cell hypertrophy and mucus production in the PASH analyses was assessed subjectively; a semi-quantitative rating scale ranged from 0 (none) to maximal (4). For direct measurements of airway wall thickening (15), tissue sections were visualized under a light field microscope at 200X magnification. Airways with a longitudinal diameter between 150-200 μm were identified and differences in airway wall thickness were determined using the imaging program Metamorph. A minimum of six independent airways per tissue section were measured.

Statistical Analysis

Data were analyzed using SPSS Version 11.0. Results are reported as group means ±SD. A One-way ANOVA determined differences among the group means and the Bonferroni post-hoc analysis determined which group means differed significantly (at a level of p≤0.05).
RESULTS

Acute Aerobic Exercise Attenuates Chemoattractant Production and Leukocyte Infiltration

Production of chemoattractants plays an important role in the recruitment and subsequent activation of immune cells into the bronchial mucosa during an inflammatory response. Elevated levels of cytokines with chemoattractant properties, including KC (CXCL8; murine homolog of human IL-8), RANTES (CCL5), MCP-1 (CCL2), and IL-12p40/p80, have been implicated in airway inflammation (16;17;18;19). Data shown in Figure 1 demonstrate that OVA-treatment of sedentary mice increased (between 6 – 35 fold) KC, RANTES, MCP-1 and IL-12p40/p80 protein levels significantly as compared with saline-treated controls. A single bout of exercise at moderate intensity attenuated (≥ 50% decrease) significantly the production of KC, RANTES, and MCP-1 in OVA-treated mice as compared with sedentary controls (Fig. 1A-C). Exercise also decreased IL-12p40/p80 protein content (Fig. 1D); however, little or no detectable IL-12p70 protein was observed in either control or experimental mice (data not shown). Sedentary and exercised saline-treated controls expressed equivalent levels of each mediator analyzed (Fig. 1).

Concomitant with these results, findings presented in Figure 2 indicate that leukocyte infiltration was increased (approximately 30-fold) significantly in OVA-treated sedentary mice as compared with saline-treated controls. OVA-treated mice that were exercised for a single bout at a moderate intensity exhibited decreases in leukocyte infiltration, including infiltration of macrophages, eosinophils, and neutrophils, when compared with sedentary controls (Fig. 2). Sedentary and exercised saline-treated controls exhibited equivalent levels of leukocyte infiltrate.
Acute Aerobic Exercise Decreases NFκB p65 Phosphorylation

The activated transcription factor NFκB regulates the expression of a wide variety of genes that encode inflammatory mediators, such as KC, MCP-1, and RANTES, and, therefore, has been implicated directly in regulating asthmatic inflammatory responses (20;21). Phosphorylation of the NFκB subunit p65 is a modification associated with enhancement of p65 transactivation potential; as such, it is an indirect measure of NFκB activation. Results presented in Figure 3 show that a single bout of exercise at a moderate intensity attenuated p65 phosphorylation in the OVA-treated lung by 50% as compared with sedentary, OVA-treated mice. Saline-treated controls exhibited levels of p65 phosphorylation that were equivalent with exercised, OVA-treated mice (Fig. 3).

Acute Aerobic Exercise Attenuates Th2-Derived Cytokine Production

Atopic asthma is considered a Th2-driven disease (22); Th2 cells express the cytokines IL-4, IL-5 and IL-13. Findings presented in Figure 4 demonstrate that OVA-treatment of sedentary mice increased IL-5 and IL-13 significantly as compared with saline controls; OVA-treatment had no affect on IL-4 levels (data not shown). A single bout of moderate intensity exercise attenuated (≥ 50% decrease) the production of IL-5 and IL-13 protein significantly in OVA-treated mice as compared with sedentary controls (Fig. 4). Saline-treated controls expressed equivalent levels of each cytokine analyzed (Fig. 4). It should be noted that the Th1-derived cytokines IL-2 and IFNγ were not detected in any experimental or control group (data not shown).
Acute Aerobic Exercise Does Not Affect AHR or Airway Remodeling

AHR to a variety of inhaled bronchoconstrictor stimuli is a hallmark of the asthmatic response. To determine the effect of a single bout of exercise on AHR in OVA-treated mice, mice were mechanically ventilated and challenged with increasing concentrations of methacholine (0-50 mg/mL). Results shown in Figure 5 demonstrate that, at the highest methacholine dose (50 mg/ml), OVA-treatment increased lung resistance (R) significantly in sedentary mice as compared with saline-treated controls. A single bout of exercise in OVA-treated mice had no effect on this response (Fig. 5).

Previous studies have suggested that airway remodeling is causal to airway hyperresponsiveness (2). To analyze the effect of a single bout of exercise on airway remodeling in OVA-treated mice, lung tissue sections were coded randomly and scored subjectively for assessment of hypertrophy/hyperplasia of the mucosal epithelium, goblet cell and mucin production; overall airway wall thickening was measured using Metamorph analysis. Representative micrographs shown in Figure 6 demonstrate that OVA-treatment in both sedentary and exercised mice increased each of these parameters as compared with saline-treated controls; however, no significant differences between OVA-treated, sedentary and OVA-treated, exercised mice were observed in scored or measured assessments (data not shown).

Acute Aerobic Exercise Attenuates PGE$_2$ Levels

Within the asthmatic lung, the prostaglandin PGE$_2$ has been implicated in the regulation of airway inflammation, Th2 cell differentiation and AHR (reviewed in 23). Data presented in Figure 7 show that OVA-treatment of sedentary mice increased PGE$_2$
levels significantly as compared with saline-treated controls. OVA-treated mice that underwent a single bout of moderate exercise, however, displayed significantly decreased ($\geq 70\%$) PGE$_2$ levels as compared with sedentary controls (Fig. 7); these levels were equivalent to those observed in saline-treated controls (Fig. 7).
DISCUSSION

In this study, sedentary mice treated acutely with OVA exhibited significant increases in asthma-related responses as compared with saline controls; these responses included production of the inflammation-related mediators KC, RANTES and MCP-1, IL-12p40/p80 and PGE2, recruitment of leukocytes, including eosinophils, into the airways, levels of Th2-derived IL-5 and IL-13, activation of NF-κB, airway remodeling, and AHR. A single bout of aerobic exercise at moderate intensity decreased significantly each of these responses with the exceptions of airway remodeling and AHR. In exercised mice, the observed increases in AHR were not a consequence of exercise-induced-bronchoconstriction; increases in resistance were measured in response to methacholine challenge at 48 hr post OVA-treatment and, therefore, reflect the persistent AHR observed in chronic asthmatics (2). Collectively, these findings suggest that airway inflammation is not required for the development of airway remodeling and AHR.

Recent work suggests that AHR can be dissociated from cellular inflammation while remaining linked with sustained airway remodeling (24;25). Specifically, Kariyawasam and co-workers and Crimi and colleagues have each shown that increases in cellular inflammation, including eosinophils, do not positively correlate with increased AHR in asthmatic patients (24;25); however, markers of airway remodeling remain associated with AHR (24). Similarly, Leigh et al have shown that, mice that undergo chronic allergen exposure, exhibit structural changes that are associated with AHR in the absence of cellular inflammation (26). In contrast, Alcorn and co-workers have indicated that attenuated airway remodeling does not impact airway inflammatory responses or AHR (27). In particular, these authors reported that neutralization of TGFβ1 responses in
the lungs of OVA-treated mice suppressed airway fibrosis while increasing AHR; neutralization had no affect on airway inflammation (27). Collectively, these studies demonstrate that airway inflammation, remodeling, and hyperresponsiveness may not be directly interrelated.

Data in the current study support and extend these previous observations. Because NFκB regulates the gene expression of a wide variety of inflammatory mediators, including KC, MCP-1, and RANTES, exercise-mediated attenuation of NF-κB activation likely led to the observed decreases in chemokine production and subsequent leukocyte recruitment into the OVA-treated lung. In addition, decreases in IL-12p40/p80, which acts as a macrophage chemoattractant (19), as well as Th2-derived IL-5 and IL-13, which promote eosinophilic inflammation (28), likely led to decreased cellular inflammation. It should be noted that there is an apparent discordance between the magnitude of exercise-mediated decreases in Th2-derived cytokines and eosinophil infiltration; such discordance suggests compensation by other cytokines and/or temporal differences in the attenuation of each of these targets. In murine asthma models, IL-12 has also been implicated in the regulation of Th1/Th2 responses (28); however, our results indicate that exercise decreases Th2 responses in OVA-treated mice via a manner that is independent of IL-12. Also, the lack of detectable IL-12p70 in both control and experimental samples may explain, in part, the lack of observed Th1 responses in our study; IL-12p70 promotes Th1 responses while IL-12p40/p80 antagonizes this action (29). Despite the reduction in inflammatory cells, mediators, and Th2-derived responses, increased airway remodeling and AHR persisted in the lungs of exercised, OVA-treated mice.
In addition to their roles in asthma-related airway inflammation, the cytokines IL-12 and IL-13 have been implicated directly in AHR pathogenesis. Gavett and colleagues and Kips et al have both demonstrated that administration of IL-12 during antigen challenge inhibits AHR in mice (30;31). With regard to IL-13, Grunig and co-workers and Wills-Karp and colleagues have both shown that reconstitution of IL-13-deficient mice with recombinant IL-13 triggered mucus hypersecretion and AHR (32;33); these studies also suggest that IL-13 can induce AHR in the absence of inflammatory cell recruitment (32;33). Our results indicate decreases in IL-12 and IL-13 within the lung do not correlate with reduced airway remodeling or AHR. The discrepancy between our results and previous findings may be explained by the differences in mouse model systems utilized. The previous studies cited above utilized antigen-treated A/J or C57BL/6 mice while the current study used the BALB/cj strain. It is well accepted that mouse strains differ in their inflammatory and pulmonary responses upon exposure to antigen (5; 34). For example, repeated antigen instillation results in increased eosinophilic inflammation, collagen deposition, and airway wall thickening in A/J and BALB/cj mice as compared with C57Bl/6 (34). Likewise, A/J and BALB/cj display a greater degree of AHR as compared with C57Bl/6 mice (5). Karp and colleagues demonstrated that the complement factor 5 (C5) gene is expressed differentially between mice that differed in their susceptibility to the development of allergen-induced AHR (35). In light of these strain differences, the interrelationship between airway inflammation, remodeling and hyperresponsiveness may be regulated via differential mechanisms. Another possibility is that inflammation primes the airway epithelium and ASM for a bronchoconstrictive response; such priming may involve desensitization of β2-adrenergic receptors, which facilitate bronchodilation, via inflammatory-mediated induction of GRK-2 (36). The temporal nature of the inflammation allows it to resolve ahead of the resultant and persistent effects on airway remodeling and AHR (24).
PGE$_2$ is considered a potent pro-inflammatory mediator that plays a role in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis; within the lung and airways, however, it is considered protective against asthma-related lung inflammation and AHR (reviewed in 23). In particular, PGE2-mediated protection against allergen-induced AHR appears to be secondary to reduced levels of cellular inflammation. For example, administration of PGE$_2$ in vivo has been shown to decrease the number of IL-4- and IL-5-producing (Th2) cells, eosinophils, and metachromatic cells within the BALF of allergen-sensitized subjects; these cell types have been implicated in the initiation and exacerbation of AHR (reviewed in 23). In addition, PGE$_2$ appears to play a role in T-lymphocyte trafficking and differentiation within the allergen-sensitized airway (23). In contrast, Liu et al have reported that PGE2 induces AHR via a signaling pathway that is dependent upon Rho/ROCK activation and Ca$^{2+}$ release (37). Results presented herein suggest that increased PGE$_2$ levels in the lungs of sedentary, OVA-treated mice do not protect against either airway inflammation, including Th2 responses, or AHR. Such conflicting results may be explained by the expression and utilization of E prostanoid receptors (EP1 – EP4) that mediate the effects of PGE$_2$ within the lung and airways. E prostanoid receptors are expressed on multiple cell types, including lymphocytes, airway epithelial cells and ASM cells; multiple E prostanoid receptor subtypes may be expressed in a single cell. Activation of the EP1 and EP3 receptors elevate intracellular calcium levels and promote effector cell activation (23). Conversely, activation of the EP2 and EP4 receptors increases intracellular cAMP concentrations, which inhibit effector cell functions (23). Liggett and co-workers have reported that activation of the EP1 receptor on ASM uncouples the $\beta_2$-AR from its G-protein complex resulting in reduced $\beta_2$-AR-desensitization (38). Penn and colleagues have also shown that the EP2
receptor regulates cytokine-mediated desensitization of the $\beta_2$-adrenergic receptor ($\beta_2$-AR) on ASM (39). Because multiple E prostanoid receptor subtypes may be expressed in a single cell, it is possible that an on-going inflammatory response would cause a multiplicity of opposing PGE$_2$-mediated actions within the lung and airways.

In our previous studies, we observed that repeated bouts of aerobic exercise at a moderate intensity attenuated both airway inflammation and remodeling in mice that had been sensitized and challenged with OVA for a total of seven weeks (7;8). In addition, OVA-treated mice from our previous studies exhibited a lesser eosinophilic response as compared with OVA-treated mice in the current study. We believe that these seemingly contrasting results between our collective studies are explained by the differences in the length of the OVA-treatment and exercise protocols. In particular, the disparity in exercise-mediated effects on airway remodeling is likely due to the difference in the number of bouts of exercise. The effects of exercise on physiological responses are dependent upon several variables, including the frequency of each bout and the total duration of the exercise protocol (40;41-45); the mechanism that underlies this disparity may involve changes in the levels of circulating hormones (e.g. glucocorticoids, catecholamines) that are released from the hypothalamic – pituitary – axis during exercise. With regard to differences in the extent of airway eosinophilia between these studies, we hypothesize that the extended OVA exposure may have had a tolerizing effect on the eosinophilic response reported in our previous papers (7;8). In support of this hypothesis, several studies have demonstrated that prolonged allergen exposure results in decreased inflammatory responses, including airway eosinophilia (46;34;47-49).
Using aerobic exercise as tool to investigate the interrelationship between airway inflammation, remodeling, and hyperresponsiveness, we have shown that airway remodeling and AHR persist in the absence of airway inflammation; this is the first report to utilize exercise as a tool to study this interrelationship. We believe that, with this novel approach, future studies will elucidate the mechanisms that underlie this interrelationship and its role in asthma pathogenesis.
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FIGURE LEGENDS

Figure 1. One bout of aerobic exercise at a moderate intensity decreased chemokine production in OVA-treated mice. 24 hr post protocol completion, BAL was performed; resulting lavagates were analyzed for changes in (A) KC, (B) RANTES, (C) MCP-1 and (D) IL-12p40/p80 via multiplex assay. Results are reported as pg/ml of respective protein (†p < 0.006 as compared with saline-treated, sedentary mice; *p < 0.04 as compared with OVA-treated, sedentary mice; n = 4 - 8 mice per group).

Figure 2. A single bout of moderate intensity aerobic exercise decreased leukocyte infiltration in OVA-treated mice. 24 hr post protocol completion, BAL was performed and resulting lavagates were analyzed for changes in total cells and eosinophils. Results were are reported as changes in cell number (†p < 0.03 as compared with saline-treated, sedentary mice; *p<0.04 as compared with OVA-treated, sedentary mice; ND – none detected; n = 4 mice per group).

Figure 3. A single bout of moderate intensity aerobic exercise decreased p65 phosphorylation in the lungs of OVA-treated mice. 24 hr post protocol completion, lungs were harvested, lysed and prepared for analyses of total and phosphorylated p65 levels via p65-specific ELISA; phosphorylated p65 levels were normalized against total p65 levels. Results are reported as fold-difference in phospho-NFκB-p65 levels as compared with saline-treated, sedentary controls (†p < 0.05 as compared with saline-treated, sedentary mice; *p<0.05 as compared with OVA-treated, sedentary mice; n = 4 mice per group).

Figure 4. One bout of moderate intensity aerobic exercise decreased Th2-derived cytokine production in OVA-treated mice. 24 hr post protocol completion, BAL was
performed and resulting lavagates were analyzed for changes in (A) IL-5 and (B) IL-13 via multiplex assay. Results are reported as pg/ml of respective protein (†p < 0.001 as compared with saline-treated, sedentary mice; *p<0.001 as compared with OVA-treated, sedentary mice; ND – none detected; n = 4 - 8 mice per group).

**Figure 5.** A single bout of moderate intensity aerobic exercise had no effect on AHR in OVA-treated mice. 48 hr post protocol completion, mice were assessed for changes in AHR upon methacholine challenge at increasing concentrations via mechanical ventilation. Results are reported as measurements of total lung resistance (R, cm H₂O/mL/s; †p < 0.04 as compared with saline-treated, sedentary mice; n = 4 - 8 mice per group).

**Figure 6.** A single bout of moderate intensity aerobic exercise had no effect on airway remodeling in OVA-treated mice. 24 hr post protocol completion, lung tissues were harvested, PASH stained and prepared for analysis of hypertrophy/hyperplasia of the mucosal epithelium, goblet cell and mucin production and overall airway wall thickening. Representative micrographs of 5 - 6 separate experiments are shown: A) sedentary; B) exercised; C) OVA-treated, sedentary; D) OVA-treated, exercised (* - airway lumen; 10X magnification, 40X inset).

**Figure 7.** One bout of moderate intensity aerobic exercise decreased PGE₂ production in OVA-treated mice. 24 hr post protocol completion, BAL was performed and resulting lavagates were analyzed for changes in PGE₂ content via ELISA. Results are reported as ng/ml (†p < 0.001 as compared with saline-treated, sedentary mice; *p<0.01, as compared with OVA-treated, sedentary; n = 6 - 8 mice per group).
Fig. 2

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*Significant difference between groups.
†Significantly different from control.
Fig. 3

Fold-difference in phospho-NF-κB p65

- Sedentary
- Exercised

†

*
Fig. 4

A. [Bar chart showing IL-5 levels with and without OVA exposure for Sedentary and Exercised groups.]

B. [Bar chart showing IL-13 levels with and without OVA exposure for Sedentary and Exercised groups.]
Fig. 5

Resistance (R) (cm H₂O/mL/s)

Methacholine (mg/mL)

- □ - Sedentary, Saline
- ● - Exercised, Saline
- △ - Sedentary, OVA
- ▼ - Exercised, OVA

† †
Fig. 6

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Saline

OVA
Fig. 7
CHAPTER 4
GENERAL DISCUSSION AND CONCLUSIONS

Data Summary

Results presented in Chapter 2 demonstrate that repeated bouts of moderate intensity aerobic exercise attenuate not only the inflammatory response in a murine model of asthma as we have previously reported, but also AHR. The data present in Chapter 3 demonstrate that an acute bout of exercise attenuates the inflammatory response, but not AHR, in the same murine model of asthma. In this chapter the mechanisms behind attenuation of AHR and inflammation will be discussed within the context of beneficial effects of acute versus chronic exercise.

It remains unclear whether inflammation and AHR are initiated and attenuated separately, in parallel, in series, or simultaneously in human asthma or a murine model of asthma. However, data presented here strongly suggests that AHR and inflammation are not attenuated in parallel but rather sequentially. The proposed mechanism involves the hypothesis that the attenuation of inflammation after acute exercise is temporally related to the attenuation of inflammation and AHR observed with chronic exercise. This hypothesis is underscored by the sequential and increased release of catecholamines and glucocorticoids. To date this lab has not specifically studied the effects of catecholamines on the asthma phenotype. Specifically the increased release of glucocorticoids and catecholamines leads to attenuated activation of NF-κB. Once NF-κB activation is significantly reduced, inflammatory mediator (chemokines / cytokines) production decreases, resulting in reduced numbers of activated immune cells being trafficked to the lungs. The prolonged attenuation of inflammatory mediators such as NF
– κB, IL-4, IL-5, IL-13, MCP-1, GM-CSF, and IL-8 (or KC in mice)\textsuperscript{79} in response to exercise leads to AHR – associated significant morphological changes in the lungs. These include: attenuation of remodeling, increases in $\beta_2$ – AR function, and decreased $\beta_2$ – AR desensitization and internalization. In Figure 1 below, a representation of the proposed mechanism of attenuation for AHR and inflammation is proposed, and this schematic also delineates how these mechanisms may be temporally linked. Figure 1 makes use of arrows to indicate whether a particular aspect of inflammation or AHR is attenuated (Red) or enhanced (Green) in response to acute and/or chronic exercise.

Figure 1. Proposed mechanism by which AHR and Inflammation are attenuated. This schematic is a representation of the proposed mechanism of attenuation of both AHR and inflammation. It compares the specific effects of acute exercise vs. chronic exercise with regard to inflammation and AHR. Arrows indicate whether an aspect of inflammation and AHR is up – regulated (Green) or down – regulated (Red) with respect to aerobic exercise.
Studies to Test the Proposed Model of Attenuation

The studies presented and discussed above have answered many questions concerning the mechanisms by which exercise exerts its beneficial effects on the asthmatic phenotype in a temporal manner. As with all science, the answering of a few questions raises many more and leads to the design of new studies to further our understanding of asthma. In this section, a kinetic study is proposed which is designed to answer the hypothesis put forth above. This study would involve subjecting mice to the chronic OVA exposure and exercise protocol detailed below in Figure 2. After each bout of exercise at both 24 and 48 hours mice will be sacrificed and AHR and inflammatory analyses will be collected. This study will help determine whether inflammation and AHR are attenuated in a temporal manner by moderate intensity aerobic exercise and elucidating the mechanism responsible for the attenuation.

Figure 2. Timeline of proposed kinetic study. This schematic represents a timeline of events for the proposed kinetics study. Mice will be divided into groups and exercised. After each bout of exercise a subset of mice will be sacrificed and airway inflammation along with AHR would be assessed.
Endogenous Hormones

It is our hypothesis that exercise augments the production of multiple hormones of the hypothalamus pituitary adrenal axis. In addition to the specific ligands focused upon in these studies and below, there may be others that are less appreciated that may also play roles in the beneficial effects of exercise. Glucocorticoids have been shown previously to increase significantly in serum in response to chronic exercise. This topic would be revisited in the proposed kinetic study outlined above to determine at what point increased release of glucocorticoids occurs in response to exercise.

Measurements will be collected twice daily to account for the diurnal release of these ligands and to examine the increased release of glucocorticoids and whether there is a tolerization effect of glucocorticoid release over time. Catecholamine measurements will also be collected to determine if exercise exerts any effects on the release of these ligands. This study will also analyze the bronchial lavage (BAL) fluid to determine if increased levels of glucocorticoids and catecholamines are found within the lung. Receptors to which glucocorticoids and catecholamines bind will also be quantified through Western blot analysis and/or radioligand binding. The hypothesis for this aspect of the kinetic study would be that from as early as 24 hours after the first bout of exercise, significant increases of glucocorticoids and catecholamines would be observed in both serum and BAL fluid. Receptor levels of these mediators within the lung may also increase in number and activity in a temporal manner in response to exercise.
Immune Infiltrate and Inflammatory Mediators

It is well established that in asthma in general and in allergic asthma in particular; an immune response is initiated via activation of NF-κB, leading to the production of inflammatory mediators and to subsequent activation of immune cells that migrate into the lungs. Thus far, the studies presented have focused on the trafficking of eosinophils, neutrophils, macrophages, and T-cells to the lung measured within the BAL fluid. In the proposed kinetic study, levels of these immune cell populations will be tracked over time within the BAL fluid. In addition, other populations of immune cells will also be examined such as, dendritic cells and regulatory T-cells by isolating cells from the BAL fluid, spleen, and lymph nodes. Mice are available expressing fluorescently labeled T-cells, both T_{H2} T-cells along with regulatory T-cells. Using these markers, it is possible through adoptive transfer to track these cells within the mouse either through flow cytometry or whole body imaging. These methods, especially whole body imaging allows the tracking of specific immune cell populations throughout the mouse temporally in response to exercise. Cytokine and chemokine expression is vital to initiate recruitment of immune cells and remodeling within the lung; therefore, concentrations of these mediators will be collected and measured via the BAL fluid during the study. It is hypothesized that exercise will cause the following effects in a temporal manner; i) decreased NF-κB activation, ii) decreased chemokines, iii) decreased infiltration of immune cells, iv) decreased inflammatory mediator expression, and v) expansion of the regulatory T-cell population within the lung.

Remodeling of the Lung

One of the more prominent features of asthma involves the remodeling of airways within the lung. It has been shown in Chapter 3 that significant airway
remodeling happens early in a murine model of human asthma. Previously, it has been shown that, with chronic exercise, significant attenuation with regards to airway remodeling does occur including airway smooth muscle thickness. The proposed kinetic study would involve isolating and sectioning lungs to determine the temporal affects of aerobic exercise on airway remodeling. An alternative assessment of measuring airway remodeling may involve measuring AHR. As the airways are remodeled especially with regards to asthma, the AHR rises. This is causes by the differential effects of airway remodeling such as, i) epithelial cell hyperplasia and hypertrophy, ii) globlet cell hyperplasia, iii) increased mucus production, iv) increased ASM and basement membrane thickness, and v) increased mucus production. It would prove useful to utilize a mechanical ventilator such as the FlexiVent to correlate the differential phases of airway remodeling to AHR. Taken together, it is hypothesized that after prolonged attenuation of the inflammatory responses significant attenuation of airway remodeling will occur. Utilizing a FlexiVent to measure AHR is hypothesized to prove useful in non-invasively assessing airway remodeling.

\[ \text{AHR and } \beta_2 - AR \]

Airway hyperresponsiveness (AHR) presents as the most obvious clinical feature of asthma, presenting as wheezing, chronic cough, and chest tightness. The receptor widely accepted being responsible for bronchodilation is the \( \beta_2 \) – adrenergic receptor (\( \beta_2 - AR \)). In the proposed study the effects of exercise on AHR, \( \beta_2 - AR \), and its downstream signaling molecules will be studied in a temporal manner. AHR will be assessed throughout the study via lung function analyses of mice connected to a mechanical ventilator (FlexiVent). As mentioned above there is significant airway remodeling in asthma and understanding where the resistance in the lung originates and
how exercise changes modulates these parameters would prove useful. The FlexiVent possesses the capabilities needed to determine if the majority of resistance lies within the central ($R_n$) or lower airways ($G$). Other mediators possessing the capability to influence AHR would be assessed including: expression levels of $\beta_2$ – AR, G – protein receptor kinase (GRK) – 2, $\beta$ – arrestin, the G – protein $G_{\alpha s}$, and cyclic adenosine monophosphate (cAMP) in both membrane and cytoplasmic extracts of the lung and ASM cells. Concentrations of these mediators would be measured via Enzyme-Linked ImmunoSorbent Assay (ELISA), radioligand binding, and Western blot analysis. GRK – 2 and $\beta$ – arrestin mediate desensitization and internalization of the $\beta_2$ – AR while increased levels of $G_{\alpha s}$ and cAMP are indicative of enhanced $\beta_2$ – AR function. It is hypothesized that the majority of $R_L$ originates from the lower airways; and that in response to chronic exercise, AHR is temporally attenuated following remodeling attenuation. Chronic exercise is hypothesized to enhance $\beta_2$ – AR function as well as attenuate $\beta_2$ – AR desensitization and internalization in a temporal fashion.

Mechanistic Approach to Test Our Proposed Model

In the preceding paragraphs, it has been hypothesized that the increased release of glucocorticoids and catecholamines in response to exercise act as the primary mediators modulating attenuation of inflammation and AHR in a murine model of asthma. Listed above are experiments to determine in a temporal fashion how exercise induces the attenuation of inflammation and AHR. However, these experiments above do not definitively determine whether glucocorticoids and/or catecholamines are responsible for these effects. Determining if increased glucocorticoid and catecholamine release is responsible for attenuating inflammation and AHR in a temporal manner in response to exercise, transgenic mice would be utilized. The ideal model for this study
would involved obtaining a cre/lox mouse model allowing the activation and silencing of catecholamine and/or glucocorticoid receptor synthesis solely in lung tissue. Using these transgenic mice, glucocorticoid and catecholamine receptor synthesis could be shut off at differential points within the sensitization and exercise protocol to determine if glucocorticoids and catecholamines are responsible for exercise – induced attenuation of inflammation and AHR.

Limitations of Our Model

Comparing human asthma to a murine model of asthma has been heavily debated. As presented in the introduction, there exist distinct similarities and differences between human asthma and modeling the disease in mice. These differences are accepted in order to gather the amount of data that is needed to increase our knowledge about asthma without being overly invasive with human subjects. One limitation of our studies is the use of OVA – albumin (OVA) as the allergen to induce an asthma phenotype. OVA was chosen due to its ability to initiate a T\textsubscript{H}2 biased response that mimics asthma as shown in the literature when this project was started\textsuperscript{10}. Currently however there are more physiologic allergens including cockroach antigen, house dust mite antigen, and ragweed that can be used to induce a model of human asthma. In future studies it would be useful to sensitize and challenge mice to each of these antigens and compare the immune response and also AHR to determine if there are differential effects caused by each antigen and to validate the model used here as an appropriate model to induce an asthma phenotype. A concern with chronically challenging mice is the induction of tolerance to the allergen. During the course of the studies examining the effectiveness of each allergen to induce an asthmatic phenotype, it would also be useful to determine which of the allergen results in the least amount of
tolerance in the mice. This could be measure through AHR and also immune cell infiltrate found in the lungs.

The other major limitation to our model concerns the day/night schedules of mice. Mice are more active at night than they are during the day hours. In the current model, mice are challenged and exercised during the day hours. This raises the question that would results differ if the mice were challenged and exercised during their normal daytime cycle. To determine this, mice would be acclimated to a reverse day/night cycle in an enclosed room within the animal facility. During the mice’s normal daytime hours, mice would be challenged and exercised in the same room to ensure they are kept in the dark and on the same day/night schedule. At the end of the protocol mice would be sacrificed and inflammatory parameters, AHR, and glucocorticoid levels would be measured and compared to our current model to determine if differing the day/night cycle of the mice has an impact on the results we have reported.

Future Directions

It is known that exercise induces the increased release of catecholamines through the up-regulation of the hypothalamus – pituitary adrenal (HPA) axis. In the context of exercise and asthma it is poorly defined what role catecholamines play. Since the airway smooth muscle (ASM) surrounding the airways is innervated by the sympathetic nervous system, it is logical that an increased release of catecholamines in response to exercise would affect ASM activity. Determining this role, studies would need to be performed in vivo and in vitro. The in vitro studies would involve sensitized and challenged mice versus controls and the study of ASM and tracheal rings at the end of the protocol. Tracheal rings would be placed in a circulating bath with standard ringer, and contractile studies would be performed by adding exogenous catecholamines as
well as catecholamine antagonists to determine if catecholamines mediate contraction. ASM isolated from the treachalis of the mice would be plated and allowed to grow. While growing; exogenous catecholamines versus controls would supplement media feeding the ASM cells. When the ASM cells reach confluence, cells would be lifted, lysed, and Western blot analysis would be performed for targets involved with AHR.

In vivo studies to determine the role of catecholamines in AHR would be more challenging. The bulk of catecholamine and glucocorticoid released in response to exercise originates from the adrenal glands located on the dorsal surface of the kidneys. Excising the adrenal glands is the most effective way to eliminate catecholamine release; however, removal of the gland may cause secondary effects that can confound the data. Implantation of a catecholamine antagonist would also prove confounding due to global inhibition. The best method to obtain a local inhibition of catecholamines would be to administer a catecholamine antagonist via inhalation. Much like the work that was presented in Chapter 2 where a $\beta_2$ – AR antagonist was nebulized, a catecholamine antagonist would be nebulized into the mice with each challenge. Once the mice are sensitized and challenged, a subset will be given the catecholamine antagonist and exercised while another group receives just the antagonist. At the end of the protocol, mice would be connected to a FlexiVent and assessed for AHR. Thereafter, ASM would be isolated and probed for targets involved with AHR. Bronchial lavage (BAL) fluid and blood would also be collected to determine if the local blockade of catecholamines influenced the inflammatory responses.

If catecholamines are found to have a profound effect on AHR and/or inflammation in a murine model of asthma, it would have implications in the treatment of human asthma. Asthma in humans is most commonly treated with a mixture of corticosteroid and $\beta_2$ – AR agonists, which after long periods of use can exhibit decreased efficacy or loss of efficacy altogether and detrimental side effects.
Catecholamines may represent an area of research to treat asthma and possibly offer new treatments with higher efficacy and without long – term side effects. If exercise alone possesses the ability to increase catecholamines to a level that is beneficial to the asthmatic phenotype; then prescribing exercise rehabilitation therapies for asthmatics could become standard practice.

How Does Our work in Mice Translate into Humans?

All of the experimental work that has been presented thus far has been an experimental atopic model of asthma in mice. As mentioned in the introduction, there are distinct similarities and differences between a murine model of human asthma and human asthma itself. There have been several studies performed in the past looking at the effect of exercise on atopic asthmatics; however, these studies have shown limited improvement in the health of the asthmatics enrolled. This limited improvement is thought to be the result of flawed and uncontrolled exercise regiments to which participants are subjected. The next advancement for this project would be to conduct a study examining the efficacy of using moderate intensity aerobic exercise as an adjunct therapy for asthma in humans. This would involve recruiting atopic asthmatics into the trial. Once the participants are properly screened; an initial visit would be scheduled. At this initial visit, spirometry measurements would be collected along with peripheral blood and exhaled breath condensate (EBC). EBC is a non – invasive method to collect fluid from the lungs to measure inflammatory mediators and protein content. The patients would also be given a workout schedule, diary, and heart rate monitor to record their workouts. At the initial visit, participants would also receive a target heart rate range that they must achieve and remain within during exercising. Participants would then go exercise on their own for 45 – 60 minutes a day three times a week. Participants would
schedule a visit at the mid–point of the exercise protocol to have spirometry measurements taken, blood drawn, EBC collected, and the heart–rate monitor downloaded. A visit would also be conducted at the conclusion of the protocol. After the conclusion of the protocol, mediators from blood and EBC would be measured. Levels of inflammatory mediators along with spirometry would be compared to both the beginning and the mid–point to determine if subjecting humans to a chronic moderate intensity aerobic exercise protocol attenuated inflammation and AHR as is seen in mouse models.

These studies have helped to advance our knowledge of how the asthmatic phenotype can be attenuated with exercise. Underappreciated immune mediators and targets might emerge in humans asthma samples during the pre–clinical trial that have not been observed in the mouse model. It is indeed true that mouse lung anatomy and human lung anatomy do differ. However, understanding the mechanisms by which exercise attenuates the inflammatory and AHR aspect of asthma also helps to uncover new therapeutic targets for the treatment of asthma. Future treatments may prove to have less undesirable side effects with a higher efficacy than current treatments. It is hoped that through understanding the role of exercise in attenuating asthma, asthmatics will understand that they can successfully exercise, thereby improving their health and reducing the dependence on their pharmacologic means of treatment. Exercise serves as a viable alternative therapy for asthma that can help significantly reduce the health care costs incurred each year stemming from asthma.
Conclusion

In conclusion, we have shown that in response to repeated bouts of exercise AHR was attenuated in OVA-treated mice as compared with controls; these effects were blocked in the presence of a β2-adrenergic receptor (AR) antagonist. Repeated bouts of exercise also decreased ASM thickness and PGE₂ production significantly in OVA-treated mice. Together, these data indicate that repeated bouts of moderate intensity aerobic exercise attenuate AHR via a mechanism that involves β2-AR. In a second study, the effects of a single bout of moderate intensity aerobic exercise on AHR and airway inflammation were delineated in OVA-treated mice. Data from this study showed that, within the lungs of OVA-treated mice, exercise attenuated inflammatory mediator production, leukocyte infiltration, and NF – κB activation as compared with controls. In contrast, a single bout of exercise had no effect on AHR and airway remodeling in OVA-treated mice. These findings suggest that a single bout of aerobic exercise at a moderate intensity attenuates airway inflammation but not AHR or airway remodeling in OVA-treated mice.

From these studies, a hypothesis was formed that moderate intensity aerobic exercise attenuates AHR and airway inflammation in a temporal manner within the asthmatic lung. This temporal attenuation is dependent upon the prolonged increased release of endogenous glucocorticoids to initiate major morphological changes within the lung to attenuate not only inflammation but also AHR. The study discussed in Chapter 3 was instrumental in illustrating that inflammation and AHR are not attenuated in parallel and that prolonged suppression of the inflammatory response is necessary to significantly attenuate AHR. The study detailed in Chapter 4 importantly showed that both inflammation and AHR are significantly attenuated after repeated bouts of exercise and that the β₂ – AR is the primary receptor responsible for mediating the attenuation of AHR in exercised mice. In conclusion, future kinetic studies are warranted to examine
the relationship between the attenuation of inflammation and AHR and to determine if differences in day/night cycle and antigen selection significantly differ from the protocols currently in use.
GENERAL LIST OF REFERENCES

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM
NOTICE OF APPROVAL WITH STIPULATIONS

DATE: March 26, 2008

TO: Lisa M. Schieber, Ph.D.
MCLM 926 0005
FAX: 975-7679

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Effects of Aerobic Exercise on Regulatory T Cell Responses in Asthma
(T.Lowder)
Sponsor: NIH
Animal Project Number: 080308434

On March 26, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>B</td>
<td>110</td>
</tr>
<tr>
<td>Mice</td>
<td>A</td>
<td>110</td>
</tr>
</tbody>
</table>

Approval is granted with the following stipulation(s):

Project registration with Occupational Health and Safety is required. Submit the registration form, available at www.healthsafe.uab.edu, to OH&S and the Animal Use Safety Information Sheet (Appendix 4 available at www.uab.edu/iacuc), listing all potentially hazardous agents that will be administered to the animals. Once the AUSI is authorized, you will be contacted by Eric Durbow (934-3538) to discuss specific safety precautions which may be necessary for the ARP care staff. Animal procurement and use of potentially hazardous agents in live animals may not occur until Mr. Durbow has informed the IACUC Office that a satisfactory discussion has occurred.

Animal use is scheduled for review one year from March 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080308434 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
Mailing Address: B10 Volker Hall VH B10
1670 University Boulevard
1530 3RD AVE S  205.934.7692
BIRMINGHAM AL 35294-00198
Schwiebert, Lisa

From: IACUC [Prov-IACUC@mail.ad.uab.edu] Sent: Friday, June 20, 2008 2:47 PM To: Schwiebert, Lisa Cc: Susan Farmer Subject: RE: IACUC stipulations

Dr. Schwiebert:

Per this correspondence, the cell line stipulation has been lifted. Thank you for your quick response to this stipulation.

Sincerely,

Chris Chandler
Project Manager
Institutional Animal Care and Use Committee (IACUC)
University of Alabama at Birmingham
Volker Hall B010
934-7880

-----Original Message-----
From: Schwiebert, Lisa [mailto:LSCHWIEBERT@PHYSIOLOGY.UAB.EDU]
Sent: Friday, June 20, 2008 11:58 AM
To: Susan Farmer Cc: IACUC; Estell, Kim
Subject: RE: IACUC stipulations

Thanks, Susan...

To follow up on our conversation, all of the cells that will be utilized in our in vitro and adoptive transfer studies will include primary cells that are isolated from our own BALB/c mice; for the transfer studies, these cells will be transferred into our own mice (BALB/c) as well...

Attached to this email is a copy of the health surveillance report for our mice, which are housed in LHRB 864, that we received from you just this week; as you know, our mice tested positive for norovirus.

If there any additional concerns, please let me know...

Thanks again - it was great talking with you! Lisa

From: Susan Farmer [mailto:sfarmer@uab.edu]
Sent: Fri 6/20/2008 11:32 AM
To: Lisa M Schwiebert
Cc: IACUC
Subject: IACUC stipulations

Hi, Lisa. I got your message and will call you but wanted to go ahead and send you this. Often just me letting IACUC know you have made contact with me and that you have received the attached information is enough to lift the stipulations. I will try
to call you this afternoon.
I hope you are enjoying summer too!

Susan

Susan Farmer, DVM, PhD Senior Clinical Veterinarian, Animal Resources Program University of Alabama at Birmingham 1717 7th Avenue South, VH B-10 Birmingham, AL 35294-0019 Phone: 205-934-5563
APPENDIX B

EDITORIAL REPRINT PERMISSIONS
My name is Matthew Hewitt and I am writing to ask for permission to use a publication that I have had accepted in the American Journal of Respiratory Cell and Molecular Biology for my thesis. I will include the citation, title, and abstract as it appears on PubMed below. If I am approved to use it then please e-mail me something that is on the official letterhead so that I may attach it to my thesis, thank you for your time and have a good day.

Matt

Am J Respir Cell Mol Biol. <javascript:AL_get(this, 'jour', 'Am J Respir Cell Mol Biol.' );> 2008 Jul 17. [Pub ahead of print] Click here to read

Acute Exercise Decreases Airway Inflammation, but not Responsiveness, in an Allergic Asthma Model.

Hewitt M


Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL, USA.
Previous studies have suggested that the asthmatic responses of airway inflammation, remodeling, and hyperresponsiveness (AHR) are interrelated; in this study, we utilized exercise to examine the nature of this interrelationship. Mice were sensitized and challenged with ovalbumin (OVA); mice were then exercised via running on a motorized treadmill at a moderate intensity. Data indicate that, within the lungs of OVA-treated mice, exercise attenuated the production of inflammatory mediators, including chemokines KC, RANTES, and MCP-1 and IL-12p40/p55. Coordinately, OVA-treated and exercised mice displayed decreases in leukocyte infiltration, including eosinophils, as compared with sedentary controls. Results also show that a single bout of exercise significantly decreased phosphorylation of the Nf-kBp65 subunit, which regulates the gene expression of a wide variety of inflammatory mediators. In addition, OVA-treated and exercised mice exhibited decreases in the levels of Th2-derived cytokines IL-5 and IL-13 and the prostaglandin PGE2, as compared with sedentary controls. In contrast, results show that a single bout of exercise had no effect on AHR in OVA-treated mice challenged with increasing doses of aerosolized methacholine (0 to 50 mg/ml) as compared with sedentary mice. Exercise also had no effect on epithelial cell hypertrophy, mucus production, or airway wall thickening in OVA-treated mice as compared with sedentary controls. These findings suggest that a single bout of aerobic exercise at a moderate intensity attenuates airway inflammation but not AHR or airway remodeling in OVA-treated mice. The implication of these findings for the interrelationship between airway inflammation, airway remodeling and AHR is discussed.

PMID: 18635813 [PubMed - as supplied by publisher]
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-----Original Message-----
From: Hewitt, Matt [mailto:MHewitt@PHYSIOLOGY.UAB.EDU]
Sent: 08 September 2008 21:48
To: Rights and Permissions (ELS)
Subject: Permission to Cite Figure From the Book THE LUNG: CLINICAL PHYSIOLOGY AND PULMONARY FUNCTION TESTS

Hello,

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham. I am writing to ask your permission to cite a figure in one of your books, "The Lung: Clinical Physiology and Pulmonary Function Tests" by Comroe, Forster, Dubois, Briscoe, and Carlsen. Specifically I would like to cite Figure 53 on page 198. I am using this figure in my thesis and need written permission to use and cite it. Please e-mail me at your earliest convinence with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt
Elsevier Limited. Registered Office: The Boulevard, Langford Lane, Kidlington, Oxford, OX5 1GB, United Kingdom, Registration No. 1982084 (England and Wales).
Hi Matt,

I am flattered. Here is a ppt file with the figures that might make document preparation easier.

Tell Lisa I said hello.

Regards,
Bill Gerthoffer

William T. Gerthoffer, Ph.D.
Professor and Chair
Department of Biochemistry & Molecular Biology
University of South Alabama, College of Medicine
307 University Boulevard
Mobile, AL 36688-0002
Phone: 251-460-6856
FAX: 251-460-6850
wgerthoffer@usouthal.edu

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham in Lisa Schwiebert's Lab, we met not too long ago when you visited. I am writing to ask your permission to cite a figure in your paper, "Signal-transduction pathways that regulate visceral smooth muscle function. III. Coupling of muscarinic receptors to signaling kinases and effector proteins in gastrointestinal smooth muscles", specifically Figure 1. I am using this figure in my thesis and need you to grant me written permission to use and cite it. Please e-mail me at your earliest convinence with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt
Matt,

No problem to cite the figure. However, you are also welcome to use one or both of the attached figures, which (I think) are nicer. Btw, are you studying GPCR signaling in the airways? I am looking for a postdoc to start sometime next year. We are working on signaling mechanisms in airway smooth muscle using in vitro and in vivo models. Specifically, the project would involve characterization of Rgs4-/- mice in asthma models. If you might be interested, forward your CV and perhaps we can talk further.

Best of luck,
Kirk

Kirk Druey
Senior Investigator
LAD/NIAID/NIH
10 Center Drive Room 11N242
Bethesda, MD 20892
ph: 301-435-8875
fax: 301-480-8384

-----Original Message-----
From: Hewitt, Matt [mailto:MHewitt@PHYSIOLOGY.UAB.EDU]
Sent: Monday, September 08, 2008 4:22 PM
To: Druey, Kirk (NIH/NIAID) [E]
Subject: Permission to Cite Figure

Dr. Druey,

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham. I am writing to ask your permission to cite a figure in your paper, "Heterotrimeric G protein signaling: role in asthma and allergic inflammation", specifically Figure 4A. I am using this figure in my thesis and need you to grant me written permission to use and cite it. Please e-mail me at your earliest convinience with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt
No problem. - Dave Nieman

Hewitt, Matt wrote:

Dr. Nieman,

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham. I am writing to ask your permission to cite a figure in your paper, "Exercise, infection, and immunity", specifically your figure illustrating the "J" curve. I am using this figure in my thesis and need you to grant me written permission to use and cite it. Please e-mail me at your earliest convience with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt

--

David C. Nieman, DrPH, FACSM, Dept HLES, Appalachian State University
PO Box 32071, 111 Rivers St., HCC 038, Boone, NC 28608
828-773-0005 (mobile); 828-262-6318 (office); 828-645-5035 (home office)
828-262-3138 (fax); e-mail: niemandc@appstate.edu
OK with me

Best wishes, Peter Barnes  
Professor Peter J Barnes DM, DSc, FRCP, FMedSci, FRS  
Head of Respiratory Medicine, Imperial College London  
Airway Disease Section, National Heart & Lung Institute, Dovehouse St, London SW3 6LY, UK  
phone: +44(0)207 351 8174; fax 0207 351 5675  
email: p.j.barnes@imperial.ac.uk  
http://www1.imperial.ac.uk/medicine/people/p.j.barnes.html

-----Original Message-----
From: Hewitt, Matt [mailto:MHewitt@PHYSIOLOGY.UAB.EDU]
Sent: 08 September 2008 21:34
To: Barnes, Peter J
Subject: Permission to Cite Figure

Dr. Barnes,

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham. I am writing to ask your permission to cite a figure in your paper, "Corticosteroid effects on cell signalling", specifically figure 3. I am using this figure in my thesis and need you to grant me written permission to use and cite it. Please e-mail me at your earliest convinence with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt
Matt,
sorry for the slow reply, I was on vacation. I am happy for you to cite the figure from IL-12p40: an inherently agonistic cytokine. Do you have interesting data regarding IL-12p40? We have a new paper coming out in JI regarding IL-12p40 and Yersinia pestis, I can send you a preprint if you like.

Andrea
Andrea Cooper, PhD
Member,
Trudeau Institute, Inc.
Saranac Lake, NY 12983
518-891-3080

>>> "Hewitt, Matt" <MHewitt@PHYSIOLOGY.UAB.EDU> 09/08/08 4:11 PM >>>
Dr. Cooper,

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham. I am writing to ask your permission to cite a figure in your paper, IL-12p40: an inherently agonistic cytokine, specifically Figure 1. I am using this figure in my thesis and need you to grant me written permission to use and cite it. Please e-mail me at your earliest convenience with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt
Matt,

I am very happy for you to use the Figure in your thesis.

Good luck with the defence!

Kind Regards,

Malcolm Johnson
Global Director of Respiratory Science,
Respiratory Medicines Development Centre,
Visiting Professor, NHLI, Imperial College, London.

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Dr. Johnson,

My name is Matthew Hewitt and I am a graduate student at the University of Alabama at Birmingham. I am writing to ask your permission to cite a figure in your paper, "Molecular mechanisms of beta(2)-adrenergic receptor function, response, and regulation", specifically Figure 3. I am using this figure in my thesis and need you to grant me written permission to use and cite it. Please e-mail me at your earliest convenience with your decision regarding this matter, thank you for your time and I look forward to hearing from you soon.

Matt