FINDING THE SIGNAL WITHIN THE NOISE:

INVESTIGATING THE INTERACTION BETWEEN HISTAMINE, EXERCISE, AND INFLAMMATION

by

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DISSERTATION ABSTRACT

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Histamine is commonly associated with immune responses, typically involved in allergic reactions. It can be released in multiple tissues, including skeletal muscle during exercise; however, it is unknown what it is specifically about exercise that causes the release of histamine. In addition, the role of histamine in inflammatory responses implies that it may be involved in the acute post-exercise inflammatory response. Histamine's role and context outside of exercise, such as its role in mediating sustained post-exercise vasodilation, has guided the creation and interpretation of the studies within this dissertation. This dissertation has two major aims, with multiple experiments addressing the first aim and one larger study addressing the second aim. The first aim was to determine what is the exercise "trigger" that causes the release of histamine during exercise in skeletal muscle. The data gathered from the studies within this aim suggest that increased skeletal muscle temperature, which occurs during exercise, may be the exercise trigger that is resulting in the observed increase in skeletal muscle histamine during exercise. Furthermore, data from Chapter V demonstrates there is a local factor or factors released from skeletal muscle during exercise that causes mast cells to degranulate more so than in resting conditions. Finally, the second aim sought to determine the role of

histaminergic signaling in the acute post-exercise inflammatory response to aerobic exercise in untrained individuals. Our data suggests that histamine H_1/H_2 receptor antagonism using over-the-counter medications does not augment the systemic inflammatory response, out to 48 hours, following a novel bout of exercise in untrained individuals. While there was no effect of histamine receptor antagonism on systemic circulating inflammatory factors, future work should investigate how histamine receptor antagonism may affect inflammatory responses in skeletal muscle where histamine is formed and released. The findings of the studies within this dissertation highlight the interaction between exercise and histamine release in skeletal muscle. Further research expanding upon these findings will help to elucidate all the factors of exercise that may influence the release of histamine in skeletal muscle and the role of histaminergic signaling in the inflammatory milieu of skeletal muscle following exercise.

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CHAPTER I
INTRODUCTION

Background and Statement of the Problem

Our laboratory has investigated cardiovascular regulation during the recovery from aerobic exercise for nearly two decades. Within the vasculature of previously exercised skeletal muscle, there is a physiological response termed sustained post-exercise vasodilation, in which blood flow leading to exercise skeletal muscle is elevated above resting levels for up to 2 hours following the cessation of exercise (65). This elevated blood flow in previously exercised skeletal muscle can also lead to systemic changes, such as a lowered mean arterial blood pressure which also can last for hours following exercise (90, 91). This lowered mean arterial blood pressure can be particularly beneficial in populations with elevated blood pressure (hypertension). With whole body aerobic exercise, this physiological response is mediated by both neural and local mechanisms. The neural mechanisms involve central resetting of the arterial baroreflex and reduced sympathetic vascular transduction (48). Whereas, our lab and others have demonstrated that the local vascular component is vastly dependent upon histamine H_1 and H_2 receptor activation. Our lab has shown the importance of histamine and histaminergic signaling, as post-exercise vasodilation is reduced by 80% following 60 mins of cycling exercise using histamine H_1/H_2 receptor antagonist over-the-counter medications (94). Furthermore, we have shown that this pharmacological intervention abolishes the post-exercise vasodilatory response following smaller muscle mass isolated exercise, such as unilateral dynamic knee extension exercise (7). Our lab has established an important role for histamine and histaminergic signaling in mediating the local

mechanisms regulating the typically observed post-exercise vasodilatory/hypotensive response following aerobic exercise. However, there still remains much to be learned about the formation of histamine in response to exercise and its role in the exercise responsome.

Histamine is a biogenic amine that is typically associated with immune and inflammatory reactions (37). While histamine concentrations in plasma are moderately elevated or not changed at all during or following exercise (37, 163); our lab has observed that histamine concentrations increase locally in active skeletal muscle during and following unilateral dynamic knee-extension exercise measured via intramuscular microdialysis (127). The source of this histamine may be from one of two sources: mast cell degranulation which releases histamine from its granules and/or *de novo* formation (104). The synthesis and release of histamine from mast cell degranulation typically occurs by antigen-immunogloblin interaction during allergic reactions, however mast cell degranulation can be initiated from non-immunologic stimuli such as hypoxia, vibration, chemokines, and other factors that are often associated with exercise (9, 55). *De novo* formation is reliant upon activation of the enzyme histidine decarboxylase (HDC), which converts the amino acid L-histidine to histamine (132, 133). This occurs secondary to increased translation, transcription, and activation of the HDC enzyme. Similar to stimulation of mast cell degranulation, histamine formation via HDC typically occurs in response to similar exercise-related stimuli such as hypoxia, changes in cytokine concentrations, and alterations in temperature among others (102, 129). In both methods of histamine formation and release, exercise related factors have been proven to be potent stimuli. While our lab has been able to demonstrate that histamine concentrations rise in

active skeletal muscle in response to exercise, it is still unknown what it is about exercise that is causing this increase in skeletal muscle histamine. There are many physiological factors that change during exercise systemically and locally in skeletal muscle that could be responsible for this histamine release. The question remains, what is it about exercise that causes the increase of skeletal muscle histamine? Is there an "exercise factor" that causes mast cells to degranulate and/or causes an increase the enzymatic activity of HDC?

In addition to histamine playing a significant role in the post-exercise vasodilatory and the post-exercise hypotensive response, our lab has also demonstrated that histaminergic signaling may impact the exercise transcriptome in skeletal muscle following exercise (126). We had subjects perform 60 minutes of dynamic unilateral knee extension exercise and obtained skeletal muscle biopsies before and 3 hours following exercise. RNA sequencing was performed on the skeletal muscle biopsy tissue to examine changes at the transcriptome level. We observed that in previously exercise skeletal muscle, over 3000 protein-coding genes were affected by the bout of exercise demonstrating a profound effect of exercise on the transcriptome of skeletal muscle. Furthermore, within these protein-coding genes affected by exercise, 795 of those genes were differentially expressed in subjects under histamine H_1/H_2 receptor antagonism. These genes included mediators and factors involved in vascular function, metabolism, and inflammation. Some of the genes that were differentially expressed are involved in the acute post-exercise inflammatory response, with some having important roles in both upregulating and mediating the pro- and anti-inflammatory responses to exercise. These

data suggest that histamine and histaminergic signaling might play an important role in the acute post-exercise inflammatory response.

Following exercise in healthy humans, there is a tight balance of pro and anti-inflammatory responses that serves to clean debris from damaged muscle, repair, and adapt for future exercise sessions (118). This inflammatory response can be magnified depending on the type of exercise and/or the training status of the individual (59). The inflammatory cascade in response to exercise has been studied for decades and continues to be investigated, as we are just beginning to understand the regulation and modulation of this delicate balance and response. As previously mentioned, our lab has shown that ingestion of histamine H_1/H_2 receptor antagonist medications results in significant changes in skeletal muscle mRNA inflammatory marker expression, suggesting a possible role for histamine and histaminergic signaling in the post-exercise inflammatory response. However, this data was localized to skeletal muscle and only measured out to 3 hours following exercise. The inflammatory response to a novel bout of exercise typically lasts 24-48 hours (164), involves a systemic inflammatory response, and we do not know how histamine receptor antagonism may affect inflammatory signaling in this longer but still considered acute post-exercise window. Given the data collected, it has provided evidence that histamine and histaminergic signaling may play a role in the post-exercise inflammatory response and antagonizing its action on receptors may impair or enhance the typical response. We observed that histamine receptor antagonism affected expression of inflammatory markers in skeletal muscle. However, we only measured out to 3 hours following exercise and the post-exercise inflammatory "window" typically lasts 24-48 hours following cessation of exercise. Therefore, the question remains to be fully

answered, does histamine receptor antagonism result in an augmented acute post-exercise inflammatory response following aerobic exercise?

The studies detailed in this dissertation were designed to provide novel insight into the overall role of histaminergic signaling in response to exercise and its role in acute post-exercise inflammation. As previously mentioned, it is known that histamine is released from skeletal muscle during exercise and that it plays a role in sustained post-exercise vasodilation, however gaps remain in our understanding the full impact of exercise induced histamine release and signaling. The studies detailed were designed to address how histamine is involved in exercise in multiple facets: including what about exercise is causing histamine to be released in skeletal muscle and how histaminergic signaling may impact inflammation following exercise. In chapter IV, we examined if increased temperature is the exercise-related factor mediating the release of histamine in skeletal muscle during and following exercise. In addition, we aimed to investigate if increasing temperatures to physiological values resulted in increased mast cell degranulation in vitro. In chapter V, utilizing in vivo and in vitro techniques, we examined if there was a/were factor(s) released locally in exercising skeletal muscle that caused increased mast cell degranulation above basal levels, in turn causing the release of histamine. To investigate the role of histaminergic signaling in post-exercise inflammation, in chapter VI we examined if H_1/H_2 receptor antagonism using over-the-counter medications augmented the acute post-exercise inflammatory response to a novel bout of exercise in untrained individuals.

Purposes and Hypotheses

This dissertation was conducted in order to test the following hypothesis:

1. In chapter IV, the purpose of these set of studies was to determine if increased skeletal muscle temperature during exercise is the "exercise factor" that results in elevated skeletal muscle histamine concentrations. We hypothesized that passively increasing skeletal muscle temperature to temperatures observed during exercise would cause an increase in skeletal muscle histamine concentrations measured in skeletal muscle dialysate. In a follow up study, we hypothesized that in-vitro, physiological step-wise increases in temperature would increase mast cell degranulation in isolated human mast cells.

2. In chapter V, the purpose of this study was to determine if there is a/are local factor(s) produced in exercising skeletal muscle that cause increased mast cell degranulation above basal levels. We collected skeletal muscle dialysate from both the exercising and resting leg during dynamic unilateral knee extension exercise. We hypothesized that dialysate collected in vivo from exercising and previously exercised skeletal muscle would increase mast cell degranulation, measured in vitro by β-hexosaminidase release, compared to dialysate from resting skeletal muscle.

3. In chapter VI, the purpose was to determine the impact of histamine receptor H_1/H_2 antagonism on the acute post-exercise inflammatory response following a

novel bout of moderate intensity aerobic exercise in untrained individuals. We obtained venous blood samples from volunteers at multiple timepoints out to 48 hours following exercise to investigate the acute inflammatory response in placebo and histamine receptor antagonism conditions. We hypothesized that histamine receptor antagonism on H_1 and H_2 receptors would decrease the acute systemic inflammatory response in a novel bout of aerobic exercise in untrained individuals compared to placebo.

Significance

Our lab has shown that histamine increases in muscle in response to exercise and we have shown an important role for histamine in a multitude of exercise responses. We have shown that histamine receptor antagonism affects post-exercise vasodilatory responses, glucose uptake, endurance performance, pain perception, changes in exercise-related transcriptome changes. In light of all we know, there still remain gaps in our knowledge in this interaction. While we show that histamine increases in skeletal muscle with exercise, we still do not understand the exercise related cellular signaling mechanisms in skeletal muscle that cause the release of histamine and how it may affect other cellular signaling processes involved with exercise. These studies will help us to further understand the impact of histamine receptor antagonism on the exercise responsome.

In addition to their significance in understanding the science of how histaminergic signaling impacts exercise and vice versa, these studies are innovative as they can

possibly elucidate the link between histamine and post-exercise inflammation, this inflammatory response being an important component of exercise-induced adaptations. We have data that suggests that histamine receptor antagonism appears to affect inflammatory markers in skeletal muscle following exercise, however we have not examined the systemic response nor a longer-term window following exercise. Our previous work only examined out to 3 hours following exercise, while the inflammatory response to exercise can extend to 48 hours following exercise. During this window of time, there is a balance between pro- and anti-inflammation that is critical following exercise. This has become a popular area of current research examining post-exercise interventions which are designed to help individuals recover faster/better. Some of these interventions include icing, supplementation with nutrition, active recovery, and other approaches. These interventions may help the participant "feel better" however they may positively or negatively influence the post-exercise inflammatory cascade of events which in turn can affect the typical exercise associated adaptations (44, 143, 48). Using state-of-the-art methods, we aim to determine how histamine influences this inflammatory milieu in skeletal muscle following exercise and if blocking the actions of histamine has deleterious (or positive) ramifications on the acute inflammatory response.

Regarding post-exercise inflammation, further understanding of how antihistamines can affect the exercise response and inflammatory processes may help to shape and inform recommendations for their use in conjunction with exercise and exercise training. The inflammatory response following exercise is a tightly regulated process consisting of both pro- and anti-inflammatory responses that can be impacted by multiple factors including diet, age, training status, medications and others (27, 92, 136).

The post-exercise inflammatory response involves a host of cytokines and other cells types that are mobilized from bone marrow and the spleen, to repair damaged skeletal muscle. This mobilization and movement into skeletal muscle is critical for proper repair and adaptations to occur, and it is possible given its nature that histamine might play a role in this immune response. Given what our lab has found thus far and what questions remain, we designed these sets of experiments in order to address the significant gaps in our knowledge regarding the interaction of histamine, exercise, and inflammation.

CHAPTER II
REVIEW OF THE LITERATURE

Histamine and Histamine Receptors

Allergic reactions are due to complex interactions between different types of inflammatory cells: including basophils, neutrophils, eosinophils, and mast cells (157). All these cells produce different inflammatory mediators and the biogenic amine histamine derived from mast cells plays an important role in allergic and inflammatory responses. Amongst these allergic responses include the maturation and activation of leukocytes and chemotaxis, the process of movement of cells being mediated by histamine (165). Histamine is considered to be a primordial signaling molecule, as it is present in single and multiple cell organisms and has a molecular mass of 111 Dalton (103, 46). Histamine is intricately involved in both pro-inflammatory and anti-inflammatory responses which is highly dependent upon the receptor subtype and the cell types that are stimulated (108). Some of the functions of histamine include neurotransmission, chemotaxis, phagocytosis, mediation of urticaria, and cell growth (18, 151). Beyond these allergic and inflammatory reactions, histamine and histaminergic signaling is involved in a vast number of cellular processes and other physiological responses. In the line of physiological responses beyond inflammation, early work from Best and colleagues discovered that histamine is formed endogenously within the lung and liver and it has a potent vasodilatory effect (12).

Histamine has four known receptor subtypes (H_1-H_4) and can act in paracrine and/or autocrine signal manners with all these receptor subtypes belonging to the G protein coupled receptor family (144, 157). These histamine receptor subtypes are

characterized by their structure, function, and their affinity to histamine. The H_1 receptor is expressed in a vast array of tissues, including nerves, endothelial cells, dendritic cells, and respiratory endothelium. Given the location of these receptors, the H_1 receptor has roles in nociception, vasodilation, and bronchoconstriction (5, 21). H_2 receptors are found in various tissues, including dendritic cells, gastric parietal cells, and vascular smooth muscle. The H_2 receptor subtype mediates gastric acid secretion, regulating cell growth and maturity, and airway mucus production (159). The use of histidine decarboxylase knockout mice ($HDC^{-/-}$) has demonstrated a critical role of H_2 receptors. In this murine line, the enzyme histidine decarboxylase is lacking, which leads to structural abnormalities of mast cells and abnormal histamine formation to where the lack of histamine leads to a downregulation of H_2 receptors, in a tissue-specific manner (106). The H_3 receptor is primarily located throughout the central nervous system, including areas such as the cerebral cortex, hippocampus, and striatum (144). It has been demonstrated that histamine signaling via H_3 receptors plays an important role in neural function and neuro-inflammatory diseases. H_4 receptors were recently discovered in the past 20 years and are primarily expressed on leukocytes (70, 168). Mast cells can be activated by histamine binding to H_4 receptors on leukocytes, which can then lead to a robust inflammatory cascade which leads to a migration of inflammatory cells to the site of inflammation. Thus, histamine and the binding of histamine to H_4 receptors has been identified as having an important role in the inflammatory response.

Sources of Histamine

Histamine has two sources that have been clearly established in the research literature. It can be released from mast cells via degranulation, which is a cellular process in which molecules (including histamine) are released from secretory vesicles found inside the cells (83). The other source of histamine is *de novo* formation via an enzyme termed histidine decarboxylase (HDC) which converts the amino acid L-histidine to histamine (65). Mast cells were first described in 1878 in a doctoral thesis by Ehrlich and given the name "Mastzellen" which literally translates to well-fed cells (82, 8). This is due to their cytoplasm being stuffed with granular material and their unique staining characteristic when examined under a microscope in which the granules are easily observed. Mast cells play an important role in inflammatory and immediate allergic reactions. Typically, mast cells are localized to tissue and not found circulating in the blood stream under normal physiological conditions (29). Depending on their tissue location, they can possess unique characteristics as the mast cells that are present in the skin are slightly unique from mast cells that are located in the gastric mucosa (98). The cytoplasm of mast cells contains 50-200 large granules that store a host of inflammatory mediators, including histamine (97). Mast cells can migrate to target tissue locations, mediated by integrins, chemokines, and cytokines. HDC is expressed in a vast number of tissues in which it plays an important role in histamine production. The molecular weight of HDC is approximately 53-55 kDa and the active form of HDC is a homo-dimer (102). HDC can be expressed in mast cells and basophils and is regulated transcriptionally with cell differentiation and maturation. In the case where histamine is synthesized by HDC and not released from mast cell degranulation, this histamine is termed 'nascent

histamine' and the histamine in tissues from this process are typically in the uM range (129). Activity of HDC can be induced due to a list of different stimuli, including endotoxin-induced inflammation, dermatitis, and allergic inflammation. Given all of this, histamine plays an important role in a host of different roles and has multiple functions ranging from inflammation to vascular permeability and even functions involved in central nervous system responses.

The Role of Histaminergic Signaling in Post-Exercise Responses

As previously mentioned, histamine is a primordial signaling molecule that can exert its effects via multiple different cell surface receptors (160). Often, since histamine is associated with allergic reactions, medications are taken to block the associated symptoms with which histaminergic signaling results in. Individuals may suffer from seasonal allergies and may take histamine receptor antagonists, commonly referred to as antihistamines, to stop the common symptoms (140). Over the counter histamine receptor antagonists such as Allegra (histamine H_1 receptor) and Zantac (histamine H_2 receptor) are among the most widely used medications globally (44). While these medications have been shown to effectively reduce the binding and signaling of histamine on its cell receptors, much is still to be learned about the role of histamine within the exercise responsome.

Of the previously discussed four histamine receptor subtypes, H_1 and H_2 receptors are the most characterized. These two receptor types are found throughout, and particularly found within skeletal muscle vasculature including, but not limited to, endothelial cells, vascular smooth muscle cells, and pericytes (100, 89). Our lab has data

demonstrating that during exercise, histamine is released from skeletal muscle (127). As previously discussed, this release can occur via mast cell degranulation and/or de novo formation via the enzyme histidine decarboxylase. Once released, this histamine can then activate local H_1 and H_2 receptors. The exercise-induced release and binding of histamine causes vasodilation via decreased intracellular calcium levels in smooth muscle (68) and increased formation of local vasodilator substances from the endothelium (156). Given the locations of these H_1 and H_2 receptors, the binding of histamine to these receptors in response to exercise, and consequently blocking them, can have significant effects on the typically observed post-exercise vasodilatory responses.

While typical physiological responses during exercise have been extensively studied, there is an intriguing phenomena post-exercise that occurs known as post-exercise hypotension. For many years, the Halliwill Lab has focused on sustained post-exercise hypotension, which is typically a result of sustained post-exercise vasodilation which is defined as increased conductance and blood flow to previously active skeletal muscle. This post-exercise phenomenon can last for several hours. By having subjects use histamine receptor antagonists during exercise, our lab has shown that about 80% of the observed sustained post-exercise vasodilation can be attributed to the activation of histamine (H_1 and H_2) receptors (86, 94, 60) . This research has culminated in a take-home message that histamine and histaminergic signaling is important in exercise-induced vascular responses. We and others have demonstrated that histamine plays an important role and is released locally in skeletal muscle in response to exercise, but there is still a gap in the literature regarding what it is about exercise that causes this increase

in skeletal muscle histamine. What is the factor or the factors that are unique to exercise that are responsible for this rise in histamine?

Exercise Factors

During exercise, there are both changes systemically as well as local changes in the exercising tissue. These can be termed as exercise "factors" as they are altered by exercise and have downstream effects on the active tissue, other tissue, and certain cell types. Some of these factors include changes in pH, increase of temperature in both the core and working skeletal muscle, increase in reactive oxygen species, and release of cytokines from exercising skeletal muscle (1, 51, 153). While there is a plethora of research on the changes that occur during exercise, which of these factors is/are the most important and how they change in response to exercise is still being investigated.

The change in pH in response to exercise and its effect on contractile properties in skeletal muscle has been extensively studied over the past century, originating in work done in skinned fibers from frogs (124). Both resistance based and aerobic based exercise have shown to shift the blood pH to more acidic, this shift being dependent both upon intensity and duration. At lower and slower running speeds, lactate is removed from the muscles as quickly as it produced, with typically no appreciable decreases in pH. Below work rates of 50-60% VO2max, muscle lactate and pH does not typically change significantly. However, it has been reported that exercise above 60% VO2max for durations longer than 30 minutes have shown significant reductions in blood and muscle pH. In addition, resistance exercise has been demonstrated to alter pH levels, however these protocols are at higher workloads, such as 60-80% of 1 rep-max.

During both aerobic and resistance based exercise, the accumulation of CO_2 and lactate can reduce cellular pH in exercising skeletal muscle, and acidosis has been shown to have a direct vasodilatory effect on vascular smooth muscle cells (88). In addition to this, alterations in pH can affect vascular K+ channels and can possibly activate sensory nerve endings within the muscle interstitium (68, 149). The changes in pH during exercise has primarily been measured in venous blood pH, however more recent literature has begun to investigate the interstitial pH changes in skeletal muscle during exercise. It can be and has been measured with near-infrared spectroscopy (NIRS) and by microdialysis combined with the use of the pH-sensitive fluorescent dye BCECF (148, 93).

In relation to aerobic based exercise, interstitial pH in exercising skeletal muscle has been shown to decrease gradually during exercise and is reduced in proportion to power output. In order to mitigate this decrease in pH, plasma bicarbonate can act to buffer the changes in pH (49). In addition, aerobic-based sport athletes have utilized sodium bicarbonate as exogenous buffering agent to mitigate the changes in pH in order to improve performance (24). Indeed, sodium bicarbonate has been used by athletes for decades as it has been shown to improve performance in cycling athletes and can be considered an ergogenic substance. While it has been shown to improve performance by possibly affecting pH concentrations, the dosage of sodium bicarbonate must be carefully selected as it can produce a great deal of gastrointestinal stress (75, 73, 87). pH has been shown to play an important role in exercise responses and altering pH utilizing buffering agents has been shown to improve performance in certain circumstances, however this evidence is not incontestable. What is incontestable is that pH is altered by exercise, is

dependent upon type, intensity, and duration of exercise, and the change in pH can have a multitude of effects on active skeletal muscle.

Another factor that is impacted or influenced by exercise is the creation of reactive oxygen species (ROS). During normal cellular function and particularly during exercise, free radicals are generated. These free radicals can be both beneficial and can also be toxic if not able to be processed appropriately by the body. When there is an accumulation of ROS, this can lead to oxidative damage or also known as oxidative stress. This can occur during exercise, and ROS can be involved in the regulation of exercise-induced mitochondrial biogenesis via peroxisome proliferator activated receptor y coactivator-1-a (PGC-1a)(79, 6). ROS generated during exercise can have been shown to have a multitude of effects on different cell types during and following exercise, ROS can be damaging to tissues if left unchecked by ROS-scavenging mechanisms (35).

Our lab has investigated the role of oxidative stress during exercise on histamine release. Subjects performed 60 minutes of unilateral dynamic knee extension exercise at 60% of peak power (125). Subjects performed this exercise under three different conditions: control, i.v. ascorbate infusion, and ascorbate infusion plus oral H_1/H_2 histamine receptor blockade. Post exercise-vasodilation did not differ between ascorbate and ascorbate plus H_1/H_2 blockade. Since it is possible that ascorbate can catalyze the degradation of histamine in vivo, a follow up study was performed with infusions of the antioxidant N-acetylcysteine prior and during exercise. Post-exercise vascular conductance was similar for control and N-acetylcysteine conditions. These data suggest that exercise-induced oxidative stress does not appear to contribute to sustained post-exercise vasodilation. Therefore, we can confidently “check off” oxidative stress as an

exercise factor that mediates histamine release and histaminergic signaling that regulates post-exercise blood flow responses. with no effect on the histaminergic signaling pathway suggested that the oxidative stress associated with exercise is not necessary for histamine release. While we have been able to rule out this factor, there are still other signals/factors that are present during exercise that may lead to this increase in histamine release. Elevated temperatures have been shown to induce mast cell degranulation as well as having transcriptional and translational effects on other cytokines/soluble factors that can be found within skeletal muscle, thus can be a likely candidate as an exercise factor causing release of histamine (97).

Both during aerobic exercise and resistance-based exercise, there are increases in both core temperature and the temperature of exercising skeletal muscle. Similar to other factors, this heat production will be highly dependent on intensity and duration. Kenny et al showed that with treadmill running exercise, intensity affected the change in temperature elevation. In subjects that ran for 15 minutes at 70% VO2 max, core temperature was elevated 0.97 degrees C and those that ran at 93% VO2max, temperature was increases by 2.17 degrees C from baseline. These observations have been corroborated from others, similar to changes in pH, highlighting a “threshold” for appreciable changes in temperature and intensity/duration dependence on changes in temperature.

In skeletal muscle, metabolic heat production is increased during exercise above resting levels and regulating temperature requires a close coordination of heat-loss responses. Exercise hyperemia increases convective heat exchange and dissipates some, but not all of the heat produced during muscular contraction. Extensive work by Saltin et

al. reported that intramuscular temperature was increased in response to aerobic unilateral dynamic knee extension exercise and that the gradient between deep and superficial skeletal muscle is maintained during submaximal exercise (128). Work from Taylor et al has indicated that intramuscular temperature increases in response to exercise and will eventually plateau at an equilibrium temperature (77). Thermosensitive elements have been suggested to exist within skeletal muscle, and muscle temperature can also modulate the discharge frequency of groups I-IV muscle afferents, which are involved in pain and pressure perception (71, 154). Increases in skeletal muscle temperature have been shown to have direct effects on skeletal muscle properties and processes involved in exercise, such as excitation-contraction coupling, enzymatic activity, as well as cross-bridge cycling which can affect force production (22, 53). In addition to its effects on properties, the increase of temperature on enzyme activity can increase the rate of reaction of most enzymes. Changes in temperature in skeletal muscle have been shown to alter activity of enzymes that are involved in skeletal muscle metabolism, including lactate dehydrogenase, citrate synthase, and hydroxy acyl dehydrogenase (40,30). Alterations in the enzymatic activity of these particular enzymes can have profound effects on metabolism and fuel utilization which then will directly impact exercise performance. Regarding HDC, in isolated cell preparations, temperature has been shown to influence the enzymatic velocity of HDC (133). With increasing temperatures, there is a linear increase in HDC activity up to a point where it reaches a plateau. Since HDC is involved in histamine production, it makes temperature and heat production from exercise in skeletal muscle a likely candidate for the observed release of histamine in skeletal muscle that has not yet been investigated. Therefore, a gap exists in the literature examining the

role of increased skeletal muscle temperature in response to exercise on skeletal muscle histamine release. Therefore, in this dissertation we propose a set of studies that will investigate if heat and local factors released in muscle cause the release of histamine in skeletal muscle during exercise.

Exercise and Inflammation

During and following exercise (whether it be aerobic or resistance), there is an inflammatory response that occurs. Within this response, there are a multitude of cytokines, molecules, and cell types that are either up-regulated or down-regulated at different time points. The response of these factors are highly dependent on the type of exercise, the time course following the exercise, as well as the status of the individual who performed the exercise (i.e. trained, untrained, healthy, unhealthy, age etc) (164) These inflammatory responses are put into distinct classifications to categorize their actions, while of course there has been some debate in regards to some specific targets and their generalizable actions.

Following muscle damaging exercise (typically having to include eccentric or lengthening components), there is ultrastructural damage to the skeletal muscle that results in many consequences. Typically, there is a reduction in force output for hours to days depending upon the severity of damage, pain and tenderness localized to the exercise area, and an increase in inflammatory cells both in the area and systemically. The strength loss that is observed after eccentric muscle damage happens immediately, and can last up to one week as has been shown in some studies (4, 145). Often, these long delays in strength loss are due to major structural damage to muscle fibers, including hallmark observations such as Z-line streaming and sarcomere disruption (146). The

inability to produce similar force from baseline stems from many of the contractile proteins being damaged and a lack of stability of the actin/myosin interaction along with titin, due to damage to the Z-line. Some studies have even shown myofibrillar necrosis with a lack of regeneration even out at 10 days following a severe protocol (158), indicating that this structural damage has a major role in force production following an eccentric exercise protocol. This ability to produce force may stem from the pain, tenderness, and swelling that is often observed during the recovery period from damaging exercise. The pain and soreness that are typically associated with recovery from muscle damaging exercise have a few potential sources. Some have argued that the mechanical strain itself, the damage that has been caused to the tissue may sensitive pain nerve fibers (nociceptors) which gives the perception of pain. There are also theories as to how this pain is perceived, whether that be truly peripherally or if it is more centrally based with "perception and sensitivity" playing a role, however this is beyond the scope of this review (69).

Certain inflammatory mediators released within skeletal muscle may cause the release of nerve growth factor, which can alter the threshold at which pain is perceived and may result in more pain. Therefore, there may be a direct causal relationship between the amount of inflammation and the amount of pain perceived. As well, chemokines released in and around the muscle may stimulate more of these mediators, exacerbating the response. Therefore, the main causes of this pain observed may be directly from the tissue damage or due to the inflammatory, or possibly a combination of both (99, 134). Some studies have shown that the pain can be observed without any evidence of structural damage, indicating that structural disruptions are not obligatory for pain to be

observed. Similar to the strength decreases, pain sensations are typically elevated immediately following muscle-damaging exercise. This pain and soreness will typically peak around 2-3 days following the eccentric damaging exercise bout; however this may slightly shift due to protocol and subjects involved. The pain and soreness usually subside by day 5-7, however as previously mentioned this could last up to 10-14 days depending on the amount of damage that was done (43).

Following both muscle damaging and moderate to high-intensity aerobic exercise, leukocytes are recruited immediately the damaged muscle tissue. Most of these leukocytes are neutrophils, they are recruited to the area from chemotactic factors such as previously mentioned pro-inflammatory cytokines which have this ability. Within minutes of completion of exercise, possibly even during exercise, both neutrophils and monocytes are recruited to the damaged tissue, monocytes can then differentiate into macrophages to work with resident macrophages that may already be present. Their primary purpose is phagocytosis as well as releasing other proteolytic enzymes and other free radicals to upregulate the inflammatory environment present in that tissue (112). Following exercise, cytokines/myokines IL-6 is rapidly upregulated, being present immediately in skeletal muscle following damaging exercise. Other pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-a) and interleukin-1 beta (IL-1B) are typically upregulated a few hours following exercise, with some showing that this can occur within the first hour. Monocyte-chemoattractant protein 1 (MCP1) can be upregulated later in the 24-hour period following an exercise bout, and may remain elevated for days following. Other cells such as mast cells can migrate into the tissue within the first hour, releasing inflammatory mediators such as histamine and tryptase (107). While anti-inflammatory cytokines are

typically thought to help resolve the inflammation later on, their up-regulation is often seen within a few hours of exercise at lower levels, and ramping up as time moves on. Certain anti-inflammatory cytokines have been shown to increase within the first hour of exercise, and still be present up to a week following the damaging exercise. Near the end of the resolution, satellite cell activation and type 2 pericytes aid in the repair of muscle by increasing differentiation of myocytes, expressing factors such as myogenin and MyoD and ultimately leading to satellite cell quiescence at the complete resolution following the bout (166). Depending on the type of exercise and the amount of damage that has occurred, this can shift the expression and localization of certain cells, however this observation of an immediate response from neutrophils and pro-inflammatory cytokines followed by a recruitment of anti-inflammatory cytokines and resolution based cell types is the typical pattern. Pournot et al had subjects perform a muscle damaging protocol involving extended running that included level, up-hill and down-hill components (63). They examined both cytokine levels and mobilized leukocytes out to 96 hours post-exercise to investigate the time course changes. In their control group that did exercise with no intervention, they observed a rapid increase immediately post exercise in IL-6, IL-10, while c-reactive protein (CRP) was not elevated until 2 days later interestingly. IL-6 had decreased back down at 24 hours as well as IL-10, however IL-1Ra had a delayed increase as discussed before. Interestingly, there were no changes in TNF-a in the post-exercise period even out to 96 hours post. Both leukocytes and neutrophils were up post-exercise in the one hour period, however both were not significantly different at 24 hours and deceased from there. This initial pro-inflammatory response followed by and upregulation in IL-1Ra and then

decrease in leukocyte count follows the typical trends observed following damaging exercise.

With respect to inflammation, pro-inflammatory and anti-inflammatory are typical categories that the cell types are placed in to. Pro-inflammatory is referring to a cytokine or molecule that: initiates, amplifies, and/or progresses the inflammatory response (109). Pro-inflammatory mediators have specific actions on their own, they are also able to signal the release or produce other key inflammatory markers via intracellular signaling mechanisms. Anti-inflammatory mediators tend to inhibit the inflammatory response or inhibit the release of pro-inflammatory mediators. Sometimes this response is to serve as a controlling mechanism to the upregulation of pro-inflammatory mediators, working as a balance to keep inflammation in check following an insult (25). There is a delicate balance between the pro- and anti-inflammatory responses. In certain conditions and situations, an imbalance with unregulated pro-inflammatory responses is often linked to low-grade inflammation and deleterious consequences if left unchecked. This is important in the anti-inflammatory response as well, particularly after exercise. A dysregulated anti-inflammatory response could be negative as it may serve as an immune system depressant, possibly not allowing proper pro-inflammatory responses to occur or not allowing proper resolution of inflammation (138). Therefore, the post-exercise up- and down-regulation of inflammation, helps to clean cell debris in damaged tissue, set the stage to repair the muscle (as well as vasculature, tendons, etc) following the exercise bout, as well as allow appropriate adaptations to occur in preparation for the next bout.

The type of exercise can affect the inflammatory response to exercise, whether it be aerobic or resistance-based exercise. Regarding aerobic exercise, there appears to be a

"threshold" in intensity that needs to be met for leukocytosis and a response of exercise related cytokines. There is debate on what this threshold might be, with different percentages stated by different authors. In a meta-analysis, Cerqueira et al found that on average this "threshold" needed to be aerobic exercise above 64% VO2max (29). While changes were observed in more moderate exercise, exercise above this percentage resulted in greater elevations in white blood cells, and pro-inflammatory cytokines such as IL-6 and IL-8 (20). Studies that utilized models of eerobic exercise that was below the 64% VO2 max did not show consistent alterations in white blood cells or pro-inflammatory cytokines. A common marker of exercise-induced muscle damage is circulating levels of creatine kinase, an enzyme found in skeletal muscle that should not be found circulating. It was noted that short low intensity aerobic exercise did not alter creatine kinase levels, and only increased after intensive and long exercise bouts (36).

There is an inflammatory response to resistance-based exercise, this type of exercise usually involves extensive damage to muscle cells with disrupted contractile structures and other cytoskeletal components. While leukocytosis occurs in response to resistance exercise, it cannot be used as a reliable marker to quantify the amount of muscle damage that has occurred (95). Typically following intense resistance exercise, there is a release of creatine kinase into the blood stream, similar to the higher intensity aerobic exercise models. Elevations of muscle specific antigens (CD11b, CD15, CD56, CD163) are typically observed over a period of hours to days following resistance exercise and this elevation is typically dependent on the intensity of the muscle damage and exercise bout (96). Cytokines such as IL-6 and IL-8, which are primarily pro-inflammatory in their circulatory nature, are typically observed following strenuous

resistance exercise (115). Certain cytokines are expressed differently depending on the intensity and type of resistance based exercise. It has been shown that the kinetics of IL-6 response differ between concentric and eccentric exercise, with greater responses observed with eccentric exercise, which is more damaging to skeletal muscle (120). In line with this, following resistance based muscle damaging exercise, there is typically soreness and tenderness, termed delayed onset muscle soreness. The exact cause and mechanism of soreness has been and is still debated, however it appears that this is due the swelling of muscle fibers that activate nocioceptors, which may or may not be directly caused by inflammatory markers (169). Further work needs to be done to fully elucidate the role of inflammatory markers on muscle soreness following strenuous resistance-based exercise.

This time course transition from a primarily pro-inflammatory environment to more anti-inflammatory and resolute phase, in specifically referring to macrophages is known as the M1 to M2 shift. This has been shown to occur following both aerobic and resistance-based type exercise (52). This M title derives from macrophage and focuses on the population of macrophages. M1 macrophages are the CD86+ population and M2 are the CD163+ population, denoting a specific marker on their cell surface. CD86+ macrophages are upregulated early following exercise, typically within a few minutes. The CD86+ macrophages phagocytose cells, release free radicals to lyse cells and can alter the redox state of the skeletal muscle to aid in initial breakdown and response (51). These macrophages are also able to increase chemotactic ability of other cells and will help recruit other phagocytotic cells to the damaged tissue. The M1 population is upregulated quickly after exercise, will stay elevated for hours and typically decreases by

the 24-48 hour mark (161). Anti-inflammatory cytokines such as IL-10 and IL-4 can upregulate the differentiation of the M2 macrophages, which shift the inflammatory profile. The M2 population express the CD163+ marker and will typically replace the M1 macrophages, dependent on the amount of tissue damage that has occurred. While stimulated by cytokines, CD163+ macrophages can also promote the release of IL-10 and stimulate production of IL-1Ra, as well as promote angiogenesis and upregulate production of glycoproteins to aid in cytoskeletal matrix remodeling (51). This helps to bring resolution to the inflammatory response, inhibiting the initial pro-inflammatory response from CD86+ macrophages. The upregulation of these CD163+ macrophages typically occurs around 24 hours, reaching peak elevations around 48 hours and staying elevated until up to 96 hours in some studies (111). All of these are targets that we intend to measure in the proposed study, as they are common exercise-related inflammatory markers and our genomic data suggests there could be alterations in the post-exercise inflammatory profile under histamine blockade.

Following exercise, there is an upregulation of both pro- and anti-inflammatory responses and both are critical for recovery and adaptation to exercise. Data from our lab demonstrates that histamine blockade affects many of the genes that are associated with this post-exercise inflammatory response. Therefore, work is warranted to further examine the impact of histamine blockade on both the systemic and local inflammatory response to exercise.

Histamine and Post-Exercise Recovery/Inflammation

Work within our lab group has expanded to look at histamine's actions on a broader scale. We have shown that exercise stimulates the release of histamine from mast

cells within skeletal muscle (127), turning on a pathway that potentially triggers a broad range of beneficial cellular adaptations in response to exercise (126, 60). Blocking the actions of histamines has been shown to alter multiple typical physiological responses that are associated with exercise. Among these exercise responses are: post-exercise hemodynamics (94, 60, 7) , glucose delivery and insulin sensitivity in skeletal muscle (41, 114) and broad mRNA and protein abundance effects during the acute recovery from exercise (126).

This broad histamine footprint on the human exercise transcriptome leads to many interesting follow up questions. Part of these nearly 800 genes that were differentially affected by histamine receptor antagonism following exercise include vascular function, metabolism, and inflammation. Within the realm of inflammation, one of the top differentially expressed protein-coding gene that was downregulated by histamine receptor antagonist was IL-6. As previously discussed, IL-6 can be released by skeletal muscle and can have both pro-inflammatory and anti-inflammatory actions. In addition to IL-6, many of the other differentially expressed protein-coding genes are involved in cytokine and chemokine signaling. Therefore, these data suggest that histamine receptor antagonism may affect the post-exercise inflammatory response. However, as previously discussed, the acute inflammatory response to exercise lasts from 24-48 hours and these protein coding gene data were only collected until 3 hours following exercise. Therefore, it is unknown if histamine receptor blockade has longer term effects on post-exercise vasodilation. As well, systemic markers of inflammation measured by venous blood draws have not been measured after exercise under histamine receptor antagonism. Our histamine transcriptome data gives promising direction that

post-exercise inflammation is impacted by histaminergic signaling and warrants further investigation. In addition, the widespread use of histamine receptor antagonists in the public suggest the need to further examine the role of histamine in exercise responses.

CHAPTER III
EXPANATION OF THE METHODOLOGY

General Experimental Approach

All studies from the dissertation were conducted on the University of Oregon Eugene Campus in Esslinger Hall. All experimental visits were conducted in the Exercise and Environmental Physiology Laboratory utilizing the Evonuk Environmental Chamber for VO_{2peak} tests and the Exercise Lab for all other experimental procedures.

The study detailed in chapter IV was conducted in 3 parts. In part 1, an intramuscular thermocouple placed in the *vastus lateralis* of volunteers was used to measure skeletal muscle temperature during 60 minutes of unilateral dynamic knee extension exercise at 60% of peak power. In part 2, a skeletal muscle microdialysis probe was implanted in the right *vastus lateralis* to collect microdialysis effluent (dialysate) before and during 60 minutes of passive heating of the leg using pulsed short-wave diathermy. In addition, utilizing similar sterile insertion technique, an intramuscular thermocouple was also placed in the right *vastus lateralis* to monitor skeletal muscle temperature during the passive heating. Central hemodynamics (heart rate and blood pressure) were measured before and during exercise. In part 3, isolated human mast cells were exposed to varying physiological temperatures to investigate the direct effects of increased temperature on mast cell degranulation to further elucidate the role of temperature produced in exercising skeletal muscle on histamine release from mast cells.

In the study detailed in Chapter V, skeletal muscle microdialysis probes were placed in both the right and left *vastus lateralis* to collect skeletal muscle dialysate from subjects before, during, and after 60 minutes of unilateral dynamic knee extension exercise at 60% of peak power. In all subjects, the right leg served as the "exercise" leg and the left leg was the "rest" leg. Central hemodynamics (heart rate and blood pressure) were measured before, during, and after exercise. In vitro, isolated human mast cells were utilized to analyze mast cell degranulation when exposed to dialysate from different timepoints and exercise conditions (exercise leg vs rest leg, pre vs during vs after exercise).

In the study detailed in chapter VI, venous blood samples were collected from subjects before and after 60 minutes of cycling exercise at 60% of their VO_{2peak} once under placebo and once under H_1 and H_2 histamine receptor antagonism (using over-the-counter medications) to investigate the effect of histamine receptor antagonism on the acute inflammatory response to aerobic exercise. Subjects were given 4 weeks between their exercise visits, visits were block-randomized for drug conditions. Venous blood samples following exercise were obtained: immediately after,6,12,24, and 48 hours following exercise. Subjects also filled out 3-day food diaries to ensure diet was not significantly different on their two visits. Central (heart rate and blood pressure) and peripheral (femoral artery blood flow) hemodynamics were measured before and after each exercise session.

Subject Overview

All studies conducted in this dissertation were approved by the Institutional Review Board at the University of Oregon (Protocols #02022017.003 for experiments in Chapter VI and V and #02172011.029 for the experiment in Chapter VI). Studies were conducted in accordance with guidelines set forth by the Office for Protection of Human Subjects at the University of Oregon. Written informed consent from all subjects subsequent to a verbal and written briefing of all experimental procedures for a given study.

Twenty-eight (twenty-one men and seven women) healthy subjects participated in studies detailed in this dissertation (28 ± 6 years, 160.5 ± 13.5 cm, 72.5 ± 15.8 kg, 24.5 ± 4.1 BMI) (mean ± SD). Subjects were deemed healthy following a standard health screening. All subjects were required to abstain from caffeine, alcohol, and exercise for at least 24hr prior to all studies. Subjects reported to the lab after an overnight fast for all studies described in chapters IV, V, and VI. No subjects were using any over-the-counter or prescription medications at the time of the study, with the exception of oral contraceptives. Female participants were studied during the early follicular phase of their menstrual cycle or during the placebo phase of their oral contraceptive. All female subjects had a negative pregnancy test prior to all studies.

Specific Methodology

Subject Characterization

In the studies detailed in this dissertation, a combination of demographic and anthropometric measures including, age, height, weight, and BMI in addition to aerobic

capacity were used to portray the subjects and generalize the study findings. Subject weight was measured using a digital scale (Sartorius model MAPPIU-150FE-L, Elk Grove IL; Chapters IV, V, VI) and height was measured using a standard stadiometer (SECA North America, Chino CA; Chapters IV, V, VI).

Passive Heating Utilizing Pulsed Short-Wave Diathermy

As previously mentioned, study 2 in chapter IV examined collected intramuscular dialysate before and during 60 minutes of direct heating. There are multiple modalities that can be used for direct, passive heating of limbs. Some of these modalities include water immersion, using hot packs, as well as water-perfused suits (102, 103, 104). One of the goals of this study was to raise muscle temperature while minimizing the hydrostatic effects of water immersion and keeping a sterile field clear for placement of the intramuscular thermocouple and microdialysis fiber. For this study, we decided to utilize pulsed short-wave diathermy (PSWD) as it is a technique that has been safely and effectively used by the athletic training community to increase skeletal muscle temperature without adverse increases to skin temperature or surrounding tissues (147, 117, 64). PSWD uses high-frequency electromagnetic currents emitted from a drumhead to heat skeletal muscle tissue, typically for therapeutic reasons. PSWD heats in a much larger area than typical ultrasound methods and increases in skeletal muscle temperature from 4-6°C have been observed utilizing different settings (81). We used the Megapulse II, which is a PSWD unit model designed by Accelerated Care Plus, seen in **Figure 3.1**. Based on previously collected pilot data from study 1 in Chapter VI, our goal was to heat skeletal muscle to an "exercising temperature" of 39.7°C. In order to do this, the settings for the PSWD unit were set to 800 pulses per second for 400 microseconds (40-48 W

average power, 150 W peak power) for 60 minutes applied directly above the vastus lateralis. The drumhead of the PSWD was approximately 2 cm from the surface of the skin for all subjects and did not make direct contact with the skin. No subjects reported any adverse local or systemic effects during or after the PSWD session. This passive heating modality also has not been reported to have any direct systemic cardiovascular effects and we did not note any significant central hemodynamic changes during the passive heating with PSWD session.

Figure 3.1. Pulsed Short Wave Diathermy Unit. For study 2 in Chapter VI, pulsed short wave diathermy was used as a passive heating modality to directly increase skeletal muscle temperature. The model used was Megapulse II (Accelerated Care Plus

Skeletal Muscle Microdialysis

Utilizing skeletal muscle microdialysis, it is possible to collect extracellular fluid in vivo. The integrity of the microdialysis fibers is maintained during exercise, allowing the ability to sample the extracellular fluid from muscle during both moderate and high intensity exercise. The modern probe for microdialysis utilizes a hollow semi-permeable membrane which can be implanted safely into different tissues including skin, brain, and skeletal muscle (31, 137). There are different permeability options based upon their molecular weight cutoff that can be selected for the probe, with higher permeabilities allowing for larger molecules to pass through and smaller permeability options being more conservative and selective. These permeability options typically range from 3 to 100 kilodaltons (kDa). This semi-permeable membrane is connected to inlet/outlet tubing and a saline solution is used with a microdialysis pump to sample the extracellular fluid which is then collected in tubes connected to the outlet tubing. For these experiments, we chose to use the 63MD model with 100 kDa cutoff utilizing a non-linear probe as it was well-tolerated by subjects during exercise and the integrity of the microdialysis fibers survived during the repeated contractions from dynamic unilateral knee-extension exercise.

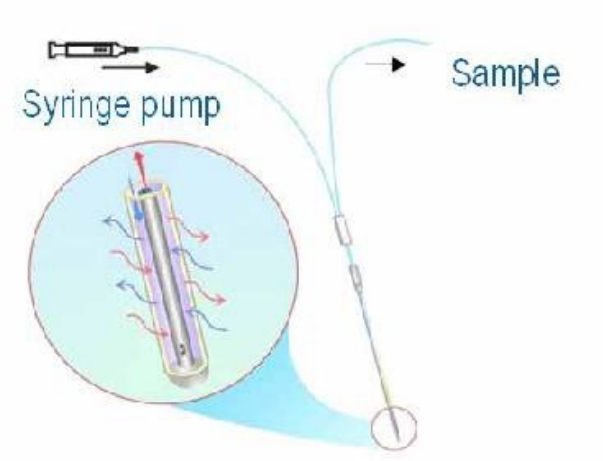

Figure 3.2. Intramuscular microdialysis fiber. The intramuscular microdialysis fiber consists of a semi-permeable membrane that is inserted into the skeletal muscle to collect extracellular fluid. Saline is pumped through inlet tubing via a syringe pump, mixes with the extracellular fluid, and dialysate (sample) is collected in a tube collected to the outlet tubing. (Note: Figure from ALEXYS Neurotransmitter analyzer, 4)

For experiments in chapters IV and V, all probes were implanted using sterile technique. The skin was anesthetized using Lignospan (Septodont Inc, PA) which is an injection mixture consisting of lidocaine hydrochloride 2% and epinephrine 1:100,000. The underlying fascia was anesthetized using (Septodont Inc, PA) which is a 4% prilocaine hydrochloride injection solution. Care was taken to ensure that prilocaine was not injected into the skeletal muscle and probes were inserted in a direction parallel with muscle fiber orientation. Muscle fiber orientation was assessed utilizing ultrasound technique before placement of fibers. The probes were held in place by covering the entry site with Tegaderm (3M), sterile transparent medical dressing. No probes ruptured or broke before, during, or after exercise in any of the subjects. The probe was perfused with a 0.9% saline at a rate of 5µl min using a microinfusion pump (CMA 400 Microdialysis pump, CMA, MA). This perfusion rate was chosen based upon successful experiments performed by our lab using this specific perfusion pump and rate.

As previously mentioned, dialysate from the skeletal muscle was collected into an outlet tube which consisted of a sterile microtube. The microtubes were covered with non-porous tape during the sampling period. For the experiment in chapter IV, samples were collected every 30 minutes before heating and every 20 minutes during passive heating. For the experiment in chapter V, dialysate samples were collected every 30 minutes before exercise, every 20 minutes during 60 minutes of exercise, and every 20 minutes following 60 minutes of recovery from exercise. For both experiments, microtube weight was documented before and after dialysate collected. Samples were stored at -80°C until analysis.

Intramuscular Thermocouple to Measure Intramuscular Temperature

For study 1 and study 2 in the experiments detailed in chapter IV, an intramuscular thermocouple probe was placed into the *vastus lateralis* to monitor intramuscular temperature. In its most basic definition, a thermocouple is a thermoelectric device used for measuring temperatures. Typically, a thermocouple is composed for two dissimilar metals that are joined together to form two junctions (105, 32). One junction is placed in the environment that is being measured, in this case the skeletal muscle, and the other junction remains at a known constant temperature. The change in temperature in the junction in the skeletal muscle is measured in millivolts as it flows through the loop, this is then measured utilizing software (Windaq) in which a given change in millivolts is equal to a change in temperature. For this experiment, we utilized the IT-17 (Physitemp, PA). We selected this particular probe for its accuracy (0.1°C in the physiological range according to the manufacturer), certification by the National Institute of Standards and Technology, and its common usage in the research

literature. This company utilizes copper and constantan as their wire metals. Sterile technique was used for the placement of the intramuscular thermocouple in to the *vastus lateralis*. The skin was anesthetized using Lignospan (Septodont Inc, PA) which is an injection mixture consisting of lidocaine hydrochloride 2% and epinephrine 1:100,000. The underlying fascia was anesthetized using (Septodont Inc, PA) which is a 4% prilocaine hydrochloride injection solution. Care was taken to ensure that prilocaine was not injected into the skeletal muscle and probes were inserted in a direction parallel with muscle fiber orientation. The probe wire is typically connected to a computer running Windaq software. For this experiment, we connected the probe wire to a portable thermocouple thermometer device to monitor skeletal muscle temperature. Temperature was constantly monitored throughout the experiment and recorded every 10 minutes during the passive heating protocol. An illustration of the intramuscular thermocouple provided by the manufacturer is below.

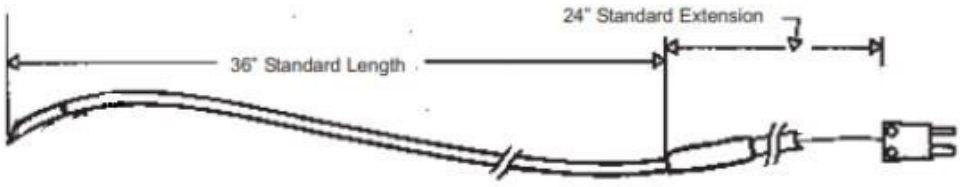

Figure 3.3. Intramuscular Thermocouple

Temperature is measured at the end of the probe, which is 36 standard length. The plug on the end of the extension was plugged in to a portable thermocouple thermometer to measure intramuscular temperature during the passive heating protocol. Illustration provided by Physitemp™

Exercise Interventions

Unilateral Dynamic Knee-Extension Exercise

Many labs, including ours, have utilized whole-body exercise such as treadmill running and cycling to examine exercise responses. There also exist exercise modalities that are aerobic in nature that require less muscle mass, such as unilateral dynamic knee-extension exercise (19, 3). This exercise model is unique and beneficial in that it isolates muscle activation to the quadriceps, allowing for targeted investigation of a single muscle group. As well, the other leg remains at rest so that intra-subject characterizations can be made. Our lab has shown an abolishment of the post-exercise vasodilatory response utilizing H_1 and H_2 histamine receptor antagonism following 60 minutes of dynamic unilateral knee extension exercise (7). This is different from the moderate intensity cycling response in which histamine receptor blockade results in an 80% reduction (94). With the abolishment observed following unilateral dynamic knee-extension exercise, the post-exercise vasodilatory response following this exercise modality is entirely histamine H_1/H_2 receptor dependent and is an optimal exercise modality for histamine related exercise research.

For study 1 described in chapter IV and the study described in chapter V, unilateral dynamic knee-extension exercise was performed on a custom-built knee extension ergometer based on a computer-controlled step-motor. This provided resistance against the subjects' lower leg on extension and allowed for passive relaxation. Power was calculated and maintained at the assigned level based on real-time measures of angular velocity and torque. Subjects were seated with their back at 60° upright and knee-extension exercise was performed with the right leg over a 45° range of motion, starting

with the leg hanging at 90° of flexion. Subjects maintained a cadence of 45 kicks per minute with visual feedback provided from custom software.

For study 1 in chapter VI and the study in chapter V, subjects performed 60 minutes of unilateral dynamic knee extension exercise at 60% of peak power. Peak power was determined during a maximal unilateral dynamic knee extension exercise test. To determine peak power, subjects performed a five-minute warm-up at a workload of 5 watts and then the workload was ramped incrementally at a rate of 3 watts per minute. Subjects were required to maintain power within 1 watt of the target workload in addition to maintaining a cadence within 5 kicks per minute. Cadence was set on a metronome to 45 kicks per minute. Subjects were encouraged to continue and performed the exercise until volitional fatigue. During the experimental visit of 60 minutes of dynamic knee extension exercise, subjects were required to maintain power within 1 watt of the target workload (60% of peak power) and also maintain a cadence within 5 kicks per minute.

Figure 3.4. Unilateral Dynamic Knee Extension Exercise. For study 1 detailed in Chapter IV and the study detailed in Chapter V, we used a custom-built ergometer for subjects to complete unilateral dynamic knee extension exercise. We have used this ergometer and methodology of exercise extensively in our lab to examine post-exercise hemodynamic responses

Peak Aerobic Power

For the study detailed in chapter VI, subjects were asked to perform an incremental cycle exercise test (Lode Excalibur, Groningen, the Netherlands) comprised of one-minute workload increments in order to determine their peak oxygen uptake (VO_{2peak}). After a two-minute warm-up period of light cycling (20-30 W), workload

increased by 25 W every minute. Subjects maintained 60 rpm throughout the test and whole-body oxygen uptake was measured with a mixing chamber (Parvomedics, Sandy, UT). The peak test was ended when subjects were no longer able to keep at least 60 rpm and subjects were given a cool-down period (50 watts) following the test. All subjects reached subjective exhaustion (rating of perceived exertion of 19-20) within the 8-12 minute period and peak aerobic power was determined to be obtained if the respiratory exchange ratio was greater than 1.10. True peak tests should be accompanied by high ratings of perceived exertion (19-20), heart rate (>95% of predicted), a respiratory exchange ratio greater than 1.0, and a plateau of VO_2 measures with increasing workloads. We utilized an open circuit technique for oxygen consumption measurement, this required knowledge of gas concentrations in the room air (inspired) and expired air. Open circuit spirometry was chosen for this study due to the ease of obtaining the measurement and the accuracy provided to determine the workload for the exercise intervention in Chapter VI.

Moderate-Intensity Dynamic (Cycle Ergometer) Exercise

For the study detailed in chapter VI, subjects returned to the lab to perform 60 minutes of seated cycling exercise on a stationary cycle ergometer at a workload corresponding to a steady state VO_2 of 60% of VO_{2peak}. This was estimated from the equation:

Estimated workload (watts) = (Target VO_2 (ml/min)/12) – 25

Steady state oxygen consumption was obtained within 3-5 minutes and the subjects remained there for the entirety of the exercise session. We chose this intensity

specifically since it produces a sustained post-exercise vasodilatory/hypotensive response that is blunted by H_1 and H_2 histamine receptor antagonism. Heart rate, blood pressure, and RPE were monitored throughout the exercise session.

Rating of Perceived Exertion

The concept of "perceived exertion" typically refers to the strain or exerted effort that is perceived by a subject regarding the work demanded on the musculoskeletal, cardiovascular, and pulmonary systems. Volunteers are typically asked to rate their exertion "right now" on a Likert scale. For the study detailed in Chapter VI, a 6-20 scale was used. The 6-20 scale was developed by Gunnar Borg in 1982 and is advantageous in that the numbers approximate heart rate (60-200 beats per minute)(15). Descriptions of perceived exertion are: 6 none; 7-8 very, very light; 9-10 very light; 11-12 fairly light; 13-14 somewhat hard; 15-16 hard; 17-18 very hard; and 19-20 very, very hard. While other exertion scales have been utilized (such as a 1-10 scale utilized in resistance training protocols), we chose the Borg 6-20 as it has been extensively used in the research literature as a reliable method to ascertain perceived exertion of subjects, particularly during aerobic exercise.

Hemodynamic Measurements

In the studies detailed in chapters IV, V, and VI all hemodynamic measurements were made in a thermoneutral room (23°C). Measurements were made with the subject supine before and after exercise and upright while performing the knee extension exercise or stationary cycling.

Heart Rate

Heart rate was monitored using a three-lead electrocardiograph (Tango+, SunTech Medical, Raleigh NC) in the experiments listed in chapters IV, V, VI. It is expressed as beats per minute (BPM).

Arterial Blood Pressure

Arterial blood pressure was measured in the right arm using an automated sphygmomanometer (Tango+, Suntech Medical, Raleigh NC). It is expressed as millimeters of mercury (mmHg). Mean arterial pressure was calculated as:

MAP = Diastolic BP + [Systolic BP – Diastolic BP/3]

Femoral Blood Flow

Our laboratory has used and has continued to use Doppler ultrasound technology for the assessment of femoral blood flow due to its lack of invasiveness and high accuracy. Classic techniques to measure blood flow in human physiology research include indicator infusion methods. These are based on a known infused indicator mixing in blood and blood flow is measured by the dilution of dye within the blood with some of these indicators being indocyanine green dye and Evans blue dye (58, 131). While easy to perform, these measurement approaches require invasive catheterization and steady-state conditions. We use Doppler ultrasound as it is a widely accepted and a reliable technique to monitor blood flow velocity. This method consists of using a probe, which produces sound waves that bounce off tissues and a 2-dimensional image is created from the difference between the outgoing vs incoming wave velocities. This image then allows for the measurement of arterial diameter and the shift in sound waves, known as the doppler

shift, is used to calculate the velocity of blood flowing past the probe. It provides high temporal resolution and continuous sampling (67). Due to its lack of invasiveness, high reliability, and consistency in accurate measurements it is used for our studies. Specifically, it was used to image skeletal muscle for microdialysis fiber/thermocouple placement in Chapter IV, microdialysis fiber placement in Chapter V, and for femoral blood flow measurements in Chapter VI.

For the study detailed in chapter VI, a linear array vascular ultrasound probe was used (9 MHz, Phillips iE33, Andover MA). An insonation angle of 60^0 was used to measure blood flow in the common femoral artery, approximately 2-3 cm proximal to the bifurcation. The Doppler ultrasound machine was interfaced with a computer running custom audio recording software. Velocity measurements were determined using an intensity-weighted algorithm (Duc2, custom software), subsequent to demodulation of forward and reverse Doppler frequencies. Femoral diameter was measured in triplicate during diastole following velocity measurements. The diameter measurement of the common femoral artery was made 2-3 cm proximal to the bifurcation of the superficial and deep femoral artery branches of the leg. Femoral blood flow was expressed in ml per min and calculated using the following equation:

$$FBF = \pi(\text{diameter}/2)^2 \times \textit{mean blood velocity} \times 60$$

Femoral blood flow is expressed in $ml*min^{-1}$, mean blood velocity is in $cm*s^{-1}$, femoral diameter is in cm, and 60 was used to convert from $ml*s^{-1}$ to $ml*min^{-1}$. Femoral vascular conductance was used to account for arterial blood pressures influence on femoral blood flow. Vascular conductance was expressed as ml per min per mmHg and calculated using the following equation.

FVC = Femoral Blood Flow/Mean Arterial Blood Pressure

Venous Blood Sampling

In the study detailed in Chapter VI, blood samples were collected using a venipuncture method to collect blood from the antecubital space of the right and left arm. Blood was collected before, immediately after, and 6, 12, 24, and 48 hours after exercise using a Safety-Lok blood collection set (Becton, Dickinson and Company, Franklin Lakes NJ). Blood was collected into 3 mL vacutainers that were coated with K2EDTA (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). At each collection point, 2 tubes were centrifuged for 10 min at 1000 RCF and plasma was extracted and frozen at -80°C until further analysis. At each time point, one tube was processed for quantification of white blood cell populations via flow cytometry (see Assays and Analysis for further details).

Oral Drugs

Oral antihistamine drugs were used in the study detailed in chapter VI. Histamine H_1 and H_2 receptors were blocked using 540 mg fexofenadine and 300 mg ranitidine. 3 subjects in the study of chapter VI were given 40 mg famotidine in place of 300 mg ranitidine. Famotidine was substituted in place of ranitidine due to a voluntary FDA recall on ranitidine. Published pharmacokinetic studies performed by others assured us that we would have similar results for H_2 receptor antagonism using 40 mg famotidine in place of 300 mg ranitidine. We have shown that this combination of fexofenadine and ranitidine blunts the sustained post-exercise vasodilation following unilateral dynamic knee extension exercise. Responses are 90% inhibited within 1 hour and remain inhibited

6 hours after administration. Time to peak concentration and half-life for the medications used: fexofenadine is 1.15 hours with a half-life of 12 hours, ranitidine is 2.2 hours with a half-life of 2.6 hours, famotidine is 1.5 hours with a half-life 3 hours. None of these drugs have any known direct cardiovascular effects when they are administered in resting situations, do not elicit any major cardiovascular changes, and do not cross the blood-brain barrier. Subjects ingested the histamine receptor antagonists with 6-8 oz of water 60 minutes prior to exercise.

Assays and Analysis

Histamine Assay. Interstitial histamine concentrations were analyzed in intramuscular dialysate collected in the study detailed in chapter IV. Interstitial histamine was measured using a standard enzyme-linked immunosorbent assay (Rocky Mountain Diagnostics, Colorado Springs, CO) and performed in accordance to the manufacturer's instructions. All samples and standards were acylated by combining them with 25 µl of acylation buffer and 25ul acylation reagent and incubating on a plate shaker at 600 rpm for 45 minutes at room temperature. 200 µl distilled water was added to all wells and the microplate was incubated on a plate shaker at 600 rpm for 15 minutes at room temperature. 25ul of the samples and standards were transferred to a 96-well microplate pre-coated with an antibody raised against human histamine. 100 µl of a histamine goat anti-serum was added to all wells. The microplate was then incubated on a plate shaker at 600 rpm for 3 hours at room temperature. The plate was then washed four times and 100 µl of an enzyme conjugate containing an anti-goat IgG was added to all wells and incubated on a plate shaker at 600 rpm for 30 minutes at room temperature. The plate was again washed and 100 µl of a tetramethylbenzidine substrate solution was added to all

wells and incubated on a plate shaker at 600 rpm for 25 minutes at room temperature. Finally, 100 µl of a stop solution containing sulfuric acid was added to all wells. The absorbance was then measured at a wavelength of 450 for each sample and plotted against the absorbance of a set of known histamine standards using a 5-parameter logistic regression.

Mast Cell Culturing Protocol. Isolated human mast cells (Kerafast LLC, Boston, MA) were cultured for experiments in Chapter IV and Chapter V. Cells arrived frozen from the manufacturer and we woke them by warming the vial of cells in a 37°C bath just until thawed. The cell suspension was transferred to a 15ml conical tube containing 5ml of warm growth medium. Warm growth medium consisted of basal medium (Gibco 10639011), penicillin-streptomycin 10,000U/ml (Gibco 15140), L-glutamine 200mM (Gibco 25030-081) and Primocin 500mg (Fisher). Cells were mixed in warm growth medium and centrifuged at 450g for 5 minutes at room temperature. Cells were resuspended in 5ml of growth medium and transferred to a T25 flask. Cells were left for 2-3 days and then transferred to a T75 flask with additional 15ml of fresh growth medium. Cells were split approximately every 3-4 days over the course of 2 weeks. After approximately two weeks and once cells were at an appropriate viability (greater than 80%) the cells were prepared for freezing for future use in experiments. Cells were then centrifuged at 600rpm for 10 minutes. Supernatant was removed and cells were resuspended in pZERVE (Protide Pharmaceuticals) to have a density of $5x10^6$ cells/ml. pZERVE is a cryopreservation solution that does not contain any dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), nor any other animal protein. This suspension was dispensed in to sterile cryotubes in 1-2ml aliquots, sealed and stored at room temperature

for 30 minutes with gentle agitation. Following this step, vials were then placed in an insulated container at -20°C for 60 minutes. Insulation was removed and vials were placed in -80°C for 60 minutes. Vials were then transferred to vapor phase of liquid nitrogen for 24 hours. Following this 24-hour incubation period, vials were finally moved to liquid phase of liquid nitrogen for storage until use for experiments. The above-mentioned waking protocol was used to prepare the frozen cells for experiments. The waking protocol typically took 1-2 weeks for cells to reach appropriate cell density and viability for experiments.

In-Vitro Mast Cell Degranulation Assay. Isolated human mast cells (Kerafast LLC, Boston MA) were used for study 3 detailed in Chapter IV and for the study detailed in Chapter V. This was a degranulation protocol that we developed in our lab based upon previously published protocols utilizing isolated mast cell lines. Mast cell degranulation was quantified in response to temperature (Chapter IV) or exposure to human skeletal muscle dialysate (Chapter V). Degranulation was determined as the release of the granule marker β-hexosaminidase. Cultured human mast cells were transferred in to wells on a 96-well plate in imaging medium buffer. For study 3 in the set of experiments in Chapter IV and the study in Chapter V, 48/80 was used as a positive control. 2 mg of compound 48/80 was dissolved in 1 ml imaging buffer. 50 ul of 2mg/ml of compound 48/80 was added to the plated mast cells in the respective positive control wells. 50 ul of imaging buffer was used as a negative control, and used to subtract background in respective experiments. For study 3 in the set of experiments detailed in Chapter IV, mast cells were exposed to three physiological temperatures to measure degranulation: 34, 37, and 39°C for 20 minutes. In the study detailed in Chapter V, mast cells were exposed to 50ul of

dialysate collected from skeletal muscle for 20 minutes at 37°C. In both set of experiments, after 20 minutes, cells were centrifuged and the supernatants removed. Supernatants were reacted with 2 mM 4-Nitrophenyl N-acetly-B-D-glucosamide (Sigma) at 37°C for 2 hours. Then, 0.5 M Tris(hydroxymethyl)aminomethane was used to stop the reaction, and the absorbance was read at 405 nm. Total content was determined by addition of Triton X-100 to lyse the cells. Degranulation is expressed as a percentage of β-hexosaminidase activity in the supernatant divided by the total (supernatant plus pellet) activity.

Inflammation Panel. In the study detailed in Chapter VI, plasma was analyzed for inflammatory cytokines in 5 subjects via a bead-based flow cytometry kit (Biolegend, LEGENDplex Human Inflammation Panel 1, multi-analyte flow assay kit). This method of cytokine identification uses beads of varying size coated with antigens specific to cytokines of interest (MCP-1, IL-8, IL-10, IL-17A). The size and fluorescence of the beads allows for separation and quantification of the cytokine by flow cytometry (Gallios, Beckman Coulter Life Science, Indianapolis IN).

Cell Staining Preparation from Whole Blood. For flow cytometry analysis, our laboratory technician Karen Needham helped to develop a cell staining protocol in conjunction with established, published literature. 2.0 ml of fresh whole blood was added to a 10x solution of red blood cell lysis buffer. This was incubated for 10 minutes at room temperature. Tubes were centrifuged at 500G for 10 minutes and supernatant was removed. The pellet was resuspended in 30mL 1x phosphate-buffered saline (PBS) and passed through a 40 micron filter into a fresh tube. The sample was then spun for 10 minutes at 500G and supernatant was removed. The pellet was then resuspended in 3 ml

PBS and cell count was quantified. Cells were diluted to $1x10^6$ cells/ml with cold PBS and 1µl violet stain per ml of cell suspension was added and incubated for 30 minutes at room temperature. Violet stained cells were diluted up to 30 ml with cold PBS and tubes were spun for 10 minutes at 500G. Supernatant was discarded, and the pellet was resuspended with 4% paraformaldehyde at 1ml per 1 million cells. After a 10-minute incubation in paraformaldehyde at room temperature this suspension was topped off to 30ml with cold PBS. This cell suspension was spun for 10 minutes at 500G, supernatant removed, and the cell pellet was resuspended in 2 mL of PBS. This was stored in the dark at 4°C until ready for staining and cytometering.

Cell Staining Protocol. Cell suspensions were re-suspended and 1.5 x 10^6 cells were transferred to individual 2.0ml microcentrifuge tubes for each staining sample plus the unstained cells. Cells were pelleted at 500G for 5 minutes. Cells were re-suspended in 100ul of staining buffer (PBS + 1% BSA + 10% FBS = Biolegend 420201 + 50 mL FBS – Gibco 26140-129, heat deactivated at 560 for 45 minutes) plus 10ul mouse IgG (Jackson Immuno – ChromPure 015-000-003). Suspensions were incubated for 30 minutes at room temperature. Cells were washed with 1mL of staining buffer, centrifuged and supernatant was discarded. 100ul of antibody staining cocktail was added to each tube and incubated for 30 minutes in the dark at room temperature. 1 mL of staining buffer was added to each tube, cells were centrifuged, and supernatant was discarded. Cell pellet was re-suspended in 500uL staining buffer and read in a flow cytometer. The following antibodies were used for the leukocyte staining protocol: CD62E, CD86, CD309, CD34, CD192, CD66B, CD14, CD16, CD3, CD19, CD56, CD45.

Statistical Analysis

Statistical inferences were drawn from a combination of paired t-tests, 1-way, and 2-way repeated measures of analysis of variance (ANOVAs) using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Fisher's Least Significant Differences was used as a post-hoc analysis where main or interaction effects were observed. For all tests, significance was set at $P < 0.05$. Variables describing the distribution of the subject population, including anthropometric and demographic measures are presented as means ± standard deviation (SD). Data describing physiological variables between conditions or over time are presented as means ± standard error of the mean (SEM) to display the precision of the interventions.

CHAPTER IV

THE ROLE OF TEMPERATURE ON EXERCISE-INDUCED SKELETAL MUSCLE HISTAMINE RELEASE

Introduction

Blood flow to previously exercised muscle remains elevated for several hours following the cessation of exercise, termed sustained post-exercise vasodilation. This results in systemic responses with mean arterial pressure being reduced below pre-exercise levels following moderate-intensity aerobic exercise (76, 60). Our lab has demonstrated that this response is highly dependent upon histamine receptor activation, as histamine H_1 and H_2 receptor antagonism using over-the-counter medications reduces this post-exercise response ~80% (94). In addition, our lab has also demonstrated that histamine concentrations within skeletal muscle increase in response to exercise (127). The source of this histamine can either be released from mast cells via degranulation and/or *de novo* formation via increased activity of the enzyme histidine decarboxylase (HDC) which converts the amino acid l-histidine to histamine (135). While we have shown that histamine is an important mediator in this sustained post-exercise vasodilatory response, the exercise "signal" that causes the increase in histamine levels has not been identified. Our lab has ruled out exercise-induced oxidative stress as a potential trigger (125), however there still remain other exercise factors that have not yet been investigated.

There are multiple exercise factors or conditions both systemically and locally in active skeletal muscle that are altered during exercise. Some of these localized responses

include a decrease in pH, the physical contraction of skeletal muscle fibers, an increase in skeletal muscle temperature, an increase in reactive oxygen species, and others (148, 148, 45). It has been well established in the research literature that during exercise, there is heat production by exercising skeletal muscle resulting in an increase in skeletal muscle temperature (54). This increase in skeletal muscle temperature is beneficial as it can increase enzymatic activity of key molecules that are necessary for appropriate exercise responses. One particular enzyme that is both important for force production in skeletal muscle and is temperature sensitive is myofibrillar ATPase (mATPase). During the cross-bridge cycle, myosin molecules binds and hydrolyzes ATP during force generation. ATPase activity is positively correlated with muscle contraction velocity, with slower fiber types containing less ATPase enzyme than fast fiber types. It has been shown that the increase in temperature in human skeletal muscle can aid in the increase of mATPase enzymatic activity, in turn allowing for greater force production that is needed with higher intensity exercise. Increases in skeletal muscle temperature have also been shown to have positive allosteric effects on rate-limiting metabolic enzymes such as phosphofructokinase (glycolysis) and cytochrome oxidase (aerobic respiration). In addition, this increased skeletal muscle temperature can also have beneficial effects on excitation-contraction coupling and mechanical contraction of muscle fibers (22). Relating back to our focus on histamine, elevated temperatures in skeletal muscle can cause the release of cytokines and soluble factors that can be found within skeletal muscle (150). This elevation of temperature in skeletal muscle could cause an increase in mast cell degranulation, which in turn would cause an increased release of histamine. Elevated temperatures have been shown to increase activity of HDC in a linear fashion up

to a point, where it then plateaus beyond what is typically physiologically observed in human skeletal muscle (132, 133). Due to the nature of the enzyme, it is possible that an increase in temperature in skeletal muscle during exercise might increase the activity of histidine decarboxylase leading to the observed increase in histamine concentrations in skeletal muscle.

Given that elevated temperatures can possibly induce human mast cell degranulation and/or alter enzymatic activity of histidine decarboxylase and it is known that increased temperature plays an important role in skeletal muscle during exercise, the study was proposed to investigate if heat production may be the "exercise signal" for the increases in intramuscular histamine observed with exercise. Therefore, the overall purpose of this study was to determine if the increase in skeletal muscle temperature is an important mediator in the upstream signaling that leads to histamine release in skeletal muscle with exercise. Our goal was to investigate the temperature of skeletal muscle during unilateral knee extension exercise and then attempt to isolate the increase in temperature apart from other exercise factors. To isolate temperature as a variable, we sought a passive heating modality which would not involve muscle contraction, alter pH, and other exercise "factors." Study 1 was operationally-driven and we aimed to investigate the rise in skeletal muscle temperature using our exercise model of dynamic unilateral knee extension exercise. In study 2, we tested the hypothesis that passively heating skeletal muscle up to 39°C using pulsed short-wave diathermy would result in increases in intramuscular histamine concentrations that are closely matched to exercising values. In study 3, a follow-up study, the purpose was to determine if increases in heat would directly cause increased degranulation of mast cells in vitro. We hypothesized that

step-wise increases in heat (matched to physiological values) would increase mast cell degranulation, measured by β-hexosaminidase release.

Methods

Subjects

For study one, five (1 female, 4 males) young healthy non-smoking subjects volunteered. For study two, we had seven (2 females, 5 males) young healthy non-smoking subjects volunteer. There was no subject that participated in both study one and study two, each study had a unique cohort of subjects. All studies were approved by the Institutional Review Board at the University of Oregon and was performed in accordance with the principles outlined by the Declaration of Helsinki. Written, informed consent was obtained from all subjects subsequent to a verbal and written briefing of all experimental procedures. Subjects were deemed healthy following a standard health history questionnaire. All subjects were required to abstain from caffeine, alcohol, and exercise for 24 h before studies. Additionally, subjects reported to the laboratory in the morning after an overnight fast. No subjects were using over-the-counter or prescription medications at the time of study, with the exception of oral contraceptives. Female participants were studied during the early follicular phase of their menstrual cycle or during the placebo phase of their oral contraceptive.

Screening Visit

During this visit, subject's physical characteristics were obtained (height, weight, body mass index) and they were familiarized with the exercise model and hemodynamic

measurements. Arterial pressure was assessed using an automated auscultatory sphygmomanometer (Tango+; SunTech Medical, Raleigh, NC, United States) after 5 min of supine rest.

Study Visit (Study 1)

Experimental approach

The goal of this experiment was to measure temperature in skeletal muscle during dynamic unilateral knee extension exercise. This was operationally driven research, considered pilot work to determine the change of skeletal muscle temperature over the course of one hour using this exercise modality. An intramuscular thermocouple was placed into the *vastus lateralis* before exercise under sterile technique to measure intramuscular temperature. Subjects then performed unilateral dynamic knee extension exercise at 60% of their peak kicking power for 60 minutes.

Study Visit (Study 2)

Experimental approach

The goal of this experiment was to passively raise skeletal muscle temperature to values observed during dynamic unilateral knee extension exercise and measure histamine concentrations in collected skeletal muscle dialysate. We had a goal to raise skeletal muscle temperature utilizing the data we collected in study 1 to determine the time course of skeletal muscle temperature during our typical protocol of unilateral knee extension exercise at 60% of peak kicking power for 60 minutes. The data from this pilot

experiment is presented in **Figure 4.1**. Skeletal muscle temperature rose rapidly and then begin to plateau at around 30 mins at approximately 39°C. Therefore, this experiment aimed to passively raise skeletal muscle temperature to the values observed during exercise while collecting skeletal muscle microdialysate, with the end goal to compare these skeletal muscle histamine concentrations to those observed from exercise.

Measurements

Pulsed Short Wave Diathermy

The diathermy unit used to increase the temperature of skeletal muscle was the Megapulse (Accelerated Care Plus-LLC, Topeka, Kansas) with an operating frequency of 27.12 MHz and a pulsed mode yielding 800 bursts per second. A picture of the Megapulse unit can be found in the "Chapter II - Explanation of the Methodology" section on Page 32. The diathermy unit consists of an induction drum coil electrode that was used to deliver the electromagnetic energy. The diathermy drum was 200 cm^2 and had a 2.5 cm space plate to protect the tissue from direct contact with the electrode. We placed the diathermy drum above the anterior aspect of the muscle belly 1 cm above the subject's skin. We positioned the drum so that the tip of the thermistor was in direct line with the center of the drum. Diathermy was applied for 60 minutes at the following settings: 800 bursts per second; 400-psecond burst duration; 850-psecond interburst interval with a peak root mean square amplitude of 150 W per burst and an average root mean square output of 48 W.

Skeletal muscle microdialysis and histamine ELISA

All probes were inserted using sterile technique. The skin was anesthetized using a lidocaine (2%) and epinephrine (1:100,000) solution (Septodont, France) and underlying fascia was anesthetized using prilocaine hydrochloride (4%) (Septodont, France). Care was taken to ensure that the lidocaine and prilocaine was not injected into the skeletal muscle. Probes were inserted in the vastus lateralis in a direction parallel with muscle fiber orientation (~19°, relative to long-axis of muscle) using a splittable introducer. After insertion, probes were held in place by covering the entry site with a sterile transparent medical dressing. Probes used to measure interstitial histamine had a 100-kDa molecular mass cutoff with a 30-mm polyarylethersulphone membrane (63 MD Catheter, MDialysis, Stockholm, Sweden). Subsequent to insertion, microdialysis probes were perfused continuously at a rate of 5 µl/min (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA). Probes used to measure histamine were perfused with a 0.9% saline solution. After insertion of the fiber and intramuscular thermocouple, dialysate was collected during 60 minutes of supine rest before heating and then every 20 minutes during heating.

Interstitial histamine was measured using an enzyme-linked immunosorbent assay (Rocky Mountain Diagnostics, Colorado Springs, CO) and performed in accordance with the manufacturer's instructions. Interstitial tryptase was measured using an enzyme-linked immunosorbent assay and performed in accordance with the manufacturer's instructions adapted for microdialysis (Kamiya Biomedical, Seattle, WA).

Intramuscular temperature

In both study 1 and study 2, an intramuscular thermocouple was placed into the *vastus lateralis* under similar sterile technique to measure skeletal muscle temperature during the passive heating protocol. The skin and fascia were anesthetized using the same lidocaine/epinephrine solution at the skin surface and prilocaine at the skeletal muscle fascia. An 18 gauge introducer Tuohy needle (Integra LifeSciences Corporation) was inserted into the vastus lateralis. The inner cannula was removed from the Tuohy needle and the thermocouple wire was threaded through the inner portion of the introducer needle. Once the thermocouple wire was in place within the skeletal muscle, the introducer Tuohy needle was removed leaving the thermocouple wire in place and it was connected to a desktop computer to monitor real time skeletal muscle temperature during the study protocol. Windaq software was utilized to record skeletal muscle temperature during the entirety of the study and temperature was also recorded on paper at 10 minute intervals.

In-Vitro Mast Cell Degranulation Assay

For study 3, degranulation of mast cells was determined as the release of the granule marker β-hexosaminidase. Human mast cells were cultured and plated on a 96-well plate in imaging medium buffer. 2 mg of compound 48/80 was dissolved in 1 ml imaging buffer. Then, to serve a as positive control, 50 ul of 2mg/ml compound 48/80 was added to plated mast cells in those respective wells. In the negative control wells, 50 ul of imaging buffer was added. Cells were then incubated at 34, 37 and 39°C for 20 minutes before being centrifuged and the supernatants removed. Supernatants were reacted with 2 mM 4-Nitrophenyl N-acetly-B-D-glucosamide (Sigma) at 37°C for 2

hours. Then, 0.5 M Tris(hydroxymethyl)aminomethane was used to stop the reaction, and the absorbance was read at 405 nm. Total content was determined by addition of Triton X-100 to lyse the cells. All test conditions were done in duplicate in a single experiment and each experiment consisted of dialysate from 1-2 subjects at all time points, imaging buffer, 48/80, and histamine. Degranulation is expressed as a percentage of β-hexosaminidase activity in the supernatant divided by the total (supernatant plus pellet) activity. Total content was determined by addition of Triton X-100 to lyse the cells

Heart Rate and Blood Pressure

For study 1 and study 2, heart rate was monitored using a three-lead electrocardiograph and arterial pressure was assessed in the right arm employing an automated sphygmomanometer (Tango+; SunTech Medical, Raleigh, NC, United States). Mean arterial pressure was calculated as (systolic – diastolic)/3 + diastolic pressure.

Statistical Analysis

Our primary outcome variables in study 1 and 2 were analyzed using a One-Way ANOVA (GraphPad Prism) to test for differences in skeletal muscle temperature over time and for histamine concentrations over time. In study 3, our primary outcome variable was analyzed using a One-Way ANOVA (GraphPad Prism) to test for differences in mast cell degranulation expressed as a percentage over different physiological temperatures. Fishers LSD was used for post-hoc analysis for all tests. Significance was set at $P < 0.05$. Data are reported as mean SEM unless stated otherwise

(e.g. SD is used in Table 4.1 and 4.2 to indicate variability in the subject pool and for the experiment performed in study 3, data shown in **Figure 4.3**).

Restults

Subject Characteristics

Subject physical characteristics and data obtained during the screening visit for study 1 are shown in Table 4.1. Subject physical characteristics and data obtained during the screening visit for study 2 are shown in Table 4.2. No subject participated in both study one and study two. Each study had a unique cohort of subjects, their characteristics listed below. For both studies. subject characteristics were similar to those obtained previously in our laboratory in young healthy subjects and consistent with recreationally active individuals.

Table 4.1 Exercise and Temperature (Study 1) - Subject Characteristics

n	5 (1F, 4M)
Age (yrs)	23 ± 3
Height (cm)	160.2 ± 8.2
Height (kg)	73.2 ± 5.1
BMI	23.3 ± 2.4

Table 4.2 Temperature and Histamine (Study 2) - Subject Characteristics

n	7 (2F, 5M)
Age (yrs)	26 ± 4
Height (cm)	168.2 ± 11.2
Height (kg)	75.4 ± 13.4
BMI	23.9 ± 2.7

Intramuscular Temperature (Study 1). Intramuscular temperature during 60 minutes of unilateral dynamic knee extension exercise is shown in **Figure 4.1**. Prior to exercise, skeletal muscle was 35.5 ± 0.4°C and was increased to 39.2 ± 0.7°C by the end of heating ($p<0.01$). By 20 minutes of exercise, temperature had significantly increased to 38.1 ± 0.1°C ($p<0.01$). Skeletal muscle temperature was significantly higher at 30 minutes and all time points beyond, compared to resting skeletal muscle temperature ($p<0.01$). There was a plateau observed in skeletal muscle temperature from 30 minutes to 60 minutes, in which no significant differences in skeletal muscle temperature from the previous timepoint were observed.

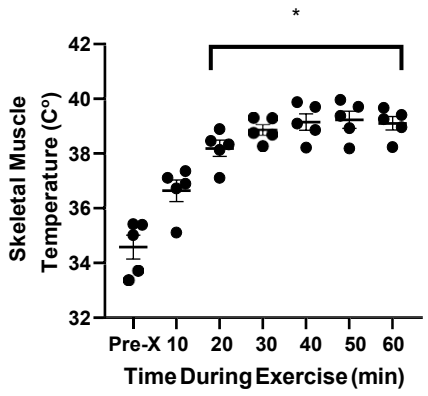

Figure 4.1. Individual data of skeletal muscle temperature during 60 minutes of one-legged dynamic knee extension exercise. Data expressed as means ± SEM. * indicates significantly different from pre ($p<0.05$)

Central Hemodynamics (Study 1). Heart rate and mean arterial pressure in response to one-hour of unilateral dynamic knee extension exercise are shown in Table 4.3.

Table 4.3. Exercise and Temperature (Study 1) - Central Hemodynamics

	Heart Rate (beats min $^{-1}$)	Mean Arterial Pressure (mmHg)
Time Point		
Pre-Exercise	66.2 ± 2.5	83.1 ± 1.5
Time During Exercise		
10 min	83.4 ± 1.4*	84.3 ± 2.2
20 min	85.6 ± 2.2*	85.2 ± 3.0
30 min	91.8 ± 3.4*	85.3 ± 3.1
40 min	91.2 ± 2.2*	85.1 ± 2.5
50 min	90.8 ± 3.5*	86.1 ± 1.4
60 min	96.3 ± 2.3*	86.2 ± 2.0

Values are expressed as mean ± SEM. * indicates significantly different from pre ($p<0.05$)

Intramuscular Temperature (Study 2). Intramuscular temperature during 60 minutes of passive heating using PSWD is shown in Figure 4.2. Prior to heating, skeletal muscle was 32.4 ± 0.4°C and was increased to 38.1 ± 0.7°C by the end of heating ($p<0.05$). Skeletal muscle temperature was significantly higher at 30 minutes and all time points beyond, compared to resting skeletal muscle temperature ($p<0.05$). Similar to the

response observed with exercise there was a plateau observed in skeletal muscle temperature from 40 minutes to 60 minutes, in which no significant differences in skeletal muscle temperature were observed.

Intramuscular Histamine Concentrations (Study 2). Intramuscular histamine concentrations during 60 minutes of passive heating using PSWD is shown in Figure 2. Intramuscular histamine increased from 2.12 ± 0.17 ng/ml at rest to 2.90 ± 0.20 ng/ml at 60 minutes of passive heating. Histamine concentrations were not different from rest at 20 minutes (p=0.9) nor at 40 minutes (p=0.6) of passive heating.

Table 4.4. Temperature and Histamine (Study 2) – Central Hemodynamics

	Heart Rate (beats min $^{-1}$)	Mean Arterial Pressure (mmHg)
Time Point		
Pre-Heating	59.2 ± 2.5	82.7 ± 2.5
Time During Heating		
10 min	59.9 ± 3.6	81.2 ± 3.1
20 min	57.7 ± 2.8	81.8 ± 3.0
30 min	59.3 ± 4.4	80.7 ± 2.6
40 min	60.9 ± 3.8	82.2 ± 3.7
50 min	56.9 ± 4.6	81.5 ± 2.0
60 min	58.3 ± 4.7	80.4 ± 3.4

Values are expressed as mean ± SEM.

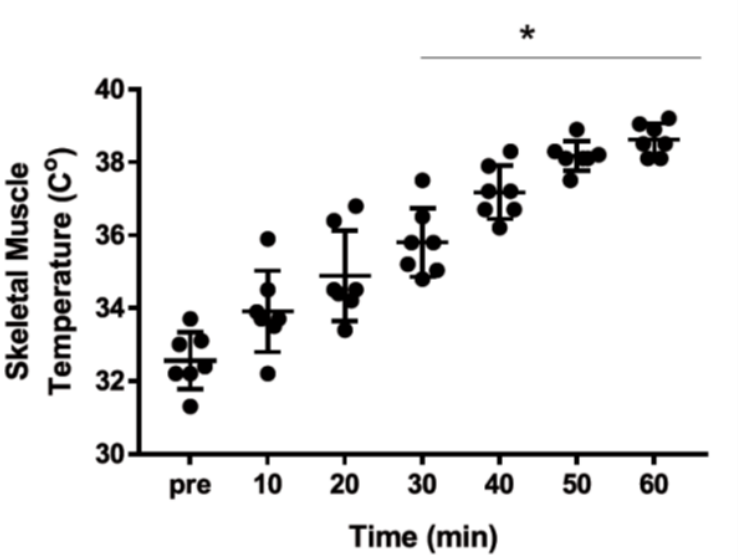

Figure 4.2. Individual data of skeletal muscle temperature during 60 minutes of pulsed short-wave diathermy. Data expressed as means ± SEM. * indicates significantly different from pre ($p<0.05$)

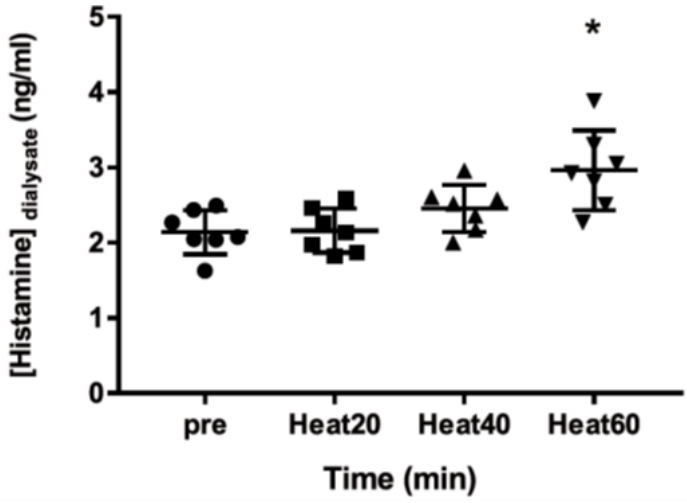

Figure 4.3. Individual data of histamine concentrations in skeletal muscle dialysate during 60 minutes of pulsed short-wave diathermy. Data expressed as means ± SEM. * indicates significantly different from pre ($p<0.05$)

Increased temperature effect on human mast cell degranulation. For the third study of this chapter, we exposed isolated human mast cells to different physiological temperatures observed at rest and during exercise. There was no significant difference in mast cell % degranulation (measured as b-hexosaminidase release) at different temperatures as shown in Figure 4.3. Compound 48/80 was used as a positive control as it directly causes mast cell degranulation.

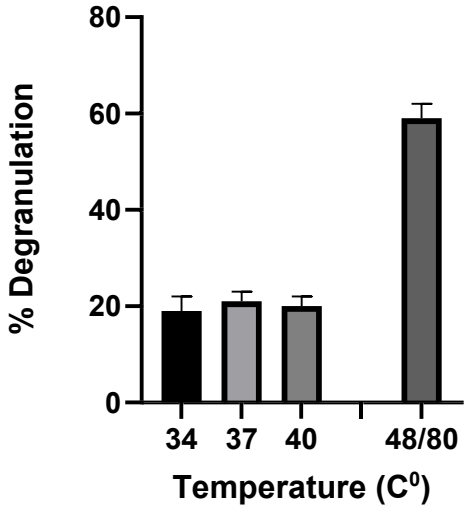

Figure 4.4. Mast cell degranulation (expressed as %) at different physiological temperatures. 48/80 was used as a positive control. Data expressed as means ± SD.

Discussion

The purpose of this study was to determine if an increase in skeletal muscle temperature mimicking what is observed during exercise is a stimulus to provoke intramuscular histamine release in skeletal muscle. In agreement with our hypothesis in study 2, passively increasing skeletal muscle temperature to exercising values resulted in increased intramuscular histamine concentrations in skeletal muscle measured via dialysate. To follow up, we designed an in-vitro experiment to investigate if physiological increases in temperature would result in increases in mast cell degranulation. In disagreement with our hypothesis in study 3, step-wise physiological increases in temperature did not result in increased mast cell degranulation. Taken

together, these data suggest that heat plays a significant role in the release of histamine in skeletal muscle during exercise. However, the in-vitro experiment suggests that the source of this histamine may be *de novo* formation via increases in the enzyme histidine decarboxylase, rather than mast cell degranulation.

It has been well established that there is a rise in skeletal muscle temperature during exercise due to heat production from muscular contractions (77). This increase in muscle temperature can vary depending on the type of exercise, the exercise environment, training status etc. This increase in skeletal muscle temperature can serve as beneficial in that it can lead to increases in enzyme activity and can also alter substrate utilization to match exercise intensity/duration (152). Temperature can also affect contractile properties of skeletal muscle including cross-bridge turnover, maximum power output, neuromuscular function, calcium sequestration, and overall recovery metabolism (74, 119, 120). Typically, during exercise in addition to temperature increasing in skeletal muscle, there are changes in other factors such as pH and build-up of metabolites. However, the difficulty lies within being able to dissect each of these factors from each other in order to isolate its effects. Our lab demonstrated that there is an increase in skeletal muscle temperature up to and a plateau effect at 39°C with dynamic unilateral knee extension exercise (**Figure 4.1**). Therefore, the goal of this study was to increase skeletal muscle temperature to "exercise levels" without contraction and to do this we utilized pulsed short-wave diathermy as our heating method.

Passive Heating Utilizing Pulsed Short-Wave Diathermy

For study 2 in this set of experiments, the goal was to increase skeletal muscle temperature up to 39°C which we had previously observed as the intramuscular temperature reached during dynamic unilateral knee extension exercise (**Figure 4.1**). There are different modalities that have been used to raise skeletal muscle temperature, some of these modalities can raise skin temperature and can also have central hemodynamic effects. Whole-body and limb water immersion has been demonstrated to effectively decrease and increase skeletal muscle temperature (162, 114). For this study we wanted to ensure we maintained a sterile field for both the intramuscular temperature and intramuscular dialysate measurement probes, as well as eliminate any hydrostatic effects that water immersion might involve. For initial pilot experiments, we used a UV heat lamp which has not been commonly used in the literature, while polarized linear light has been utilized (34). With this UV heat lamp modality, we noted significant increases in skin temperature, and it appeared it was not able to penetrate to the depth of the skeletal muscle. In addition, subjects expressed discomfort in the rise in skin temperature. Therefore, after a thorough literature search on heating modalities we chose pulsed short-wave diathermy.

Short wave diathermy provides electromagnetic radiation that can either be delivered in a continuous or pulsed manner, both having benefits in the athletic training community (50). We elected to use pulsed short-wave diathermy in this experiment as it has been shown to effectively raise skeletal muscle temperature without any significant changes in central hemodynamics (155). As seen in **Figure 4.2**, there was a quite rapid increase in skeletal muscle temperature utilizing pulsed short-wave diathermy. There was

a significant increase in skeletal muscle temperature by 30 minutes and then we observed a "plateau" effect similar to what we observed during unilateral dynamic knee-extension exercise. In addition, the resting temperature and slope of rise in skeletal muscle temperature was comparable to exercise. Therefore, we are confident that we were able to mimic the rise in skeletal muscle temperature that is observed in single leg exercise without significant changes in skin temperature or in central hemodynamics (Table 4.4). Our research adds to the body of literature demonstrating the safety and efficacy of pulsed short-wave diathermy's ability to increase skeletal muscle temperature.

Heating and Skeletal Muscle Histamine

Our lab has previously shown that histamine concentrations within skeletal muscle increase in response to exercise (127). These data indicate that certain factors released or altered within skeletal muscle are causing its release from mast cells and/or de novo formation via histidine decarboxylase. Using infusions of the antioxidant N-acetylcysteine prior and during exercise with no effect on the histaminergic signaling pathway suggested that the oxidative stress associated with exercise is not necessary for histamine release (125). While we have been able to "rule out" this factor, there are still other signals that are present during exercise that may lead to this increase in histamine release. This experiment investigated if heat might be one of the factors that leads to the increase in histamine that is observed with exercise.

Pulsed short-wave diathermy increased skeletal muscle temperature to values observed during dynamic unilateral knee extension exercise, shown in **Figure 4.1** and **Figure 4.2**. Histamine concentrations measured in skeletal muscle dialysate during our heating protocol demonstrated that there appears to be a "threshold" for this increase in

histamine levels. At 20 and 40 minutes, there was not a significant increase in histamine levels in skeletal muscle dialysate compared to pre-heating (**Figure 4.3**). However, at 60 minutes there was a 38% increase ($p<0.05$) in skeletal muscle dialysate levels which is where skeletal muscle temperature had reached 39°C. This was our "goal temperature" as this is where skeletal muscle temperature plateaus during kicking exercise. This increase in skeletal muscle dialysate levels in response to heating is comparable to the percentage increased observed in skeletal muscle dialysate levels observed during exercise, which is around ~41% (127). Given that we were able to isolate temperature without measurably affecting other exercise factor variables, these data suggest that the increase in temperature in skeletal muscle during exercise plays a significant role in histamine release as we observed similar increases.

The Effect of Temperature on Human Mast Cells and Histidine Decarboxylase

As previously mentioned, there are two sources of histamine that are observed in human tissue. Mast cells can degranulate in response to multiple factors which in turn releases histamine from its vesicles into circulation and the surrounding tissue (43). Another source is de novo formation via histidine decarboxylase which converts histidine to histamine. Histidine decarboxylase is induced by and can be increased in response to a variety of stimuli (83). As discussed, it has been demonstrated that increases in temperature can cause a linear increase in histidine decarboxylase levels. Therefore, it is logical to hypothesize that the increase in skeletal muscle temperature might be increasing the degranulation of mast cells, increasing histidine decarboxylase activity, or both. In study 3, we set out to examine if isolated human mast cells degranulate in response to temperature increases.

As seen in Figure 4.4, we exposed isolated human mast cells to differing physiological temperatures (34, 37, and 40°C). Given that we observed skeletal muscle temperature increases from 34 to 39°C with pulsed short-wave diathermy and exercise, we were confident these temperatures would be appropriate to utilize. Interestingly, in contradiction to our hypothesis, changes in temperature did not result in significant changes in mast cell degranulation in isolated human mast cells (**Figure 4.4**). We had an expected and robust response in mast cell degranulation in the presence of compound 48/80, which served as a positive control to ensure that the mast cells being utilized are viable. The fact that we did not see differences in degranulation in response to varying temperatures suggests that it might be an increase in histidine decarboxylase activity with increases in skeletal muscle temperature, rather than direct effects on mast cell degranulation. However, we recognize that our isolated human mast cell preparation is different than intact mast cells which are found in human skeletal muscle and surrounded by other cell types. There is a possibility that there is other cell cross-talk in human skeletal muscle during heating that could result in mast cell degranulation, which we would not observe in an isolated mast cell preparation. Certain cell types present in nearby tissue may be contributing to the degranulation of mast cells or increases in histidine decarboxylase in response to the rise in temperature. Follow-up experiments should examine histidine decarboxylase activity during heating to further elucidate the role of temperature on histidine decarboxylase activity.

Summary and Bridge

Both whole body and single-leg aerobic exercise initiates a sustained post-exercise vasodilatory response that is dependent upon histaminergic signaling. We have

shown that histamine is released in skeletal muscle during exercise. The findings from this study demonstrate that the increase in temperature in skeletal muscle might be one of the primary causes for this histamine release. In vitro experiments with isolated mast cells suggest that this may be due to an increase in histidine decarboxylase activity, rather than heat directly causing mast cell degranulation. Chapter V will build upon the observations of these experiments to further understand the exercise "signal" that causes the release of histamine. We will attempt to identify if there is a circulating factor that is released locally in exercising skeletal muscle that is not present in resting tissue.

CHAPTER V

THE EFFECT OF DIALYSATE FROM EXERCISING SKELETAL MUSCLE ON MAST CELL DEGRANULATION

Introduction

Following a bout of aerobic exercise, blood flow to previously exercised muscle remains elevated for several hours. This sustained post-exercise vasodilation is mediated by both central neural mechanisms and local vascular mechanisms (60). Our lab has demonstrated that histamine plays a significant role in this post-exercise vasodilatory response. Blockade of histamine H_1 and H_2 receptors with over-the-counter medications blunts this response with whole-body exercise (94) and abolishes it with isolated small muscle-mass exercise (7). This culmination of work suggests that this phenomenon is histaminergic in origin and may be independent of the neural mechanisms.

This impact of H_1/H_2 histamine receptor blockade demonstrates that histamine receptor activation is essential for the sustained post-exercise vasodilatory response. Histamine acting as a ligand on these receptors appears to be critical since blocking its actions can blunt or abolish the phenomenon. Investigations of histamine levels in arterial and venous plasma have been conflicted, as some have shown no changes during exercise and others have shown increases (62, 101). Our lab has demonstrated that interstitial histamine concentrations are increased during and following exercise in skeletal muscle, measured by intramuscular dialysis fibers (127). The source of this histamine can be released from mast cells via degranulation and/or increased activity of the enzyme histidine decarboxylase which converts the amino acid L-histidine to histamine. While

we have shown that histamine levels increase in skeletal muscle in response to exercise, it is still not known what it is about exercise that causes the release of histamine. Our lab has ruled out exercise-induced oxidative stress as a potential trigger (125), however there still remain other exercise "factors" that have not yet been investigated.

There are multiple exercise factors or conditions systemically and locally that are altered during exercise. Some of these localized responses include a decrease in pH, the physical contraction of skeletal muscle fibers, an increase in skeletal muscle temperature, and others (65, 45, 121). Considering local factors within skeletal muscle, cytokines and other cell types have been shown to be released directly from exercising/exercise muscle termed "myokines". These can then bind to different types of cells to have further downstream effects. Mast cells are sensitive to a multitude of factors, including cytokines IL-4 and IFN-y which have been shown to be released from exercising/previously exercised skeletal muscle (137, 17). It is possible that the release of a single or multiple cytokines might be inducing mast cell degranulation, leading to the observed increase histamine concentrations in skeletal muscle in response to exercise. Furthermore, it is quite probable that is response is tightly regulated locally and these released cytokines may not have an effect in resting tissue. To date, no one has investigated if there is or are local factors released in exercising/exercised skeletal muscle that cause mast cell to degranulate more so than baseline levels that is not present in resting tissue.

As stated, given that exercise increases histamine levels locally in active skeletal muscle, it is possible that there are local factors released in exercising/exercised skeletal muscle that cause mast cell degranulation leading to the rise in skeletal muscle histamine levels we have observed. This study was proposed to investigate there is a local

factor/factors that is/are present in exercising skeletal muscle that induce mast cell degranulation that is not present in resting tissue. It is possible that there is a factor released in skeletal muscle that serves as the "exercise signal" for the increases in intramuscular histamine observed with exercise. Our goal was to obtain interstitial fluid from skeletal muscle from both the resting and exercising leg during and following unilateral dynamic knee extension exercise and examine its effects on mast cell degranulation. In this study, we tested the hypothesis that skeletal muscle dialysate collected from exercising and previously exercised skeletal muscle in vivo would increase mast cell degranulation, measured in vitro by β-hexosaminidase release from isolated human mast cells.

Methods

Subjects

For this study nine (2 females, 7 males) young healthy non-smoking subjects volunteered. This study was approved by the Institutional Review Board at the University of Oregon and was performed in accordance with the principles outlined by the Declaration of Helsinki. Written, informed consent was obtained from all subjects subsequent to a verbal and written briefing of all experimental procedures. Subjects were deemed healthy following a standard health history questionnaire. All subjects were required to abstain from caffeine, alcohol, and exercise for 24h before the study visit. Additionally, subjects reported to the laboratory in the morning after an overnight fast. No subjects were using over-the-counter or prescription medications at the time of study, with the exception of

oral contraceptives. Female participants were studied during the early follicular phase of their menstrual cycle or during the placebo phase of their oral contraceptive.

Screening Visit

During this visit, subject's physical characteristics were obtained (height, weight, body mass index) and they were familiarized with the exercise model and hemodynamic measurements. Arterial pressure was assessed using an automated auscultatory sphygmomanometer (Tango+; SunTech Medical, Raleigh, NC, United States).

Study Visit

Experimental approach

The goal of this experiment was to obtain skeletal muscle microdialysate from the resting and exercise leg during dynamic unilateral knee extension exercise to then expose to cultured human mast cells. An intramuscular microdialysis fiber was placed into both the right and left leg in the *vastus lateralis* under sterile technique. The subjects then performed dynamic unilateral knee extension exercise with their right leg at 60% of their pre-determined peak power for 60 minutes. Skeletal muscle dialysate was collected before, during, and after exercise at different time points. Then, cultured human mast cells were exposed to dialysate in order to determine if there is a circulating factor that is localized to exercising muscle and time dependent that impacts mast cell degranulation.

Measurements

Peak Power Assessment

The peak power assessment visit was used to determine peak power output during a unilateral dynamic knee extension peak exercise test performed to volitional fatigue. Dynamic knee extension exercise during both the peak and exercise visits was performed using a custom-built knee extension ergometer based on a computer-controlled step-motor that provided resistance against the subject's lower leg. Based on real-time measured of angular velocity and torque, power was calculated, and a feedback loop maintained measured power at the assigned level. Subjects were seated with their back at 60° upright and knee-extension exercise was performed with the right leg over a 45° range of motion, starting with the leg hanging at ~90° of flexion. Subjects were asked to maintain a cadence of 45 kicks min^{-1} while being provided with visual feedback of both kicking cadence and range of motion. Workload was ramped incrementally at a rate of 3 watts min. During the dynamic knee extension exercise test subjects reached peak power in 9.5 ± 0.4 min (range 7 - 13 min).

Dynamic Knee Extension Exercise

Subjects returned to the lab after their peak power test to perform 60 minutes of unilateral dynamic knee extension exercise at 60% of their peak power. The exercise was performed at 60% of peak power and a cadence of 45 kicks per minute. Power was ramped at the onset of exercise to 60% peak power over the first 10 minutes. Power output was recorded continuously throughout the 60 minutes of unilateral dynamic knee extension exercise. For all subjects in this study, the right leg served as the "exercise" leg and the left leg served as the "resting" leg.

Skeletal muscle microdialysis

All probes were inserted using sterile technique. The skin was anesthetized using a lidocaine (2%) and epinephrine (1:100,000) solution (Septodont, France) and underlying fascia was anesthetized using prilocaine hydrochloride (4%) (Septodont, France). Care was taken to ensure that the lidocaine and prilocaine was not injected into the skeletal muscle. Probes were inserted in the vastus lateralis in a direction parallel with muscle fiber orientation (~19°, relative to long-axis of muscle) using a splittable introducer. After insertion, probes were held in place by covering the entry site with a sterile transparent medical dressing. Probes used to measure interstitial histamine had a 100-kDa molecular mass cutoff with a 30-mm polyarylethersulphone membrane (63 MD Catheter, MDialysis, Stockholm, Sweden). Subsequent to insertion, microdialysis probes were perfused continuously at a rate of 5 μl/min (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA). Probes used to obtain skeletal muscle microdialysate were perfused with a 0.9% saline solution. Following insertion of the fiber, dialysate was collected immediately before exercise, at 20 minute intervals during 60 minutes of dynamic unilateral kicking exercise, and at 20 minute intervals during 60 minutes of supine recovery.

In-Vitro Mast Cell Degranulation Assay

Degranulation of mast cells was determined as the release of the granule marker β-hexosaminidase utilizing an in-vitro plate assay. Human mast cells (Kerafast) were cultured and cells were plated at 1 x 10^6 cells/ml in wells of a 96 well plate in imaging medium (50 ul total volume). 2 mg of compound 48/80 was dissolved in 1 ml imaging buffer. To serve as positive control, 50 ul of 2mg/ml compound 48/80 was added to

plated mast cells in those respective wells. In the negative control wells, 50 ul of imaging buffer was added. In the experimental wells, 50ul of skeletal muscle dialysate from each time point was added. In the histamine wells, plated cells were exposed to a physiological dose of histamine 10^{-3} mM, in order to determine the direct effect of histamine on mast cell degranulation. Cells were then incubated at 37°C for 20 minutes before being centrifuged and the supernatants removed. Supernatants were reacted with 2 mM 4-Nitrophenyl N-acetly-B-D-glucosamide (Sigma) at 37°C for 2 hours. Then, 0.5 M Tris(hydroxymethyl)aminomethane was used to stop the reaction, and the absorbance was read at 405 nm. Total content was determined by addition of Triton X-100 to lyse the cells. All test conditions were done in duplicate in a single experiment and each experiment consisted of analysis of dialysate from 1-2 subjects at all time points, imaging buffer (negative control), 48/80 (positive control), and histamine. Degranulation is expressed as a percentage of β-hexosaminidase activity in the supernatant divided by the total (supernatant plus pellet) activity. Total content was determined by addition of Triton X-100 to lyse the cells

Heart Rate and Blood Pressure

For this study, heart rate was monitored using a three-lead electrocardiograph and arterial pressure was assessed in the right arm employing an automated sphygmomanometer (Tango+; SunTech Medical, Raleigh, NC, United States). Mean arterial pressure was calculated as (systolic – diastolic)/3 + diastolic pressure. Heart rate and arterial pressure was obtained before exercise, at 10 minute intervals during exercise, and 20 minute intervals during recovery from exercise.

Statistical Analysis

Our primary outcome variable for this study was mast cell degranulation expressed as a percentage and was analyzed using a Two-Way ANOVA (Prism, GraphPad, San Diego, CA) to test for differences in mast cell degranulation (expressed as percentage) over time and between legs (resting vs. exercise). A One-Way ANOVA was used to determine significant differences in central hemodynamic measurements (heart rate and blood pressure) before and during exercise. Fishers LSD test was used for post-hoc analysis. Significance was set at $P < 0.05$. Data are reported as mean SEM unless stated otherwise (e.g. SD is used in Table 5.1 to indicate variability in the subject pool).

Results

Subject Characteristics

Subject physical characteristics and data obtained during the screening visit for study 1 are shown in Table 5.1. For this study, subject characteristics were similar to those obtained previously in our laboratory in young healthy subjects and consistent with recreationally active individuals.

Table 5.1 Subject Characteristics

n	9 (2F, 7M)
Age (yrs)	25 ± 3
Height (cm)	154.2 ± 7.2
Height (kg)	71.1 ± 4.3
BMI	23.5 ± 2.1

Central Hemodynamics. Heart rate and mean arterial pressure in response to one-hour of dynamic unilateral knee extension exercise are shown in Table 5.2. There was a significant increase in heart rate at 10 minutes into exercise ($p<0.05$) compared to pre-exercise that was sustained throughout the 60 minutes of exercise. Mean arterial pressure was no significantly different at any timepoint during exercise, compared to pre-exercise.

Table 5.2. Central Hemodynamics

	Heart Rate (beats min)	Mean Arterial Pressure (mmHg)
Time Point		
Pre-Exercise	63.1 ± 2.3	85.1±1.5
Time During Exercise		
10 min	88.2± 3.1*	84.3±2.2
20 min	88.8± 2.3*	84.3±2.5
30 min	92.0± 3.3*	86.5±3.5
40 min	93.6± 2.9*	86.3±3.4
50 min	91.9± 3.4*	87.2±2.4
60 min	93.6± 2.9*	86.9±1.0

Values are expressed as mean ± SEM. * indicates significantly different from pre-exercise ($p<0.05$)

Mast Cell Degranulation Response. Degranulation of mast cells (expressed as %) in response to skeletal muscle dialysate collected before, during, and after exercise is shown

in **Figure 5.1**. There was no significant difference in mast cell degranulation percentage in response to dialysate collected pre-exercise between the resting and exercise leg. There was a significant increase ($p<0.05$) in the percentage of mast cells that degranulated in response to the dialysate at 40 and 60 minute time points of exercise and the post-exercise 20 minute timepoint from the exercise leg, compared to pre-exercise values. Compared to pre-exercise values, there was no difference in mast cell degranulation in response to the dialysate from the resting leg at any timepoint. There was a significant difference in mast cell degranulation between the exercise and resting leg dialysate at 40 and 60 minute timepoint of exercise and post-exercise 20 ($p<0.05$).

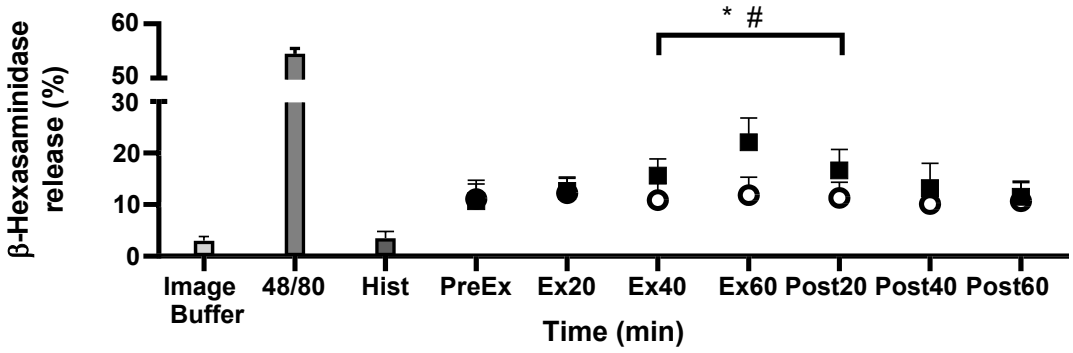

Figure 5.1. Mast cell degranulation in response to skeletal muscle dialysate collected during and after exercise. Image buffer served to subtract out background activity and 48/80 served as a positive control. All experiments used histamine in designated control wells to examine the effects of histamine directly on mast cell degranulation. Open circles (o) denote dialysate collected from the resting leg, black boxes (■) denote dialysate collected from the exercise leg. Data expressed as means ± SEM. * indicates significantly different from PreEx. # indicates significantly different from resting leg. ($p<0.05$)

Discussion

The purpose of this study was to determine if there is a/are local soluble factor(s) released in skeletal muscle during exercise that can cause mast cell degranulation. To do this, we had subjects perform 60 minutes of unilateral dynamic knee extension exercise at 60% of their peak power and collected intramuscular dialysate from both the resting leg and exercise leg before, during, and after exercise. We then exposed cultured mast cells to this collected dialysate to examine degranulation of mast cells (expressed as a percentage). We hypothesized that we would observe a greater percentage of mast cells degranulating in response to dialysate collected from exercising skeletal muscle compared to dialysate collected from resting skeletal muscle. In agreement with our hypothesis, there was a significant increase in mast cell degranulation in response to dialysate obtained from the exercise leg while no change compared to pre-exercise was observed in response to dialysate obtained from the resting leg. In addition, we observed differences in mast cell degranulation in response to dialysate collected from the exercise leg near the end of exercise and near the beginning of recovery, compared to the similar timepoints in the resting leg. Taken together, these data suggest that there is a/are local factor(s) that are released in locally in skeletal muscle in response to exercise that can cause mast cell degranulation, ultimately leading to histamine release.

Our lab has previously shown that histamine concentrations within skeletal muscle increase in response to exercise (127). These data indicate that certain factors released or altered within skeletal muscle are causing its release from mast cells and/or de novo formation via histidine decarboxylase. Using infusions of the antioxidant N-acetlycysteine prior and during exercise with no effect on the histaminergic signaling

pathway suggested that the oxidative stress associated with exercise is not necessary for histamine release (155). While we were able to deem this factor as not a exercise probable trigger for mast cell degranulation, there are still other factors that are present due to exercise that may lead to this increase in histamine release. This experiment investigated if there might be a/are local factor(s) released in skeletal muscle that serve as a "trigger" for histamine release.

Local Factors in Skeletal Muscle and Histamine

This study provides unique insight into the exercise-related trigger that causes the release of histamine in skeletal muscle in response to exercise. Our lab has previously shown that there in an increase in skeletal muscle histamine concentrations in response to exercise that continues post-exercise. Using skeletal muscle microdialysis, we attempted to examine if there might be factors released from skeletal muscle that can cause mast cell degranulation, leading to increased histamine concentrations. As seen in **Figure 5.**1, we observed that there was an increased percentage of mast cells that degranulated in response to dialysate that was obtained from the exercising skeletal muscle as opposed to dialysate from resting muscle. It can be noted that we did not see this at the 20 minute mark of exercise, but did at the 40 and 60 minute mark. This suggests that there is a time or intensity component and that the release of these factors may not be immediately released in response to exercise. This persisted to 20 minutes following exercise, however no significant differences in mast cell degranulation were observed with dialysate from 40- and 60-minutes post-exercise. This indicates that in response to exercise, skeletal muscle is releasing a cytokine or other soluble factor that is causing mast cells to

degranulate at a higher rate than that at rest. Our observations point to this being a localized response that appears to be quickly resolved following the cessation of exercise.

While it has been known for quite some time that exercise causes a release of cytokines and other factors from skeletal muscle in response to exercise, the nomenclature of these being classified as "myokines" has been quite recent (26). It has been shown that skeletal muscle is an immunogenic producer of cytokines which then can exert endocrine functions as well as autocrine on local cells. These myokines released in response to exercise can have local effects on metabolism, angiogenesis, and muscle growth. In addition, these myokines can also have systemic effects acting on other target organs such as adipose tissue, liver, brain, and others (10). Therefore, factors released by skeletal muscle in response to exercise can have significant effects both locally and systemically, indicating a powerful role for skeletal muscle in intra- and intercellular signaling. In our study, it appears that myokines or other local factors released in response to exercise are causing mast cell degranulation. A multitude of different cytokines and other stimulating factors can bind to mast cell receptors, resulting in degranulation and the release of histamine.

The Effect of Histamine on Human Mast Cells

As previously mentioned, there are a few different cytokines and factors that can directly bind to mast cells to cause degranulation, releasing histamine. Mast cells express a multitude of receptors that are both excitatory and inhibitory. For example, most mast cells express toll-like TLR receptors which LPS (a major surface molecule of gram-negative bacteria) can bind to and cause degranulation (130). In addition, it has been shown that mast cells can express histamine receptors and that histamine can bind to

these receptors, potentially leading to degranulation. This is serving in an autocrine signaling manner, in which histamine is mediating histamine release from mast cells. While there is an array of histamine receptors found throughout the human body (H_1-H_4), mast cells from different tissues express different receptors (70). For example, it has been shown that human skin mast cells express H_2 and H_4 receptors, but not H_3 receptors (85). Mast cells that are present in skeletal muscle tissue have not been extensively studied in regards to their receptor types and properties that are similar/unique to other mast cells.

Our goal of this experiment was to examine if there is a/are local factor(s) released in exercising skeletal muscle that can cause increased mast cell degranulation. Part of our experiment involved exposing our mast cells to physiologically relevant concentrations of histamine. The purpose of this was to "rule out" that histamine was acting on the mast cells to cause degranulation. As seen in Figure 5.1, the percentage of mast cells that degranulated in response to histamine was similar to the imaging buffer, which serves as a negative control or to subtract out background activity. With this data regarding histamine, it suggests that the mast cell line that we were using does not express histamine receptors or that they are not responsive to histamine. In our pilot work, we tested multiple different concentrations of histamine. These concentrations included 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9}mM. In none of these concentrations did we observe notable increases in mast cell degranulation compared to imaging buffer exposure, serving as a negative control. It is possible that in vivo, skeletal muscle mast cells in fact do express histamine receptors and can degranulate in response to histamine. The expression of these histamine receptors on mast cells is most likely highly dependent on the tissue that the mast cells are found in. Further work should investigate mast cells that

are present in skeletal muscle tissue, classifying their receptors and specific characteristics to elucidate their role in exercise responses. In our experiment, we did not observe any significant degranulation of mast cells in response to physiological doses of histamine. Therefore, in our experiment we can confidently rule out that histamine in skeletal muscle dialysate was causing mast cell degranulation in our isolated mast cell line, and it appears that there are other cytokines or circulating factors in the skeletal muscle that are causing the degranulation response we observed.

Could It Be Complement?

Nearly 100 years ago, complement was discovered as a component of plasma that enhances the opsonization (coating of a particle to enhance phagocytosis) of bacteria by antibodies and facilitates antibody-dependent killing of bacteria. It gets its nomenclature of "complement" in that is complementary to that of humoral immunity. It is comprised of membrane-bound regulators and receptors as well as different plasma proteins that can interact with different cell types of the immune system. The C1 complex is formed when an antigen is bound to an antibody and then it can break in to C2a and C4b fragments, this is part of the classical pathway (95, 96). Then, C3 convertase can hydrolyze leaving C3b and C3a fragments which then cleave to C5a and C5b which can form a cylindrical membrane and attack cells in a direct inflammatory manner.

Both C3a and C5a have a range of functions on their own, one of these being mast cell degranulation (42). C3a and C5a can bind to mast cells and drive intracellular signaling processes, ultimately leading to mast cell degranulation and histamine release. C3a has a molecular weight of ~10kDa while C5a has a molecular weight of ~113kDa (2, 115). The skeletal muscle microdialysis fibers utilized for this study were 100kDa so we

can presume that if C5a was present in the tissue, it was not able to be collected in the skeletal muscle effluent. However, at ~10kDa, C3a is small enough to pass through and could have been present in the dialysate we collected.

Aerobic based exercise has been shown to increase components of the complement system. Sanz et. al had subjects complete exhaustive, aerobic exercise and obtained blood draws before and following observed (141). They observed that there were increases in all components of the complement system, including a 45% increase in circulating C3a levels. Consistent with this finding, Smith et al had individuals perform aerobic exercise and obtained venous samples before and after exercise (158). In addition, they examined the long-term effects of aerobic exercise on complement component expression. They observed that following short-term aerobic exercise, there was an increase in the classical pathway of the complement system, with increases in C3 and C4, with specific increase in C3a. They did note that highly trained individuals had lower C3 levels and showed that untrained individuals had a more exaggerated C3a response compared to trained individuals following exhaustive aerobic exercise. For our study, all subjects were considered untrained and were only moderately active, therefore we would expect robust complement activation in response to exercise.

It appears that C3a and other components of the complement system have direct effects on mast cells and can cause degranulation and the subsequent release of histamine. In addition to this, others have demonstrated that aerobic exercise (which is the model that we used) mounts an immune response with an upregulation of the complement system. Therefore, it is plausible that the complement system is playing

some role in the increased mast cell degranulation we observed, in the skeletal muscle dialysate from exercising muscle when exposed to mast cells compared to resting values.

Future experiments could help to elucidate the role of the complement system response to aerobic exercise and how it may affect mast cell degranulation. The protocol could be repeated again under similar circumstances, however both venous blood samples and dialysate could be collected before, during, and following exercise. There are assays to identify components of the complement system (C3a and C5a which are of interest) along with others, from collected venous blood samples. In addition, transcriptomic analysis of collected skeletal muscle dialysate could examine how complement might be changing directly in skeletal muscle, and if we observe similar or different changes locally as compared to systemic changes. It appears from the data thus far that this is a highly localized response, but it would be of interest to examine if our model results in a systemic wide response of some factors (such as complement, which have been observed) in addition to their local responses. Given the nature of activation of complement from aerobic exercise and their role in mast cell degranulation, further work is warranted to examine the role of the complement system and its possible interaction with histamine and exercise.

Future Experiments

This study has furthered our knowledge regarding the interaction of exercise and histamine release in skeletal muscle, our data indicating that there is a factor or factors released by exercising skeletal muscle that appears to be highly localized that causes mast cells to degranulate. We observed that dialysate fluid collected from exercising skeletal muscle was unique from dialysate fluid from resting muscle in that it caused a higher

percentage of mast cells to degranulate in response. In addition, there appears to be a time component to this release. We noted a significant difference in responsiveness of mast cells to collected dialysate from the latter portion of exercise and immediately after, however by 40 minutes of recovery this factor or factors did not appear to be present. This transient, robust rise in skeletal muscle in response to exercise is interesting and deserves further investigation.

An "omics" approach would be a logical next step to further understand what it is that is being released in skeletal muscle in response to exercise. This approach would involve repeating this experiment, however analysis of the collected dialysate would involve investigating the transcriptome of this dialysate, a technique that our lab has done before in collaboration with our Genomics Core Facility on our campus. Utilizing this approach could elucidate what factor or factors might be upregulated in response to exercise that is unique to exercising skeletal muscle, and not present or responsive in resting tissue. In addition, it would be of interest to examine if there are measurable increases in histidine decarboxylase in skeletal muscle dialysate, to further investigate the impact of exercise on this enzyme. The timepoints of every 20 minutes during 60 minutes of exercise are appropriate time points to collect, and recovery of 60 minutes seems to be sufficient to capture the transient responses that are occurring in skeletal muscle.

Perspectives and Considerations

From this experiment, we can gather that exercise is causing skeletal muscle to release a/multiple soluble factor that results in mast cell degranulation, this response being highly localized. We utilized an isolated human mast cell line for the mast cell degranulation assay. While we are confident in the data that we obtained, we have to be

cautious in the overall conclusions and scope that we take from this. Mast cells in different tissues of the body express different receptors, such as the mast cells in skin have slightly different structures and receptors than mast cells in the gut. The isolated mast cells we utilized were not tissue-specific and were blood CD34+ derived (80).

We did observe significant differences in the degranulation of mast cells in response to skeletal muscle dialysate collected from the exercising leg as opposed to the resting leg. However, these mast cells used in this study could differ than the mast cells that are expressed in human skeletal muscle tissue. We are not able to ascertain how different or how similar they may be in comparison to the mast cells that are present in human skeletal muscle tissue. Therefore, we need to be cautious in our interpretation of what we observed in vitro might be slightly different than what is happening in vivo. Regardless, we are confident in our findings that there is something unique that is released locally in exercising skeletal muscle that is not observed in resting tissue, which has profound effects on mast cell degranulation and the release of histamine.

Summary and Bridge

Both whole body and single-leg aerobic exercise initiates a sustained post-exercise vasodilatory response that is dependent upon histaminergic signaling. We have shown that the dialysate in exercising/exercised skeletal muscle is unique from resting, as we observed different responses of mast cell degranulation. This suggests that the "signal" for histamine release appears to be quite localized and confined to the skeletal muscle that is working. While this "signal" might be localized, we have demonstrated profound systemic effects that histaminergic signaling contributes to. Another line of research we have begun to examine is the role of histaminergic signaling on the post-

exercise inflammatory response. We have published data that demonstrates that histamine H_1 and H_2 antagonism augments the mRNA expression of some proteins in skeletal muscle following exercise. Among these are some that are involved in the post-exercise inflammatory response. In Chapter VI, we will further examine the role that histaminergic signaling has on the acute post-exercise inflammatory response in untrained individuals following a novel bout of aerobic exercise.

CHAPTER VI

EFFECT OF HISTAMINE-RECEPTOR ANTAGONISM ON THE ACUTE INFLAMMATORY RESPONSE TO AEROBIC CYCLING EXERCISE

Introduction

Following a bout of novel exercise, there is an acute inflammatory response consisting of an increase of circulating white blood cells (leukocytes) (123, 131). This initial response immediately following exercise is typically considered "pro-inflammatory" and the following resolving component is typically considered "anti-inflammatory" (47). During both of these phases, cytokines are released from damaged muscle tissue (termed myokines) and from other non-active tissues. A balance of pro-inflammatory and anti-inflammatory cytokines is necessary for the proper repair of damaged tissue and for appropriate adaptation responses to occur (145). Dysregulated pro-inflammatory responses can lead to chronic diseases, and often exercise can be used as a therapeutic option as it has anti-inflammatory effects (116, 61). Both resistance exercise and aerobic exercise has been demonstrated to elicit leukocytosis, much depending on intensity and duration (56). While much has been discovered regarding this acute post-exercise inflammatory response, newer literature is investigating interventions which may serve to enhance or dampen this inflammatory response.

One important mediator in inflammatory responses is histamine (11). Histamine can be released from many tissues; our lab has shown that it can be released by skeletal muscle in response to exercise. The source of this histamine can be either from *de novo* production via the enzyme histidine decarboxylase and/or mast cell degranulation in

which histamine is synthesized and released from mast cells. Histamine has the ability to produce many different physiological responses, including the contraction and dilation of certain smooth muscle (68). It initiates the margination of capillary endothelial cells to increase vascular permeability and allows larger molecules to pass through, that typically cannot (72). Histamine also aids in chemotaxis, a physiological mechanism to recruit various cell types to the site of injury or damaged tissue (66, 28).

Our lab has shown an important role in histamine and histaminergic signaling in regulating post-exercise vascular responses, including post-exercise vasodilation and post-exercise hypotension. Antagonism of histamine H1 and H2 receptors with over-the-counter medications blunts these responses with whole-body exercise (94) and abolishes it with isolated small muscle-mass exercise (7). Due to its critical importance in these vascular responses and given its nature in inflammatory responses, histamine and histaminergic signaling could be necessary for appropriate acute post-exercise inflammatory responses.

In addition to vascular responses, our lab has also shown that antagonism of histamine H_1 and H_2 receptors significantly alters the exercise transcriptome in skeletal muscle of humans following a bout of exercise (126). Exercise using dynamic unilateral knee extension exercise causes the differential expression of over 3000 protein coding genes in exercised skeletal muscle. We have shown that histamine receptor antagonism alters the expression of 795 of these genes that are typically affected by exercise. Among these genes are some that are involved in both the pro- and anti-inflammatory response to exercise. Therefore, it appears that histamine receptor antagonism affects RNA level expression changes in skeletal muscle that are related to inflammatory responses.

However, it is unknown if histamine receptor antagonism affects the circulating cytokine expression profile typically observed following a novel bout of exercise.

Therefore, the purpose of this study was to gain insight into histamine's role in the acute post-exercise inflammatory response in untrained individuals performing a novel bout of exercise. Specifically, human subjects performed 60 minutes of stationary cycling at 60% of their VO_{2peak}. Circulating leukocytes and cytokines were measured periodically for 48 hours after exercise. Subjects performed the exercise two times, once under placebo condition and once under a condition where H_1 and H_2 receptor antagonism with over-the-counter histamine receptor antagonist medications. It was hypothesized that histamine receptor antagonism would decrease the inflammatory response to novel aerobic exercise.

Methods

Subjects

For this study seven (2 females, 5 males) young healthy non-smoking subjects volunteered. This study was approved by the Institutional Review Board at the University of Oregon and was performed in accordance with the principles outlined by the Declaration of Helsinki. Written, informed consent was obtained from all subjects subsequent to a verbal and written briefing of all experimental procedures. Subjects were deemed healthy following a standard health history questionnaire. All subjects were required to abstain from caffeine, alcohol, and exercise for 24 h before the study visit. Additionally, subjects reported to the laboratory in the morning after an overnight fast. No subjects were using over-the-counter or prescription medications at the time of study,

with the exception of oral contraceptives. Female participants were studied during the early follicular phase of their menstrual cycle or during the placebo phase of their oral contraceptive.

Screening Visit

During this visit, subject's physical characteristics were obtained (height, weight, body mass index) and they were familiarized with the exercise model and hemodynamic measurements. Arterial pressure was assessed using an automated auscultatory sphygmomanometer (Tango+; SunTech Medical, Raleigh, NC, United States).

Experimental approach

The goal of this experiment was to obtain venous blood samples from untrained subjects after performing 60 minutes of stationary cycling, one exercise test under placebo conditions and one exercise test under histamine H1 and H2 receptor antagonism conditions. Exercise test visits were separated by at least 30 days. A food diary was provided to the subjects prior to the first testing visit. The food diary encompassed the 24-h prior to the exercise visit as well as the 48-hr observation period following exercise. Participants reported to the laboratory in the morning after an overnight fast. After a confirmed negative pregnancy test for female subjects, subjects were block randomized in to one of two testing orders. One order was placebo being the first visit, histamine receptor blockade for the second visit. The other order was histamine receptor blockade for the first visit, placebo for the second visit. Venous blood draws were obtained prior to

exercise and at selected time points over the course of 48 hours of recovery from exercise. Figure 6.1 below is a timeline of the study.

Figure 6.1. Inflammation Study timeline. The 48-hr study and observational period was conducted twice after the subject consumed either Placebo or Blockade (Rx). Each blood drop (🩸) indicates a time point at which a venous blood sample was obtained.

Following an initial blood draw, subjects consumed the placebo or blockade pills orally with 6 oz of water and laid supine for 60 minutes. Subjects then performed 60 minutes of stationary cycling at 60% of peak oxygen uptake, which was previously determined with a peak oxygen uptake test. Exercise of this intensity produces a robust sustained post exercise vasodilatory response (59). Venous blood samples were obtained immediately after exercise, then at 6, 12, 24, and 48 hours after the completion of exercise.

Histamine H1 and H2 Receptor Antagonism.

Subjects consumed either placebo or histamine receptor antagonist medications, these consisted of fexofenadine and ranitidine for 5 subjects, fexofenadine and famotidine for 2 subjects. Oral administration of 540 mg of fexofenadine, a selective H1-receptor antagonist, reaches peak plasma concentrations within 1 hour and has a 12-hour half-life. Oral administration of 300 mg ranitidine, a selective H2-receptor antagonist, reaches peak plasma concentration within 2 hours and has a 3-hour half-life. This dosage of

histamine receptor antagonists results in ~90% inhibition of H1 and H2 receptors lasting for 6-hours after administration. Due to an FDA voluntary recall on ranitidine, 2 subjects received famotidine which is a selective H2 receptor antagonist. Oral administration of 40 mg famotidine, a selective H2 receptor antagonist, reaches peak plasma concentration within 2 hours and has a 3-hour half-life, with pharmacokinetics similar to ranitidine. These H_1 and H_2 receptor antagonism medications do not alter blood flow, heart rate, or blood pressure at rest.

Placebo

The placebos were manufactured by Zeebo, free of any active ingredients and mainly containing microcrystalline cellulose.

Measurements

Blood Sampling. Venous blood (9ml) was collected from a superficial vein in the antecubital space into three 3-ml Vacutainers (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes NJ, USA) for collection plasma (K2 EDTA anticoagulant). Blood was then aliquoted for separation and quantification of white blood cell populations via flow cytometry (Gallios, Beckman Coulter Life Sciences, Indianapolis IN, USA) using the manufacturers data acquisition software (Kaluza, Beckman Coulter Life Sciences, Indianapolis IN, USA). Separate aliquots were centrifuged at 1,275 RCF and stored in cryovials at -80C until analysis.

Cell Staining Protocol for Flow Cytometry. Cell suspensions were re-suspended and 1.5 x 10^6 cells were transferred to individual 2.0ml microcentrifuge tubes for each

staining sample plus the unstained cells. Cells were pelleted at 500G for 5 minutes. Cells were re-suspended in 100ul of staining buffer (PBS + 1% BSA + 10% FBS = Biolegend 420201 + 50 mL FBS – Gibco 26140-129, heat deactivated at 560 for 45 minutes) plus 10ul mouse IgG (Jackson Immuno – ChromPure 015-000-003). Suspensions were incubated for 30 minutes at room temperature. Cells were washed with 1mL of staining buffer, centrifuged and supernatant was discarded. 100ul of antibody staining cocktail was added to each tube and incubated for 30 minutes in the dark at room temperature. 1 mL of staining buffer was added to each tube, cells were centrifuged and supernatant was discarded. Cell pellet was re-suspended in 500uL staining buffer and read in a flow cytometer. The following antibodies were used for the leukocyte staining protocol: CD62E, CD86, CD309, CD34, CD192, CD66B, CD14, CD16, CD3, CD19, CD56, CD45.

Circulating Cytokine Analysis. Plasma was also analyzed for inflammatory cytokines via a bead based flow cytometry kit (Biolegend, LEGENDplex Human Inflammation Panel 1, multianalyte flow assay kit). This method of cytokine identification uses beads of varying size coated with antigens specific to cytokines of interest (MCP-1, IL-10, IL-17A, IL-18,). The size and fluorescence of the beads allows for separation and quantification of the cytokine by flow cytometry (Gallios, Beckman Coulter Life Sciences, Indianapolis IN, USA.)

Hemodynamic Measurements

All resting measurements were made pre- and post-exercise with the subjects in the supine position. Subjects were asked to remain quiet and relaxed during the pre and post-exercise hemodynamic measurements. Heart rate and blood pressure during exercise were made with the subjects in the upright position on the stationary cycle ergometer.

Heart Rate and Blood Pressure. Arterial blood pressure was measured in the right arm using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh NC, USA). Hear rate was monitored using a three-lead electrocardiograph (Tango+, SunTech Medical, Raleigh NC, USA). Heart rate and blood pressure were obtained before, during 60 minutes of cycling exercise, and post-exercise.

Femoral Blood Flow. Femoral artery blood flow velocity was measured via duplex ultrasonography. A linear-array vascular ultrasound probe (9 MHz, Phillips iE33, Andover, MA., USA) and an insonation angle of 60 was used to measure blood flow in the common femoral artery, approximately 2-3 cm proximal to the bifurcation. The Doppler ultrasound machine was interfaced with a computer running custom audio recording software. Velocity measurements were determined using an intensity-weighted algorithm (custom software), subsequent to demodulation of forward and reverse Doppler frequencies. Leg blood flow was calculated as cross-sectional area multiplied by femoral mean blood velocity and reported in ml per min. Femoral vascular conductance was calculated by dividing femoral blood flow by mean arterial pressures and expressed as ml per min per mmHg.

Statistical Analysis

Our primary outcome variables for this study (leukocytes, neutrophils, granulocytes, monocytes, and cytokines) were analyzed using a Two-Way ANOVA (GraphPad Prism) to test for differences in cell populations over time and between conditions (placebo vs. blockage). A Two-Way ANOVA was used to determine significant differences in central and peripheral hemodynamic measurements during and following exercise. Fisher's LSD test was used for post-hoc analysis. Significance was set at $P < 0.05$. Data are reported as mean SEM unless stated otherwise (e.g. SD is used in Table 6.1 to indicate variability in the subject pool).

Results

Subject Characteristics

Subject physical characteristics and data obtained during the screening visit are shown in Table 6.1. For this study, subject characteristics were similar to those obtained previously in our laboratory.

Table 6.1 Subject Characteristics

n	7 (2F, 5M)
Age (yrs)	24 ± 3
Height (cm)	161.9 ± 10.2
Height (kg)	75.1 ± 9.7
BMI ($kg*m^{-2}$)	23.2 ± 2.8

Values are means ± SD.

Pre-exercise Hemodynamics. Pre-exercise heart rate and mean arterial blood pressure are shown in **Table 6.2.** Both heart rate and mean arterial pressure did not differ between control and H1/H2 blockade conditions. Pre-exercise femoral blood flow and femoral vascular conductance are shown in **Table 6.3.** Femoral blood flow did not differ between control and H1/H2 blockade conditions. Likewise, femoral vascular conductance did not differ between control and H1/H2 blockade conditions.

Post-exercise Hemodynamics. Table 6.2 shows heart rate and mean arterial blood pressure during recovery from exercise. Heart rate was not different between control and H_1/H_2 antagonist conditions. In addition, mean arterial pressure did not differ between control and H_1/H_2 blockade conditions. Absolute values for femoral blood flow and femoral vascular conductance are shown in **Table 6.3.**

Table 6.2. Central Hemodynamics

	Heart Rate (beats min $^{-1}$)		Mean Arterial Pressure (mmHg)	
Time Point	Placebo	H_1/H_2 Blockade	Placebo	H_1/H_2 Blockade
Pre-Exercise	63.1 ± 2.3	65.4 ± 2.1	85.1±2.5	87.9±1.3
Time Post Exercise				
20 min	68.8± 2.3	71.3± 2.3	79.3±2.7*	84.3±2.5
40 min	67.2± 2.9	68.2± 2.9	80.9±2.3*	86.1±1.8
60 min	66.6± 2.9	67.6± 2.9	82.1±2.5*	84.9±1.2

Table 6.3. Absolute Femoral Blood Flow and Femoral Vascular Conductance

	Femoral Blood Flow (ml min $^{-1}$)		Femoral Conductance (ml min $^{-1}$ mmHg)	
Time Point	Placebo	H_1/H_2 Blockade	Placebo	H_1/H_2 Blockade
Pre-Exercise	202 ± 18	220 ± 15	2.2 ± 0.4	2.1 ± 0.9
Time Post Exercise				
20 min	360 ± 26*	244 ± 10	4.1 ± 1.1*	2.9 ± 1.3
40 min	352 ± 18*	231 ± 15	4.0 ± 0.8*	2.7 ± 0.6
60 min	320 ± 15*	235 ± 12	3.8 ± 1.2*	2.4 ± 0.9

Values are expressed as mean ± SEM. * indicates significantly different from pre-exercise ($p<0.05$)

Circulating Leukocytes. There was no difference in leukocyte count pre-exercise between placebo and blockade conditions. Exercise caused an increase in circulating leukocytes immediately after exercise (time effect $p<0.05$). Compared to pre-exercise, immediately after exercise there was a 27 ± 3.2% rise in leukocytes in the control condition, and a 38 ± 1.5% rise in leukocytes in the blockade condition. There were no differences between placebo and blockade (drug effect $P = 0.69$) nor was there a pattern of change over time between Placebo and Blockade (drug X time interaction $P=0.98$) (**Figure 6.2**).

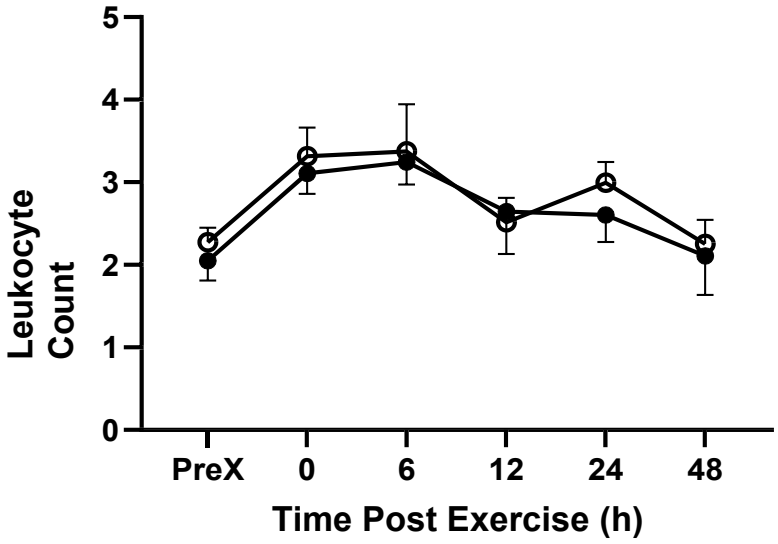

Figure 6.2. Total Leukocyte count per ml of blood during 48-hour of recovery from cycling exercise. Data expressed as means ± SEM. ME = main effect ($p<0.05$) * indicates significantly different from placebo ($p<0.05$) Open circles (o) blockade, closed circles (•) placebo

Circulating Neutrophils. There was no difference in neutrophil count pre-exercise between placebo and blockade. Exercise caused an increase in circulating neutrophils immediately after exercise (time effect $p<0.05$). Compared to pre-exercise, immediately after exercise there was a 34 ± 4.2% rise in neutrophils in the control condition, and a 30 ± 5.3% rise in neutrophils in the blockade condition. There were no differences between placebo and blockade (drug effect $P = 0.85$) nor was there a pattern of change over time between Placebo and Blockade (drug X time interaction $P=0.90$) (**Figure 6.3**).

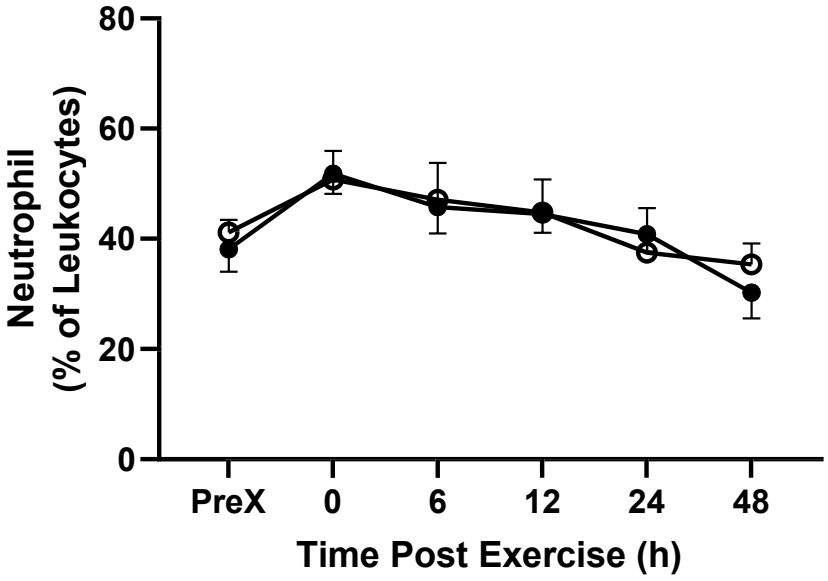

Figure 6.3. Neutrophil count as percent of leukocytes during 48-hour of recovery from cycling exercise. Data expressed as means ± SEM. ME = main effect ($p<0.05$) Open circles (o) blockade, closed circles (•) placebo

Granulocytes and Monocytes

Granulocytes and monocyte subpopulations were not different between drug conditions or over time (Table 6.4). There was no significant difference for drug, time, or interaction in any of the monocyte subpopulation in this analysis.

Table 6.4. Granulocyte and monocyte sub-populations during recovery from exercise

		Time after exercise (h)						P-Value		
		Pre	0	6	12	24	48	Drug	Time	Interact
Gran (%)	Placebo	41.1 ±7.2	42.0 ±8.1	52.3 ±6.9	50.9 ±8.1	44.6 ±4.0	42.1 ±7.2	0.32	0.34	0.94
	Blockade	44.5 ±5.7	52.1 ±5.3	54.0 ±7.0	50.4 ±6.5	43.4 ±10.3	45.4 ±7.1			
Mon I (%)	Placebo	0.61 ±0.2	0.78 ±0.5	3.21 ±5.1	0.52 ±0.3	0.87 ±0.7	0.40 ±0.2	0.88	0.88	0.99
	Blockade	0.50 ±0.4	0.82 ±0.2	0.46 ±0.3	1.21 ±0.6	1.15 ±0.5	1.15 ± 0.6			
Mon II (%)	Placebo	0.18 ±0.4	0.22 ±0.2	2.25 ±2.9	0.88 ±0.1	0.78 ±0.2	1.13 ±1.8	0.72	0.90	0.99
	Blockade	0.15 ±0.2	0.23 ±0.2	1.33 ±0.3	1.07 ±0.2	0.24 ±0.2	0.53 ± 0.2			
Mon III (%)	Placebo	0.58 ±0.2	0.24 ±0.2	0.22 ±0.2	0.28 ±0.1	0.18 ±0.1	0.64 ±0.2	0.58	0.97	0.99
	Blockade	0.38 ±0.2	0.30 ±0.1	0.10 ±0.1	0.24 ±0.1	0.12 ±0.2	0.16 ±0.2			

* indicates significant difference

Plasma Cytokine Levels

Plasma cytokine levels were analyzed in 5 subjects, shown in Table 6.5. There was a significant main effect of time in circulating MCP-1 cytokine levels (p=0.04) and a trending effect of drug (p=0.12). There was also a significant main effect of time in circulating IL-10 cytokine levels (p=0.01) however no significant effect of drug (p=0.82). There was no significant effect of time, drug, or interaction in circulating IL-18 or IL-17a cytokine levels.

Table 6.5. Plasma cytokine changes during recovery from exercise (n=5)

		Time after exercise (h)						P-Value		
		Pre	0	6	12	24	48	Drug	Time	Interact
MCP-1 (pg/mL)	Placebo	142.5 ±9.8	171.7± 9.2	154.7 ±3.8	151.2 ±15.8	153.2 ±6.5	148.5 ±6.1	0.12	0.04*	0.98
	Blockade	155.8 ±11.7	180.1± 9.4	169.9 ±9.1	159.6 ±13.2	155.3 ±9.2	155.6 ±8.3			
IL-18 (pg/mL)	Placebo	54.9 ±21.4	45.4 ±15.4	33.6 ±8.0	42.9 ±10.1	33.6 ±9.3	39.1 ±9.7	0.68	0.95	0.67
	Blockade	36.1 ±5.5	50.9 ±8.3	55.7 ±24.2	44.1 ±10.8	47.9 ±10.6	32.3 ±7.7			
IL-10 (pg/mL)	Placebo	27.5 ±3.3	28.9 ±2.6	31.6 ±4.3	26.2 ±3.3	26.2 ±4.9	42.3 ±5.8	0.82	0.01*	0.90
	Blockade	28.2 ±3.1	29.1 ±3.0	27.4 ±4.6	26.5 ±4.8	32.1 ±5.0	42.5 ±3.2			
IL-17a (pg/mL)	Placebo	121.4 ±15.6	110.9 ±6.3	103.2± 1.1	104.3 ±2.1	105.6 ±3.6	107.5 ±5.1	0.68	0.74	0.51
	Blockade	106.2 ±2.4	117.9 ±11.1	110.5 ±5.4	110.8 ±5.3	107.8 ±2.9	107.3 ±3.7			

* indicates significantly difference ($p<0.05$)

Discussion

The purpose of this study was to determine the effect of histamine receptor H_1/H_2 antagonism on the acute post-exercise inflammatory response. The main finding was that there was no significant difference in the acute post-exercise inflammatory response under histamine receptor H_1/H_2 antagonism. We did note a significant effect of time in many of the systemic circulating inflammatory factors, indicating that our exercise model induces a systemic inflammatory response. Overall, the data suggest that histamine

receptor antagonism does not affect the acute post-exercise inflammatory response following aerobic exercise in untrained individuals.

Acute Post-Exercise Inflammatory Response and Histamine

To our knowledge, this is the first known investigation to examine the link between histamine and the systemic inflammatory response following a bout of aerobic exercise. Our exercise protocol was selected for two reasons, one of them relating directly back to histamine. We have shown robust post-exercise vasodilatory responses in individuals who perform 60 minutes of stationary cycling at 60% VO2 peak. This response is blunted by 80% when this exercise is performed under histamine receptor H_1/H_2 blockade (94), indicating a critical role for histamine and histaminergic signaling in this response. Second, this intensity and duration of exercise has been shown to elicit an inflammatory response in untrained individuals. Previous studies have used this exercise modality and have demonstrated systemic leukocytosis and increase in white blood cells systemically within 12 hours of exercise. Therefore, we are confident in the research design in its ability to elucidate the role of histaminergic signaling in the post-exercise inflammatory response to this type of exercise.

We observed a notable increase in circulating leukocytes and neutrophils immediately after exercise that persisted until 6 hours following exercise. However, there were no differences between placebo or blockade condition in leukocyte and neutrophil counts (**Figures 6.2 and 6.3**). We also did not note any significant differences of drug condition on circulating granulocytes or monocyte sub-populations. We did note a significant effect of time in certain circulating cytokines such as MCP-1 and IL-10 (Table 6.6), however no effect of drug nor an interaction. The significant upregulation of these

cytokines, in addition to leukocyte and neutrophil counts demonstrate an acute inflammatory response to this exercise model. Our data shows that while we did have an acute inflammatory response to our exercise model, this inflammatory response is not affected by histamine receptor H_1 and H_2 antagonism.

Ranitidine vs Famotidine and Sustained Post-Exercise Vasodilation

Sustained post-exercise vasodilation can last upwards of several hours during the recovery period following a bout of aerobic exercise. This response is typically absent following short duration exercise (7). Sustained post-exercise vasodilation is usually studied following large muscle-mass dynamic exercise ranging from 30-60 minutes. The dependence of this response is on the duration and intensity of exercise, consistently observed and greater in magnitude following moderate intensity (50-60%) and prolonged (30-60 minutes)(59).

Our lab has been using ranitidine as a histamine H2 receptor antagonist, in combination with fexofenadine as the histamine H1 receptor antagonist. We typically use 300 mg of ranitidine HCl (Brand name: Zantac) as this oral dose has been shown to adequately block H2 receptors with a peak plasma concentration at approximately 2.2 hours and a 2.6 hour half-life. Furthermore, the responses are >90% inhibited within 60 minutes and remain inhibited for 6hrs after administration (20). This blockade prevents a decrease in smooth muscle intracellular calcium levels that usually occur with histaminergic signaling. We enrolled and completed 5 subjects in the study using fexofenadine and ranitidine for their drug visit. During our study, the FDA issued a voluntary recall on ranitidine on the market due to possible contamination. Even though none of our batch or lot number ranitidine was affected, in caution we decided to move to

another H_2 receptor antagonist. We chose famotidine (brand name: Pepcid AC) as it is a selective H_2 receptor antagonist with similar pharmacokinetics for 2 subjects.

In line with other research from our lab, we demonstrated a significant blunting of the sustained post-exercise vasodilatory response under blockade conditions, as seen in Table 6.3. This blunted response was similar regardless of H2 receptor antagonist (ranitidine vs famotidine). Following exercise under placebo conditions, there was a significant increase in femoral blood flow out to 60 minutes following exercise. At 60 minutes, there was still a 16% increase in femoral blood flow and a 30% increase in femoral vascular conductance, compared to pre-exercise values. Under histamine H_1/H_2 receptor antagonism, there were no significant differences in femoral blood flow or femoral vascular conductance at any timepoint following exercise, compared to pre-exercise. This did not differ depending on the H_2 receptor antagonist. Immediately after exercise, with fexofenadine/ranitidine the change from pre-exercise was about 4% and with fexofenadine/famotidine approximately 3%. Examining mean arterial pressure, blood flow, and femoral conductance data, there does not appear to be a differential effect on post-exercise hemodynamics due to famotidine compared to ranitidine. However, given the continuation of FDA recall on ranitidine, further work should be done on famotidine, to ensure and verify its similarity to ranitidine in the ability to blunt the sustained post-exercise vasodilatory response in order to continue our work on the effect of histamine receptor antagonism on the exercise responsome.

Role of Histamine in Capillary Permeability/Systemic Inflammation

We noted that histamine H_1/H_2 receptor antagonism, specifically with fexofenadine/ranitidine, resulted in an augmented acute post-exercise systemic

inflammatory response. Leukocytes and neutrophils were present in higher counts and percentages over the time course following exercise. Subjects performed the same workload for the same amount of time, in similar environmental conditions so we are confident that their exercise sessions were similar in intensity between placebo and blockade. It is possible that these antagonist medications are influencing capillary permeability which is then leading to the augmented systemic circulating factor response we observed.

The vasculature is composed of vascular endothelial cells and vascular mural cells, such as vascular smooth muscle cells and pericytes (122, 142). These functionally interact with each other and coordinate a variety of vascular functions. Vascular permeability is determined by two major factors: blood flow and endothelial barrier function (142). Histamine-induced vascular permeability is mediated by intracellular signaling pathways, such as the phosphorylation of VE-cadherin and other critical proteins in the endothelial barrier (33, 167). An increase in vascular permeability will allow larger solutes and molecules to easily pass from inside the vessel to the interstitial space and vice versa.

In response to challenging exercise (both aerobic and resistance exercise based) there is a release of white blood cells (leukocytosis) that migrate to the damaged skeletal muscle tissue. Leukocytes and neutrophils are typically found within 1 hour in skeletal muscle, following cessation of intense exercise. These leukocytes have many sources as they respond to the stressor of exercise. This mobilization of white blood cells can come from bone marrow and the spleen, where they demarginate through blood vessel walls to then enter the blood stream circulation, to then migrate to the damaged tissue. There are a

multitude of chemotactic signals in skeletal muscle that act to bring these leukocytes, neutrophils, granulocytes, and other cell types to the affected tissue. As with their mobilization, many of these cell types are not able to easily pass through blood vessel walls to then enter tissue. Histamine can aid in the margination and diapedesis, allowing these cell types to move from the blood, through the vessel wall, and then to enter tissue. This movement of leukocytes in to the affected tissue is essential for proper skeletal muscle adaptation and remodeling that occurs in response to a bout of intense exercise.

With our model, following a bout of intense exercise under placebo conditions, we hypothesized that histamine may be acting to increase vascular permeability to allow circulating factors which are circulating in the blood stream that are typically too large to pass through vasculature, to then enter skeletal muscle or other tissues. Following a bout of intense exercise under histamine H_1/H_2 receptor antagonism, vascular permeability may be affected to where these circulating factors are not able to enter skeletal muscle tissue and cross barriers as easily, therefore leaving them in systemic circulating longer than under placebo conditions. We did not note any significant differences of circulating factors between placebo and blockade conditions. However, we do not know what is happening locally in previously exercise skeletal muscle under histamine receptor antagonism. Being able to obtain skeletal muscle biopsies following exercise to compare blockade to placebo would help to elucidate if there are few leukocyte-type cells in previously exercise skeletal muscle under blockade conditions versus placebo conditions. Future work should investigate this as well as further examine the role of histamine on capillary permeability following exercise to elucidate the mechanisms of what we observed in this study.

This reduction of sustained post-exercise vasodilation with histamine receptor antagonism is consistent and in line with previous research from our lab. While we did not find differences in the circulating inflammatory marker profile of subjects under histamine receptor antagonism compared to placebo, we recognize that the acute inflammatory response to aerobic exercise is a complicated process in which some individuals show more robust responses than others. There great deal of variability in the inflammatory response to exercise in humans which can be affected by many things such as external environmental factors, diet, and many others. Further work should be done to gather a larger pool of subjects to further examine the role of histaminergic signaling on acute post-exercise inflammatory responses.

Perspectives and Considerations

From this experiment, we have shown that our model of aerobic exercise utilizing 60 minutes of stationary cycling at 60% VO2 peak induces an acute inflammatory response. However, our data suggest that histamine H_1/H_2 receptor antagonism does not appear significantly augment this acute post-exercise inflammatory response following aerobic exercise in untrained individuals.

While we did note significant differences in systemic circulating inflammatory factors, it is unknown what is happening within skeletal muscle. We have data showing out to 3 hours following exercise, beyond that is unknown. It could be possible that antagonizing the effects of histamine on histamine H_1/H_2 receptors could be affecting the inflammatory response in skeletal muscle. Neutrophil invasion and release of cytokines locally in skeletal muscle play an important role in mediating the inflammatory response, and could be affected by histaminergic signaling. Further work should perform this type

of study and obtain skeletal muscle biopsies at certain intervals out to 48 hours, to understand what might be changing locally in muscle in regards to the acute inflammatory response and its interaction with histamine. This work would involve immunohistochemical staining to identify cell types in skeletal muscle and protein abundance analysis to investigate if histaminergic signaling affects translation of proteins that are involved in the acute inflammatory response.

Summary

This study has addressed how antihistamine medications may impact the acute inflammatory response to aerobic exercise in untrained individuals. This study demonstrated that blocking histaminergic signaling on H_1 and H_2 receptors did not alter the systemic inflammatory response of leukocytes and neutrophils following aerobic exercise. Blocking histaminergic signaling on H_1 and H_2 receptors did not appear to affect the expression of circulating cytokines that are typically associated with the aerobic acute post-exercise inflammatory response. of histamine in the post-exercise. Future research should seek to investigate the role of histaminergic signaling in post-exercise inflammation locally in skeletal muscle.

CHAPTER VII
CONCLUSIONS AND FUTURE DIRECTIONS

The studies of this dissertation focus on and build upon our current knowledge regarding the interaction of histamine and exercise. Our lab has clearly established a role for histamine and histaminergic signaling in the post-exercise responsome. This role includes vascular responses such as sustained-post exercise vasodilation, post-exercise hypotension and others such as glucose uptake in skeletal muscle (8, 63, 123). In addition to these, recent evidence from our lab suggests the role of histaminergic signaling in mRNA expression in previously exercised skeletal muscle (136). While we have shown that histamine and exercise have an intimate interaction with each other, these studies addressed questions that still remained in order to better understand the role of histamine in the exercise responsome.

Summary of Key Findings

Over the past two decades, our lab has created models to examine the effects of histamine receptor antagonism on exercise responses. Most of these have been aerobic exercise based, such as cycling or unilateral dynamic knee extension exercise. During dynamic aerobic exercise, there are a multitude of changes occurring in skeletal muscle, including changes in temperature, changes in pH, release of myokines, among others (138, 26). Regarding this, we wanted to know what is it about exercise that causes the release of histamine? It has been shown that histamine is released from skeletal muscle in response to exercise. Since many local factors are altered with exercise, we do not which one or combination of these factors are causing the release of histamine in skeletal

muscle. Therefore, we performed studies, detailed in Chapter IV and V, in an attempt to identify if temperature or local factors released by muscle in response to exercise, is the trigger that leads to the release of histamine in skeletal muscle.

Data from chapter VI suggests that the increase in skeletal muscle temperature appears to play an important role in the release of histamine from skeletal muscle. In vitro experiments suggest this release of histamine may be the influence of temperature on enzymatic kinetics of histidine decarboxylase rather than direct mast cell degranulation. In addition, the data in Chapter V suggests that there is a local factor released in skeletal muscle in response to exercise that causes mast cells to degranulate. This response appears to be localized as we did not see an effect on mast cell degranulation from skeletal muscle dialysate collected from resting tissue. Another gap we sought to investigate was the effect of histamine receptor antagonism on the acute inflammatory response to exercise. Our lab has published promising data that blocking the actions of histamine on H1 and H2 receptors alters gene expression in skeletal muscle, particularly genes that are involved in inflammation (136). However, it was only measured to 3 hours post-exercise in skeletal muscle. The study detailed in Chapter VI built upon this, aimed to investigate the role of histaminergic signaling on the acute post-exercise inflammatory response in untrained individuals after a bout of novel exercise. We found that following a novel bout of exercise, untrained individuals had a robust systemic rise in circulating leukocytes, neutrophils, and some inflammatory cytokines. When these same volunteers exercised under histamine H_1/H_2 receptor antagonism, the acute inflammatory response was not augmented. In all, we have made significant progress in our understanding of the

interaction of histamine, exercise, and inflammation. And these experiments have set the stage for future experiments to address questions that still remain from these studies.

During exercise, the creation and release of energy in exothermic reactions results in metabolic heat production in skeletal muscle (58). During both aerobic and resistance exercise, it has been observed that skeletal muscle temperature increases due to increased metabolic activity. This change in temperature has been shown to have a multitude of effects in skeletal muscle, altering cross-bridge cycling dynamics and metabolic activity (169, 125). Therefore, these set of experiments investigated if an increase in skeletal muscle temperature, which happens during exercise, is the stimulus for histamine release in skeletal muscle. In order to passive raise skeletal muscle to "exercising temperatures", we utilized passive short-wave diathermy, a non-invasive method of raising skeletal muscle temperature (36). We were successful in increasing skeletal muscle temperature up to values observed during dynamic unilateral knee extension exercise, as seen in **Figure 4.2**. Analysis of the dialysate collected from skeletal muscle revealed a significant increase in histamine concentrations near the end of the heating protocol when temperature was approximately 39°C. This rise in skeletal muscle histamine concentration was similar in magnitude to what we had previously observed in dialysate collected from exercising skeletal muscle (155). The final question of this study remained, is temperature directly causing mast cells to degranulate? Previous literature has suggested that changes in temperature can cause mast cells to degranulate (171). To answer this question, we utilized isolated human mast cells in an in-vitro experimental approach. We exposed isolated human mast cells to 3 different physiological temperatures. We did not observe any significant differences in mast cell degranulation in

when exposed to higher and lower temperature conditions, as seen in **Figure 4.3**. These data suggest that the histamine release observed in related to temperature increases in skeletal muscle might be of *de novo* origin, rather than direct effects of temperature on mast cell degranulation.

While our data suggested temperature plays a significant role, we wanted to further understand what is changing in skeletal muscle as opposed to resting tissue that causes the release of histamine. The research literature has demonstrated that cytokines and other cell types can be release by skeletal muscle in response to exercise. These cytokines can have both paracrine and autocrine effects, affecting tissues such as the liver and brain (121, 120) and the skeletal muscle (122). The question that framed this experiment in context of what we already know was, "is there or are there factors released locally in skeletal muscle during exercise that cause mast cell degranulation?" In the experiment in this chapter, we were curious if there is a factor or factors that are released in skeletal muscle in response to exercise that cause increased mast cell degranulation that are not present in resting skeletal muscle. To investigate this, we had subjects perform 60 minutes of unilateral dynamic knee extension exercise at 60% of their peak power. We once again used skeletal muscle microdialysis fibers to collect dialysate from the *vastus lateralis* of the exercising leg and the *vastus lateralis* of the resting leg. Of interest, we noted a significant increased mast cell degranulation percentage when exposed to dialysate from exercising skeletal muscle compared to pre-exercise values (**Figure 5.1**). This significant response persisted out to 20 minutes of recovery from exercise. The percentage of mast cells that degranulated from dialysate from the resting leg was not different at any timepoint compared to pre-exercise. We

noted significant differences in mast cell degranulation between the exercising and resting dialysate collected at 40 and 60 minutes during exercise and 20 minutes after exercise.

Following a bout of exercise, there is a local and systemic inflammatory response which is responding to the muscle damaged by the exercise. This response is mediated by a multitude of factors such as myokines that are released by skeletal muscle (56) along with the response of bone marrow and the spleen to release leukocytes to migrate to the damage tissue (133). Histamine is known to be intimately involved in the inflammatory process, therefore the purpose of this experiment was to investigate how using over-the- counter histamine receptor antagonist medications would affect the systemic inflammatory response to a novel bout of moderate intensity aerobic exercise. Previous work from our lab suggested that histaminergic blockade may impact the inflammatory response to a bout of exercise (126). For this study, we utilized a model of exercise that both elicits a significant sustained post-exercise vasodilatory response that is mediated by histaminergic signaling and has been shown to elicit a robust inflammatory response in untrained individuals. Venous blood draws were obtained at multiple points following out to 48 hours post-exercise, once under placebo conditions and once under histamine H_1/H_2 receptor antagonism.

In our model, we found that histamine H_1/H_2 receptor

antagonism did not augment the systemic response of circulating leukocytes and neutrophils compared to placebo conditions, as shown in Figures **6.2** and **6.3.** Going back through 3-day food recall logs, subjects consumed similar foods under both conditions and exercised at the same intensity under both conditions. For this study, we had to use two different H_2

receptor antagonists due to a voluntary FDA recall on ranitidine. Some subjects received famotidine instead of ranitidine for the H_2 receptor antagonist. We noted that the blunting of post-exercise vascular responses were similar between drug conditions. We recognize that the human inflammatory response to exercise is quite variable, and future studies should further investigate the role of histaminergic signaling on the inflammatory response to aerobic exercise with a larger subject pool.

The work presented in this dissertation, in concert with others from our lab, has demonstrated an important role for histamine in exercise responses. Histaminergic signaling has important roles in endurance exercise, blood flow regulation, skeletal muscle glucose uptake. Our models and findings complement and add to the history of histamine research, helping to further understand the role and importance of histamine and exercise responses. We have helped increase our knowledge, however there are still gaps that remain regarding exercise and histaminergic signaling.

Future Directions and Experiments

Our systemic blockade could be affecting other unknown pathways both during and following exercise, which warrant further investigation. Histamine plays an important role in the many physiological responses to exercise, and this dissertation has shown that certain aspects of exercise trigger the release and that histaminergic signaling may be involved in the systemic inflammatory response to exercise.

While we have shown that temperature appears to be an important exercise trigger for the release of histamine, there could be other factors in skeletal muscle causing this release. An insightful follow up experiment that would be a logical next step would be to

examine how temperature affects HDC enzymatic activity. While there is some published literature that has examined it (143, 144), in scientific rigor this should be revisited and should keep to physiological temperatures that are typically observed in human skeletal muscle. Also, while we established a clear role for temperature and have ruled out oxidative stress, there are still other exercise factors that remain to be investigated. So where do we go from here?

pH is a factor that is highly influenced by exercise, as there are decreases in pH in skeletal muscle with higher intensity exercise. It is plausible that an increased presence of hydrogen ions in an acidic environment may affect mast cell degranulation. Therefore, pH could also be playing a role as an "exercise factor" that causes the release of histamine during exercise. It would be quite difficult to alter pH levels in muscle without affecting other factors. However, a buffering agent with direct measurements of skeletal muscle pH levels during exercise could be useful in a similar experiment where skeletal muscle dialysate could be collected to then investigate skeletal muscle histamine levels. In addition, in-vitro experiments could be valuable in investigating if mast cells degranulate in response to physiological changes in pH in their culture media. These additional studies will help to further investigate the exercise factor or factors that result in exercise-induced increases in skeletal muscle histamine concentrations.

In addition, the contraction of skeletal muscle contraction in itself may cause mast cells to degranulate independent of other factors. During skeletal muscle contraction, actin-myosin crossbridge cycling is a dynamic process that involves the hydrolysis of ATP and other enzymes. This dynamic process affects other cell types in skeletal muscle that are critical for force production. Mast cells have been shown to be present in long-

term cultures of skeletal muscle of rats (30). Histamine concentrations can be biochemically measured in the dish in which the single or multi-fiber muscle preparations are prepared. Given this, it is possible to extend this to an examination of human model fiber preparation. This would involve multi-fiber isolation from resting skeletal muscle biopsy obtained from humans. Then using a setup in which one end of the fiber bundle is attached to a force-transducer and the other end is secured, it is possible to contract the muscle at different velocities and covering force ranges from 0.5-1000mN (24). This preparation is in solution that is pH, enzyme, and temperature controlled. Histamine concentrations could be measured in the culture media, to examine if histamine is released from skeletal muscle fiber contraction and if this is velocity/force dependent.

The microdialysis fibers we used for the study in Chapter V had a molecular cut-off weight of 100kDa, so the factor or factors causing the increased mast cell degranulation must be smaller than this. In all, this study has demonstrated that there is a factor or multiple factors that are released in exercising skeletal muscle that cause increased mast cell degranulation. These are localized to the exercising tissue and appear to be resolved within 40 minutes of the cessation of exercise. A logical future direction would be an "omics" approach to investigate what this factor or factors may be. The transcriptomics approach allows for biological samples to be investigated for the characterization and quantification of biological molecules within that sample (126). The experiment could be repeated, with skeletal muscle microdialysis fibers placed in both the exercising and resting leg. The exercise transcriptome could be investigated from the collected dialysate samples, allowing us to evaluate factors that might be upregulated during exercise and that are unique to exercising skeletal muscle, opposed to resting

skeletal muscle. Furthermore, the size of the microdialysis fibers allows for a more "focused" approach as we know that the factors we observed in this study are smaller than 100kDa. Larger molecular weight cut-off probes could be used to further investigate factors that may be released in skeletal muscle in response to exercise that are larger than 100kDa, as it is highly likely that there are factors larger than this that can directly cause mast cell degranulation, such as C5a which was discussed in Chapter V

Even though we did not find statistical significance of our blockade condition in the inflammatory study, it still has promising future directions. An important follow up study should involve obtaining skeletal muscle biopsies at time points out to 48 hours following this type of exercise under these conditions. We were able to quantify many of the systemic, circulating factors that are typically associated with the acute post-exercise inflammatory response. However, another portion of the inflammatory response is localized to the skeletal muscle that was exercised with invading neutrophils, myokines released from the muscle, and other proteins that are either upregulated or downregulated in response to exercise. Having the localized skeletal muscle data in conjunction with the systemic, circulating factors data would be complementary and tell a more complete story of the role of histaminergic signaling on the acute inflammatory response to exercise. Overall, these studies have answered many questions and provide us direction and guidance for further experiments regarding the interaction between histamine, exercise, and inflammation.

APPENDIX A: INFORMED CONSENT DOCUMENTS

Chapter IV

INFORMED CONSENT FORM

TITLE: Exercise, histamine receptors, and vascular function (Experiment: Muscle temperature)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

When humans exercise, there is an increase in blood flow to the skeletal muscle. This increase in blood flow lasts for several hours after exercise as a result of vasodilation of the blood vessels. Previous studies have shown that this vasodilation is largely the result of a release of histamine. Histamine is released from cells called mast cells, but the stimulus that activates this release of histamine from mast cells is still debated. The purpose of this study is to determine if heat is a possible stimulus for the release of histamine from mast cells during exercise. This protocol will explore the changes in temperature of both muscle and skin with dynamic knee extension exercise. This topic is both clinically and scientifically important. As such, this study is currently funded by the National Institutes of Health. You have been asked to participate in this study because you are a young healthy individual, who is either sedentary or recreationally active and free from any known cardiovascular disease.

What will happen in the study?

Prescreen

1. You will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon for an initial visit. This visit will take approximately one hour. You will meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. You will be asked questions about your health history that will help the investigators to determine if you fit the inclusion criteria for the study.

Exercise Test Visit

1. You will return to Dr. Halliwill's lab for an exercise test visit. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. This visit will take approximately one hour. When you arrive in the lab your height, weight and resting blood pressure will be measured. You will be given a physical activity questionnaire, which will allow investigators to determine your activity level. The questionnaire will take approximately 15 minutes to complete. You will also be asked

to fill out a Medical History Form to be used in case of an emergency. This document will be shredded after you complete the study.

2. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the exercise test visit (and again during the study visit). For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.
3. A small needle will be used to obtain a blood sample from a vein near your elbow, as they would for a routine blood test at the doctor's office. The blood samples will be used for DNA analysis to determine which versions of specific genes you have related to the processing of histamine. The samples will either be analyzed by ourselves or by a commercial lab that will only receive coded samples with no other identifying information.
4. During the exercise test visit, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. The electrical activity of the muscle in your legs will also be monitored by electromyogram electrodes on the skin of the back of your leg. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm. The pH and tissue oxygenation of your working leg will be measured by a noninvasive near-infrared spectroscopy (NIRS) probe placed on the surface of your skin.
5. You will perform one-legged knee extension exercise while seated on an exercise machine that measures how hard you are working. This exercise is like kicking a soccer ball over and over again. After a 5-minute warm-up, you will be asked to maintain a selected kicking rate as the machine increases how hard you work every minute until you reach your peak exercise capacity. It normally takes 10 to 15 minutes for people to reach their peak effort. There is no win/lose threshold or specific value that participants need to achieve; participants simply need to do their best. While you exercise you will be wearing a mouth piece and nose clip. The total time for either test (including placement of electrodes on your skin, warm-up, exercise, and cool-down) is approximately thirty minutes.
6. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit. Some examples of discomfort include fatigue and muscle soreness.
7. This session will serve to familiarize you with the procedures to be used on the study day. It will also establish your peak exercise capacity on the exercise machine and therefore will be used to establish the appropriate workload for the exercise session on the study day.

Study Visit

1. You will then return to Dr. Halliwill's laboratory to participate in the study visit, which will be between 7 and 10 days after the exercise test visit. This testing session will take approximately 3 hours.
2. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. In addition, you will need to refrain from consuming caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 24

hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. If you use oral contraceptives, you should take this when you normally do so.

3. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the study visits.
4. During the study visit, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm. The electrical activity of the muscle in your legs will also be monitored by electromyogram electrodes on the skin of the back and front of your leg.
5. During the study visit, you will perform one-legged knee extension exercise while seated on an exercise machine that measures how hard you are working. During this exercise session you will be asked to maintain a selected kicking rate for 60 minutes. The pH and tissue oxygenation of your working leg will be measured by a noninvasive near-infrared spectroscopy (NIRS) probe placed on the surface of your skin.
6. Before and after the exercise session, a small probe (ultrasound Doppler probe) will be held over an area of skin on your groin-hip intersection to image your femoral artery. The ultrasound Doppler probe uses ultrasound waves to measure blood flow in these arteries. The femoral artery will be studied for 10 minutes before you exercise and for two hours after you exercise.
7. You will undergo the following procedure. You will have a small probe (this is called a "thermocouple") placed into the vastus lateralis (outer thigh). First, the area of skin where the probe will enter will be numbed with a local anesthetic (prilocaine and lidocaine/epinephrine). Then a small needle will be placed through the skin and into the muscle. The small probe will be passed through the needle, and then the needle will be withdrawn, leaving the small probe in your muscle (about 1-2 inches). The probe will remain in place throughout the rest of the study. There may be some discomfort during the insertion of the small probes into your muscle. We will use a local anesthetic to numb skin where the probes will be inserted to minimize this discomfort, but you may feel pressure or a dull ache in the muscle as the needle moves through the muscle. The purpose of the thermocouple is to measure the temperature within the muscle. The probe will be placed in your muscle for the duration of the study (3 hours).
8. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the study visit.

How long will I be in the study?

You will be in the study for 3 days (initial visit, exercise test day, and one study visit). The first visit will last one hour. The exercise test visit will last one hour. The study day will last three hours. You will need to refrain from eating for 2 hours prior to the exercise test day and the study day.

What are the risks of the study?

There is some minor discomfort associated with the initial exercise test and exercise sessions, including temporary fatigue and muscle soreness. These sensations resolve

within minutes after the test is completed. There is the possibility of some residual muscle soreness in the few days following the initial exercise test and each exercise session.

Thermocouples: There may be some discomfort during the insertion of the small probe into your muscle. We will use a local anesthetic to numb skin where the probes will be inserted to minimize this discomfort, but you may feel pressure or a dull ache in the muscle as the needle moves through the muscle. At the end of the study, the probe will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study, but you may feel some muscle soreness for several days. You will be given some ibuprofen (e.g. Advil or Motrin) to relieve some of the initial soreness. Although the small probes are sterile, there is a slight risk of infection at the sites where the probes were placed. You will be instructed on how to keep the area clean for a day or two following the study and will need to inform the researchers immediately if you have any redness or swelling in the area. The researchers will also ask you to return to the lab the day after the study visit so they can see how the site is healing.

Blood sampling for genotyping: In total, 10 ml of blood will be withdrawn, which is less than a tablespoon. The blood sample will be used for genetic analysis to determine which versions of specific genes you have related to the processing of histamine. The blood sample will be labeled with a "subject identification number" or ID number, without any personally identifying information. The names associated with each ID number will be kept in a locked file cabinet in Dr. Halliwill's office area. The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. You have the right to withdraw your blood sample from research in the future should you decide to do so, but once the list of names has been destroyed, it will be impossible for the researchers to know which blood sample is yours. Blood samples will be kept for up to five years. After five years, the samples will be destroyed by discarding them into a biohazard waste container to be collected and destroyed by the Environmental Hazard and Safety department at the University of Oregon. Any excess samples will be discarded and destroyed in the same manner. Blood samples without any personally identifying information may be sent to a third-party lab for genetic analysis. Once the third-party lab has performed the analysis, they will destroy any remaining blood sample. The genetic data from this analysis, without any personally identifying information, may be stored by the researchers indefinitely. There may exist potential risks of insurability, employability, and social discrimination if the results of individual genetic data were to be made known to others. To safeguard against this risk, your genetic data will be stored by an ID number only and your name or other personally identifiable information. The genetic data, without any personally identifiable information, may be used in other research and by other parties studying the human genetics of histamine in collaboration with this study. Any data that may be published in scientific journals will not reveal the identity of the participants. Since the implications of our research are unknown, you will not be told the results, even if there might be some potential benefit to

you. We will not reveal any genetic data to your physicians, or any other health care provider. A new Federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against you based on your genetic information. Be aware that this new Federal law does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance. GINA also does not protect you against discrimination if you have already been diagnosed with the genetic disease being tested. Although we have made every effort to protect your identity, there is a small risk of loss of confidentiality. If the results of these studies of your genetic makeup were to be accidentally released, it might be possible that the information we will gather about you as part of this study could become available to an insurer or an employer, or a relative, or someone else outside the study. Even though there are discrimination protections in both Oregon law and Federal law, there is still a small chance that you could be harmed if a release occurred. If you decided not to participate in the DNA sampling aspects of this research protocol, it will not compromise your ability to participate in the other aspects of the research.

Prilocaine and Lidocaine/Epinephrine (brand name Citanest and Lignospan): The risks associated with prilocaine and lidocaine/epinephrine are similar in character to those observed with other local anesthetics. Some of the more common adverse reactions include nervousness, dizziness, blurred vision, tremor, drowsiness, tinnitus, numbness, disorientation, hypotension, nausea and vomiting.

Tracking of Taxable Income: Please note, compensation from participation in Human Subjects Research studies is taxable income. If your compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. The University requires its departments to track participant compensation and may contact you to complete a Form W-9 for tax reporting purposes. Because of the federal and University tracking requirements, your name will be associated with participation in research. Department and University administrators will have access to this information, but will not have access to research data.

The study may include risks that are unknown at this time.

Are there benefits to taking part in this study?

This study will not make your health better. Electrocardiogram and other measurements are not being conducted for diagnostic purposes. You will be provided with the results from your blood test for glucose and cholesterol, as well as your body composition information. The results will not be reviewed by a physician. However, if results fall outside of the normal range, you will be informed that you should consult your primary care physician for additional medical evaluation. The purpose of this study is to provide more information on what causes blood flow increases to occur after exercise. This protocol will explore whether older adults exhibit the same blood flow responses after exercise that young adults do. Our hope is that by better understanding the physiology of how the human body responds to exercise, we will be better able to use exercise in the prevention and treatment of diseases such as hypertension (high blood pressure) and other

forms of cardiovascular disease.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not pay for any tests or procedures that are done just for this research study. You will get $75 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $15 per hour that you complete.

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 600-4337.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with the University of Oregon.

You do not waive any liability rights for personal injury by signing this form. Biomedical research involves some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. For example, if the investigators determine the study is overly stressful for you (as indicated by body language and an elevated heart rate and/or blood pressure) they may stop you from participating in the study. Or if you express pain or discomfort beyond what is normally expected, the investigators stop you from participating in the study.

If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the State of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

General Counsel
Office of the President
1226 University of Oregon
Eugene, OR 97403-1226
(541) 346-3082

Research Compliance Services
5237 University of Oregon
Eugene, OR 97403-5237
(541) 346-2510

A law called the Oregon Tort Claims Act may limit the amount of money you can receive from the State of Oregon if you are harmed.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill's office area.

Research Compliance Services and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. All blood and muscle dialysate fluid samples will be destroyed when study results are published or 5 years after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

You will be asked to complete a Medical History Form on the study day which will list personal identifying information so that in the unlikely event of a medical emergency in which we would activate the emergency medical system, we would be able to provide this information to emergency healthcare providers. This document would be retained in a locked file cabinet in Dr. Halliwill's lab for up to a week after the study day, after which it is placed in a locked confidential documents disposal bin which is emptied by a secure shredding service.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5219 University of Oregon, Eugene, OR 97403, 541/346-2510.

Your initial indicates that you have read and understand the information provided above about the genetic testing, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty.

_____

(Initial of Participant) (Date)

If you would choose to abstain from the genetic testing portion of this study, please initial below. There will be no penalty if you choose to abstain, and you will still be able to participate in all other aspects of the study.

_____

(Initial of Participant) (Date)

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____

(Signature of Participant) (Date)

_____

(Signature of Individual Obtaining Consent) (Datee

Informed Consent Form

TITLE: Exercise, histamine receptors, and vascular function (Experiment: Heating Muscle)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

When humans exercise, there is an increase in blood flow to the skeletal muscle. This increase in blood flow lasts for several hours after exercise as a result of vasodilation in the blood vessels. Previous studies have shown that this vasodilation is largely the result of a release of histamine. Histamine is released from mast cells, but the stimulus that activates this release of histamine from mast cells is still debated. The purpose of this study is to determine if heat is a possible stimulus for the release of histamine from mast cells during exercise. This protocol will explore the changes in temperature of both muscle and skin with external heating of the leg to simulate what happens during exercise. This topic is both clinically and scientifically important. As such, this study is currently funded by the National Institutes of Health. You have been asked to participate in this study because you are a young healthy individual, who is either sedentary or recreationally active and free from any known cardiovascular disease.

What will happen in the study?

Prescreen

1. You will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon for an initial visit. This initial visit will take approximately one hour. You will meet with one of the investigators of the study to discuss the project, to see the

laboratory, and to read this form. You will be asked questions about your health history that will help the investigators to determine if you fit the inclusion criteria for the study.

Study Visit

1. You will return to Dr. Halliwill's lab for a study visit. This visit will take approximately four hours. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. In addition, you will need to refrain from consuming caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 24 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. If you use oral contraceptives, you should take this when you normally do so.
2. When you arrive in the lab your height, weight and resting blood pressure will be measured. You will be given a physical activity questionnaire, which will allow investigators to determine your activity level. The questionnaire will take approximately 15 minutes to complete. You will also be asked to fill out a Medical History Form to be used in case of an emergency. This document will be shredded after you complete the study.
3. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the study visit. For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.
4. A small needle will be used to obtain a blood sample from a vein near your elbow, as they would for a routine blood test at the doctor's office. The blood samples will be used for DNA analysis to determine which versions of specific genes you have related to the processing of histamine. The samples will either be analyzed by ourselves or by a commercial lab that will only receive coded samples with no other identifying information.
5. Your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. The electrical activity of the muscle in your legs will also be monitored by electromyogram electrodes on the skin of the back of your leg. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm.
6. A small probe (ultrasound Doppler probe) will be held over an area of skin on your groin-hip intersection to image your femoral artery. The ultrasound Doppler probe uses ultrasound waves to measure blood flow in these arteries. This will occur periodically throughout the study.
7. You will undergo the following procedure. You will have a small probe (this is called a "thermocouple") placed into the vastus lateralis (outer thigh). First, the area of skin where the probe will enter will be numbed with a local anesthetic (prilocaine and lidocaine/epinephrine). Then a small needle will be placed through the skin and into the muscle. The small probe will be passed through the needle, and then the needle will be withdrawn, leaving the small probe in your muscle (about 1-2 inches). The probe will remain in place throughout the rest of the study. There may be some discomfort during the insertion of the small probes into your muscle. We will use a

local anesthetic to numb skin where the probes will be inserted to minimize this discomfort, but you may feel pressure or a dull ache in the muscle as the needle moves through the muscle. The purpose of the thermocouple is to measure the temperature within the muscle. The probe will be placed in your muscle for the duration of the study (four hours).

8. For this particular study, we are looking at three different types of heating modalities: infrared heating, ultrasound and pulsed short-wave diathermy. For the infrared heating, you will have a heating lamp placed close to the skin of your thigh (there will be no direct contact between the lamp and the surface of the skin). The purpose of this is to increase the temperature of your muscle to around 100ºF. As skin temperatures above 111ºF can feel painful to some, we will keep your skin temperature below 108ºF. For comparison, the temperature of water in hot tubs is routinely 102-104ºF. We will heat your thigh for one hour. For the ultrasound heating procedure, an ultrasound probe will be placed directly on the skin of your thigh. For the short-wave pulse diathermy, the heating coil will be placed close to the skin of your thigh, however it will not make contact with the surface of your skin. During this time of heating, you may feel an itching sensation as time progresses, but this is a normal response. If you experience any pain or burning, please inform the investigator immediately.
9. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the study visit.

How long will I be in the study?

You will be in the study for 2 days (initial visit and one study visit). The first visit will last one hour. The study day will last 4 hours. You will need to refrain from eating for 2 hours prior to the study visit.

What are the risks of the study?

Heating: Heating of the skin of your thigh can become painful and there is a risk of burns or lesions to the skin. Discomfort does not generally occur below 111ºF, and damage does not generally occur below 113ºF. We will keep your temperature below these levels, but if you experience any pain or burning, please inform the investigator immediately. For this study, the goal is to get your muscle to around 100ºF. We will be using different modalities, all of which have been safely used for decades by physical therapists and physicians. We will be using ultrasound heat therapy, which involves using sound waves to heat up your muscle. We also plan to use pulsed short-wave diathermy, which uses electromagnetic waves, delivered in a pulsatile manner in order to raise the temperature in your muscle. These methodologies have been extensively researched and have data in peer-reviewed articles supporting both their safety and efficacy.

Thermocouple: There may be some discomfort during the insertion of the small thermocouple probe into your muscle. We will use a local anesthetic to numb skin where the probes will be inserted to minimize this discomfort, but you may feel pressure or a dull ache in the muscle as the needle moves through the muscle. At the end of the study, the probe will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study, but you

may feel some muscle soreness for several days. You will be given some ibuprofen (e.g. Advil or Motrin) to relieve some of the initial soreness. Although the small probes are sterile, there is a slight risk of infection at the sites where the probes were placed. You will be instructed on how to keep the area clean for a day or two following the study and will need to inform the researchers immediately if you have any redness or swelling in the area. The researchers will also ask you to return to the lab the day after the study visit so they can see how the sites are healing.

Blood sampling for genotyping: In total, 10 ml of blood will be withdrawn, which is less than a tablespoon. The blood sample will be used for genetic analysis to determine which versions of specific genes you have related to the processing of histamine. The blood sample will be labeled with a "subject identification number" or ID number, without any personally identifying information. The names associated with each ID number will be kept in a locked file cabinet in Dr. Halliwill's office area. The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. You have the right to withdraw your blood sample from research in the future should you decide to do so, but once the list of names has been destroyed, it will be impossible for the researchers to know which blood sample is yours. Blood samples will be kept for up to five years. After five years, the samples will be destroyed by discarding them into a biohazard waste container to be collected and destroyed by the Environmental Hazard and Safety department at the University of Oregon. Any excess samples will be discarded and destroyed in the same manner. Blood samples without any personally identifying information may be sent to a third-party lab for genetic analysis. Once the third-party lab has performed the analysis, they will destroy any remaining blood sample. The genetic data from this analysis, without any personally identifying information, may be stored by the researchers indefinitely. There may exist potential risks of insurability, employability, and social discrimination if the results of individual genetic data were to be made known to others. To safeguard against this risk, your genetic data will be stored by an ID number only and your name or other personally identifiable information. The genetic data, without any personally identifiable information, may be used in other research and by other parties studying the human genetics of histamine in collaboration with this study. Any data that may be published in scientific journals will not reveal the identity of the participants. Since the implications of our research are unknown, you will not be told the results, even if there might be some potential benefit to you. We will not reveal any genetic data to your physicians, or any other health care provider. A new Federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against you based on your genetic information. Be aware that this new Federal law does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance. GINA also does not protect you against discrimination if you have already been diagnosed with the genetic disease being tested. Although we have made every effort to protect your identity, there is a small risk of loss of confidentiality. If the results of these studies of your genetic makeup were to be accidentally released, it might be possible that

the information we will gather about you as part of this study could become available to an insurer or an employer, or a relative, or someone else outside the study. Even though there are discrimination protections in both Oregon law and Federal law, there is still a small chance that you could be harmed if a release occurred. If you decided not to participate in the DNA sampling aspects of this research protocol, it will not compromise your ability to participate in the other aspects of the research.

Prilocaine and Lidocaine/Epinephrine (brand name Citanest and Lignospan): The risks associated with prilocaine and lidocaine/epinephrine are similar in character to those observed with other local anesthetics. Some of the more common adverse reactions include nervousness, dizziness, blurred vision, tremor, drowsiness, tinnitus, numbness, disorientation, hypotension, nausea and vomiting.

Tracking of Taxable Income: Please note, compensation from participation in Human Subjects Research studies is taxable income. If your compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. The University requires its departments to track participant compensation and may contact you to complete a Form W-9 for tax reporting purposes. Because of the federal and University tracking requirements, your name will be associated with participation in research. Department and University administrators will have access to this information, but will not have access to research data.

The study may include risks that are unknown at this time.

Are there benefits to taking part in this study?

This study will not make your health better. Electrocardiogram and other measurements are not being conducted for diagnostic purposes. You will be provided with the results from your blood test for glucose and cholesterol, as well as your body composition information. The results will not be reviewed by a physician. However, if results fall outside of the normal range, you will be informed that you should consult your primary care physician for additional medical evaluation. The purpose of this study is to provide more information on what causes blood flow increases to occur after exercise. This protocol will explore whether older adults exhibit the same blood flow responses after exercise that young adults do. Our hope is that by better understanding the physiology of how the human body responds to exercise, we will be better able to use exercise in the prevention and treatment of diseases such as hypertension (high blood pressure) and other forms of cardiovascular disease.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not pay for any tests or procedures that are done just for this research study. You will get $75 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $15 per hour that you

complete.

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 600-4337.

What are my rights if I take part in this study?

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You do not waive any liability rights for personal injury by signing this form. Biomedical research involves some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. For example, if the investigators determine the study is overly stressful for you (as indicated by body language and an elevated heart rate and/or blood pressure) they may stop you from participating in the study. Or if you express pain or discomfort beyond what is normally expected, the investigators stop you from participating in the study.

If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the State of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

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(541) 346-2510

A law called the Oregon Tort Claims Act may limit the amount of money you can receive from the State of Oregon if you are harmed.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a “subject identification number”. The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill’s office area.

Research Compliance Services and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. All blood and muscle dialysate fluid samples will be destroyed when study results are published or 5 years after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

You will be asked to complete a Medical History Form on the study day which will list personal identifying information so that in the unlikely event of a medical emergency in which we would activate the emergency medical system, we would be able to provide this information to emergency healthcare providers. This document would be retained in a locked file cabinet in Dr. Halliwill's lab for up to a week after the study day, after which it is placed in a locked confidential documents disposal bin which is emptied by a secure shredding service.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

Your initial indicates that you have read and understand the information provided above about the genetic testing, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty.

_____

(Initial of Participant) (Date)

If you would choose to abstain from the genetic testing portion of this study, please initial below. There will be no penalty if you choose to abstain, and you will still be able to participate in all other aspects of the study.

_____

(Initial of Participant) (Date)

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____

(Signature of Participant) (Date)

_____

(Signature of Individual Obtaining Consent) (Date

Chapter V

INFORMED CONSENT FORM

TITLE: Exercise, histamine receptors, and vascular function (Experiment: Exercise and Microdialysis)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

When humans exercise, there is an increase in blood flow to the skeletal muscle. This increase in blood flow lasts for several hours after exercise as a result of vasodilation in the blood vessels. Previous studies have shown that this vasodilation is largely the result of a release of histamine. Histamine may also be an important signal for stimulating blood vessel growth (angiogenesis) in skeletal muscle as a consequence of exercise training. The purpose of this study is to determine if exercise-related histamine release stimulates the growth of new blood vessels. This protocol will use fluid collected from your skeletal muscle before, during, and after exercise to determine the effect of histamine on angiogenic properties. This topic is both clinically and scientifically important. As such, this study is currently funded by the National Institutes of Health. You have been asked to participate in this study because you are a young healthy individual, who is either sedentary or recreationally active and free from any known cardiovascular disease.

What will happen in the study?

Prescreen

1. You will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon for an initial visit. This initial visit will take approximately one hour. You will meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. You will be asked questions about your health history that will help the investigators to determine if you fit the inclusion criteria for the study.

Exercise Test Visit

2. You will return to Dr. Halliwill's lab for an exercise test visit. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. This visit will take approximately one hour. When you arrive in the lab your height, weight and resting blood pressure will be measured. You will be given a physical activity questionnaire, which will allow investigators to determine your activity level. The

questionnaire will take approximately 15 minutes to complete. You will also be asked to fill out a Medical History Form to be used in case of an emergency. This document will be shredded after you complete the study.

3. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the exercise test visit (and again during the study visit). For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.
4. A small needle will be used to obtain a blood sample from a vein near your elbow, as they would for a routine blood test at the doctor's office. The blood samples will be used for DNA analysis to determine which versions of specific genes you have related to the processing of histamine. The samples will either be analyzed by ourselves or by a commercial lab that will only receive coded samples with no other identifying information.
5. During the exercise test visit, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. The electrical activity of the muscle in your legs will also be monitored by electromyogram electrodes on the skin of the back of your leg. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm.
6. You will perform one-legged knee extension exercise while seated on an exercise machine that measures how hard you are working. This exercise is like kicking a soccer ball over and over again. After a 5-minute warm-up, you will be asked to maintain a selected kicking rate as the machine increases how hard you work every minute until you reach your peak exercise capacity. It normally takes 10 to 15 minutes for people to reach their peak effort. There is no win/lose threshold or specific value that participants need to achieve; participants simply need to do their best. While you exercise you will be wearing a mouth piece and nose clip. The total time for either test (including placement of electrodes on your skin, warm-up, exercise, and cool-down) is approximately thirty minutes.
7. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit. Some examples of discomfort include fatigue and muscle soreness.
   This session will serve to familiarize you with the procedures to be used on the study day. It will also establish your peak exercise capacity on the exercise machine and therefore will be used to establish the appropriate workload for the exercise session on the study day.

Study Visit

1. You will return to Dr. Halliwill's lab for a study visit. This visit will take approximately six hours. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. In addition, you will need to refrain from consuming caffeine (for example, coffee, tea, Red Bull, coke, etc.) or medications (except oral contraceptives) for 24 hours prior to the study and abstain from alcohol

or exercise for 24 hours prior to the study. If you use oral contraceptives, you should take this when you normally do so.

2. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the study visit. For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.
3. A small needle will be used to obtain a blood sample from a vein near your elbow, as they would for a routine blood test at the doctor's office. The blood samples will be used to determine circulating levels of angiogenic factors. The samples will either be analyzed by ourselves or by a commercial lab that will only receive coded samples with no other identifying information.

4. Your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. The electrical activity of the muscle in your legs will also be monitored by electromyogram electrodes on the skin of the back of your leg. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm.
5. During the study visit, you will perform one-legged knee extension exercise while seated on an exercise machine that measures how hard you are working. During this exercise session you will be asked to maintain a selected kicking rate for 60 minutes. The pH and tissue oxygenation of your working leg will be measured by a noninvasive near-infrared spectroscopy (NIRS) probe placed on the surface of your skin.
6. You will undergo the following procedure. You will have two small probes (called "microdialysis fibers") placed into the vastus lateralis (outer thigh). First, the area of skin where each probe will enter will be numbed with a local anesthetic (prilocaine and lidocaine/epinephrine). Then a small needle will be placed through the skin and into the muscle. The small probe will be passed through the needle, and then the needle will be withdrawn, leaving the small probe in your muscle (about 1-2 inches). This will be done for each of the two probes. The probes will remain in place throughout the rest of the study. There may be some discomfort during the insertion of the small probes into your muscle. We will use a local anesthetic to numb skin where the probes will be inserted to minimize this discomfort, but you may feel pressure or a dull ache in the muscle as the needle moves through the muscle. The thermocouple is used to measure the temperature within the muscle. All the probes will be in placed in your muscle for four hours.
7. The microdialysis probes will be used to infuse saline into your muscle. This will allow us to collect a sample of the fluid surrounding the muscle before, during, and after you exercise.
8. At the end of the study visit, the probes will be withdrawn from your leg and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study, but you may feel some muscle soreness for several days. You will be given some ibuprofen (e.g. Advil or Motrin) to relieve some of the initial soreness. Although the small probes are sterile, there is a slight risk of

infection at the sites where the probes were placed. You will be instructed on how to keep the area clean for a day or two following the study and will need to inform the researchers immediately if you have any redness or swelling in the area.

9. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the study visit.

How long will I be in the study?

You will be in the study for 3 days (initial visit, one exercise test visit, and one study visit) for a total of 8 hours. The first visit will last one hour. The exercise test visit will also last one hour. The study day will last six hours. You will need to refrain from eating for 2 hours prior to the study visit.

What are the risks of the study?

Microdialysis probes: There may be some discomfort during the insertion of the small probes into your muscle. We will use a local anesthetic to numb skin where the probes will be inserted to minimize this discomfort, but you may feel pressure or a dull ache in the muscle as the needle moves through the muscle. At the end of the study, the probe will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study, but you may feel some muscle soreness for several days. You will be given some ibuprofen (e.g. Advil or Motrin) to relieve some of the initial soreness. Although the small probes are sterile, there is a slight risk of infection at the sites where the probes were placed. You will be instructed on how to keep the area clean for a day or two following the study and will need to inform the researchers immediately if you have any redness or swelling in the area. The researchers will also ask you to return to the lab the day after the study visit so they can see how the sites are healing. In addition, there is the risk that the microdialysis fibers will break while they are in the muscle and no longer function. It is possible for subjects to have an allergic reaction to the microdialysis fibers and/or the fluid being infused through the fibers.

Blood sampling for genotyping: In total, 10 ml of blood will be withdrawn, which is less than a tablespoon. The blood sample will be used for genetic analysis to determine which versions of specific genes you have related to the processing of histamine. The blood sample will be labeled with a "subject identification number" or ID number, without any personally identifying information. The names associated with each ID number will be kept in a locked file cabinet in Dr. Halliwill's office area. The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. You have the right to withdraw your blood sample from research in the future should you decide to do so, but once the list of names has been destroyed, it will be impossible for the researchers to know which blood sample is yours. Blood samples will be kept for up to five years. After five years, the samples will be destroyed by discarding them into a biohazard waste container to be collected and destroyed by the Environmental Hazard and Safety department at the University of Oregon. Any excess samples will be discarded and destroyed in the same manner. Blood samples without any

personally identifying information may be sent to a third-party lab for genetic analysis. Once the third-party lab has performed the analysis, they will destroy any remaining blood sample. The genetic data from this analysis, without any personally identifying information, may be stored by the researchers indefinitely. There may exist potential risks of insurability, employability, and social discrimination if the results of individual genetic data were to be made known to others. To safeguard against this risk, your genetic data will be stored by an ID number only and your name or other personally identifiable information. The genetic data, without any personally identifiable information, may be used in other research and by other parties studying the human genetics of histamine in collaboration with this study. Any data that may be published in scientific journals will not reveal the identity of the participants. Since the implications of our research are unknown, you will not be told the results, even if there might be some potential benefit to you. We will not reveal any genetic data to your physicians, or any other health care provider. A new Federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against you based on your genetic information. Be aware that this new Federal law does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance. GINA also does not protect you against discrimination if you have already been diagnosed with the genetic disease being tested. Although we have made every effort to protect your identity, there is a small risk of loss of confidentiality. If the results of these studies of your genetic makeup were to be accidentally released, it might be possible that the information we will gather about you as part of this study could become available to an insurer or an employer, or a relative, or someone else outside the study. Even though there are discrimination protections in both Oregon law and Federal law, there is still a small chance that you could be harmed if a release occurred. If you decided not to participate in the DNA sampling aspects of this research protocol, it will not compromise your ability to participate in the other aspects of the research.

Prilocaine and Lidocaine/Epinephrine (brand name Citanest and Lignospan): The risks associated with prilocaine and lidocaine/epinephrine are similar in character to those observed with other local anesthetics. Some of the more common adverse reactions include nervousness, dizziness, blurred vision, tremor, drowsiness, tinnitus, numbness, disorientation, hypotension, nausea and vomiting.

Tracking of Taxable Income: Please note, compensation from participation in Human Subjects Research studies is taxable income. If your compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. The University requires its departments to track participant compensation and may contact you to complete a Form W-9 for tax reporting purposes. Because of the federal and University tracking requirements, your name will be associated with participation in research. Department and University administrators will have access to this information, but will not have access to research data.

The study may include risks that are unknown at this time.

Are there benefits to taking part in this study?

This study will not make your health better. Electrocardiogram and other measurements are not being conducted for diagnostic purposes. You will be provided with the results from your blood test for glucose and cholesterol, as well as your body composition information. The results will not be reviewed by a physician. However, if results fall outside of the normal range, you will be informed that you should consult your primary care physician for additional medical evaluation. The purpose of this study is to provide more information on what causes blood flow increases to occur after exercise. This protocol will explore whether older adults exhibit the same blood flow responses after exercise that young adults do. Our hope is that by better understanding the physiology of how the human body responds to exercise, we will be better able to use exercise in the prevention and treatment of diseases such as hypertension (high blood pressure) and other forms of cardiovascular disease.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not pay for any tests or procedures that are done just for this research study. You will get $120 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $15 per hour that you complete.

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 600-4337.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with the University of Oregon.

You do not waive any liability rights for personal injury by signing this form. Biomedical research involves some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. For example, if the investigators determine the study is overly stressful for you (as indicated by body language and an elevated heart rate and/or blood pressure) they may stop you from

participating in the study. Or if you express pain or discomfort beyond what is normally expected, the investigators stop you from participating in the study.

If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the State of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers:

General Counsel
Office of the President
1226 University of Oregon
Eugene, OR 97403-1226
(541) 346-3082

Research Compliance Services
5237 University of Oregon
Eugene, OR 97403-5237
(541) 346-2510

A law called the Oregon Tort Claims Act may limit the amount of money you can receive from the State of Oregon if you are harmed.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill's office area.

Research Compliance Services and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. All blood and muscle dialysate fluid samples will be destroyed when study results are published or 5 years after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

You will be asked to complete a Medical History Form on the study day which will list personal identifying information so that in the unlikely event of a medical emergency in which we would activate the emergency medical system, we would be able to provide this information to emergency healthcare providers. This document would be retained in a locked file cabinet in Dr. Halliwill's lab for up to a week after the study day, after which it is placed in a locked confidential documents disposal bin which is emptied by a secure shredding service.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5219 University of Oregon, Eugene, OR 97403, 541/346-2510.

Your initial indicates that you have read and understand the information provided above about the genetic testing, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty.

_____

(Initial of Participant) (Date)

If you would choose to abstain from the genetic testing portion of this study, please initial below. There will be no penalty if you choose to abstain, and you will still be able to participate in all other aspects of the study.

_____

(Initial of Participant) (Date)

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____

(Signature of Participant) (Date)

_____

(Signature of Individual Obtaining Consent) (Date)

Chapter VI

INFORMED CONSENT FORM

TITLE: EXERCISE, INFLAMMATION, AND HISTAMINE (AEROBIC EXERCISE)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done? When humans exercise, it generates pro- and anti-inflammatory signals. Histamine is released by exercising muscles and has the potential to either trigger or modulate the inflammatory response. The long-term goal of this research protocol is to better understand the link between exercise, histamine, and inflammatory signaling so that we can determine how and when to intervene in the inflammatory process, preserving the beneficial and avoiding the deleterious effects of inflammation in the context of exercise training and physical rehabilitation from injury. This topic is both clinically and scientific important. You have been asked to participate in this study because you are a healthy individual who does not participate in routine aerobic or endurance exercise.

What will happen in the study?

Initial Visit

You will arrive at Dr. Halliwill's laboratory in Esslinger Hall at the University of Oregon for an initial visit. This initial visit will take approximately half an hour. You will meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. Your height and weight and resting blood pressure will be measured, and you will be asked questions about your health history. You will be given a physical activity questionnaire, which will allow investigators to determine your activity level.

Fitness Test Visit

1. You will return to Dr. Halliwill's laboratory for an exercise test to determine your fitness level. The total time for this visit is approximately one hour. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. In addition, you will need to refrain from consuming caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 12 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. If you use oral contraceptives, you should take this when you normally do so.
2. If you are a woman who can still become pregnant, you will be asked to undergo a

pregnancy test during the exercise test visit (and again during the study visits). For this test, you will be asked to collect a sample of urine in the women's restroom near the physiology lab. If the test is "positive," indicating that you are pregnant, you will not be allowed to participate and will be advised to see your physician or the University of Oregon Health Center.

3. Aerobic Fitness Test: You will pedal on an exercise bicycle while wearing a mouth piece, nose clip, and electrocardiogram electrodes (heart rhythm monitor). If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. After a 5-minute warm-up, you will be asked to maintain a selected pedaling rate as pedaling resistance (work) is increased every minute until you reach your maximum exercise capacity. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm. This is to measure your overall aerobic fitness level. It normally takes 10 to 15 minutes for people to reach their maximum effort. This session will establish your maximal exercise tolerance on a bike and will be used to establish the appropriate workload for the exercise session on the study visits.
4. You should notify the investigator immediately if you feel any significant discomfort or concern about your well-being at any time during the initial visit. Some examples of discomfort include fatigue and muscle soreness.

Study Visits

1. You will then return to Dr. Halliwill's laboratory to participate in the two study visits, one of which will be about a week after the Exercise Test visit, and the other will be about a month after that. The study visits will last about 5 hours each. You will be given a food log to track what foods you consume prior to these visits, and you will be asked to eat similar foods before each visit.
2. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival. In addition, you will need to refrain from consuming caffeine (for example, coffee, tea, red bull, coke, etc.) or medications (except oral contraceptives) for 12 hours prior to the study and abstain from alcohol or exercise for 24 hours prior to the study. If you use oral contraceptives, you should take this when you normally do so.
3. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the study visits.
4. During the study visits, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a woman, the adhesive patches used for this test will be placed on you by one of the women in the lab. Your blood pressure will be measured at periodic intervals by the inflation of a blood pressure cuff around your arm.
5. On one of the study visits you will be given a 540 mg dose of fexofenadine hydrochloride (brand name Allegra) and a 300 mg dose of ranitidine hydrochloride (brand name Zantac). On the other study visit you will be given placebo tablets that look like the Allegra and Zantac. You will not know which pills you are taking on either study day. The 540 mg dose of fexofenadine hydrochloride is greater than the 180 mg over-the-counter recommended. This amount of fexofenadine is still safe for

humans as single doses up to 1980 mg and 690 mg two times per day for a month showed no ill outcomes. The 300 mg dose of ranitidine hydrochloride s in-line with the manufacturers recommended dose.

6. During the study visits, you will pedal on a bicycle at a moderate rate for 1 hour.
7. Before and after the exercise session, a small probe (ultrasound Doppler probe) will be held over an area of skin on your groin-hip intersection to image your femoral artery. The ultrasound Doppler probe uses ultrasound waves to measure blood flow in these arteries. The femoral artery will be studied for 10 minutes before you exercise and for one hour after you exercise.
8. Before and after the exercise session, an elastic band placed around your calf will be used to measure changes in leg volume in response to inflation of a pressure cuff above your knee. The pressure cuff above your knee will be inflated for several minutes at a time to a pressure which will feel snug but not uncomfortable. It is used to limit blood return out of your lower leg, but will not limit blood from flowing into your lower leg.
9. Blood sampling. A small needle will be used to obtain a blood sample from a vein near your elbow, as they would for a routine blood test at the doctor's office. This will be done at the beginning and end of the study visit. There will also be a blood draw at the follow up visits: 6 hours, 12 hours, 24 hours and 48 hours after exercise. The blood samples will be used to measure markers of inflammation. You should not donate blood or volunteer for another research study where blood will be drawn for 8 weeks following completion of the study. The 8-week period is recommended to allow your body to reproduce the blood that was taken during the study days.

Follow-up Visits

You will be asked to return for four follow-up visits at 6, 12, 24, and 48 hours after each of the two Experiment Visits. During each Follow-Up Visit, the researchers will obtain another blood sample,The total time for each follow-up visit is 15 minutes when blood is being obtained.

How long will I be in the study?

You will be in the study for a total of 12 sessions. These sessions include the Initial Visit, the Fitness Test Visit, the two Study Visits, and a total of 8 Follow-up Visits (4 after each Study Visit). Your total involvement would be about 14.5 hours.

What are the risks of the study?

1. Exercise Test. There is some minor discomfort associated with exercise testing, including temporary fatigue, shortness of breath, and muscle soreness. These sensations resolve within minutes after the test is completed. There is the possibility of some residual muscle soreness in the few days following the exercise test. There is also the risk of a heart attack or death during an exercise test. The risk of a complication requiring hospitalization is about 1 incident in 1000. The risk of a heart attack during or immediately after an exercise test is less than 1 incident in 2500. The risk of death during or immediately after an exercise test is less than 1 incident in

10,000. In the unlikely case of a life-threatening heart rhythm, the laboratory is equipped with an Automatic Electronic Defibrillator that is in the same room where the study is taking place. In the event of an emergency, 911 will be called and we will direct an ambulance to the correct location.

2. Blood sampling. In total, about 360 ml of blood will be withdrawn over the course of the experiments, which is a less than those associated with standard blood donation programs, where 450-500ml of blood (half a quart) is routinely withdrawn. You should not donate blood or volunteer for another research study where blood will be drawn for 8 weeks following completion of the study. The 8-week period is recommended to allow your body to reproduce the blood that was taken during the study days.
3. Tracking of Taxable Income. Please note, compensation from participation in Human Subjects Research studies is taxable income. If your compensation totals $600 or more in a calendar year, the University is required to report the income to the IRS. The University requires its departments to track participant compensation and may contact you to complete a Form W-9 for tax reporting purposes. Because of the federal and University tracking requirements, your name will be associated with participation in research. Department and University administrators will have access to this information, but will not have access to research data.

The study may include risks that are unknown at this time.

Are there benefits to taking part in this study?

This study will not make your health better. Measurements are not being conducted for diagnostic purposes. The results will not be reviewed by a physician. The purpose of this study is to provide more information on how humans respond to exercise. Our hope is that by better understanding the physiology of how the human body responds to exercise, we will be better able to use exercise in the prevention and treatment of diseases such as hypertension (high blood pressure) and other forms of cardiovascular disease.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not pay for any tests or procedures that are done just for this research study. You will get $220 for participating in this study. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, the amount of money you receive will be pro-rated at a rate of $15 per hour that you complete.

Who is funding this research study?

This study is being funded by the American Heart Association (AHA).

Who can answer my questions?

You may talk to Dr. John Halliwill at any time about any question you have on this study. You may contact Dr. Halliwill by calling the Department of Human Physiology at (541) 600-4337.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with the University of Oregon.

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If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

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A law called the Oregon Tort Claims Act may limit the amount of money you can receive from the State of Oregon if you are harmed.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a “subject identification number”. The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Halliwill’s office area.

Research Compliance Services and/or authorized representatives of the Food and Drug Administration (FDA) or National Institute of Health (NIH) may need to review records

of individual subjects. As a result, they may see your name, but they are bound by rules of confidentiality not to reveal your identity to others.

The list of names will be destroyed when study results are published or 24 months after your participation, whichever comes first. All blood and muscle samples will be destroyed when study results are published or 5 years after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

You will be asked to complete a Medical History Form on the study day which will list personal identifying information so that in the unlikely event of a medical emergency in which we would activate the emergency medical system, we would be able to provide this information to emergency healthcare providers. This document would be retained in a locked file cabinet in Dr. Halliwill's lab for up to a week after the study day, after which it is placed in a locked confidential documents disposal bin which is emptied by a secure shredding service.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5219 University of Oregon, Eugene, OR 97403, 541/346-2510. Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____

(Signature of Participant) (Date)

_____

(Signature of Individual Obtaining Consent) (Date)

APPENDIX B STANDARD OPERATING PROCEDURES ASSAYS

Leukocyte isolation and preparation for flow cytometry

___Take a vial of PFA from the freezer to thaw (may need to put in a beaker of water to accelerate)

___Make dilution of 10x RBC lysis buffer (brown bottle in fridge) - 3 ml + 27 ml H2O in a green lid tube

___Add 2.0 ml fresh whole blood to RBC lysis buffer.____Incubate **10 min** at RT

___Wipe down shaft of pipet with disinfectant towel to remove blood splatter

___Spin tube at 500 x G for 10 min (program 1)

___Pour off supernatant into beaker with a splash of bleach in hood.

___Resuspend in 30 mL 1x PBS from fridge. Pipet to break up cell pellet.

___Pass through 40 micron filter into fresh tube.

___Spin 10 min @ 500G, discard super into bleach beaker

___Resuspend in 3-5 ml PBS :_____ml **(R)**

___In a tiny tube, mix 10 ul trypan blue with 10 ul cell suspension.____Load 10ul to each slide well

___Insert slide into countess and zoom to adjust focus (cells with blue edge with white center)

___Count both sides of slide, record cells/ml alive and %alive. Average two sides.

Alive:_____,_____Average_____cells/ml **(A)**

Initials:_____

% alive:_____,_____Average_____%

___Divide 1,000,000 by **A** cells/ml =_____ml for 1,000,000 cells **(B)**

___(One or two unstained samples total per subject-week is enough)Remove **B** amount of suspension to microcentrifuge tube to save as unstained. Store in fridge until fixing step

___Remaining cells □ **R** – **B** =_____ml x **A** cells/ml=

_____cells **(C)**

*Only subtract **B** if you have removed a sample for "unstained"*

___Dilute cells to 1 x 106 cells/ml with cold PBS (if **C** is 4.2x106 cells, bring volume up to 4.2 ml) **(D)**

___If there's no reconstituted violet dye less than 2 weeks old in the freezer, make a fresh tube by adding 50 ul DMSO to lyophilized stain (both DMSO and stain are in Invitrogen pouch in freezer. Label stain with date after reconstituting).

___Add 1 ul violet stain per ml of cell suspension (same as 1 ul per million cells – volume **D** but **in ul.** Swirl and incubate in the dark at RT for **30 min.**

___Retrieve unstained cells from fridge (if unstained cells were collected at this time point).

___Fill violet stained cells up to 30 ml with PBS to dilute stain before spinning

___Spin tubes 10 min @ 500G, discard super

____To each tube, add 1 ml 4% PFA per million cells (volume **D**). Pipet to break up cell clumps.

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___Incubate at RT in dark for **10 min**

___Top off cell suspension to 30 ml w/ PBS to dilute PFA**.**

___Spin and discard super into **Formaldehyde Waste Bottle.**

___Resuspend cells in 2.0 mL PBS. Transfer to 2mL tube with label.

___Store in the dark in the fridge until ready for staining & cytometering (up to one week)

___Make sure remaining PFA and violet dye solutions are returned to freezer

___Blood beaker with 10% bleach can be washed down the sink 20 minutes after the last addition

Subject ID_____Time point_____

Date_____Initials:_____

Alive:_____,_____Average (A):_____cells/mL

% alive:_____,_____Average:_____%

R:_____mL

B:_____mL C:_____cells D:_____mL (added

_____mL)

1,000,000/avg live cells avg live cells x remaining vol C/106 mL

Leukocyte Staining for Flow Cytometry

Channel/Antibody/Stock solution(ug/ul)/Antibody volume(ul)/Stain Buffer(ul)/Master Cocktail

1 FL1-10 Unstained 0 100

2-9 FL1 CD**62E** 0.75ug .100 7.5 60

FL2 CD**86** 1.56ug .200 7.8 62.4

FL3 CD**309** 0.125ug .025 5 40

FL4 CD**34** 5ug .200 25 200

FL5 CD**192** 0.156ug .200 .78 6.24

FL6 CD**66B** 0.5ug .200 2.5 20

FL7 CD**14** 0.625ug .200 3.13 25

FL8 CD**16** 0.156ug .200 .78 6.24

FL9 CD**3** 0.156ug .200 .78 6.24

CD**19** 0.0625ug .050 1.25 10

CD**56** 0.156ug .200 .78 6.24

FL10 CD**45** 0.75ug .100 7.5 60

Histamine ELISA

Sample preparation and acylation

1. Pipette 25 µL of standards, 25 µL of controls, 25 µL of plasma samples, 10 µL of urine samples, or 50 µL of supernatant from the release test* into the respective wells of the Reaction Plate.

2. Add 25 µL of Acylation Buffer to all wells.

3. Add 25 µL of Acylation Reagent to all wells.

4. Incubate for 45 min at RT (20-25°C) on a shaker (approx. 600 rpm).

5. Add 200 µL of distilled water to all wells.

6. Incubate for 15 min. at RT (20-25°C) on a shaker (approx. 600 rpm).

2. Take 25 µL of the prepared standards, controls and samples for the Histamine ELISA

Histamine ELISA

1. Pipette 25 µL of the acylated standards, controls and samples into the appropriate wells of the Histamine Microtiter Strips.

2. Pipette 100 µL of the Histamine Antiserum into all wells and cover plate with Adhesive Foil.

3. Incubate for 3 hours at RT (20-25°C) on a shaker (approx. 600 rpm).

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4. *Alternatively: shake* the Histamine Microtiter Strips briefly by hand and incubate for 15 – 20 hours at 2 – 8 °C.

5. Remove the foil. Discard or aspirate the contents of the wells and wash each well 4 times thoroughly with 300 µL Wash Buffer. Blot dry by tapping the inverted plate on absorbent material.

6. Pipette 100 µL of the Enzyme Conjugate into all wells.

7. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).

8. Discard or aspirate the contents of the wells and wash each well 4 times thoroughly with 300 µL Wash Buffer. Blot dry by tapping the inverted plate on absorbent

material.

9. Pipette 100 µL of the Substrate into all wells and incubate for 20-30 min at RT (20-25°C) on a shaker (approx. 600 rpm). *Avoid exposure to direct sun light!*

10. Add 100 µL of the Stop Solution to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.

11. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm with a reference wavelength between 620 nm and 650 nm.

Mast Cell Culturing protocols

To make growth medium:

- Remove 50 ml from basal medium (Gibco 10639011) bottle and store in sterile conical tube
- Add entire contents of nutrient supplement (included w/ basal), 5 ml pen/strep (Gibco 15140), 5 ml L-glutamine (Gibco 25030-081), 1 ml Primocin (Fisher)
- Store at 4° (Primocin only good for 3 months at 4°. Add fresh primocin as needed.)

Waking cells

- Warm vial of cells in 37 degree bath just until thawed
- Wash tube with 70% ethanol
- Transfer cell suspension to 15 ml conical tube containing 5 ml warm growth medium
- Mix cells and centrifuge (450 x g at RT for 5 min) to pellet
- Resuspend cells in 5 ml growth medium and transfer to a T25 flask
- Leave cells alone for ~3 days then transfer to a T75 with additional 15 mL fresh media.
- It takes about a week for cells to recover after thawing
- Split cells every 3-4 days (Mondays and Fridays)

Passage/feeding – cells must be split or fed every 2-3 days. Split 1:2 – 1:5 depending on needs.

- To split, add 40 mL medium to each flask and mix. Transfer 20 ml from each flask to two new flasks (n=3)
- To feed, transfer suspension to a 50 ml conical tube and centrifuge at 450 x g for 5 min at RT. Pull off half the supernatant (10 ml), add 10 ml fresh growth medium, resuspend, transfer to new growth flask

Freezing

- Count cells to determine viability (should be greater than 80%)
- Centrifuge cells at 600 rpm for 10 minutes. Remove supernatant
- Resuspend in enough pZERVE (protide pharmaceuticals) to have a density of 5 x 10^6 cells/ml.
- Dispense suspension into sterile cryotubes in 1-2ml aliquots. Seal and store at RT for 30 min with gentle agitation
- Place vials in an insulated container (styrofoam works) in -20 for 1 hr.

- Remove insulation and place vials in -80 for 1 hour (*Do not store in -80 for more than 2 hrs)
- Transfer vials to vapor phase of LN2 for 24 hrs. Move vials to liquid phase
- *as an alternative, try doing one vial the traditional way (IPA canister in -80 for overnight, then transfer to liquid LN2)

Degranulation Assay

- Plate cells at 1 x 10^6 cells/ml in wells of a 96 well plate in imaging medium (50 ul total volume) See recipe below.
- Plate imaging medium only in blank wells
- Dissolve 2 mg compound 48/80 (H_20 soluble up to 50 mg/ml, store dry at -20) in 1 ml imaging buffer, sterile filter
- In the positive control wells, add 50 ul of 2 mg/ml compound 48/80 to a final concentration of 1 mg/ml
- In experimental wells, add 50 ul microdialysate in triplicate wells
- After 20 minutes*, remove suspension from all wells, pellet cells, pull off supernatant and transfer to new tube (need not be sterile). Keep the pellet. (*for initial trial, do only 48/80 and sample at 5, 20, 60 minutes)
- React supernatant with substrate, 2 mM **4-Nitrophenyl N-acetyl-β-D-glucosaminide** diluted in 0.2M citrate buffer (soluble to 5 mg/ml. Do 4.25 mg in 1 ml and add 15 ul per well) for 2 hr at 37 C
- Stop reaction with 50 ul 1 M Tris-Cl, pH 9
- Measure absorbance at 405 nm
- To determine total B-hexosaminidase content, lyse cells in pellet with Triton – X 100 (0.06%).
- React lysate with 2 mM substrate for two hrs at 37 C
- Measure absorbance at 405 nm.
- Subtract background signal and present data as percent of total B-hexosaminidase activity.

Imaging buffer (250mL)

142 mM NaCl 58.44 g/mol ➔ 2.0746 g

5 mM $NaHCO_3$ 84.01 g/mol ➔ 0.105 g

10 mM HEPES 238.3 g/mol ➔ 0.5958 g

16 mM Glucose 180.16 g/mol ➔ 0.7206 g

2 mM KCl 74.55 g/mol ➔ 0.0373 g

2 mM $CaCl_2$ 219.08 g/mol ➔ 0.1095 g

1 mM $MgCl_2$ 203.31 g/mol ➔ 0.0508 g

0.1% BSA ➔ 0.25 g

pH to 7.3 w/ NaOH

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