SUSTAINED POST-EXERCISE VASODILATION: HISTAMINERGIC MECHANISMS AND ADAPTATIONS

by

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DISSERTATION ABSTRACT

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Blood flow to the previously active skeletal muscle remains elevated for several hours following an acute bout of aerobic exercise and is dependent on activation of H_1 and H_2 histamine receptors. Many questions remain unanswered in humans regarding the mechanisms mediating this sustained post-exercise vasodilation and what benefits come of this physiological phenomenon. The studies detailed in this dissertation were designed to examine the upstream mechanisms and explore a potential benefit associated with sustained post-exercise vasodilation.

In chapter IV, we examined if oxidative stress is the upstream exercise-related factor mediating sustained post-exercise vasodilation. Intravenously infusing the antioxidant ascorbate blunted sustained post-exercise vasodilation, and this reduction was similar in magnitude to that observed with $H_1\backslash H_2$ blockade. However, ascorbate may directly degrade histamine and may also inhibit its formation. Therefore, we conducted a follow-up study to verify the findings in study 1. In this study, we intravenously infused n-acetylcysteine, a potent antioxidant with no known histaminergic interactions. We found that n-acetylcysteine had no effect on sustained post-exercise vasodilation, indicating that exercise-induced oxidative stress is not the exercise related factor mediating sustained post-exercise vasodilation.

In chapter V, we attempted to measure interstitial histamine in an effort to demonstrate that exercise induces the local formation of histamine in previously active skeletal muscle. We found that histamine is increased in the interstitial fluid within skeletal muscle during and after exercise. Additionally, we determined that *de novo* synthesis via histidine decarboxylase contributes to the rise in histamine during and following exercise. We also demonstrated a possible role of mast cells as an additional mechanism augmenting histamine in skeletal muscle. Collectively, these studies demonstrate that histamine is the ligand activating histamine receptors and activation is due to the induction of histidine decarboxylase and mast cell activation.

In chapter VI, we attempted to determine if histamine receptor activation contributes to the expression of pro- and anti-angiogenic growth factors during the recovery from exercise. Our preliminary findings indicate that activation of histamine receptors may play a role in the expression of pro-angiogenic growth factors during the recovery from acute aerobic exercise.

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DEDICATION

For my precious daughter Quinn and my amazing wife Sarah….

You mean the world to me….

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

LIST OF EQUATIONS

Equation Page

CHAPTER I

INTRODUCTION

BACKGROUND

An acute bout of aerobic exercise elicits profound changes in the cardiovascular and metabolic systems of the human body. These changes ensure that the essential cardiovascular and metabolic demands of exercise are met, and that the overall function of these systems is never compromised. But what happens after exercise? Do these systems function at levels observed prior to exercise or are there more prolonged alterations that subserve some regulatory mechanism or phenotypic adaptation? These are fundamental questions that are often overlooked in human integrative physiology.

For over a decade our laboratory has studied cardiovascular regulation during the recovery from aerobic exercise. Original studies examined the mechanisms regulating the known blood pressure lowering effect of exercise, post-exercise hypotension (74, 77, 78, 80). Research on post-exercise hypotension has evolved over the past several decades from Fitzgerlad's personal account of hypotension following exercise (57) to studies describing the alterations in cardiac output and vascular resistance, and more recently to mechanistic studies describing how and why these changes occur.

Arterial blood pressure is equal to cardiac output multiplied by peripheral vascular resistance. Thus, alterations in cardiac output and/or peripheral vascular resistance can drive the reduction in blood pressure observed following aerobic exercise. Studies investigating the mechanisms regulating cardiac output and peripheral vascular resistance gave rise to a phenomenon termed sustained post-exercise vasodilation. As one can imagine, it is this physiological phenomenon that reduces peripheral vascular

resistance during the recovery from exercise. Thus, without an equal and opposite rise in cardiac output to compensate for the robust peripheral vasodilation, arterial blood pressure will fall. Recently, our laboratory has focused its efforts on exploring the mechanisms and potential adaptations related to sustained post-exercise vasodilation, but we still acknowledge that it is a component of the larger hemodynamic pattern that occurs during the recovery from exercise.

Both neural and local vascular mechanisms contribute to sustained post-exercise vasodilation. Central resetting of the arterial baroreflex and reduced sympathetic vascular transduction comprise the neural component of sustained post-exercise vasodilation (78), whereas the local vascular component is entirely dependent on H_1 and H_2 histamine receptor activation. McCord and colleagues were the first to demonstrate that sustained post-exercise vasodilation is reduced by ~80% following 60 min cycling exercise using systemic H_1/H_2 histamine receptor blockade (119, 125, 126). More recently our laboratory has demonstrated that systemic H_1/H_2 histamine receptor blockade abolishes post-exercise vasodilation following unilateral dynamic knee extension exercise, suggesting that vasodilation following small muscle mass exercise is entirely histamine receptor dependent (16). Unpublished data from our laboratory supports this conclusion as the arterial baroreflex and sympathetic vascular transduction appear unchanged following dynamic knee extension exercise.

Aside from the contribution to post-exercise hypotension, does histamine receptor mediated sustained post-exercise vasodilation subserve any cardio-metabolic adaptations within the skeletal muscle? Data collected recently from our laboratory demonstrated that systemic H_1/H_2 histamine receptor blockade reduces oxygen consumption across the

previously exercised leg, and occurred in the absence of changes in arteriovenous oxygen difference or oxygen extraction. This suggests that an unknown metabolically active process is exquisitely linked to activation of histamine receptors during the recovery from exercise. Presently, it is unclear what this process is, but it was recently suggested that histamine receptor mediated sustained post-exercise vasodilation may contribute to microvascular remodeling through induction of pro-angiogenic growth factors (74). This and other potential adaptations associated with histamine receptor activation and sustained post-exercise vasodilation have not been examined thoroughly.

STATEMENT OF PROBLEM

The underlying mechanisms mediating sustained post-exercise vasodilation remain unresolved. The findings using H_1/H_2 histamine receptor blockade suggest that sustained post-exercise vasodilation is linked inherently to histaminergic signaling pathways. The logical assumption is that exercise induces the release and/or formation of histamine where it can then activate histamine receptors thus driving sustained post-exercise vasodilation. However, histamine concentrations in both plasma and whole-blood are unchanged following exercise (119, 125, 126) suggesting that histamine may be released locally in the previously active skeletal muscle without spillover into the circulation. This notion is supported by the observation that the vasodilation occurs only within the previously exercise skeletal muscles using the unilateral dynamic knee extension exercise model (16). To date, intramuscular histamine has never been measured during or following acute aerobic exercise in humans.

If histamine is the ligand activating histamine receptors during the recovery from exercise, where is it coming from? Histamine can be formed locally within skeletal

muscle through mast cell activation (129, 201) or formed *de novo* via the inducible enzyme, histidine decarboxylase (55). Thus, several competing or perhaps complimentary mechanisms may increase histamine formation in skeletal muscle during the recovery from exercise.

The question regarding the origins of histamine formation within skeletal muscle can be taken one step further; what upstream exercise related factor might be driving post-exercise histamine formation? Oxidative stress, increased temperature, and the local release of myokines have been proposed as mediators of sustained post-exercise vasodilation through histamine dependent mechanisms (74).

Sustained post-exercise vasodilation is a robust phenomenon. Acutely, it contributes to post-exercise hypotension and increases glucose delivery to the previously active skeletal muscle. Moreover, several recent studies have demonstrated that the magnitude of post-exercise hypotension is related to the long-term blood pressure reductions observed with chronic exercise training in pre-hypertensives (84, 115). These observations reveal that repeated activation of histamine receptors and subsequent vasodilation associated with acute aerobic exercise may contribute to various cardio-metabolic adaptations associated with chronic exercise training.

It is well known that chronic exercise induces microvascular growth and remodeling within skeletal muscle (50, 161). This expansion of the skeletal muscle microvasculature, also known as angiogenesis, is dependent on the acute release of pro- and anti-angiogenic growth factors within the previously exercise skeletal muscle (150). Interestingly, histamine contributes to angiogenesis in conditions such as wound healing (143) and cancer growth (59). Furthermore, histamine has been shown to upregulate pro-

angiogenic growth factors such as vascular endothelial growth factor (64, 65) and matrix metalloproteinase 2 (45). Thus, it is plausible that the angiogenesis associated with exercise training may result from recurring activation of histamine receptors and upregulation of pro-angiogenic growth factors.

The studies detailed in this dissertation were designed to answer several lines of inquiry relating to the histaminergic mechanisms and potential adaptations of post-exercise histamine receptor activation and sustained post-exercise vasodilation. In chapter IV, we examined if oxidative stress is the exercise-related factor mediating sustained post-exercise vasodilation. In chapter V, we attempted to measure interstitial histamine in an effort to demonstrate that exercise induces the local formation of histamine in previously active skeletal muscle. Additionally, we wanted to determine if the source of histamine was mast cells or *de novo* synthesis via histidine decarboxylase. In chapter VI, we attempted to determine if histamine receptor activation contributes to the expression of pro- and anti-angiogenic growth factors during the recovery from exercise.

HYPOTHESES

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

1. In chapter IV, study 1, we hypothesized that intravenous administration of the potent antioxidant ascorbate would blunt sustained post-exercise vasodilation. Moreover, this reduction would be similar in magnitude to that observed with H_1/H_2 histamine receptor blockade. In the follow-up study, we hypothesized that intravenous n-acetylcysteine administration would similarly inhibit sustained post-exercise vasodilation.

2. In chapter V, we hypothesized that interstitial histamine would be augmented in skeletal muscle during and after 1 hr of unilateral dynamic knee extension exercise. Additionally, we hypothesized that this observed increase was the result of *de novo* synthesis via histidine decarboxylase and not mast cell activation.
3. In chapter VI, we hypothesized that compared with control subjects, the mRNA expression of pro-angiogenic growth factors would be blunted during the recovery from exercise in subjects who received H_1/H_2 histamine receptor blockade.

SIGNIFICANCE

The primary goal of this dissertation is to examine the mechanisms and potential adaptations associated with post-exercise histamine receptor activation and sustained post-exercise vasodilation. Delineating the underlying mechanisms and potential adaptations will set the stage for future investigations that can determine if this phenomenon that occurs acutely during the recovery from exercise contributes to the phenotypic changes observed with chronic exercise training. We can then develop physiological and pharmacological interventions designed to exploit the histaminergic signaling pathway and related adaptations. These interventions could then be used to improve vascular health in patients suffering from vascular diseases such as peripheral artery disease.

CHAPTER II

REVIEW OF THE LITERATURE

INTRODUCTION

Two distinct vasodilatory phenomenon occur during the recovery from an acute bout of exercise (74, 77, 114). The first, known as immediate post-exercise vasodilation, occurs during the initial seconds of recovery and can last upwards of 20 mins. The second, sustained post-exercise vasodilation, is more prolonged that its immediate counterpart, lasting upwards of two hours into the recovery period. While these phenomena both occur during the recovery from exercise, each has unique and independent regulatory mechanisms.

Immediate Post-exercise Vasodilation

Immediate post-exercise vasodilation occurs following both isometric and dynamic exercise. The primary variable influencing immediate post-exercise vasodilation is the duration of exercise but the type and intensity of the exercise are also important. For example, by using varying intensities and duration of isometric contractions of the quadriceps muscle, Wesche (195) documented that immediate post-exercise vasodilation was more pronounced and longer in duration following exercise that is more sustained and that requires a greater effort. The elevation in blood flow observed by Wesche was transient, lasting ~10 sec following low intensity, short duration exercise and lasting upwards of only ~22 sec following more severe isometric exercise. Walløe and Wesche later conducted a similar study in which they used intermittent isometric contractions of the quadriceps muscle performed at varying levels of intensity and duration (190). The post-exercise vasodilation reported in this study was longer than previously observed but

was still quite transient lasting ~150 sec following the highest workloads performed for 6 min. These findings are relatively universal with respect to isometric exercise as several other studies have reported similar findings. The immediate post-exercise vasodilation observed following short duration dynamic exercise is longer lived and more pronounced than isometric exercise. For example, Bangsbo and colleagues reported that blood flow remained elevated for 20 mins following exhaustive unilateral dynamic knee extension exercise lasting 3 min in duration (13). Together, these studies demonstrate that a relationship exists between the time, type, and intensity of exercise performed and the duration and magnitude of immediate post-exercise vasodilation.

The mechanisms contributing to immediate post-exercise vasodilation are multifactorial and complex. It should be noted that this vasodilatory response is not mediated by mechanisms related to oxygen consumption within the previously active skeletal muscle (13, 133).

As observed with the onset of exercise, the muscle pump may also contribute to immediate post-exercise vasodilation. Mechanical compression of the arterial blood supply by contracting skeletal muscles may induce inherently a reactive hyperemic response. This is the most likely mechanism mediating immediate post-exercise vasodilation following prolonged isometric exercise, but may contribute to the rapid (seconds) vasodilation following rhythmic isometric exercise or dynamic exercise.

Much of the immediate vasodilatory response can be attributed to alterations in the neural control of the vasculature and local vasodilator signals. During exercise sympathetic outflow is elevated but is reduced during the immediate recovery period. Direct neural recordings of muscle sympathetic outflow in humans via

microneurography, indicate that nerve activity is reduced rapidly following isometric handgrip exercise, thus attenuating vascular resistance and increasing blood flow to the previously active skeletal muscle (180). Numerous local vasodilators have been implicated as potential mediators of immediate post-exercise vasodilation (14). For example, adenosine uptake inhibition via dipyridamole administration augmented post-isometric contraction hyperemia whereas inhibiting adenosine formation via ecto-5-nucleotidase adenine reduced it, suggesting that adenosine contributes to immediate post-exercise vasodilation (110). Additionally, nitric oxide has been suggested to contribute to immediate post-exercise vasodilation. Using intra-arterial infusions of the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine, Rådegran and Saltin, documented a 34% reduction in femoral blood flow following short duration high intensity dynamic knee extension exercise (165). Prostanoids and histamine may also contribute to immediate post-exercise vasodilation. Using an experimental model that consisted of prolonged isometric exercise and blood flow restriction, Morganroth et al. documented that immediate post-exercise vasodilation was reduced via prostanoid synthesis inhibition or via H_1 and H_2 histamine receptor antagonism (134). However, using a similar experimental approach but with a lesser exercise intensity to that of Morganroth et al., Honig and Daniel demonstrated that immediate post-exercise vasodilation was unaffected by antagonism of H_1 and H_2 histamine receptors (39). The discrepancy between these two studies is unclear but Honig and Daniel contend that the findings by Morganroth et al. may be related to an inflammatory response associated with vascular injury induced by the use of a more intense exercise protocol coupled with blood flow restriction.

Taken together, these investigations clearly indicate that the mechanisms governing immediate post-exercise vasodilation are indeed multifactorial. Reductions in sympathetically mediated vasomotor tone and the numerous local vasodilators associated with immediate post-exercise vasodilation point towards a possible "carry-over" effect from the preceding exercise bout. More specifically, the multiple and redundant vasodilatory mechanisms thought to contribute to exercise hyperemia may carry-over into the early recovery period.

Sustained Post-exercise Vasodilation

Unlike its short-lived counterpart, sustained post-exercise vasodilation can last upwards of several hours into the recovery period. In humans, sustained post-exercise occurs following prolonged aerobic exercise but is absent following short duration resistance exercise (16). Post-exercise hypotension has been observed following moderate duration whole body resistance exercise, but appears to be mediated by reductions in cardiac output as vascular resistance is usually unchanged or even elevated (21, 121, 169, 187). Interestingly, sex appears to be an important determinant of sustained post-exercise vasodilation following resistance exercise as it is more often observed in females (163).

The majority of studies investigating sustained post-exercise vasodilation have utilized large muscle-mass dynamic exercise (e.g. cycling) ranging from 30 to 60 min in duration. Similar to the dependence of immediate post-exercise vasodilation on the duration and intensity of exercise, sustained post-exercise vasodilation is consistently observed and greater in magnitude following moderate intensity (50% - 60% VO_{2peak}), prolonged (30 - 60 min) exercise (80). Recently, our laboratory has shown that a

unilateral dynamic knee extension exercise model also induces sustained post-exercise vasodilation. While smaller in magnitude compared with large muscle-mass exercise, the vasodilation observed following 1hr of knee extension exercise at 60% peak power is reproducible and is quite robust (16).

During exercise, there are well known vascular changes that occur within and beyond the active skeletal muscle (114). Likewise, regional vasomotor changes also occur during the recovery from exercise. Sustained post-exercise vasodilation occurs primarily within the vascular beds perfusing previously active skeletal muscle, but also occurs, to a lesser extent, in the inactive skeletal muscle following large muscle-mass exercise (76, 125). However, our laboratory has recently demonstrated that 60 min of moderate intensity unilateral dynamic knee extension exercise induces sustained post-exercise vasodilation in *only* the previously active leg (16). Blood flow was similar to pre-exercise levels in the contralateral leg. This divergence from the typical vascular changes following large muscle-mass exercise is likely related to the amount of active skeletal muscle and afferent nerve activation during exercise, as will be discussed in a subsequent section.

A series of papers from our laboratory have also provided evidence regarding blood flow in the splanchnic, renal, and cutaneous circulations during sustained post-exercise vasodilation. Pricher and colleagues reported that both splanchnic and renal vascular conductance was reduced during exercise as expected, but returned to pre-exercise levels following 60 min cycling exercise (160). Wilkins et al. reported that cutaneous vascular conductance was elevated early in the recovery period but returned to pre-exercise levels thereafter. To my knowledge, only one study has examined the

cerebral circulation during the recovery from exercise when arterial blood pressure was reduced and skeletal muscle blood was presumably elevated. Willie et al. documented that cerebral blood velocity and autoregulation are unchanged following 60 min moderate intensity cycling exercise (199). This finding is not surprising considering the ability of the cerebral vasculature to maintain blood flow during modest, non-syncopal hypotension.

MECHANISMS OF SUSTAINED POST-EXERCISE VASODILATION

Several investigators have laid the physiological groundwork with respect to the mechanisms regulating sustained post-exercise vasodilation. These investigators and their elegant studies have together constructed the framework that integrates the various mechanistic components mediating sustained post-exercise vasodilation. However, some of the inner workings currently remain unclear. The current evidence suggests that critical changes occur within the central baroreflex network and locally within the skeletal muscle vasculature and are referred to as the neural and local vascular components of sustained post-exercise vasodilation.

Neural Component: Arterial Baroreflex Resetting and Reduced Vascular Transduction

A number of early investigations reported that neurovascular regulation is altered during the recovery from aerobic exercise. It was not until recently that these post-exercise changes were found to be related to the well-known neural changes that occur *during* exercise. Floras and colleagues were the first to provide direct evidence of the discreet neurovascular changes that occur during the recovery from exercise in humans (58). Using microneurography, they documented a 34% reduction in muscle sympathetic nerve activity following 45 min of moderate treadmill running. The absence of reflex

tachycardia and augmented sympathetic outflow despite a clear reduction in arterial blood pressure was suggestive of an exercise-induced sympathoinhibition via altered baroreflex regulation and/or reduced sympathetic vascular transduction. Along these lines, Halliwill and colleagues later demonstrated in humans that the arterial baroreflex is indeed reset following large muscle-mass aerobic exercise (78). Specifically, they documented a 29% reduction in muscle sympathetic nerve activity following 60 min of cycle exercise at 60% VO_{2peak}. This dramatic reduction in baseline sympathetic outflow was due to a downward and leftward shift of the arterial baroreflex stimulus response curve and occurred without changes in sympathetic baroreflex gain. However, in a different study, Halliwill and colleagues showed that carotid-cardiac baroreflex gain is augmented during the recovery from aerobic exercise, possibly as mechanism to restrain the reduction in arterial blood pressure (79). This exercise-induced sympathoinhibition via altered sympathetic baroreflex regulation has also been documented in normotensive (130) and spontaneously hypertensive rats (107, 112). However, in contrast to the findings in humans, sympathetic baroreflex gain is attenuated in rats (107, 130).

Unraveling the central pathways that mediate resetting of the baroreflex during and following exercise has been a central focus of research within the last decade. It was not until recently that we developed a clear understanding of these central pathways in the medulla and their interactions during and after exercise. Much of our understanding regarding resetting of the baroreflex during exercise was due to the elegant work of Jeff Potts (158). The central theme of this research focused on the inhibition of second order barosensitive neurons by afferent input from active skeletal muscle. The afferent neurons project from skeletal muscle to the nucleus tractus solitarii where they release substance

P which then activates neurokinin 1 receptors on γ-aminobutyric acid (GABA) inhibitory interneurons. Upregulation of GABAergic interneuron activity inhibits increased baroreceptor afferent input to the nucleus tractus solitarii. This inhibition normalizes nucleus tractus solitarii activity output to the caudal ventrolateral medulla, thus reducing inhibitory input to the rostral ventrolateral medulla and increasing sympathetic outflow.

Chen and colleagues built upon these observations regarding muscle afferents and baroinhibition in the nucleus tractus solitarii, but focused their efforts on the exercise recovery period (31). Their work also centers on the GABAergic inhibition of barosensitive neurons via substance P release by skeletal muscle afferents and neurokinin 1 receptor activation. Chen and colleagues first demonstrated an obligatory role for substance P and neurokinin 1 receptors by reducing post-exercise hypotension by 37% via nucleus tractus solitarii microinjection of a neurokinin 1 receptor antagonist (32). As expected, antagonism of neurokinin 1 receptors also blocked the exercise pressor response, but had no effect on pre-exercise blood pressure. To further delineate the mechanisms underlying the baroinhibition in the nucleus tractus solitarii, they recorded GABAergic inhibitory post-synaptic currents and documented a large reduction in spontaneous activity in second order barosensitive neurons following aerobic exercise (30), which is suggestive of a reduced GABA inhibitory input. Interestingly, antagonism of neurokinin 1 receptors reduced the inhibitory post-synaptic currents on second order barosensitive neurons following sham exercise, but had no effect following aerobic exercise, suggesting that the neural response to endogenously released substance P and neurokinin 1 receptor activation is inhibited during the recovery from exercise. Because substance P has been shown to cause neurokinin 1 receptor internalization in the spinal

cord of rats (124), Chen and colleagues proposed that this may be the mechanism preventing GABAergic inhibition of second order barosensitive neurons following exercise. They confirmed this hypothesis by immunofluorescence labeling of neurokinin 1 receptors located on GABA inhibitory neurons within the nucleus tractus solitarii and visually confirming receptor internalization following aerobic exercise, but not sham exercise (30). Taken together, the arterial baroreflex is reset during the recovery from exercise by increased afferent input from active skeletal muscle which then increases the release of substance P and subsequent activation of neurokinin 1 receptors located on GABA inhibitory neurons. Prolonged activation of neurokinin 1 receptors causes receptor internalization and blunted GABAergic inhibition of second order barosensitive neurons during the recovery from exercise. In turn, nucleus tractus solitarii activity output to the caudal ventrolateral medulla in increased, thus augmenting inhibitory input to the rostral ventrolateral medulla and reducing sympathetic outflow to the peripheral vasculature.

In addition to resetting of the arterial baroreflex, Halliwill and colleagues also documented in humans, a reduced capacity to transduce sympathetic outflow into vasoconstriction following exercise (78). A reduction in sympathetic vascular transduction can be mediated by pre-synaptic inhibition, changes in the synaptic cleft, and post-junctional inhibition. In humans, it appears that the reduction in sympathetic vascular reduction is not mediated post-junctionally as the vasoconstrictor response to the intra-arterial infusion of the α_1-agonist phenylephrine and the α_2-agonist clonidine is intact during the recovery from 60 min cycle exercise (75). This suggests that the reduction in sympathetic vascular transduction is likely mediated by pre-synaptic inhibition or through a mechanism in the synaptic cleft that increases norepinephrine

metabolism or reuptake. In contrast to the findings in humans, DiCarlo and co-workers demonstrated that the vascular responsiveness to the intravenous infusion of phenylephrine is attenuated following aerobic exercise, using an isolated aortic ring preparation (94) and in several intact animal models (95, 154, 168). The inconsistency between the human and non-human investigations is unclear, but may be related to the local vascular mechanisms thought to mediate sustained post-exercise vasodilation as will be discussed in a subsequent section.

Taken together, resetting of the arterial baroreflex due to discreet changes in the cardiovascular control centers in medulla induce sustained post-exercise vasodilation through reductions in sympathetic vasoconstrictor tone. Additional sympathoinhibition is mediated through the inability to transduce sympathetic outflow into vasoconstriction. Thus, these two neural changes that occur during the recovery from aerobic exercise compromise the "neural component" of sustained post-exercise vasodilation. These post-exercise changes have been reviewed recently by our laboratory (74) and are summarized in **Figure 2.1**.

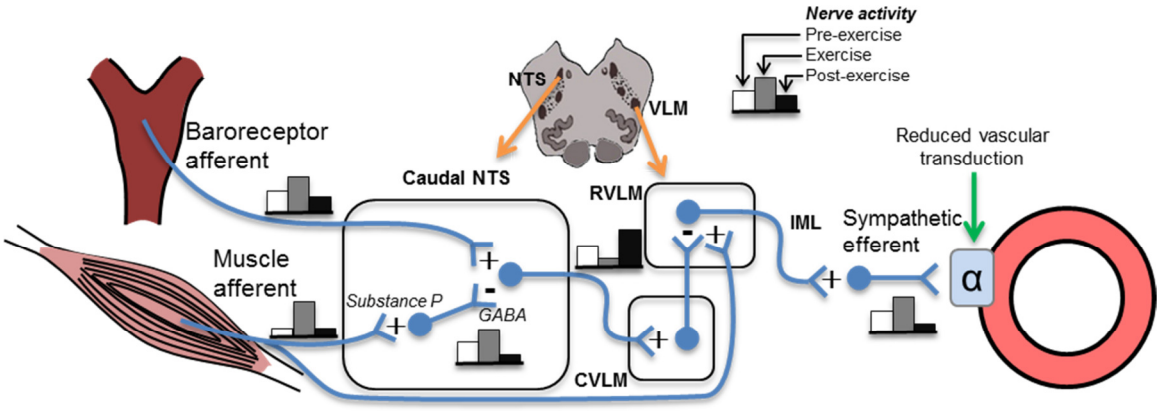

Figure 2.1. Neural Component of Sustained Post-exercise Vasodilation.
Baroreflex resetting via discreet changes in the nucleus tractus solitarii of the medulla and reduced sympathetic vascular transduction are highlighted as mediators of sustained post-exercise vasodilation. From (74).

Local Vascular Component: Histamine Receptor Activation

In 2001, Halliwill presented a "laundry list" of local vasodilators that could mediate sustained post-exercise vasodilation (80). Potential candidates included in this list were nitric oxide, prostanoids, adenosine, and several others. As presented elegantly by the late John T. Shepard, a proposed mediator of hyperemia must meet the following criteria: 1) the substance(s) or its precursor(s) should be present in skeletal muscle; 2) the substance(s) should have access to the muscle resistance vessels; 3) the concentration in the interstitial fluid must be capable of causing and maintaining vasodilation, and there should be a close relationship between the interstitial fluid concentration and the blood flow; and, 4) the substance(s) should be capable of producing dilatation of arterioles on topical application. Each of the candidate vasodilator substances meets these requirements and has also been implicated as a mediator of exercise hyperemia. Since Halliwill's "laundry list," we have developed a better understanding of the local vasodilator mechanisms that have no role in mediating sustained post-exercise vasodilation, but have also identified a principal mechanism. This section will summarize the investigations that have attempted, without success, to identify the local vascular mechanism and will present the current view of the local vascular component mediating sustained post-exercise vasodilation.

One of the most studied local vasodilator substances is nitric oxide which is formed through the actions of several nitric oxide synthase isoforms (185). Endothelial nitric oxide synthase is thought to be primarily responsible for nitric oxide formation within the vascular endothelium in response to acute exercise (114). Exercising hemodynamic factors such as shear stress, circumferential stretch, and paracrine factors

are thought to increase endothelial nitric oxide synthase activity during exercise. Nitric oxide has been implicated in mediating exercise hyperemia, but its role remains controversial (34, 175). Is nitric oxide formation augmented following exercise? Jungersten et al. documented that plasma nitrate, a major stable end product of nitric oxide metabolism was augmented for 2 hrs following prolonged cycle exercise (106). This finding coupled with those in animal models suggesting that nitric oxide blunts α-adrenergic responsiveness during the recovery from exercise (154, 168) lead Halliwill and colleagues to examine the contribution of nitric oxide to sustained post-exercise vasodilation (76). Using N^G-monomethyl-L-arginine to inhibit systemic nitric oxide formation, Halliwill and colleagues demonstrated in healthy humans that peripheral vasodilation persisted during the recovery from 60 min cycle exercise. Thus, it does not appear that nitric oxide contributes to sustained post-exercise vasodilation.

Next on the laundry list of local vasodilators to be examined were the prostanoids. Enzymatic breakdown of membrane bound arachidonic acid via cyclooxygenase-1 induces prostanoid formation within the vascular endothelium. Exercise and the immediate recovery period increases sheer stress along the vascular tree within the skeletal muscle and is a potent stimulus for the endothelial formation of prostanoids (111). Moreover, Cowley et al. inhibited cyclooxygenase-1 via ingestion of 1800 mg of aspirin and reduced both calf and forearm blood flood early into the exercise recovery period (36). Further, 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin hydrolysis, is elevated early in the recovery period following short duration high intensity aerobic exercise (171). Given these prior findings, the prostanoids were an ideal candidate to mediate sustained post-exercise vasodilation. However, Lockwood and co-workers

demonstrated that the systemic inhibition of cyclooxygenase-1 via 10 mg kg^{-1} oral ibuprofen had no effect on femoral vascular conductance through 90 min of exercise recovery (118). Thus, it does not appear that the exercise-induced formation of prostanoids contributes to sustained post-exercise vasodilation.

In 2005, our understanding of the local vascular component was significantly advanced with the finding that a histaminergic mechanism was responsible for sustained post-exercise vasodilation. This was quite unexpected given that it was not on Halliwill's original laundry list of potential vasodilators. According to laboratory folklore, the initial experimental endeavors regarding a histaminergic mechanism were purely serendipitous.

The initial evidence in humans regarding a histaminergic mechanism was provided by Lockwood and colleagues (119). They demonstrated that systemic blockade of histamine H_1 receptors via high dose (500 mg) fexofenadine significantly blunted the rise in femoral vascular conductance through 90 min of recovery from 60 min moderate intensity cycle exercise. Moreover, blockade of histamine H_2 receptors via high dose ranitidine (300mg), also significantly blunted the rise in femoral vascular conductance through 90 min of recovery following cycle exercise (125). Combined blockade of H_1 and H_2 histamine receptors reduces sustained post-exercise vasodilation by ~80% following 60 min cycling exercise in both sedentary and trained individuals (74, 126). More recently, our laboratory has demonstrated that combined H_1/H_2 histamine receptor blockade abolishes sustained post-exercise vasodilation following 60 min of unilateral dynamic knee extension exercise and that the vasodilation occurs only in the vascular beds perfusing the previously active skeletal muscle (16).

An explanation for the discrepant findings regarding histamine receptor mediated sustained post-exercise vasodilation following large muscle mass exercise versus small muscle mass exercise is lacking, but the divergence is likely related to the active muscle required to perform exercise. As previously discussed, muscle afferent activation during exercise mediates resetting of the arterial baroreflex and associated sympathoinhibition during the recovery from large muscle mass exercise. Presumably, there would be less afferent feedback during small muscle mass exercise and therefore resetting of the arterial baroreflex and associated sympathoinhibition would be limited. Recent unpublished observations from our laboratory support this conclusion as the arterial baroreflex and sympathetic vascular transduction appear unchanged following 60 min unilateral dynamic knee extension exercise. Additionally, sustained post-exercise vasodilation is observed only in the vasculature of the previously active leg following unilateral dynamic knee extension exercise, whereas blood flow to active and non-active regions is elevated following cycling exercise in the absence of elevated circulating histamine concentrations. This suggests that there are muscle mass dependent variations in the neural component contributing to sustained post-exercise vasodilation following small versus large muscle mass exercise.

Activation of histamine receptors following exercise is the *sine qua non* of the local vascular mechanism of sustained post-exercise vasodilation. However, one important question remains - what is the ligand activating histamine H_1 and H_2 receptors during the recovery from exercise? The obvious choice is histamine, which has long been recognized for its potent vasodilator effects and long been supported as an intrinsic regulator of exercise hyperemia (178).

Over a century ago, Barger and Dale isolated beta-iminazolylethylamine (i.e. histamine) from ergot fungi (15). Dale and Laidlaw later found that histamine had several physiological effects such as smooth muscle contraction of the ileum and a shock-like syndrome when injected into animals (37, 38). It was not until 1927, when Best and colleagues discovered that histamine is formed endogenously within the liver and lung and that it had a potent vasodilatory effect (18). Needless to say, countless studies have since then built upon these seminal studies identifying histamine and its physiological role.

Is histamine the ligand responsible for activating histamine H_1 and H_2 receptors during the recovery from exercise? Histamine meets all of the criteria outlined earlier with the exception of number three - the concentration in the interstitial fluid must be capable of causing and maintaining vasodilation, and there should be a close relationship between the interstitial fluid concentration and the blood flow. To our knowledge, interstitial histamine concentrations have never been measured in skeletal muscle during or following aerobic exercise. Thus, it still remains unclear if histamine is indeed the ligand activating histamine receptors following exercise.

While interstitial concentrations have never been measured in skeletal muscle during or after exercise, several studies have measured histamine in circulation. Early studies by Anrep and colleagues reported that plasma histamine is elevated in the venous blood draining the active skeletal muscle using blood flow restricted and free flowing experimental models (3–5). More recent studies in healthy humans have provided further evidence that histamine is elevated in whole blood (25, 82) and serum (47), but is unchanged in arterial or venous plasma (82, 83, 132). Studies in our laboratory have

failed to document any rise in plasma or whole-blood histamine during the recovery from exercise (119, 125, 126). It is unclear why our studies vary from others but may be related to experimental methodologies or histamine assay techniques. Histamine quantitation is technically challenging due the numerous assays available and associated variability (66) and to a half-life of just under two minutes (98). Moreover, it is possible that histamine is taken up by basophils when in circulation (149) or that histamine receptor sensitivity is increased during the recovery from exercise. Evidence recently collected in our laboratory points towards another explanation for the lack of elevated circulating histamine. Barrett-O'Keefe et al. documented that blood flow was elevated in the vascular bed perfusing the previously active quadriceps muscles in the exercised leg, whereas the blood flow in the contralateral, unexercised, leg was unchanged following dynamic knee extension exercise (16), which suggests that histamine is released locally within the skeletal muscle without spillover into the circulation.

Histamine formation in skeletal muscle could be augmented during the recovery from exercise through two mechanisms, mast cell degranulation or *de novo* synthesis via histidine decarboxylase (74). Mast cells originate from hematopoietic progenitor cells in red bone marrow and circulate in an immature form until deposition in various tissues (129). Once in the tissue, mast cells will develop functional and structural characteristics under the influence of various factors within local environment. Several exercise-related factors such as oxidative stress (183), cytokine release, (129) and elevated temperatures (67) have been shown in other contexts to induce mast cell degranulation in an antigen-independent manner. Alternatively, histidine decarboxylase could augment histamine synthesis in skeletal muscle through decarboxylation of the amino acid histidine (149,

192). Endo and colleagues have provided clear evidence that exercise augments both the gene expression and activity of histidine decarboxylase in mice (10, 55). Oxidative stress (90) and the transcription factor, hypoxia-inducible factor-1α (101) are associated with upregulated transcription of histidine decarboxylase. Histidine decarboxylase also appears to function optimally at elevated temperatures and an acidic pH, both conditions are associated with exercise (177). Lastly, a study by DeForrest et al. suggests that shear stress may increase *de novo* histamine synthesis within blood vessels through a shear stress dependent mechanism (41). Taken together, there are several lines of evidence suggesting that exercise-related factors could increase histamine formation through mast cell activation and/or *de novo* synthesis via histidine decarboxylase.

Figure 2.2 illustrates the local vascular component of sustained post-exercise vasodilation, as recently reviewed by our laboratory (74). The activation of histamine receptors during the recovery from exercise is the key mechanism of sustained post-exercise vasodilation. However, the ligand activating histamine receptors remains unclear, but the most likely candidate is histamine which may be formed locally within skeletal muscle through various mechanisms. Further, the factors known to stimulate histamine formation through the aforementioned mechanisms and that are also associated with exercise are yet to be examined.

PHYSIOLOGICAL ADAPTATIONS AND SUSTAINED POST-EXERCISE VASODILATION

Why are histamine receptors activated during the recovery from exercise? The answer to this question is the last piece of the physiological puzzle that our laboratory has been recently investigating. It is well understood that chronic exercise training induces a

number of systemic cardio-metabolic adaptations in humans. By convention, physiologists assume that the exercise bout per se, is the main factor mediating these exercise adaptations. What happens after exercise? Could the exercise bout be the stimulus that "primes" the system and the recovery period serve as the conduit for the true phenotypic adaptations to occur? We are just now beginning to unravel the answer to this and many other questions regarding the adaptations associated with histamine receptor activation and sustained post-exercise vasodilation.

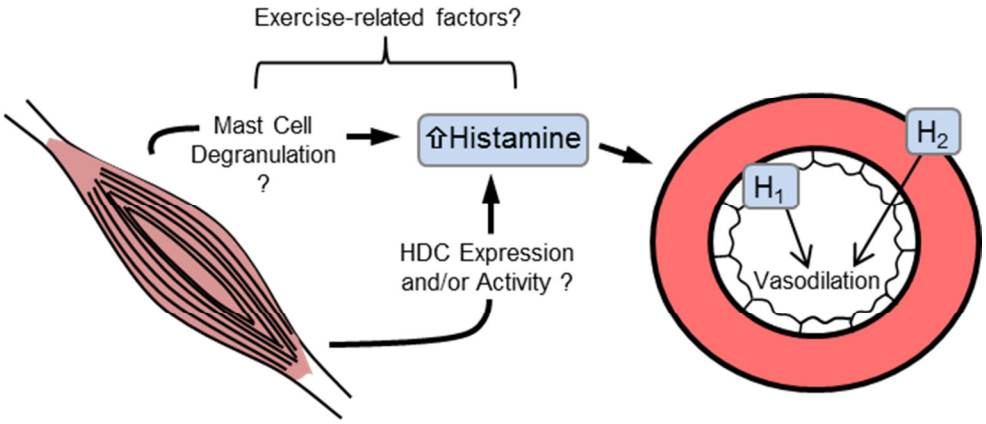

Figure 2.2 Local Vascular Component of Sustained Post-exercise Vasodilation Histamine receptor activation is highlighted as the primary vascular mechanism of sustained post-exercise vasodilation. The unanswered questions regarding intramuscular histamine formation are also highlighted. From (74).

Angiogenesis

We have recently speculated that histamine receptor mediated sustained post-exercise vasodilation is linked to exercise-induced angiogenesis (74). Sustained post-exercise vasodilation is not related to whole-body oxygen consumption (198). However, we have shown recently that H_1/H_2 histamine receptor antagonism reduces leg oxygen uptake following cycling exercise (54), suggesting that post-exercise histamine receptor

activation and subsequent vasodilation may be related to some unknown metabolically active process.

Exercise promotes growth and remodeling of the capillary network (angiogenesis) within skeletal muscle (70, 161, 196). Skeletal muscle angiogenesis is beneficial as it augments nutritive blood flow due to a decrease in red blood cell transit time, decreased diffusional path length, and an increased capillary surface area for diffusion (70). This expansion of muscle capillarity and nutritive blood flow should increase oxygen extraction and muscle performance. The mechanisms promoting angiogenesis in skeletal muscle remain unclear, but are thought to involve mechanical mechanisms such as shear stress and muscle overload/stretch, but may also be related to other metabolic factors and hypoxia (50, 51). Irrespective of the exercise related stimulus, angiogenesis depends critically on the balance of pro- and anti-angiogenic growth factors that are released in response to acute and chronic exercise (150).

Expansion of the skeletal muscle capillary network is dependent on a set of highly coordinated cellular events that respond to pro- and anti-angiogenic growth factors. The physical process of capillary remodeling can occur due to sprouting or intussusceptive (splitting) angiogenesis. In general, sprouting angiogenesis involves five key steps: 1) increased capillary permeability; 2) endothelial cell proliferation; 3) degradation of basement membrane & interstitial matrix; 4) migration and extension of endothelial cells; and, 5) vessel stabilization and perfusion (1, 70). Angiogenesis via splitting angiogenesis is far less understood owing to its relatively recent discovery in human skeletal muscle. The current understanding is that pillar-like structures migrate intralumnially within the blood vessel where they connect with one another, thus creating two separate blood

vessels in parallel. Importantly, both forms of angiogenesis have been shown to occur in response to elevated blood flow and muscle overload, which are models used to dissect the exercise-related mechanisms that induce capillary growth and remodeling (49).

Mechanical, metabolic, and hypoxic stimuli contribute to sprouting and intussusceptive angiogenesis in response to exercise through the release of pro- and anti-angiogenic growth factors. The most highly studied growth factor is vascular endothelial growth factor (VEGF). However, many other factors are thought to contribute to exercise-induced angiogenesis. Matrix metalloproteinases, nitric oxide synthase, and monocyte chemotactic protein are just a few of the known pro-angiogenic growth factors that respond to acute exercise. It is important to note that the capillary remodeling in skeletal muscle is dependent on the angiogenic balance, which is the equilibrium state of pro- vs anti-angiogenic growth factors. If the balance is in favor of the pro-angiogenic growth factors then the muscle capillary network will remodel and expand, whereas capillary regression will occur if the balance favors the anti-angiogenic factors (150). Potent anti-angiogenic growth factors include thrombospondin 1 and endostatin, but many others have been identified. Importantly, acute aerobic exercise upregulates both pro- and anti-angiogenic factors within the previously active skeletal muscle. For example, acute aerobic exercise upregulates VEGF mRNA in animal models (19, 81) and humans (69, 170) which translates into long-term elevations in skeletal muscle VEGF content (68). Conversely, thrombospondin 1 mRNA is also upregulated following acute exercise (151), which is likely "applying the physiological brake" in response to the robust expression of pro-angiogenic factors.

In addition to its potent vasodilator effects, histamine also induces angiogenesis in pathological and physiological settings. Histamine was first implicated as a potent angiogenic stimulus in the late 1960's (202) and has since been studied in the angiogenic settings of wound healing, cancer development, and pregnancy (59, 100, 143). The angiogenic effect of histamine is due the induction of pro-angiogenic growth factors such as VEGF (64, 65) or matrix metalloproteinase 2 (44), and can be inhibited via histamine receptor antagonism, blockade of histamine formation, or both (142, 149). Additionally, recent evidence suggests that histamine may directly induce angiogenesis irrespective of growth factor stimulation (162). Thus, there is ample evidence that histamine can directly or indirectly promote angiogenesis. Does the activation of histamine receptors during the recovery from exercise contribute capillary remodeling through the upregulation of angiogenic growth factors? Curiously, the temporal expression VEGF mRNA following acute exercise tracks closely with temporal pattern of sustained post-exercise vasodilation. While this observation is not causative by any means, it does provide "food for thought."

Taken together, exercise-induced angiogenesis within the skeletal muscle capillary network is a highly complex process. Exercise related factors such as elevated shear stress induce the release of pro- and anti-angiogenic factors that can then influence the angiogenic cascade. There is ample evidence that suggests that histamine receptor activation during the recovery from exercise may contribute to exercise-induced angiogenesis. This notion remains untested.

CHAPTER III

METHODS

SUBJECT CHARECTERIZATION

Studies conducted in this dissertation were approved by the Institutional Review Board at the University of Oregon (Protocol #02172011.029). 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 was obtained from all subjects subsequent to a verbal and written briefing of all experimental procedures for a given study.

Sixty-four (38 men, 26 women) healthy subjects participated in studies detailed in this dissertation (age 22 ± 3 yrs, height 174 ± 8 cm, weight 72 ± 10 kg, body mass index 23.5 2.4 kg m^2, 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 24h prior to all studies. Subjects reported to the laboratory after an overnight fast for the study described in chapter IV and following a 2h fast for studies described in chapters 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. For the studies detailed in chapters IV and V, female participants were studied during the early follicular phase of their menstrual cycle or during the placebo phase of their oral contraceptive. For the study detailed in chapter VI, women were studied irrespective of menstrual cycle phase. All female subjects had a negative pregnancy test prior to all studies.

GENERAL EXPERIMENTAL APPROACH

The studies detailed in chapters IV and V were conducted on the University of Oregon Campus in Esslinger Hall. The study detailed in chapter VI was conducted in the Center for Medical Education and Research at Sacred Heart Medical Center.

The study detailed in chapter IV was conducted in two parts. In study 1, subjects were studied under three conditions: control, ascorbate alone, and ascorbate plus H_1 and H_2 histamine receptor blockade. In the follow-up study, n-acetylcysteine was infused in lieu of ascorbate and subjects did not receive antihistamines. Central and peripheral hemodynamics were measured before and after 1 hr unilateral dynamic knee extension exercise within respective drug conditions.

In the study described in chapter V, subjects were studied under resting conditions or having performed 1 hr unilateral dynamic knee extension exercise. Skeletal muscle microdialysis probes were implanted in the subjects right *vastus lateralis* and used to deliver drugs of interest and to collect the microdialysis effluent (dialysate). Central and peripheral hemodynamics were measured before and after 1hr seated rest or knee extension exercise.

In the study detailed in chapter VI, skeletal muscle biopsies were obtained from the *vastus lateralis* in control subjects and those who received $H_1$and H_2 histamine receptor blockade. A pre-exercise biopsy was obtained from the non-exercised leg and two biopsies were obtained from the previously exercised leg. Central and peripheral hemodynamics were measured before and after 1 hr unilateral dynamic knee extension exercise.

UNILATERAL DYNAMIC KNEE-EXTENSION EXERCISE

Studies described in this dissertation made use of a unilateral dynamic knee extension ergometer. Unilateral knee extension ergometry was originally developed at the Copenhagen Muscle Research Centre in Copenhagen, Denmark. Unilateral dynamic knee extension exercise was first used in a seminal publication by Per Andersen and the late Bengt Saltin (2). Since then, countless studies examining the physiology of exercise have utilized this exercise model. Unilateral dynamic knee extension exercise is unique in that it isolates muscle activation to the *quadriceps femoris* and allows for passive knee flexion, thus allowing for targeted investigation of a single muscle group.

Historically our laboratory has utilized whole-body aerobic exercise such as cycling to investigate sustained post-exercise vasodilation. Moderate intensity cycling exercise of 1 hr in duration elicits a robust vasodilatory response during the recovery from exercise that is reduced by ~80% via H_1 and H_2 histamine receptor blockade (119, 125, 126). In contrast, unilateral dynamic knee-extension exercise of 1 hr in duration elicits a vasodilatory response during the recovery from exercise that is abolished via H_1 and H_2 histamine receptor blockade (119, 125, 126). The differential effects of histamine receptor blockade between exercise modalities appear to be due to the absence of baroreflex resetting and reduced sympathoinhibition following dynamic knee-extension exercise (unpublished observation). Thus, sustained post-exercise vasodilation following unilateral dynamic knee-extension exercise is entirely histamine receptor dependent and therefore provides optimal conditions to investigate the local vascular contribution.

Unilateral dynamic knee-extension exercise was performed using 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 measures 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.

Peak power was determined during maximal dynamic knee extension exercise test. Following a five minute warm-up at a workload of 5 watts, workload was ramped incrementally at a rate of 3 watts min^{-1}. Subjects were required to maintain power within 1 watt of the target workload and also maintain cadence within 5 kicks min^{-1}. Subjects performed the exercise until volitional fatigue.

Moderate intensity unilateral dynamic knee extension exercise was utilized in each study detailed within this dissertation. Power was ramped at the onset of exercise to 60% peak power over the first 15 min for studies described in chapters IV and V. Power was ramped at the onset of exercise to 60% peak power over the first 5 min for the study described in chapter VI. Subjects were required to maintain power within 1 watt of the target workload and also maintain cadence within 5 kicks min^{-1}.

HEMODYNAMIC MEASUREMENTS

In the studies detailed in chapters IV, V, and VI, resting hemodynamic measurements were made with the subjects in the supine position and in a thermoneutral room (~23°C). Some hemodynamic measurements were made with the subjects seated at 60° upright and while performing the knee extension exercise or resting.

Heart Rate

Heart rate was monitored using a three lead electrocardiograph (Datex-Ohmeda Cardiocap/5, GE Healthcare, Tewksbury, MA, USA) for study 1 in chapter IV. Due to technical difficulties, a different three lead electrocardiograph was used for the follow-up study in chapter IV and for all other studies (Tango+, SunTech Medical, Raleigh, N.C., USA).

Arterial Blood Pressure

Arterial blood pressure was measured in the right arm using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh, N.C., USA). Mean arterial pressure was calculated as:

$$MAP = Diastolic\ BP + \left[\frac{Systolic\ BP - Diastolic\ BP}{3}\right]$$

Equation 3.1. Mean Arterial Pressure

Femoral Blood Flow

Although there are a number of techniques that can be used to assess regional blood flow, our laboratory uses Doppler ultrasound technology. Prior to the widespread adoption of Doppler ultrasound, most physiologists relied on other techniques such as plethysmography or indicator methods to measure regional blood flow in humans.

Plethysmography is non-invasive, relatively inexpensive, and provides reasonable estimates of regional blood flow. Blood flow is based on the rate of limb distension following the occlusion of venous outflow. The major disadvantages to this technique is that it cannot differentiate blood flowing to various tissues (skin vs. muscle) and is also subject to inaccurate measurements due to unintentional occlusion of arterial inflow at pressures thought to occlude only venous outflow (24).

Indicator methods are classic techniques utilized in human integrative physiology research. This approach is based on the infusion of an indicator in a known concentration or temperature and subsequent measurement of the indicator following the infusion and thorough mixing within the blood. Various indicators have been utilized in humans such as indocyanine green dye, Evans blue dye, and ice-cold saline. Regarding dye indicators, blood flow is measured by the dilution of the dye within the blood. Thus, regional blood flow is assumed to be proportional to the dye concentration in the blood relative to the known concentration prior to infusion (167). Thermodilution is based on the same principle but depends on the temperature change within a vessel during the constant infusion of ice-cold saline. Regional blood flow is assumed to be proportional to the temperature deflection within the blood (167). The indicator methods have been used to measure regional blood flow in humans with high accuracy at rest and during exercise (2, 62, 105). These methods are fairly easy to perform but require invasive catheterization and steady-state conditions. Moreover, the cost associated with indicators dyes such as indocyanine green can be high.

Doppler ultrasound derived blood flow velocities correlate highly with the thermodilution technique (166). Unlike indicator methods, Doppler ultrasound provides high temporal resolution and continuous sampling as it does not require steady state conditions and is completely non-invasive (167). However, the equipment can be expensive and a highly skilled technician is needed to obtain accurate and reproducible measurements. Similar to venous occlusion plethysmography, Doppler ultrasound cannot differentiate between the blood perfusing various tissues within the region of interest. This limitation can be overcome by simultaneously measuring regional blood flow via

Doppler ultrasound and tissue specific blood flow. Given the aspects outlined for the various techniques available to measure regional blood flow in humans, Doppler ultrasound is the ideal technique for use in our studies.

Femoral artery blood flow velocity was measured via duplex ultrasonography. A linear-array vascular ultrasound probe (10 MHz, GE Vingmed System 5, Horton, Norway) was used for studies in chapters IV and V while a different linear array vascular ultrasound probe was used for chapter VI (9 MHz, Phillips iE33, Andover, MA., USA). An insonation angle of 60° was used always to measure blood flow in the common femoral artery, approximately 2-3 cm proximal to the bifurcation. The Doppler ultrasound machines were 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. Femoral diameter was measured in triplicate during diastole following velocity measurements. Femoral blood flow was expressed in ml per min and calculated using the following:

$$FBF = \pi(\frac{d}{2})^2 \times v \times 60$$

Equation 3.2. Femoral Blood Flow

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:

$$FVC = \frac{Femoral\ Blood\ Flow}{Mean\ Arterial\ Blood\ Pressure}$$

Equation 3.3. Femoral Vascular Conductance

Cutaneous Blood Flow

Laser-Doppler flowmetry was used to determine red blood cell flux, an index of skin blood flow for the studies described in chapter IV. Laser-Doppler flowmetry was used in conjunction with Doppler ultrasound to simultaneously measure skin and femoral blood flow. In doing so, we can ensure that any alteration in post-exercise femoral vascular conductance due to the intervention was not mediated by changes in the cutaneous vasculature.

Laser-Doppler flowmetry uses Doppler technology that is based on laser light emission into the skin at depth of ~1mm. The light is scattered by tissue and moving red blood cells and collected by the probe and transmitted to photo detector where it is processed electronically to derive the laser Doppler flux signal. Given the depth and non-specific laser emission, laser-Doppler flowmetry assesses blood flow normally in the microvasculature (i.e. arterioles, capillaries, and venules).

Laser-Doppler probes (DRT4, Moor Instruments LTD, Devon, UK) were placed anteriorly, on the midline, and halfway between the inguinal ligament and patella on both the active and inactive leg. Cutaneous vascular conductance was calculated by dividing laser Doppler flux by mean arterial pressure and normalized to maximal flux values measured during local heating to 43°C to account for the variability between laser Doppler sites (109, 197).

INTRAVENOUS CATHETERIZATION

Intravenous catheterization was utilized in the studies described in chapter IV. A 22G intravenous catheter (ProtectIV Safety I.V. Catheter, Smiths-Medical, Dublin OH, USA) was inserted into a peripheral vein within the antecubital space of the subjects left

arm. The intravenous catheter was kept patent through the use of a continuous saline drip (60 ml hr^{-1}). Venous blood was collected immediately after placement, prior to any drug infusion, and immediately following exercise.

SKELETAL MUSCLE MICRODIALYSIS

Skeletal muscle microdialysis is a powerful, yet complex technique that is used to obtain biological samples from the extracellular fluid compartment *in vivo*. The origin of microdialysis technique is attributable to the "push pull" cannula, which was used to study cerebral tissue chemistry (61). The technique was later refined with the advent of the "dialytrode" which was introduced by Delgado and colleagues in 1971 (42). Ungerstedt and Pycock developed what is thought to be the modern microdialysis probe consisting of a hollow semi-permeable membrane which they implanted in the brain of conscious, freely moving rats (189).

The modern skeletal muscle microdialysis technique allows for the continuous monitoring of substances that are associated with metabolism, blood flow regulation, and other physiological events using the principle of diffusion (120). A unique quality of microdialysis is the localization of fluid sampling and drug delivery to the interstitial space. In general, fluid sampling and drug delivery will occur within 1 cm^3 of the microdialysis probe (86).

Microdialysis probes are constructed using two different designs; non-linear (**Figure 3.1.**) and linear. While the nomenclature of the probes lacks creativity the design of the probes does not. The semi-permeable membrane is made from a variety of materials such as polyarylethersulphone and regenerated cellulose. Membranes are designated by their molecular weight cutoff which identifies the "pore" size in which

substances in the interstitial space can traverse. Molecular weight cutoff can vary from 3 kilodaltons to 100 kilodaltons. The membrane is usually attached to inlet/outlet tubing that is made of a soft malleable plastic such as polyurethane.

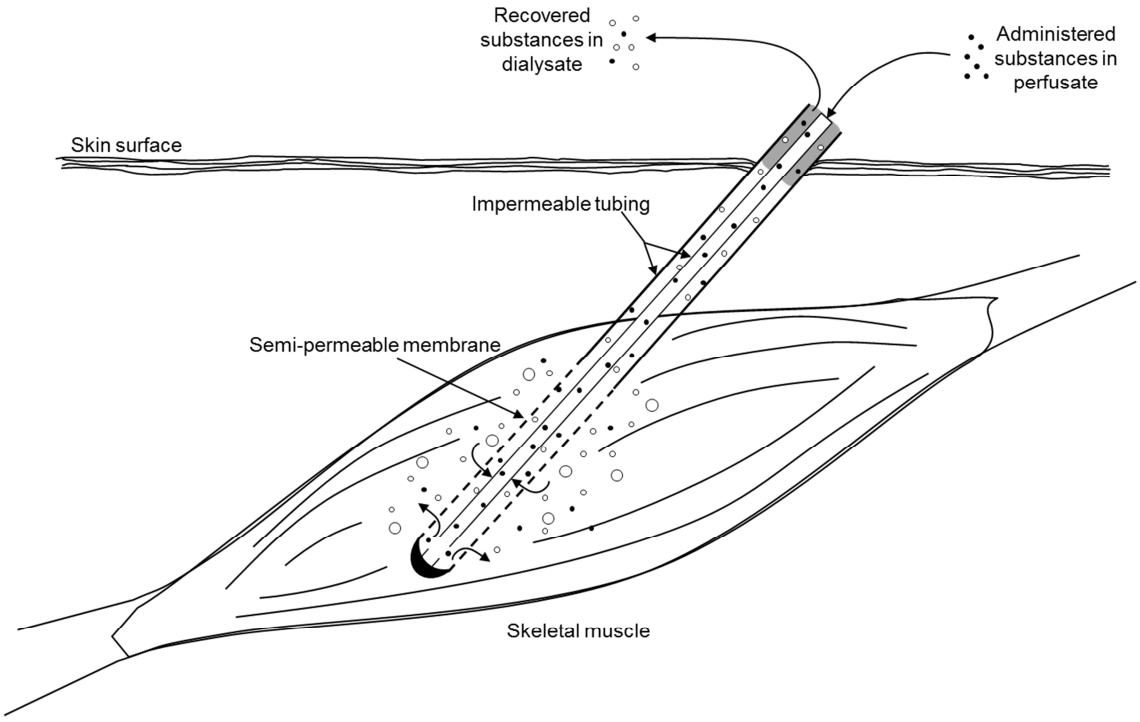

Figure 3.1. Non-linear Skeletal Muscle Microdialysis Probe Schematic
Filled circles denote substances perfused into the interstitial space. Small open circles denote substances capable of traversing the semi-permeable membrane. Large open circles denote substances that are too large to traverse the semi-permeable membrane. This schematic was adapted from artwork developed originally by Chris Minson and colleagues.

The recovery of substances from the interstitial space is dependent on 1) perfusion rate, 2) probe length, 3) probe membrane characteristics, and 4) the rate of diffusion in the interstitial space (108, 122, 139). The first three factors are usually fixed within an experiment and therefore do not affect recovery across a study, but can vary greatly between studies and need to be taken into account when determining recovery. The rate

of diffusion is influenced by a number of factors and can vary greatly both between and within studies. The diffusion of substances is dependent on: tortuosity, volume fraction, blood flow, and perfusate flow rate (86, 108, 122, 139). Experiments with dynamic conditions such as exercise or pharmacological interventions can greatly alter the factors that influence diffusion. Thus, an *in vivo* calibration should be performed in such conditions in order to account for changes in probe recovery.

Given the complex technical factors associated with microdialysis we used the best available approach for the studies described in chapter V. We chose to use the non-linear skeletal muscle microdialysis probe as it is better tolerated by human subjects when performing exercise and the membrane is better able to withstand the force exerted onto the probe during vigorous muscle contractions. All probes were implanted using sterile technique. The skin and underlying fascia were anesthetized using 1% lidocaine HCL (Hospira Worldwide, Lake Forest, IL, USA) buffered with sodium bicarbonate (Hospira Worldwide, Lake Forest, IL, USA). Care was taken to ensure that the lidocaine was not injected into the skeletal muscle. Probes were implanted in the *vastus lateralis* in a direction parallel with muscle fiber orientation (~19°, relative to long-axis of muscle) using a splitable introducer. After implantation, probes were held in place by covering the entry site with a sterile transparent medical dressing.

A probe with a 30 mm polyarylethersulphone membrane and a 20 kilodalton molecular weight cutoff (63 MD Catheter, MDialysis, Stockholm, Sweden) was used to measure interstitial histamine and to assess interstitial fluid flux. The probe was perfused with 0.9% saline at rate of 5µl min^{-1} using a microinfusion pump (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA, USA). Because perfusion rates

above 0.3 µl min^{-1} do not allow for complete equilibration with the interstitial fluid, the internal reference technique (179) was used to for *in vivo* calibration of recovery by adding 0.40 µCi per ml of tritiated histamine [^3H] to the perfusate. Additionally, ethanol (5 mM) was added to the perfusate in order to assess interstitial fluid flux via the ethanol washout technique. A detailed description of the internal reference and ethanol washout techniques and associated analyses are provided in subsequent sections. As detailed in a subsequent section, specific drugs were also added to the perfusate in some of the implanted probes. Interstitial tryptase was measured using a probe with a 30 mm polyarylethersulphone membrane and a 100 kilodalton molecular weight cutoff (71 High Cut-off Brain MD Catheter, MDialysis, Stockholm, Sweden). The probe was perfused with 0.9% saline at rate of 5µl min^{-1} using a microinfusion pump (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA, USA). Tritiated water (^3H_2O) was added to the perfusate to help assess probe stability during exercise but was not used to calibrate for probe recovery.

Dialysate was collected into microtubes every 30 min throughout the studies. Microtubes were covered with non-porous tape during each sampling period in order to prevent ethanol evaporation. Microtube weight was documented before and after dialysate collection in order to assess fluid loss and estimate perfusion rate of the probe. A small volume (10 µL) of the dialysate was pipetted immediately after collection for scintillation counting. The remaining volume of dialysate was stored at -20°C for the remainder of the study. Thereafter, the samples were stored at -80°C until analysis.

SKELETAL MUSCLE BIOPSY

All skeletal muscle biopsies were performed by Dr. Hans C. Dreyer, an expert muscle physiologist. All biopsies were performed in the *vastus lateralis* under sterile technique. The first biopsy was taken just prior to unilateral dynamic knee extension exercise from the left, non-exercising leg. The second biopsy was taken immediately following exercise. The final biopsy was taken from the same incision as the previous, but at a site directly opposite to that of the prior biopsy. The post-exercise biopsies were taken immediately after and three hours following the completion of exercise. The skin and underlying fascia were anesthetized using 1% lidocaine HCL (Hospira Worldwide, Lake Forest, IL, USA). A 5 mm Bergström biopsy needle was inserted through a small incision made in the skin and muscle fascia. A 140 cc syringe and tubing were attached to an aspiration port on the biopsy needle in order apply negative pressure in the biopsy needle and increase the sample recovered. A single suture is placed after the biopsy is complete. The suture is also covered with bacitracin and a sterile transparent dressing to help prevent infection during the healing process. Harvested skeletal muscle tissue was blotted and removed of any adipose tissue and snap frozen in liquid nitrogen. When applicable, a small portion of the muscle sample was removed for future histological analysis. These tissue samples were snap frozen in isopentane cooled with liquid nitrogen and stored at -80°C until analysis.

PHARMACOLOGICAL INTERVENTIONS

Oral Drugs

Oral antihistamine drugs were used in study 1 of chapter IV and the study detailed in chapter VI. Histamine H_1 and H_2 receptors were blocked using 540 mg fexofenadine

and 300 mg ranitidine. This combination of fexofenadine and ranitidine inhibits sustained post-exercise vasodilation following unilateral dynamic knee extension exercise (16). This dosage of oral fexofenadine has been shown to selectively block H_1 receptors (time to peak concentration 1.15 h and half-life 12 h), while the dose of oral ranitidine has been shown to selectively block H_2 receptors (time to peak plasma concentration 2.2 h and 2.6 h half-life) (63, 173). Responses are 90% inhibited within 1 h and remain inhibited 6 h after administration (22, 63). Fexofenadine and ranitidine do not appear to cross into the central nervous system or possess sedative actions (22). Furthermore, these drugs do not have any direct cardiovascular effects in the absence of histamine receptor stimulation (i.e., when given under normal resting conditions, these drugs do not elicit any changes in heart rate, blood pressure, or smooth muscle tone) (126). Subjects ingested the histamine receptor antagonists with water 1 hr prior to exercise in all studies.

Intravenous Drugs

Systemic intravenous drugs were used in the studies detailed in chapter IV. Ascorbate (Bioniche Pharma, Morgantown, WV, USA) used in study 1 in chapter IV was diluted in saline and infused intravenously for 20 min prior to exercise (primer dose, 0.5 g kg^{-1} body weight) and during 60 min of dynamic knee extension exercise (maintenance dose, 0.017 g kg^{-1} body weight). The primer dose of ascorbate was diluted with saline to a final volume of 60 ml and infused at a rate of 3.0 ml min^{-1} while the loading dose was diluted with saline to a final volume of 30 ml and infused at a rate of 0.5 ml min^{-1}. Both doses of ascorbate were infused along with a continuous saline drip (60 ml hr^{-1}). This pharmacological approach has been used in humans to significantly increase plasma ascorbate concentrations (17, 56, 99). Furthermore, ascorbate has been used successfully

to reduce both direct and indirect markers of oxidative stress following exhaustive aerobic exercise in humans (9). Ascorbate does not appear to have any direct cardiovascular effects under resting conditions in healthy young adults (17, 56, 99). Ascorbate is a water-soluble scavenger of reactive species such as superoxide and peroxynitrite (73) and has a half-life of 30 min when administered intravenously (46, 153).

N-acetylcysteine was infused in the follow-up study detailed in chapter IV. N-acetylcysteine (Acetadote ®, Cumberland Pharmaceuticals Inc., Nashville, TN, USA) was diluted in saline and infused intravenously for 15 min prior to exercise (primer dose, 125 mg kg^{-1} hr^{-1}) and during 60 min of dynamic knee extension exercise (maintenance dose, 25 mg kg^{-1} hr^{-1}). The primer dose of n-acetylcysteine was diluted with saline to a final volume of 45 ml and infused at a rate of 3.0 ml min^{-1} while the maintenance dose was diluted with saline to a final volume of 60 ml and infused at a rate of 1.0 ml min^{-1}. Both doses of n-acetylcysteine were infused along with a continuous saline drip (60 ml hr^{-1}). This pharmacological approach has been used in humans to significantly increase plasma n-acetylcysteine concentrations prior to and during exercise (20, 127, 128). Furthermore, n-acetylcysteine has been used successfully to alter blood redox status in both short-term high intensity and prolonged cycle exercise in humans (127, 128). N-acetylcysteine does not appear to have any direct cardiovascular effects under resting conditions in healthy young adults (144). N-acetylcysteine is potent water-soluble pharmacological antioxidant with a half-life of ~5 hrs. It can directly scavenge reactive species such as hydrogen peroxide and hydroxyl radical and can also indirectly reduce reactive species by augmenting glutathione production via intracellular cysteine formation (35, 73, 176).

Microdialysis Drugs

Drugs delivered via muscle microdialysis were used in studies described in chapter V. Pyrilamine maleate (1mM) was used to block H_1 histamine receptors and cimetidine (3mM) was used to block H_2 histamine receptors (Sigma-Aldrich, St. Louis, MO, USA). These doses have been used previously in our lab to reduce successfully sustained post-exercise vasodilation (155). α-fluoromethylhistidine dihydrochloride (200μM) was infused to irreversibly inhibit histidine decarboxylase activity (193). This dose was extrapolated from studies using α-fluoromethylhistidine dihydrochloride to inhibit histidine decarboxylase in cell culture (181, 200). 10 mM of the mast cell degranulater, compound 48/80 (Sigma-Aldrich, St. Louis, MO, USA), was infused in a total of four subjects to verify the diffusive ability of tryptase through the 100 kilodalton membrane. This dose has been used successfully in our lab to activate mast cells and produce localized vasodilation within the skeletal muscle.

ASSAYS AND ANALYSIS

Oxidative Stress Analysis

Plasma F_2 Isoprostanes Assay. Plasma F_2-isoprostanes were used as an *in vivo* biomarker of exercise-induced oxidative stress for study 1 in chapter IV. Countless indirect biomarkers of oxidative stress exist but most are considered inaccurate and/or obsolete (e.g. thiobarbituric acid reactive substances assay) (73). Oxidative stress via free radicals can damage biomolecules such as DNA, lipids, and proteins (73, 131). Indirect biomarkers of oxidative stress are typically byproducts of free radical mediated damage. F_2-isoprostanes are prostaglandin-like substances that result from lipid peroxidation, specifically free radical mediated peroxidation of arachidonic acid (131). It is important

to note that this is mediated independent of cyclooxygenase enzyme activity. F_2-isoprostanes are considered the ideal biomarker of oxidative stress and have been assayed within the context exercise (141).

Venous blood collected into a vacutainer® and immediately centrifuged at 1,300 RCF for 10 minutes. Blood plasma (500 µl) was then aliquoted into cryogenic vials, immediately snap frozen in liquid nitrogen, and stored at -80°C. Butylated hydroxytoluene (0.005%) was added to each 500µl plasma sample prior to snap freezing to prevent *ex vivo* oxidation. Total plasma F_2-isoprostanes were assayed using gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) using stable isotope dilution with [$^2H_4$]-15-F_{2t}-Isoprostane as the internal standard (Vanderbilt University Eicosanoid Core Laboratory). Gas chromatography/negative ion chemical ionization mass spectrometry is considered the gold standard assay for F_2-isoprostanes (172).

Dialysate Analysis

Microdialysis Probe Recovery. The internal reference method (179) was used to continuously monitor probe recovery when determining interstitial histamine concentration. This technique requires the use of a radioisotope added to the microdialysis perfusate. The internal reference method assumes that the loss of radioisotope from the perfusate is equal to the recovery of the target analyte from the interstitial space. Thus, we can account for any change in analyte concentration that is not caused by a change in true interstitial concentration.

Tritiated histamine [^3H] (0.40 µCi ml) was added to the perfusate. 10 µl of perfusate and dialysate were pipetted into 5 ml polypropylene scintillation vials

containing 3 ml of scintillation cocktail (Ultima Gold, Shelton CT, USA). The scintillation vials were then capped and vortexed for 20 sec. Radioactivity was then measured (counts min^{-1}) in duplicate using a liquid scintillation counter (Beckman LS 6000SC, Brea, CA, USA). Probe recovery was then calculated using the following equation:

$$Probe\ Recovery\ \% = \frac{Perfusate\ activity - Dialysate\ activity}{Perfusate\ activity}$$

Equation 3.4. Probe Recovery via the Internal Reference Technique

Assessing prove recovery when analyzing for tryptase was performed using a different method due to a number of issues. First, radiolabelled tryptase is unavailable, thus using the internal reference technique to account for probe recovery was of limited use. Second, granular storage of tryptase in mast cells occurs at a pH of ~5.5 and is also thought to be dependent on mast cell heparin (72). Thus, tryptase may be converted from its tetrameric structure into active monomers when released into the interstitial space at a neutral pH and with low heparin concentrations. Third, the ELISA used to quantitate tryptase utilized the majority of the dialysate sample leaving ~10 - 20 µl remaining. Therefore, we decided to measure the total protein in a given sample to account for changes in probe recovery during and following exercise. This method has not been validated but was the only method available given the limited volume of dialysate remaining after measuring tryptase.

Total protein was assayed using a 96-well based kit (Quant-iT™, Invitrogen, Eugene OR, USA). 200 ul of a working solution containing 100 ul of Quant-iT™ protein reagent and 20 ml Quant-iT™ protein buffer was added to a black 96-well microplate. 5 ul of dialysate was then added to each well. The fluorescent signal of each well was then

measured using an excitation of 485 nm and an emission of 590 nm (Synergy HT, Biotek, Winooski, VT, USA) and plotted against the fluorescent signal of a set of known protein standards using a 5-paramter logistic regression.

Interstitial Fluid Flux via Ethanol Washout. Interstitial fluid flux was assessed via the ethanol washout technique. Introduced by Hickner and colleagues, the ethanol washout technique was originally developed to measure relative changes in blood flow in close vicinity of the implanted microdialysis probe (86, 88, 89). To do this, ethanol is added to the perfusate where it can diffuse freely into the interstitial space, cells, and microcirculation (86). Thus, the rate in which ethanol had cleared once diffused into the area around the probe is thought to relate directly to local blood flow. Calculating the ratio of ethanol remaining in the dialysate to that in the perfusate is thought to represent an inverse surrogate of microvascular blood flow. In other words, the lower the ethanol outflow-inflow ratio, the higher the microvascular blood flow.

The ethanol washout technique was originally validated in human and non-human models using a number of techniques to manipulate skeletal muscle blood flow (85–87, 89). However, Rådegran et al. reported that the ethanol washout technique may reflect changes in probe recovery rather than microvascular blood flow, as the femoral artery infusion of the adenosine failed to reduce the ethanol outflow-inflow ratio at rest and during knee extension exercise (164). Given the complex nature and multiple mechanisms governing the interstitial environment, ethanol washout most likely reflects a number of factors within interstitial space such as blood flow, vascular permeability, and volume fraction. Thus, we believe that ethanol washout technique in general measures interstitial fluid flux, but can still be used as a surrogate marker of local perfusion.

Importantly, local blockade of H_1/H_2 histamine receptors via microdialysis attenuates the reduction in the ethanol outflow-inflow ratio during the recovery from exercise (155). This suggests that the mutability of interstitial fluid (and thus local perfusion) is inherently dependent on histamine receptor activation during the recovery from exercise. Thus, the ethanol washout technique should be useful in pharmaco-dissecting the possible mechanisms increasing local histamine formation and mediating sustained post-exercise vasodilation.

The washout of ethanol from the microdialysis probe was measured using a modified alcohol dehydrogenase enzymatic assay (89, 157). The assay uses alcohol dehydrogenase to catalyze the oxidation of ethanol using nicotinamide adenine dinucleotide (NAD^+) as the electron acceptor. The enzymatic reaction yields acetaldehyde, the oxidized form of ethanol. 150 ul of a glycine-hydrazine buffer (pH 8.9) containing .5 μM NAD^+ was added to a standard black 96-well microplate. Ethanol was measured in the perfusate, where 5mM ethanol (Sigma-Aldrich, St. Louis, MO, USA) was added, and in all dialysate samples. 2ul dialysate or perfusate and 10 ul alcohol dehydrogenase were then added to each well and the plate was incubated in the dark, at room temperature (~23°C) for 1 hr. The fluorescent signal of each well was then measured using an excitation of 360 nm and an emission of 460 nm (Synergy HT, Biotek, Winooski, VT, USA) and plotted against the fluorescent signal of a set of known ethanol standards using a 5-paramter logistic regression. The resulting ethanol concentrations were used to calculate the ethanol outflow-inflow ratio using the following equation:

$$EtOH\ outflow - inflow\ = \frac{[EtOH]Dialysate}{[EtOH]Perfusate}$$

Equation 3.5. Ethanol Outflow-inflow Ratio

Tryptase Assay. Interstitial tryptase was measured using a standard "sandwich" enzyme-linked immunosorbent assay and performed in accordance to the manufactures instructions with slight modification (Kamiya Biomedical, Seattle, WA, USA). 50 ul dialysate was added to a 96-well microplate pre-coated with an antibody raised against human tryptase. 100 ul of a detection reagent containing a biotin-conjugated antibody specific to tryptase was then added to each well. The plate was incubated for 1 hr at 37°C and then washed using a standard wash buffer. 100 ul of a secondary detection reagent containing avidin conjugated to horseradish peroxidase was then added to each well. The plate was again incubated for 1 hr at 37°C and then washed using a standard wash buffer. Finally, a tetramethylbenzidine substrate solution was added to each well and incubated for ~3 min at 37°C. The enzyme-substrate reaction was then terminated by adding 50 ul of a stop solution containing sulfuric acid. The absorbance was then measured at a wavelength of 450 (Synergy HT, Biotek, Winooski, VT, USA) for each sample and plotted against the absorbance of a set of known tryptase standards using a 5-paramter logistic regression.

Histamine Assay. Interstitial histamine was measured using a standard "competitive" enzyme-linked immunosorbent assay (Rocky Mountain Diagnostics, Colorado Springs, CO, USA) and performed in accordance to the manufactures instructions. The assay was performed in two phases - the acylation phase and the enzyme-linked immunosorbent assay phase. All samples and standards were acylated by combining them with 25 µl acylation buffer and 25µl acylation reagent and incubating on a plate shaker (600 rpm) for 45 min at room temperature. 200 µl distilled water was then added to all wells and the microplate was incubated on a plate shaker (600 rpm) for 15 min at room temperature.

25 µl of the acylated samples and standards were transferred to a 96-well microplate pre-coated with an antibody raised against human histamine. 100 ul of a histamine goat anti-serum was added to all wells. The microplate was then incubated on a plate shaker (600 rpm) for 3 hrs at room temperature. The plate was then washed four times and 100 µl of an enzyme conjugate containing an anti-goat IgG conjugated with peroxidase was added to all wells and incubated on a plate shaker (600 rpm) for 30 mins at room temperature. The plate was again washed four times and 100 µl of a tetramethylbenzidine substrate solution was added to all wells and incubated on a plate shaker (600 rpm) for 25 min at room temperature. Finally, 100 ul of a stop solution containing sulfuric acid was added to all wells. The absorbance was then measured at a wavelength of 450 (Synergy HT, Biotek, Winooski, VT, USA) for each sample and plotted against the absorbance of a set of known histamine standards using a 5-paramter logistic regression.

Gene Expression Analysis

mRNA expression was evaluated for the study detailed in chapter VI. We utilized next-generation RNA sequencing to probe gene expression on a transcriptome-wide level. RNA sequencing provides a comprehensive index of all of the genes expressed in the transcriptome at the time when the host tissue and cells were obtained (191). Thus, RNA sequencing technology allows us to exhaustively and precisely examine transcripts related to acute exercise, angiogenesis, and histamine receptor activation. Additionally, RNA sequencing will allows us to interrogate additional genes and pathways unrelated to angiogenesis but that may respond to acute exercise and histamine receptor activation so that we can generate novel hypothesis driven research questions.

Gene expression analysis via RNA sequencing was performed at the University of Oregon Genomics Core Facility following RNA isolation by the Muscle Physiology Laboratory. The following sections were written in consultation with the Muscle Physiology Laboratory and the Genomics Core Facility.

RNA Isolation. Approximately 15 mg of skeletal muscle tissue was homogenized in Eppendorph RNase-free tubes containing 1 ml TRI reagent. The sample was then separated using 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. The resulting RNA pellet was washed twice in 75% alcohol, dried, and subsequently dissolved in 1.5 µl 0.1 mM EDTA for each 1 mg of starting skeletal muscle tissue.

RNA Sequencing. Following RNA isolation, sequencing was carried using the following standardized workflow within the Genomics Core Facility: 1) RNA preparation 2) cDNA library preparation 3) sequencing and 4) bioinformatics analysis. Because mRNA is only 1 - 5% of total RNA, an isolation procedure is necessary to ensure that the sample is not contaminated with non-polyadenylated mRNA (e.g. ribosomal). Pure mRNA was isolated from the total RNA preparation using a commercially available kit (Dynabeads® mRNA Purification Kit, Life Technologies, Eugene, OR, USA). Input RNA yield and integrity was analyzed prior to library construction via automated capillary electrophoresis (Fragment Analyzer, Advanced Analytical Technologies, Inc., Ames, IA, USA) coupled with a High Sensitivity RNA Analysis Kit (DNF-491, Advanced Analytical Technologies, Inc., Ames, IA, USA).

The cDNA library preparations was performed using a KAPA Stranded RNA-Seq Library Preparation Kit (Kapa Biosystems, Boston, MA, USA), which is designed specifically for Illumina Sequencers. Briefly, isolated mRNA was first fragmented by

heating each sample to 94°C in the presence of magnesium. Double-stranded cDNA was then synthesized from the fragmented RNA using a two-step process which includes 1st strand cDNA synthesis via random priming and 2nd strand cDNA synthesis which converts the cDNA:RNA hybrid to double-stranded cDNA. Adapters were then ligated to the ends of the cDNA preceding library amplification using high-fidelity, low-bias polymerase chain reaction. Constructed libraries were validated using the High Sensitivity NGS Fragment Analysis Kit (DNF-486, Advanced Analytical Technologies, Inc., Ames, IA, USA) and quantitated using a Qubit 2.0 fluorometer coupled with a High Sensitivity DNA assay kit (Life Technologies, Eugene, OR, USA).

Subsequent to cDNA library preparation, flowcells were loaded with samples and clusters were formed and replicated for each oligo. Flowcells were then placed in the sequencer (Illumina HiSeq 2000, Illumina, San Diego CA, USA) and fluorescently tagged nucleotides were competitively attached to complimentary bases within each cluster. Single-end sequencing was performed at a length of 100 nucleotides.

Following the read process, adapters were removed and sequences were tiled and aligned to the reference human genome. RNA sequencing is in the final stages of analysis. Thus, the bioinformatics component of data analysis is yet to be completed.

CHAPTER IV

SUSTAINED POST-EXERCISE VASODILATION, OXIDATIVE STRESS, AND HISTAMINE RECEPTOR ACTIVATION

INTRODUCTION

Blood flow to the previously active skeletal muscle remains elevated for several hours following an acute bout of aerobic exercise (74, 77, 114). This sustained post-exercise vasodilation occurs following both whole-body exercise (78, 79, 154) and isolated small muscle-mass exercise (16). In humans, following large muscle-mass exercise, sustained post-exercise vasodilation results from a combination of histamine H_1 and H_2 receptor activation (119, 125, 126) alterations in the central neural control of sympathetic outflow (78), and a reduced ability to transduce sympathetic nerve activation into vasoconstriction (75). In contrast, H_1 and H_2 receptor blockade abolishes sustained post-exercise vasodilation following small-muscle mass exercise, suggesting that this phenomenon is primarily histaminergic in origin and may occur independent of the neural mechanisms (16). The upstream signaling that drives sustained post-exercise histaminergic vasodilation remains undetermined, but presumably relies on the release or formation of histamine within the skeletal muscle vascular bed. Histamine may be released by mast cells via degranulation. Alternatively, local histamine formation may be increased post-exercise through *de novo* synthesis in non-mast cells by the inducible enzyme histidine decarboxylase. Exercise-related factors such as increased intramuscular temperature and oxidative stress have been proposed to stimulate local histamine release or formation within the previously active skeletal muscle (74), but these theories are untested. Notably, oxidative stress has been linked to mast cell degranulation using both

in vitro and *in vivo* experimental models (146–148), and has also been linked to induction of histidine decarboxylase in vitro (90).

By common definition, oxidative stress is a condition in which pro-oxidants overwhelm antioxidant defense mechanisms resulting in macromolecular damage and altered redox signaling and control (104, 182). In humans, Dillard and colleagues showed that acute whole-body aerobic exercise increases oxidative stress (43). This was first demonstrated indirectly by increased lipid peroxidation, and subsequently confirmed in rats (40) and later in humans (8) using electron paramagnetic spectroscopy. Involvement of whole body exercise is not a prerequisite for oxidative stress, as unilateral dynamic knee extension exercise similarly induces oxidative stress within the exercising muscle group (11, 12).

Thus, we propose that there may be a distinct mechanistic link between exercise-induced oxidative stress and sustained post-exercise histaminergic vasodilation. Therefore, the purpose of this study was to determine if acute exercise-induced oxidative stress is an important part of the upstream signaling that leads to sustained post-exercise vasodilation via activation of histamine receptors. In study 1, we tested the hypothesis that the infusion of ascorbate, a potent antioxidant, would inhibit sustained post-exercise vasodilation, and that this inhibition would be similar in magnitude to that observed with histamine receptor antagonism in prior studies. Further, we hypothesized that H_1 and H_2 blockade in combination with ascorbate would not further reduce the vasodilation beyond the effect of ascorbate alone. Given the potential for ascorbate to interact directly with histamine we conducted a follow-up study using an alternative antioxidant, n-acetylcysteine. The purpose of the follow-up study was to determine if the findings from

study 1 were due to the blunting of exercise-induced oxidative stress and thus inactivation of a histaminergic signaling pathway or due to the direct degradative action of ascorbate on histamine. We tested the hypothesis that the infusion of the potent antioxidant, n-acetylcysteine would, similar to ascorbate, inhibit sustained post-exercise vasodilation.

METHODS

Subjects

Nineteen young healthy subjects were invited to participate in this study. Seven men and 4 women participated in study 1 whereas 5 men and 3 women participated in the follow-up study. 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 prior to the study. Additionally, subjects reported to the laboratory after an overnight fast. 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. This study was approved by the Institutional Review Board at the University of Oregon and was performed in accordance to the principles outlined by the Declaration of Helsinki.

Screening Visit

The screening visit was used to determine peak power output during a unilateral dynamic knee extension exercise test performed to volitional fatigue. Dynamic knee-

extension exercise during all 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 measures 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^{-1}. During the dynamic knee extension exercise test subjects reached peak power in 9.9 ± 0.7 min (range 7 - 13 min).

Experimental Approach

Study 1. Following the screening visit, subjects were required to visit the laboratory on three separate occasions and were studied within three different conditions: 1) control 2) ascorbate infusion and 3) ascorbate infusion plus H_1/H_2 receptor blockade. Conditions were randomized, counterbalanced, and were separated by at least 7 days. Leg hemodynamics were assessed during each visit before and after 60 min of unilateral dynamic knee extension exercise performed at 60% of peak power and a cadence of 45 kicks min^{-1}. Power was ramped at the onset of exercise to 60% peak power over the first 15 min. Power output was recorded continuously throughout 60 min dynamic knee extension exercise.

Ascorbate (Bioniche Pharma, Morgantown, WV, USA) was diluted in saline and infused intravenously for 20 min prior to exercise (primer dose, 0.5 g kg^{-1} body weight) and during 60 min of dynamic knee extension exercise (maintenance dose, 0.017 g kg^{-1}

body weight). The primer dose of ascorbate was diluted with saline to a final volume of 60 ml and infused at a rate of 3.0 ml min^{-1} while the maintanence dose was diluted with saline to a final volume of 30 ml and infused at a rate of 0.5 ml min^{-1}. Both doses of ascorbate were infused along with a continuous saline drip (60 ml hr^{-1}). This pharmacological approach has been used in humans to significantly increase plasma ascorbate concentrations (17, 56, 99). Furthermore, ascorbate has been used successfully to reduce both direct and indirect markers of oxidative stress following exhaustive aerobic exercise in humans (9). Ascorbate does not appear to have any direct cardiovascular effects under resting conditions in healthy young adults (17, 56, 99). Ascorbate is a water-soluble scavenger of reactive species such as superoxide and peroxynitrite (73), and has a half-life of 30 min when administered intravenously (46, 153).

For study 1, histamine H_1 and H_2 receptors were blocked using 540 mg fexofenadine and 300 mg ranitidine. This combination of fexofenadine and ranitidine reduces sustained post-exercise vasodilation by ~86% following unilateral dynamic knee extension exercise (16). This dosage of oral fexofenadine has been shown to selectively block H_1 receptors (time to peak concentration 1.15 h and half-life 12 h), while the dose of oral ranitidine has been shown to selectively block H_2 receptors (time to peak plasma concentration 2.2 h and 2.6 h half-life) (63, 173). Responses are 90% inhibited within 1 h and remain inhibited 6 h after administration (22, 63). Fexofenadine and ranitidine do not appear to cross into the central nervous system or possess sedative actions (22). Furthermore, these drugs do not have any direct cardiovascular effects in the absence of histamine receptor stimulation (i.e., when given under normal resting conditions, these

drugs do not elicit any changes in heart rate, blood pressure, or smooth muscle tone) (126). Subjects ingested the histamine receptor antagonists with water 1 hr prior to exercise. On the control day, saline was infused in lieu of ascorbate and subjects did not receive fexofenadine or ranitidine.

Follow-up Study. Following the screening visit, subjects were required to visit the laboratory on two separate occasions and were studied within two different conditions: 1) control 2) n-acetylcysteine infusion. We chose to not add a third condition combining n-acetylcysteine and H_1/H_2 receptor blockade as the follow-up study was conducted in order to determine if the findings from study 1 were related to the antioxidant effect of ascorbate or were due to a direct interaction with histamine. Conditions were randomized, counterbalanced, and were separated by at least 7 days. Leg hemodynamics were assessed during each visit before and after 60 min of unilateral dynamic knee extension exercise performed at 60% of peak power and a cadence of 45 kicks min^{-1}. Power output was ramped at the onset of exercise to 60% peak power over the first 15 min. Power output was recorded continuously throughout 60 min dynamic knee extension exercise. N-acetylcysteine (Acetadote ®, Cumberland Pharmaceuticals Inc., Nashville, TN, USA) was diluted in saline and infused intravenously for 15 min prior to exercise (primer dose, 125 mg kg^{-1} hr^{-1}) and during 60 min of dynamic knee extension exercise (maintenance dose, 25 mg kg^{-1} hr^{-1}). The primer dose of n-acetylcysteine was diluted with saline to a final volume of 45 ml and infused at a rate of 3.0 ml min^{-1} while the maintenance dose was diluted with saline to a final volume of 60 ml and infused at a rate of 1.0 ml min^{-1}. Both doses of n-acetylcysteine were infused along with a continuous saline drip (60 ml hr^{-1}). This pharmacological approach has been used in humans to significantly increase

plasma n-acetylcysteine concentrations prior to and during exercise (20, 127, 128). Furthermore, n-acetylcysteine has been used successfully to alter blood redox status in both short-term high intensity and prolonged cycle exercise in humans (127, 128). N-acetylcysteine does not appear to have any direct cardiovascular effects under resting conditions in healthy young adults (144). N-acetylcysteine is potent water-soluble pharmacological antioxidant with a half-life of ~5 hrs. It can directly scavenge reactive species such as hydrogen peroxide and hydroxyl radical and can also indirectly reduce reactive species by augmenting glutathione production via intracellular cysteine formation (35, 73, 176). Histamine receptor blockade was not used in this study.

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 all hemodynamic measurements. Room temperature remained thermoneutral (~23°C) throughout the study. All measurements were identical between study 1 and the follow-up study with the exception of venous blood sampling.

Heart Rate and Blood Pressure. Arterial blood pressure was measured in the right arm using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh, NC, USA). Heart rate was monitored continuously using a three lead electrocardiograph (Datex-Ohmeda Cardiocap/5, GE Healthcare, Tewksbury, MA, USA) for study 1 and was monitored using a three lead electrocardiograph in the follow-up study (Tango+, SunTech Medical, Raleigh, NC, USA). Heart rate and blood pressure were also measured during 60 min dynamic knee extension exercise.

Femoral Blood Flow. A pressure cuff (Hokanson E20 Rapid Cuff Inflator, D. E.

Hokanson, Inc., Bellevue, WA. USA) was placed on both legs, immediately distal to the patella and inflated to 250 mmHg to ensure that blood flow measured in the femoral artery was indicative of blood flow to the thigh region. Femoral artery blood flow velocity was measured via duplex ultrasonography. A linear-array vascular ultrasound probe (10 MHz, GE Vingmed System 5, Horton, Norway) 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. For study 1, velocity measurements were made at an average depth of 1.63 ± 0.01 cm and were thin-beam corrected, based on a known beam-width of 2.21 mm which resulted in an average correction factor of 0.766 ± 0.001, as outlined by Buck et al. (23). For the follow-up study, velocity measurements were made at an average depth of 1.72 ± 0.01 cm and were thin-beam corrected which resulted in an average correction factor of 0.765 ± 0.0001. Femoral diameter was measured in triplicate during diastole following velocity measurements. The average femoral artery diameter was 8.89 ± 0.03 mm for study 1 and 9.09 ± 0.04 mm for the follow-up study. 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.

Skin Blood Flow. Laser-Doppler flowmetry (DRT4, Moor Instruments LTD, Devon, UK) was used to determine red blood cell flux, an index of skin blood flow. Laser-

Doppler probes were placed anteriorly, on the midline, and halfway between the inguinal ligament and patella on both the active and inactive leg. Cutaneous vascular conductance was calculated by dividing laser Doppler flux by mean arterial pressure and normalized to maximal flux values measured during local heating to 43°C to account for the variability between laser Doppler sites (109, 197).

Blood Sampling and Analysis. A 22 gauge intravenous catheter was placed in the subjects left arm and was used for infusions and blood sampling. Venous blood was collected and analyzed for only study 1. Venous blood was sampled before exercise preceding all infusions and immediately post-exercise. Venous blood was collected into a vacutainer® and centrifuged at 1,300 RCF within five minutes. Blood plasma (500 µl) was then aliquoted into cryogenic vials, immediately snap frozen in liquid nitrogen, and stored at -80°C. Butylated hydroxytoluene (0.005%) was added to each 500µl plasma sample prior to snap freezing to prevent *ex vivo* oxidation. Total plasma F_2-isoprostanes were assayed using gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) using stable isotope dilution with [$^2H_4$]-15-F_{2t}-Isoprostane as the internal standard (Vanderbilt University Eicosanoid Core Laboratory). Plasma F_2-isoprostanes were used as an indirect biomarker of exercise-induced oxidative stress.

Electromyography. Surface electromyography (Z03 EMG preamplifiers, Motion Lab Systems, Baton Rouge, La., USA) was used to ensure the subjects right quadriceps and posterior thigh muscles were not activated during knee flexion and to ensure that the muscles in the non-exercised leg remained inactive during exercise. Electromyography probes were placed on the anterolateral aspect of the thigh 8 - 10 cm above the patella and posteriorly one-third of the distance between the popliteal fossa and ischial tuberosity

on both the active legs and inactive legs. Electromyography probes were placed in parallel with the pennation angle of the skeletal muscle fibers. The electromyography probes were integrated with custom built software in order to provide visual feedback to investigators during performance of knee extension exercise.

Statistical Analyses

Preliminary statistical analysis indicated that our primary outcome variables did not vary by sex for study 1. As such, all subsequent analyses for study 1 were performed after grouping data for both men and women. Given the limited number of female subjects in the follow-up study, the primary outcome variables were not assessed by sex. All analyses for the follow-up study were performed after grouping data for both men and women.

The statistical analysis was identical for both studies. Baseline differences between conditions were analyzed using a one-way mixed model analysis of variance with repeated measures. Exercise responses were also analyzed using a one-way mixed model analysis of variance with repeated measures. Our primary outcome variables during the recovery from exercise were analyzed using a stepwise regression and carried out with SAS Proc GLMSELECT (SAS version 9.2; SAS Institute Inc. Cary, NC, USA). As opposed to the traditional approach which would use ANOVA to test for differences between conditions at discrete time points during the recovery from exercise, our stepwise approach allows the examination of both linear and quadratic relationships across time and tests whether or not these relationships differ between conditions. Independent variables remained in the model if a minimum *P*-value threshold was met (*P*

< 0.15). Significance was set at $P < 0.05$. Data are reported as mean ± SEM unless stated otherwise (e.g., SD is used in Table 1 to indicate the variability in the subject pool).

RESULTS

Subject Characteristics

Subject physical characteristics and data obtained during the screening visit are shown in **Table 4.1**. Subject characteristics are similar to those obtained previously in our laboratory in young healthy subjects and consistent with recreationally active individuals.

Table 4.1. Subject Characteristics

	Study 1	Follow-up Study
n	11	8
Age (yrs)	22.2 ± 1.9	20.6 ± 2.3
Height (cm)	173 ± 9	178 ± 8
Weight (kg)	72.8 ± 11.4	75.0 ± 14.0
Body mass index (kg m^{-2})	24.0 ± 2.8	23.5 ± 3.4
Baecke sport index (arbitrary units)	3.0 ± 0.7	3.3 ± 0.7
Physical activity index (MET hr^{-1} $week^{-1}$)	88 ± 54	39 ± 17
Peak power output (W)	35.2 ± 6.4	34.8 ± 6.9

Values are mean ± SD. MET, metabolic equivalents.

Study 1

Pre-exercise Hemodynamics. Pre-exercise heart rate and mean arterial blood pressure are shown in **Table 4.2**. Both heart rate ($P = 0.3$) and mean arterial pressure ($P = 0.9$) did not differ across the three conditions of control, ascorbate, and ascorbate plus H_1/H_2 blockade. As shown in **Table 4.3**, cutaneous vascular conductance within both the active leg and inactive leg did not differ across the three conditions ($P = 0.9$). Pre-exercise

femoral blood flow and femoral vascular conductance are shown in **Figure 4.1**. Within the active leg, femoral blood flow did not differ ($P = 0.6$) across the three conditions. Likewise, within the inactive leg, femoral blood flow did not differ across the three conditions ($P = 0.6$). Within the active leg, femoral vascular conductance did not differ across the three conditions ($P = 0.6$). Likewise, within the inactive leg, femoral vascular conductance did not differ across the three conditions ($P = 0.6$).

Exercise Responses. Power output during steady state dynamic knee extension exercise was similar for the three conditions of control (20.8 ± 1.3 W), ascorbate (20.0 ± 1.1 W), and ascorbate plus H_1/H_2 blockade (19.9 ± 1.4 W) ($P = 0.1$). Power output for each condition was within 2 W of the estimated 60% workload (21 W). Compared with the control condition (96.8 ± 1.0 mmHg), mean arterial blood pressure was higher for the ascorbate condition (100.0 ± 1.3 mmHg, $P < 0.05$), but was similar with the ascorbate plus H_1/H_2 blockade condition (98.2 ± 1.3 mmHg, $P = 0.3$). Mean arterial pressure did not differ for ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.2$). Heart rate was similar for the three conditions of control (90.6 ± 3.3 beats min^{-1}), ascorbate (95.5 ± 4.2 beats min^{-1}), and ascorbate plus H_1/H_2 blockade (90.9 ± 3.8 beats min^{-1}) ($P = 0.1$).

Oxidative Stress. Pre-exercise F_2-isoprostane concentration did not differ across the three conditions (control 87.1 ± 8.7 pg ml^{-1}; ascorbate 88.7 ± 8.2 pg ml^{-1}; ascorbate plus H_1/H_2 blockade 84.7 ± 7.2 pg ml^{-1}; $P = 0.4$). Compared with pre-exercise, F_2-isoprostane concentration was not elevated immediately following dynamic knee extension exercise for all three conditions ($P = 0.4$). Likewise, post-exercise F_2-isoprostane concentrations

did not differ across the three conditions (control 84.0 ± 7.7 pg ml^{-1}; ascorbate 94.3 ± 11.0 pg ml^{-1}; ascorbate plus H_1/H_2 blockade 88.7 ± 10.1 pg ml^{-1}; $P = 0.4$).

Post-exercise Hemodynamics. Table 4.2 shows heart rate and mean arterial blood pressure during recovery from exercise. Compared with the control condition, mean arterial blood pressure was similar for the ascorbate condition ($P = 0.7$) and the ascorbate plus H_1/H_2 blockade condition ($P = 0.8$). Mean arterial pressure was not different between ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.6$). Compared with the control condition, heart rate was similar for the ascorbate condition ($P = 0.4$) and the ascorbate plus H_1/H_2 blockade condition ($P = 0.8$). Heart rate was not different between ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.5$).

As shown in **Table 4.3**, cutaneous vascular conductance in the active leg was similar across all three conditions during the recovery from exercise ($P > 0.15$). However, in the inactive leg, cutaneous vascular conductance was lower with ascorbate plus H_1/H_2 blockade than either the control or the ascorbate conditions ($P < 0.05$).

Figure 4.1 shows femoral blood flow and femoral vascular conductance during the recovery from exercise. In the active leg, the increase from pre-exercise in femoral blood flow at 1 h post-exercise was reduced for both the ascorbate (13 ± 7 %, $P < 0.05$) and ascorbate plus H_1/H_2 blockade (15 ± 7%, $P < 0.05$) conditions compared with the control condition (37 ± 7 %). The change in femoral blood flow did not differ between ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.8$). Likewise, the increase from pre-exercise in femoral vascular conductance at 1 h post-exercise was reduced for the both the ascorbate (14 ± 8 %, $P < 0.05$) and ascorbate plus H_1/H_2 blockade (18 ± 7%, $P < 0.05$) conditions compared with the control condition (43 ± 9 %).

Table 4.2. Central Hemodynamics

	Heart Rate (beats min^{-1})			Mean Arterial Pressure (mmHg)		
Time Point	Control	Ascorbate	Ascorbate + H_1/H_2 Blockade	Control	Ascorbate	Ascorbate + H_1/H_2 Blockade
Pre-exercise	57.1 ± 0.5	60.1 ± 0.7	56.7 ± 0.7	83.0 ± 0.6	82.9 ± 0.8	82.6 ± 0.6
Time post-exercise						
20 min	60.8 ± 1.0*	61.6 ± 1.4	61.6 ± 1.2*	81.4 ± 0.7	83.1 ± 1.2	81.6 ± 0.7
40 min	57.8 ± 0.9	59.5 ± 1.2	58.6 ± 1.0*	80.8 ± 0.7*	81.7 ± 1.2	81.7 ± 0.7
60 min	56.6 ± 0.8	58.4 ± 1.1	57.7 ± 1.2	82.3 ± 1.1	84.3 ± 1.3	81.1 ± 0.7
80 min	56.7 ± 1.0	57.3 ± 1.1*	56.8 ± 1.2	83.2 ± 0.9	83.7 ± 1.2	83.4 ± 0.6
100 min	56.2 ± 0.8	57.4 ± 0.8*	57.2 ± 1.0	85.3 ± 0.8*	84.0 ± 1.1	83.9 ± 0.7
120 min	56.4 ± 0.9	57.4 ± 0.8*	56.5 ± 0.9	85.3 ± 0.9*	85.3 ± 1.1	85.0 ± 0.7*

Values are mean ± SEM, *$P < 0.05$ vs. pre-exercise.

Table 4.3 Cutaneous Vascular Conductance

	Inactive Leg			Active Leg		
Time Point	Control	Ascorbate	Ascorbate + H_1/H_2 Blockade	Control	Ascorbate	Ascorbate + H_1/H_2 Blockade
Pre-exercise	8.3 ± 1.6	10.8 ± 2.5	7.8 ± 1.1	10.0 ± 2.4	14.6 ± 4.5	9.9 ± 1.9
Time post-exercise						
20 min	10.7 ± 2.2	10.0 ± 1.1	8.0 ± 1.4*	20.3 ± 3.3	25.1 ± 4.6	21.3 ± 4.2
40 min	10.0 ± 2.4	8.5 ± 1.0	7.2 ± 1.2*	18.6 ± 3.9	20.3 ± 3.4	17.4 ± 3.2
60 min	8.4 ± 1.4	7.8 ± 1.0	7.2 ± 1.1*	15.9 ± 3.5	16.2 ± 2.7	15.7 ± 3.2
80 min	8.5 ± 1.4	7.8 ± 1.1	6.6 ± 1.0*	14.0 ± 3.1	14.1 ± 2.2	13.9 ± 2.8
100 min	7.5 ± 1.1	7.7 ± 1.2	6.6 ± 0.9*	11.7 ± 2.6	13.0 ± 2.4	13.1 ± 2.9
120 min	7.8 ± 1.4	7.1 ± 0.9	6.9 ± 0.9*	10.8 ± 2.5	12.4 ± 2.2	12.3 ± 2.7

Data are presented as percentage of maximal cutaneous vascular conductance. Values are mean ± SEM. *$P < 0.05$ vs. control and ascorbate conditions.

The change in femoral vascular conductance did not differ between ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.7$).

In the inactive leg, the femoral blood flow change from pre-exercise to 1h post-exercise did not differ between the ascorbate (-1 ± 5 %, $P = 0.3$) and control (6 ± 2 %) conditions, and also between the ascorbate plus H_1/H_2 blockade (4 ± 5 %, $P = 0.8$) and control conditions. The change in femoral blood flow did not differ between ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.4$). Likewise, the femoral vascular conductance change from pre-exercise to 1h post-exercise did not differ between the ascorbate (-1 ± 5 %, $P = 0.2$) and control (7 ± 4 %) conditions, and also between the ascorbate plus H_1/H_2 blockade (8 ± 6 %, $P = 0.9$) and control conditions. The change in femoral vascular conductance did not differ between ascorbate and ascorbate plus H_1/H_2 blockade conditions ($P = 0.2$)

Follow-up Study

Pre-exercise Hemodynamics. Pre-exercise heart rate and mean arterial blood pressure are shown in **Table 4.4**. Compared with the control condition heart rate was greater for the n-acetylcysteine condition ($P < 0.05$). Mean arterial pressure did not differ between the two conditions ($P = 0.9$). As shown in **Table 4.5**, cutaneous vascular conductance within both the active leg and inactive leg did not differ between the two conditions ($P = 0.9$). Pre-exercise femoral blood flow and femoral vascular conductance are shown in **Figure 4.2**. Within the active leg, femoral blood flow did not differ ($P = 0.9$) between the two conditions. Likewise, within the inactive leg, femoral blood flow did not differ between the two conditions ($P = 0.9$). Within the active leg, femoral vascular conductance did not differ between the two conditions ($P = 0.9$). Likewise, within the

inactive leg, femoral vascular conductance did not differ between the two conditions (P = 0.9).

Exercise Responses. Power output during steady state dynamic knee extension exercise was similar for the control (21.2 ± 0.6 W) and n-acetylcysteine conditions (21.1 ± 0.6 W, P = 0.3). Power output for each condition matched the estimated 60% workload (21 W). Mean arterial blood pressure was similar between control (100.8 ± 1.6 mmHg) and n-acetylcysteine conditions (101.0 ± 1.9 mmHg, P = 0.8). Compared with the control condition (92.5 ± 1.6 beats min^{-1}), heart rate was higher for n-acetylcysteine condition (101.5 ± 1.6 beats min^{-1}, $P < 0.05$).

Table 4.4. Central Hemodynamics

	Heart Rate (beats min^{-1})		Mean Arterial Pressure (mmHg)	
Time Point	Control	n-acetylcysteine	Control	n-acetylcysteine
Pre-exercise	59.7 ± 0.9	63.8 ± 1.0†	86.0 ± 0.7	86.9 ± 1.2
Time post-exercise				
20 min	61.5 ± 1.3	70.4 ± 1.9*†	87.1 ± 1.0	88.6 ± 0.9
40 min	59.3 ± 1.3	66.6 ± 1.5†	86.8 ± 0.9	88.6 ± 1.0
60 min	56.9 ± 1.2	64.5 ± 1.4†	87.8 ± 0.7	89.0 ± 1.1
80 min	56.0 ± 1.4*	63.8 ± 1.4†	88.8 ± 0.8	91.0 ± 0.9*
100 min	57.1 ± 1.4	64.4 ± 1.4†	89.2 ± 0.7*	91.3 ± 1.1*
120 min	57.2 ± 1.6	63.7 ± 1.4†	90.2 ± 0.9*	92.1 ± 1.0*

Values are mean ± SEM. *$P < 0.05$ vs. pre-exercise; †$P < 0.05$ vs. control

Post-exercise Hemodynamics. Table 4.4 shows heart rate and mean arterial blood pressure during recovery from exercise. Mean arterial blood pressure did not differ between control and n-acetylcysteine conditions ($P = 0.8$). Compared with the control condition, heart rate was higher for the n-acetylcysteine condition throughout the recovery from exercise ($P < 0.05$).

Table 4.5. Cutaneous Vascular Conductance

	Inactive Leg		Active Leg	
Time Point	Control	n-acetylcysteine	Control	n-acetylcysteine
Pre-exercise	7.5 ± 0.4	7.1 ± 0.5	8.2 ± 0.7	8.8 ± 0.7
Time post-exercise				
20 min	7.1 ± 0.6	7.3 ± 0.5	11.7 ± 1.6	19.7 ± 1.9*†
40 min	6.5 ± 0.5	7.0 ± 0.6	10.0 ± 1.3	16.3 ± 1.1*†
60 min	5.9 ± 0.5	6.8 ± 0.6	9.5 ± 1.2	15.2 ± 1.2*†
80 min	5.7 ± 0.5	6.2 ± 0.7	9.4 ± 1.4	13.1 ± 0.9*
100 min	5.6 ± 0.5	6.7 ± 0.6	9.0 ± 1.3	11.9 ± 1.2
120 min	5.7 ± 0.5	6.9 ± 0.7	8.8 ± 1.4	12.6 ± 1.3

Data are presented as percentage of maximal cutaneous vascular conductance. Values are mean ± SEM. *$P < 0.05$ vs. pre-exercise; †$P < 0.05$ vs. control

Cutaneous vascular conductance during the recovery from exercise is shown in **Table 4.5**. Compared with the control condition, cutaneous vascular conductance in the active leg was higher for the n-acetylcysteine condition through the first 60 min of exercise recovery ($P < 0.05$). In the inactive leg, cutaneous vascular conductance did not

differ between control and n-acetylcysteine conditions throughout the recovery from exercise ($P = 0.6$).

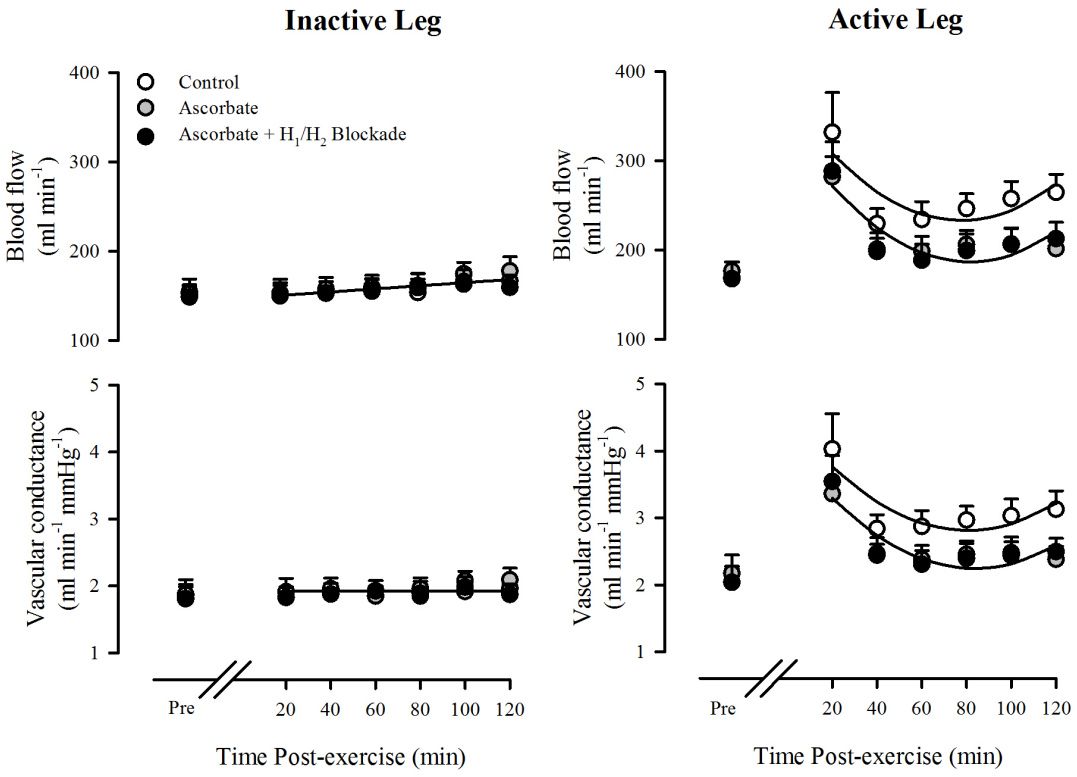

Figure 4.1. Femoral Blood Flow & Femoral Vascular Conductance
Femoral blood flow and femoral vascular conductance are shown for study 1 in both the inactive (left panels) and active (right panels) leg prior to exercise and throughout two hours of recovery. Open circles, control condition; Gray circles, ascorbate condition; Closed circles, ascorbate plus H_1/H_2 blockade. Parallel regression lines for femoral blood flow and femoral vascular conductance (right panels) indicate a significant drug effect ($P < 0.05$) for ascorbate and ascorbate plus H_1/H_2 blockade conditions versus control condition during the recovery from exercise. Solitary regression lines (left panels) indicate the absence of main effects or interactions for the control, ascorbate, and ascorbate plus H_1/H_2 blockade conditions during the recovery from exercise.

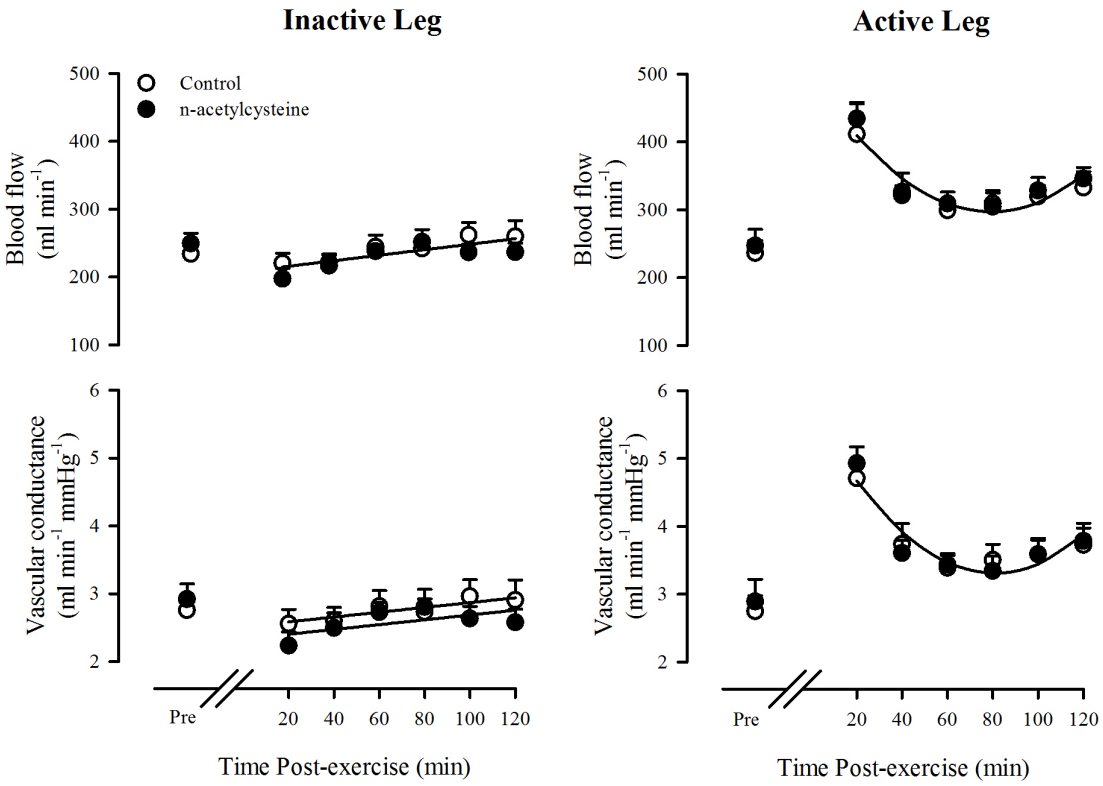

Figure 4.2. Femoral Blood Flow & Femoral Vascular Conductance
Femoral blood flow and femoral vascular conductance are shown for the follow-up study in both the inactive (left panels and active (right panels) leg prior to exercise and throughout two hours of recovery. Open circles, control condition. Closed circles, n-acetylcysteine condition. Parallel regression lines for femoral vascular conductance (bottom, left panel) indicate a significant drug effect ($P < 0.05$) for n-acetylcysteine condition versus control condition during the recovery from exercise. Solitary regression line for femoral blood flow (upper, left panel) indicates the absence of main effects or interactions for the control and n-acetylcysteine conditions during the recovery from exercise. Solitary regression lines (right panels) indicate the absence of main effects or interactions for the control and n-acetylcysteine conditions during the recovery from exercise.

Figure 4.**2** shows femoral blood flow and femoral vascular conductance during the recovery from exercise. In the active leg, the increase from pre-exercise in femoral blood flow at 1 h post-exercise did not differ between control (22 ± 4 %) and n-acetylcysteine (18 ± 7%, $P = 0.4$) conditions. Likewise, the increase from pre-exercise in femoral

vascular conductance at 1 h post-exercise did not differ between control (22 ± 4 %) and n-acetylcysteine (16 ± 8 %, $P = 0.4$) conditions.

In the inactive leg, the femoral blood flow change from pre-exercise to 1h post-exercise did not differ between control (1 ± 4 %) and n-acetylcysteine (-6 ± 4 %, $P = 0.1$) conditions. Likewise, the femoral vascular conductance change from pre-exercise to 1h post-exercise did not differ between control (-0.4 ± 5 %) and n-acetylcysteine (-10 ± 4 %, $P = 0.1$) conditions.

DISCUSSION

The purpose of this study was to determine if exercise-induced oxidative stress contributes to sustained post-exercise vasodilation. In agreement with our hypothesis for study 1, we observed that the infusion of the potent antioxidant, ascorbate, inhibited sustained post-exercise vasodilation. The degree of inhibition we observed with ascorbate was similar to what we have found in our previous work utilizing H_1/H_2 receptor blockade. Furthermore, the inhibition produced by ascorbate was not augmented with the addition of H_1/H_2 receptor blockade. In disagreement with our hypothesis for the follow-up study, we observed that sustained post-exercise vasodilation was unaffected by the infusion of the potent antioxidant, n-acetylcysteine. Taken together, these results suggest that the inhibition of sustained post-exercise vasodilation by ascorbate was due to a direct degradative effect on histamine and not to the blunting of exercise-induced oxidative stress as n-acetylcysteine, an antioxidant with no known histaminergic interaction, had no effect on sustained post-exercise vasodilation.

Sustained Post-exercise Vasodilation and Exercise-Induced Oxidative Stress

Our laboratory has demonstrated in several studies that sustained post-exercise

vasodilation is largely inhibited by H_1/H_2 receptor blockade following either large muscle mass exercise such as cycling (119, 125, 126), or more recently following unilateral dynamic knee-extension (16). There are several advantages to the use of the single-leg model, including the availability of a "control" non-exercising limb. We therefore chose to utilize the dynamic knee-extension exercise model in order to isolate the histaminergic component of sustained post-exercise vasodilation and to directly assess the role of exercise-induced oxidative stress.

It is well understood that acute aerobic exercise increases oxidative stress that results predominantly from reactive oxygen and nitrogen species formation within contracting skeletal muscle (159). Because the local formation of oxidative stress is augmented within active skeletal muscle during dynamic knee extension exercise (11, 12) and given the relationship between oxidative stress and histamine formation in other settings (90, 183), we proposed that exercise-induced oxidative stress may contribute to sustained post-exercise vasodilation.

Given independently or in combination, ascorbate and H_1/H_2 receptor blockade each inhibit sustained post-exercise vasodilation to a similar extent, suggesting that under normal exercise conditions oxidative stress could activate a histaminergic signaling mechanism located upstream of histamine receptors. However, this interpretation is complicated due to a degradative interaction between ascorbate and histamine. Several early studies provided evidence of the histamine degradative potential of ascorbate (28, 29, 96, 97, 136, 186). Uchida and colleagues later demonstrated that ascorbate degrades histamine through a selective and direct interaction at the imidazole ring (188). Ascorbate may also reduce histamine through inhibition of histidine decarboxylase, the enzyme that

catalyzes the formation of histamine from the amino acid L-histidine (48, 145). Several investigations have also provided evidence of the histamine-reducing effect of ascorbate in humans. Clemetson was the first to demonstrate that three days of oral administration of 1g ascorbate reduced blood histamine concentrations (33). Similar results were found later using chronic ascorbate administration (102, 103). Importantly, a recent study demonstrated that the acute intravenous administration of high dose ascorbate, an approach similar to that used in our study, reduced serum histamine concentrations in patients with allergic and infectious diseases (71). These studies provide evidence that ascorbate may reduce histamine concentrations *in vivo*. It is important to note that the combination of ascorbate and H_1/H_2 receptor blockade does not further reduce sustained post-exercise vasodilation which supports our interpretation these that interventions block a single common pathway for vasodilation with ascorbate inhibiting the upstream formation of histamine and H_1/H_2 receptor blockade preventing activation of histamine receptors.

We conducted the follow-up study to determine if the inhibition of sustained post-exercise vasodilation observed in study 1 was the result of blunted exercise-induced oxidative stress or the result of histamine degradation by ascorbate. We designed the study to pharmaco-dissect the parallel pathways affected by ascorbate. We isolated the oxidative stress pathway by intravenously infusing n-acetylcysteine, a potent antioxidant with no known histaminergic interactions. In contrast to the findings in study one using ascorbate, blood flow to the previously active skeletal muscle remained elevated throughout recovery and was nearly identical to that observed in the control condition. These findings support the view that ascorbate inhibits sustained post-exercise

vasodilation through histamine degradation and not through oxidative stress related signaling mechanisms.

Emerging Mechanisms of Sustained Post-exercise Vasodilation

Significant progress has been made recently in identifying the mechanisms that mediate sustained post-exercise vasodilation. While both neural and local vascular mechanisms contribute to sustained post-exercise vasodilation following whole-body exercise, the local vascular mechanism (i.e. histamine receptor activation) predominates following small muscle mass exercise. By using the dynamic knee extension exercise model to isolate the histaminergic component of sustained post-exercise vasodilation, we are now able to better dissect the signaling mechanisms that drive local histamine formation and to eventually identify the source of histamine. New and colleagues recently reported that lipid hydroperoxides, an indirect marker of oxidative stress, and systemic vascular conductance were elevated in parallel throughout 120 min of exercise recovery. The authors proposed that sustained post-exercise vasodilation and associated hypotension may be linked to exercise-induced oxidative stress. Our findings indicate that exercise-induced oxidative stress has no affect and that another upstream exercise-related factor is driving this response.

Histamine is notoriously difficult to measure when released or produced locally because it is metabolized quickly by histamine-N-methyltransferase and/or diamine oxidase or taken up by basophils in the bloodstream. Several studies in our lab have reported that histamine measured in both plasma and whole-blood is unchanged during the recovery from exercise despite a clear histamine receptor-mediated vasodilation (119, 125, 126). While the results from this study failed to provide a mechanistic link between

oxidative stress and sustained post-exercise vasodilation, they do provide evidence that histamine is indeed the ligand activating histamine receptors during the recovery from exercise. One can test a proposed physiological mechanism by inhibiting or augmenting one or more of critical components that mediate the response. Blockade of histamine at the receptor level inhibits sustained post-exercise vasodilation following dynamic knee extension exercise (16). By inhibiting local histamine formation with ascorbate sustained post-exercise vasodilation was inhibited to a similar degree to that observed with H_1/H_2 blockade, thus providing additional evidence that this physiological response is indeed histamine dependent.

Cutaneous Circulation

Sustained post-exercise histaminergic vasodilation is not related to changes in cutaneous vascular conductance following large-muscle mass exercise (119, 125, 126, 197). However, we measured cutaneous vascular conductance to ensure that any alteration in post-exercise femoral vascular conductance due to ascorbate, H_1/H_2 blockade, or n-acetylcysteine administration was not mediated by changes in the cutaneous vasculature. Importantly, the administration of ascorbate, H_1/H_2 blockade, or n-acetylcysteine had no effect on cutaneous vasculature in the active leg. However, within the inactive leg, cutaneous vascular conductance was reduced slightly in the ascorbate plus H_1/H_2 blockade condition. This is interesting given that systemic H_1 and/or H_2 receptor blockade has no effect on thigh cutaneous vascular conductance following whole-body exercise (119, 125, 126). It is not completely clear why cutaneous vascular conductance in the inactive leg is reduced with ascorbate plus H_1/H_2 receptor blockade in the present study. It is possible that histamine receptor activation in the cutaneous

circulation varies between the type of exercise (whole-body vs. small-muscle mass exercise) and the location measured (active vs. inactive leg). Regardless, the small reduction in cutaneous vascular conductance observed in the ascorbate plus H_1/H_2 blockade condition did not influence regional blood flow as femoral vascular conductance was similar to control and ascorbate conditions during recovery from exercise.

Considerations

Several experimental considerations warrant discussion. First, we chose to intravenously infuse ascorbate and n-acetylcysteine to reduce exercise-induced oxidative stress. These experimental approaches have been used successfully in humans to pharmaco-dissect the interactions between oxidative stress and physiological regulatory mechanisms (17, 20, 56, 99, 127, 128, 156). It is always prudent to consider whether such interventions might alter central or local vascular regulatory mechanisms, thus confounding the interpretation of an experiment. Along these lines, it is unlikely that ascorbate administration affected central control of the vasculature as ascorbate is unable to freely traverse the blood-brain barrier. This is further supported by Bell *et al*. who documented that muscle sympathetic nerve activity, an index of central sympathetic outflow, is unchanged following high dose ascorbate administration (17). Furthermore, baseline blood flow and femoral vascular conductance did not differ across our three conditions indicating both the central and local vascular regulatory mechanisms were unaffected with ascorbate administration. The infusion of n-acetylcysteine is used less often in human studies investigating the influence of oxidative stress on cardiovascular regulatory mechanisms. However, a recent study using an infusion protocol similar to

ours reported that both arterial blood pressure and femoral vascular conductance are unaffected with high dose intravenous n-acetylcysteine (144). We reported similar findings, thus suggesting n-acetylcysteine does not have a direct effect on cardiovascular regulatory mechanisms. Second, the effect of H_1/H_2 receptor blockade alone on sustained post-exercise vasodilation was not examined in this current investigation. We felt there was no added benefit to include this condition as we have previously established the efficacy of H_1/H_2 receptor blockade to inhibit sustained post-exercise vasodilation following unilateral dynamic knee extension exercise (16). Third, plasma F_2-isoprostanes were measured in only study 1 and were not increased with exercise and were also unaffected by ascorbate administration. We do not believe that this reflects a lack of exercise-induced oxidative stress and the inability of ascorbate to scavenge reactive species. Our results more likely reflect the use of remote venous blood sampling to assess oxidative stress induced by moderate intensity small muscle-mass exercise. While these considerations are valid points of discussion, our experimental approaches represent the best methods available to test our hypothesis in humans and we remain confident that these considerations do not limit or undermine our interpretation.

Perspectives

Pharmaco-dissecting the control systems and pathways that regulate the human cardiovascular system is inherently complex. Mechanistic studies provide an added level of complexity. We are often limited by the invasiveness of the experimental protocol or the limited availability of pharmacological agents that are available for use in humans. Most importantly, the pharmacological agents used in human investigations may affect physiological systems other than that targeted for investigation. Thus, conclusions drawn

from these studies may be inadvertently incorrect or misleading. Thus, understanding the pharmacology and potential the "side effects" of the chosen drug is critical. The number of human studies investigating the link between oxidative stress and pathophysiological conditions has increased tremendously within the last decade. Additionally, recent studies have also provided unique insight between acute and chronic cardiovascular and metabolic changes that occur as a result of exercise-induced oxidative stress. These studies often use antioxidants of various forms to pharmaco-dissect these interactions. The mechanistic links drawn from the results of these studies may not necessarily reflect the true nature of the response as the antioxidants may have unintentional consequences. Thus, understanding the pharmacology of the antioxidants and the multiple systems and pathways they may affect is essential in human investigations.

Summary & Bridge

Acute aerobic exercise promotes a sustained post-exercise histaminergic vasodilation within the vasculature perfusing the previously active skeletal muscle. This study contributes further to the understanding of the mechanisms that drive this response. The findings from this study demonstrate that exercise-induced oxidative stress does not contribute to sustained post-exercise vasodilation. While the systemic infusion of n-acetylcysteine did not affect sustained post-exercise vasodilation, ascorbate, an antioxidant with the unique ability to catalyze the degradation of histamine, inhibited the vasodilation to a similar degree of that observed in our previous work utilizing H_1/H_2 receptor blockade. This observation provides the first line of evidence that histamine is likely released locally within the previously exercised muscle and where it can then activate H_1/H_2 receptors leading to sustained post-exercise vasodilation. Chapter V will

build upon this observation and attempt to directly measure interstitial histamine within skeletal muscle during and following exercise. Further, we will attempt to identify the specific source of local histamine formation.

CHAPTER V

EXERCISE AND HISTAMINE FORMATION: IMPLICATIONS FOR SUSTAINED POST-EXERCISE VASODILATION

INTRODUCTION

Blood flow to previously active skeletal muscle remains elevated for several hours following an acute bout of aerobic exercise (74, 77, 114). This sustained post-exercise vasodilation occurs following both whole-body exercise (78, 79, 154) and isolated small muscle-mass exercise (16). In humans, both central neural mechanisms and local vascular mechanisms contribute to sustained post-exercise vasodilation following large muscle mass exercise. Conversely, H_1 and H_2 histamine receptor blockade abolishes sustained post-exercise vasodilation following unilateral dynamic knee extension exercise, suggesting that this phenomenon is primarily histaminergic in origin and may occur independent of the neural mechanisms following isolated small muscle mass exercise (16).

The reduction in blood flow observed with H_1/H_2 histamine receptor blockade illustrates that histamine receptor activation is a critical step in the signaling process of sustained post-exercise vasodilation. Presumably, histamine is the ligand activating H_1/H_2 histamine receptors during the recovery from exercise. However, studies in our laboratory and others have failed to document any rise in arterial and venous plasma or whole-blood histamine during the recovery from exercise (82, 83, 119, 125, 126, 132). Conversely, several investigations have reported that histamine is elevated in whole blood (25, 82) and serum (47). It is unclear why there are such conflicting reports, but the varied findings may be related to experimental methodologies or assay techniques used to

measure histamine concentrations. Histamine quantitation is technically challenging owing to a half-life of just under two minutes (98). Moreover, there is clear variability and bias between the numerous assays available (66). Nevertheless, evidence collected recently in our laboratory points towards another explanation for the lack of elevated circulating histamine following exercise. Barrett-O'Keefe et al. documented that blood flow was elevated in the vascular bed perfusing the previously active quadriceps muscles following unilateral dynamic knee extension exercise, whereas blood flow in the unexercised leg was unchanged (16), suggesting that histamine is released locally within the skeletal muscle without spillover into the circulation. To date, histamine has not been measured locally in skeletal muscle during or following acute aerobic exercise.

Histamine could be elevated in skeletal muscle in response to exercise through two histaminergic mechanisms. First, in skeletal muscle, mast cells are located adjacent to blood vessels and nerves and also within connective tissue (129, 201). Degranulation of these mast cells could augment histamine release within the skeletal muscle during or following exercise. Alternatively, histamine can be synthesized *de novo* in non-mast cells by the inducible rate-limiting enzyme, histidine decarboxylase, which catalyzes the formation of histamine via decarboxylation of the amino acid L-histidine (135, 192). Augmented gene expression and/or activity of histidine decarboxylase in response to acute aerobic exercise could increase histamine formation within previously active skeletal muscle. Both mast cell degranulation and histidine decarboxylase induction are thought to increase intramuscular histamine in mice during prolonged exercise (10, 55, 140, 201). However, these studies have never measured blood flow in their studies, nor

have they quantitated skeletal muscle histamine or tryptase, a marker of mast cell degranulation, during or after exercise.

The purpose of this project was twofold. First, we wanted to quantitate interstitial histamine concentrations in skeletal muscle during and after exercise. Second, we wanted to delineate the histaminergic mechanism responsible for local histamine formation within previously active skeletal muscle. This purpose was addressed in two separate studies. In study 1, we tested the hypothesis that interstitial histamine is elevated in skeletal muscle during and following 1h unilateral dynamic knee extension exercise and that blockade of histidine decarboxylase, via the irreversible inhibitor α-fluoromethylhistidine, would inhibit this rise. Additionally, we tested the hypothesis that that blockade of histidine decarboxylase would blunt the histamine receptor dependent flux of interstitial fluid, a surrogate marker of local blood flow, during the recovery from exercise and that this effect would be similar in magnitude to that observed with local H_1/H_2 histamine receptor blockade. In study 2, we tested the hypothesis that mast cells are not activated during or following 1h unilateral dynamic knee extension exercise.

METHODS

Subjects

Twenty-nine subjects were invited to participate in this study. Five men and five women participated in study 1, whereas eleven men and eight women participated in study 2. 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 prior to the study. Additionally, subjects

reported to the laboratory 2h post-prandial. 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. This study was approved by the Institutional Review Board at the University of Oregon and was performed in accordance to the principles outlined by the Declaration of Helsinki.

Screening Visit

The screening visit was used to determine peak power output during a unilateral dynamic knee extension exercise test performed to volitional fatigue. Dynamic knee-extension exercise during all 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 measures 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^{-1}.

Experimental Approach

Following the screening visit, subjects were randomly assigned to sham or exercise conditions for both studies. Subjects in the exercise condition performed 60 min of unilateral dynamic knee extension exercise at 60% of peak power and a cadence of 45 kicks min^{-1}. Power was ramped at the onset of exercise to 60% peak power over the first

15 min. Power output was recorded continuously throughout 60 min dynamic knee extension exercise. Subjects in the sham group sat upright for 60 min. Skeletal muscle microdialysis probes were implanted 2.5h prior to exercise or upright rest and used to sample interstitial fluid from the *vastus lateralis*. Microdialysis effluent (dialysate) was collected every 30 min prior to, during, and following exercise or upright rest. Hemodynamic measurements were made prior to exercise and every 30 min throughout the 90 min exercise recovery period.

Probe Implantation. All probes were implanted using sterile technique. The skin and underlying fascia were anesthetized using 1% lidocaine HCL (Hospira Worldwide, Lake Forest, IL, USA) buffered with sodium bicarbonate (Hospira Worldwide, Lake Forest, IL, USA). Care was taken to ensure that the lidocaine was not injected into the skeletal muscle. Probes were implanted in the vastus lateralis in a direction parallel with muscle fiber orientation (~19°, relative to long-axis of muscle) using a splitable introducer. After implantation, probes were held in place by covering the entry site with a sterile transparent medical dressing.

Probe Specifications. In study 1, all probes had a 20 kilodalton molecular weight cutoff with a 30 mm polyarylethersulphone membrane (63 MD Catheter, MDialysis, Stockholm, Sweden) and were used to measure interstitial histamine and interstitial fluid flux. In study 2, probes had a 100 kilodalton molecular weight cutoff with a 30 mm polyarylethersulphone membrane (71 High Cut-off Brain MD Catheter, MDialysis, Stockholm, Sweden) and was used to measure interstitial tryptase, which is a gold standard marker of mast cell activation (91).

Perfusate & Dialysate. Subsequent to implantation, microdialysis probes were attached

to microinfusion pump (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA, USA and set to perfuse at a rate of 5μl min^{-1}. All probes study 1 were perfused with 0.9% saline that also contained 0.40 μCi ml^{-1} of tritiated histamine [^{3}H] and 5 mM ethanol. Tritiated histamine was used for the *in vivo* calibration of probe recovery (179) and ethanol was used to assess interstitial fluid flux via the ethanol washout technique (88). The microdialysis probe targeting tryptase in study 2 was perfused with 0.9% saline.

Several drugs were added to the perfusate in study 1. Pyrilamine maleate (1mM) was used to block H_1 histamine receptors and cimetidine (3mM) was used to block H_2 histamine receptors (Sigma-Aldrich, St. Louis, MO, USA). These doses have been used previously in our lab to locally block H_1/H_2 receptors and reduce successfully sustained post-exercise vasodilation (155). α-fluoromethylhistidine dihydrochloride (α-FMH, 200 μM) (Santa Cruz Biotechnology, Dallas TX, USA) was used to irreversibly inhibit histidine decarboxylase activity (193). This dose was extrapolated from studies using α-fluoromethylhistidine dihydrochloride to inhibit histidine decarboxylase in cell culture (181, 200).

Microtubes were covered with non-porous tape during each sampling period in order to prevent dialysate and ethanol evaporation. Microtube weight was documented before and after dialysate collection in order to assess fluid loss and estimate perfusion rate of the probe. Dialysate was stored at -20°C for the duration of the study. Thereafter, the samples were stored at -80°C until analysis.

Dialysate Analysis

Interstitial histamine was measured using a "competitive" enzyme-linked immunosorbent assay (Rocky Mountain Diagnostics, Colorado Springs, CO, USA) and

performed in accordance to the manufactures instructions. Interstitial tryptase was measured using a standard "sandwich" enzyme-linked immunosorbent assay and performed in accordance to the manufactures instructions with slight modification (Kamiya Biomedical, Seattle, WA, USA).

In probes measuring interstitial histamine, 10 µl of perfusate and dialysate were pipetted into 5 ml polypropylene scintillation vials containing 3 ml of scintillation cocktail (Ultima Gold, Shelton CT, USA) immediately after the sampling period. Radioactivity was then measured (counts min^{-1}) in duplicate using a liquid scintillation counter (Beckman LS 6000SC, Brea, CA, USA) and used to determine probe recovery using **Equation 3.4**. Assessing probe recovery when analyzing for tryptase was performed using a commercially available fluorescence assay (Quant-iT™, Invitrogen, Eugene OR, USA) and performed according to manufacturer's instructions.

Interstitial fluid flux was assessed by measuring ethanol washout from the interstitial space. Ethanol in the perfusate and dialysate was measured using a modified alcohol dehydrogenase enzymatic assay (89, 157) as previously performed in our laboratory (155). The assay uses alcohol dehydrogenase to catalyze the oxidation of ethanol using nicotinamide adenine dinucleotide as the electron acceptor.

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 all hemodynamic measurements. Room temperature remained thermoneutral (~23°C) throughout the study.

Heart Rate and Blood Pressure. Arterial blood pressure was measured in the right arm using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh, NC, USA). Heart rate monitored using a three lead electrocardiograph (Tango+, SunTech Medical, Raleigh, NC, USA). Heart rate and blood pressure were also measured during 60 min dynamic knee extension exercise.

Femoral Blood Flow. Femoral artery blood flow velocity was measured via duplex ultrasonography. A linear-array vascular ultrasound probe (10 MHz, GE Vingmed System 5, Horton, Norway) 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. Velocity measurements were made at an average depth of 1.56 ± 0.04 cm for study 1 and a depth of 1.68 ± 0.02 cm for study 2. Velocities were thin-beam corrected, based on a known beam-width of 2.21 mm which resulted in an average correction factor of 0.766 ± 0.0002 for study 1 and 0.766 ± 0.0002 for study 2, as outlined by Buck et al. (23). Leg blood flow was calculated as cross-sectional area multiplied by femoral mean blood velocity and reported in ml per min. The average femoral artery diameter was 8.69 ± 0.08 mm for study 1, and 8.78 ± 0.07 mm for study 2. Femoral vascular conductance was calculated by dividing femoral blood flow by mean arterial pressures and expressed as ml per min per mmHg.

Statistical Analysis

The statistical analysis was identical for study 1 and study 2. Preliminary statistical analysis indicated that our primary outcome variables did not vary by sex. As such, all subsequent analyses were performed after grouping data for both men and women. Our primary outcome variables during the recovery from exercise were analyzed using a two-way mixed model analysis of variance with repeated measures (JMP Pro 10; SAS Institute Inc. Cary, NC, USA). We used *a priori* contrasts to examine specific condition-time interactions. General interactions were examined using Tukey's post hoc procedure. Significance was set at $P < 0.05$. Data are reported as mean ± SEM unless stated otherwise (e.g., SD is used in Table 1 to indicate the variability in the subject pool).

RESULTS

Subject Characteristics

Subject physical characteristics and data obtained during the screening visit are shown in **Table 5.1**. Subject characteristics are similar to those obtained previously in our laboratory in young healthy subjects and are consistent with recreationally active individuals

Study 1

Pre-exercise Hemodynamics. Absolute systemic hemodynamics prior to exercise are shown in **Table 5.2**. Both heart rate ($P = 0.9$) and mean arterial pressure ($P = 1.0$) did not differ between sham and exercise conditions. Femoral blood flow did not differ between sham and exercise conditions ($P = 0.9$). Likewise, femoral vascular conductance did not differ between sham and exercise conditions ($P = 0.9$).

Table 5.1. Subject Characteristics

	Study 1		Study 2	
	Sham	Exercise	Sham	Exercise
n	4	6	9	10
Age (yrs)	22.0 ± 1.6	20.1 ± 2.2	23.4 ± 5.2	21.4 ± 1.8
Height (cm)	170 ±8	174 ± 4	179 ± 9	173 ± 6
Weight (kg)	67.3 ± 13.4	68.3 ± 6.5	76.4 ± 13.3	68.6 ± 6.8
Body mass index ($kg\ m^{-2}$)	23.1 ± 2.9	22.4 ± 2.8	23.6 ± 2.4	22.6 ± 1.2
Baecke sport index (arbitrary units)	3.1 ± 0.6	3.6 ± 0.6	3.1 ± 0.7	2.9 ± 0.8
Physical activity index ($MET\ hr^{-1}\ week^{-1}$)	29.8 ± 7	60 ± 27.9	32.6 ± 10	39.7 ± 21
Peak power output (W)	35.7 ± 15.6	29.9 ± 4.1	24.6 ± 6.0	17.3 ± 2.7

Values are mean ± SD. MET, metabolic equivalents.

Exercise & Sham Responses. Power output during steady state dynamic knee extension exercise for the exercise condition was (15.5 ± 0.7 W). Power output was within 2 W of the estimated average 60% workload (17.2 W). Compared with the sham condition (74.2 ± 2.0 beats min^{-1}), heart rate was higher for the exercise condition (98.9 ± 4.4 beats min^{-1}) ($P < 0.05$). Compared with the sham condition (86.0 ± 5.2 mmHg), mean arterial blood pressure did not differ with the exercise condition (96.3 ± 5.2 mmHg, $P = 0.2$).

Post-exercise Hemodynamics. Absolute systemic hemodynamics for sham and exercise conditions are shown in **Table 5.2**. The change from pre-exercise in heart rate at 1h post-exercise did not differ between the sham (2 ± 1%) and exercise conditions (4 ± 3%, $P = 0.5$). Likewise, the change from pre-exercise in mean arterial pressure at 1h post-exercise

did not differ between the sham (-3 ± 1%) and exercise conditions (0 ± 2%, $P = 0.2$). The change from pre-exercise in femoral blood flow at 1h post-exercise was negligible for the sham condition (-2 ± 12 %) compared with the exercise condition (36 ± 4%, $P < 0.05$). Likewise, the change from pre-exercise in femoral vascular conductance at 1h post-exercise was negligible for the sham condition (-1 ± 10 %) compared with the exercise condition (36 ± 4%, $P < 0.05$).

Interstitial Histamine Concentrations. Histamine concentrations for control and H_1/H_2 blockade probes were grouped together and referred to collectively as "control."

Raw histamine concentrations for sham and exercise conditions are shown in the left panels of **Figure 5.1**. In the sham condition, histamine did not differ between control and α-FMH probes prior to upright rest ($P = 0.9$). Likewise, histamine did not differ between control and α-FMH probes during ($P = 0.9$) and following ($P = 0.9$) upright rest. In the exercise condition, histamine did not differ between control (3.0 ± 0.6 ng ml^{-1}) and α-FMH (2.9 ± 0.6 ng ml^{-1}) probes prior to exercise ($P = 0.8$). Compared with pre-exercise, histamine was elevated during exercise in the control (7.2 ± 0.6 ng ml^{-1}, $P < 0.05$) and α-FMH probes (5.4 ± 0.6 ng ml^{-1}, $P < 0.05$).

Compared with the control probes, histamine was lower during exercise in the α-FMH probes ($P < 0.05$). Compared with pre-exercise, histamine was elevated during the recovery from exercise in the control probes (4.1 ± 0.5 ng ml^{-1}, $P < 0.05$), but not in the α-FMH probes (2.9 ± 0.6 ng ml^{-1}, $P = 0.9$). Compared with the control probes, histamine was lower in α-FMH probe during the recovery from exercise ($P < 0.05$).

Table 5.2. Study 1 Hemodynamics

	Time (min)			
Variable	Pre	30	60	90
Heart rate (beats min^{-1})				
Sham	61.0 ± 1.7	60.7 ± 1.8	62.5 ± 1.4	62.0 ± 1.7
Exercise	58.0 ± 3.0	63.5 ± 2.4*	62.1 ± 2.5	60.6 ± 2.5
Mean arterial pressure (mmHg)				
Sham	84.9 ± 2.8	79.9 ± 3.1	81.9 ± 3.0	85.0 ± 3.1
Exercise	86.5 ± 1.6	83.0 ± 2.6	86.2 ± 1.8	85.7 ± 3.0
Femoral blood flow (ml min^{-1})				
Sham	194.8 ± 21.2	172.3 ± 17.0	207.6 ± 37.8	232.3 ± 26.9
Exercise	214.7 ± 11.4	318.7 ± 16.9*†	337.6 ± 13.3*	366.5 ± 14.7*
Femoral Vascular Conductance (ml min^{-1} mmHg)				
Sham	2.2 ± 0.1	2.1 ± 0.1	2.4 ± 0.3	2.6 ± 0.2
Exercise	2.4 ± 0.1	3.8 ± 0.1*†	3.9 ± 0.1*†	4.2 ± 0.1*†

Values are mean ± SEM. *P < 0.05 vs. pre-exercise. †P < 0.05 vs. sham within time period

Corrected histamine concentrations for sham and exercise conditions are shown in the right panels of **Figure 5.1**. In the sham condition, histamine did not differ between control and α-FMH probes prior to upright rest ($P = 0.9$). Likewise, histamine did not differ between control and α-FMH probes during ($P = 0.8$) and following ($P = 0.3$) upright rest.

In the exercise condition, histamine did not differ between control (13.4 ± 1.5 ng ml^{-1}) and α-FMH (11.9 ± 1.8 ng ml^{-1}) probes prior to exercise ($P = 0.3$). Compared with pre-exercise, histamine did not differ during exercise in the control probes (15.0 ± 1.5 ng ml^{-1}, $P = 0.3$) and in the α-FMH probes (10.1 ± 1.8 ng ml^{-1}, $P = 0.3$). Compared with the control probes, histamine during exercise was lower in α-FMH probes ($P < 0.05$). Compared with pre-exercise, histamine did not differ following exercise in the control probes (13.6 ± 1.4 ng ml^{-1}, $P = 0.8$), but was reduced in the α-FMH probes (8.3 ± 1.6 ng ml^{-1}, $P = 0.05$). Compared with the control probes, histamine was lower in the α-FMH probes ($P < 0.05$) during the recovery from exercise.

Interstitial Fluid Flux. **Figure 5.2** shows the percent change of interstitial fluid flux from pre-exercise to during and following exercise for control, H_1/H_2 blockade, and α-FMH probes in both sham and exercise conditions. The greater the percent reduction is equivalent to a greater flux of interstitial fluid and is an inverse surrogate marker of local perfusion.

In the sham condition, compared with control probes, the percent change of interstitial fluid flux from pre-rest to upright rest did not differ from H_1/H_2 blockade ($P = 0.9$) and α-FMH probes ($P = 0.9$). Likewise, the percent change of interstitial fluid flux from pre-rest to upright rest did not differ between H_1/H_2 blockade and α-FMH probes (P

= 0.3). Compared with control probes, the percent change of interstitial fluid from pre-rest to the recovery period did not differ from H_1/H_2 blockade (P = 0.3) and α-FMH probes (P = 0.6). Likewise, the percent change of interstitial fluid flux from pre-rest to the recovery period did not differ between H_1/H_2 blockade probes and α-FMH probes (P = 0.8).

In the exercise condition, compared with the control probes (-115 ± 23 %), the percent change of interstitial fluid flux from pre-exercise to exercise tended to be lower for H_1/H_2 blockade (-50 ± 30 %, P = 0.1) and α-FMH probes (-65 ± 26 %, P = 0.1). The percent change of interstitial fluid flux from pre-exercise to exercise did not differ between H_1/H_2 blockade and α-FMH probes (P = 0.6). Compared with control probes (-44 ± 13 %), the percent change of interstitial fluid flux from pre-exercise to the recovery period was lower for H_1/H_2 blockade (-7 ± 14 %, $P < 0.05$) and α-FMH probes (-5 ± 13 %, $P < 0.05$). The percent change of interstitial fluid flux from pre-exercise to the recovery period did not differ between H_1/H_2 blockade probes and α-FMH probes (P = 0.9).

Study 2

Pre-exercise Hemodynamics. Absolute systemic hemodynamics prior to exercise are shown in **Table 5.3**. Both heart rate (P = 0.3) and mean arterial pressure (P = 0.4) did not differ between sham and exercise conditions. Femoral blood flow did not differ between sham and exercise conditions (P = 1.0). Likewise, femoral vascular conductance did not differ between sham and exercise conditions (P = 1.0).

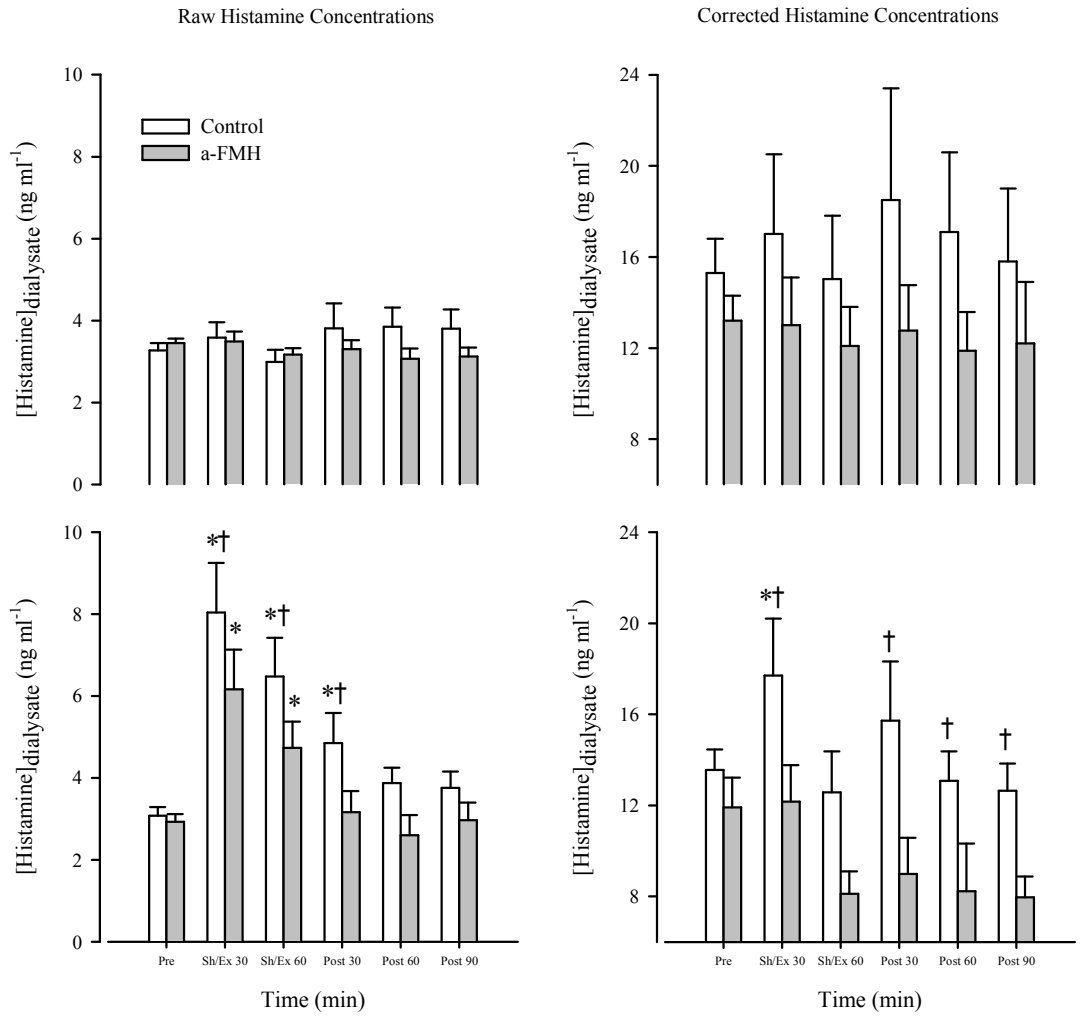

Figure 5.1. Interstitial Histamine Concentrations
Raw histamine is shown in the left panels and corrected histamine concentrations in the right panels. Top panels, sham condition; Lower panels, exercise condition. Open bars, control; Gray bars, α-FMH. *P < 0.05 vs. pre-exercise; † P < 0.05 vs. α-FMH

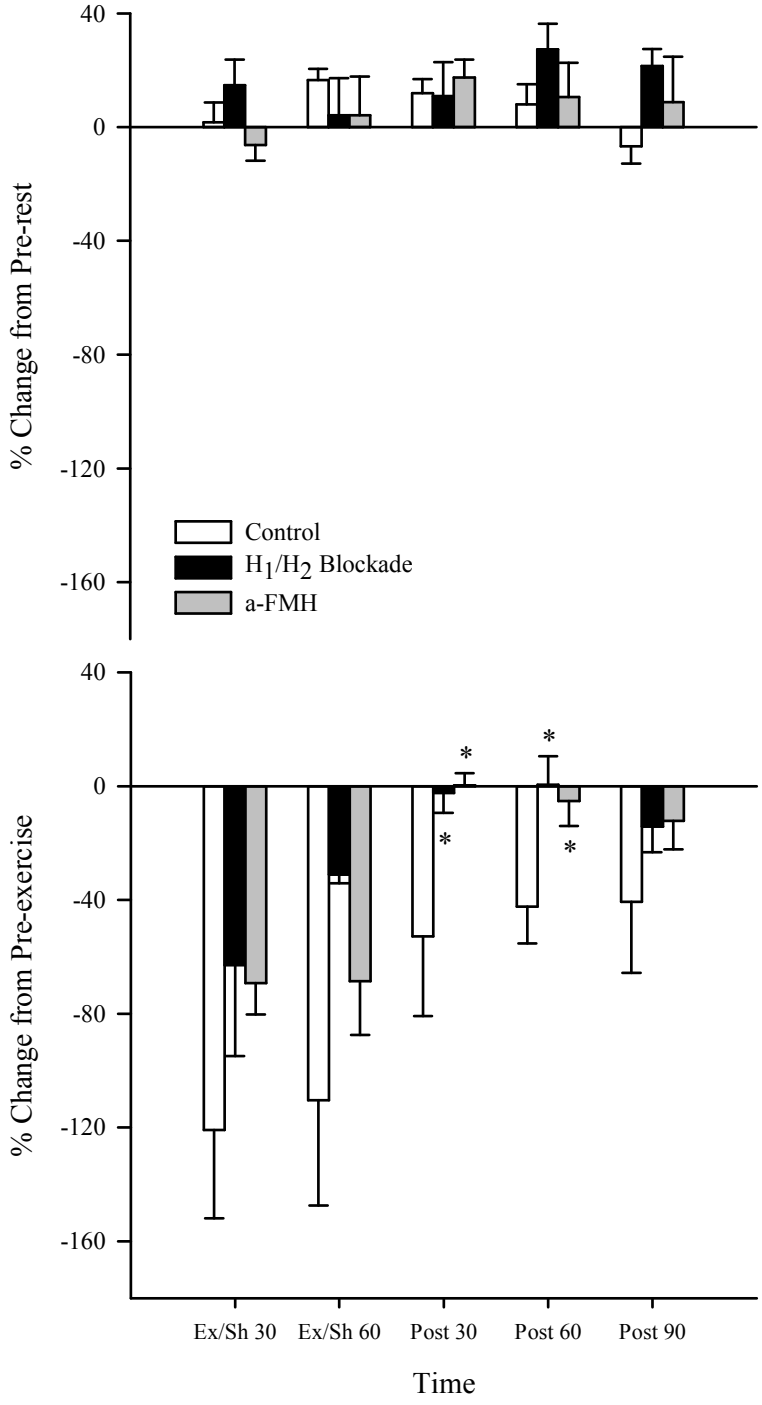

Figure 5.2. Interstitial Fluid Flux
Interstitial fluid flux is shown as the percent change from pre-rest (top panel) or pre-exercise (bottom panel). Open bars, control probe; Closed bars, H_1/H_2 Blockade; Gray bars, α-FMH. *P < 0.05 vs. control probe

Exercise & Sham Responses. Power output during steady state dynamic knee extension exercise was (16.7 ± 0.7 W). Power output was within 1 W of the average 60% workload (17.2 W). Compared with the sham condition (65 ± 1.0 beats min^{-1}), heart rate was higher for the exercise condition (107.2 ± 4.2 beats min^{-1}) ($P < 0.05$). Compared with the sham condition (93.0 ± 3.2 mmHg), mean arterial blood pressure did not differ with the exercise condition (97.4 ± 1.8 mmHg, $P = 0.2$).

Post-exercise Hemodynamics. Absolute systemic hemodynamics for sham and exercise conditions are shown in **Table 5.3**. The change from pre-exercise in heart rate at 1 h post-exercise did not differ between the sham (4 ± 1%) and exercise conditions (4 ± 1%, $P = 0.9$). Likewise, the change from pre-exercise in mean arterial pressure at 1 h post-exercise did not differ between the sham (-2 ± 2%) and exercise conditions (0 ± 2%, $P = 0.4$). The increase from pre-exercise in femoral blood flow at 1 h post-exercise was negligible for the sham condition (-2 ± 5 %) compared with the exercise condition (37 ± 4%, $P < 0.05$). Likewise, the increase from pre-exercise in femoral vascular conductance at 1 h post-exercise was negligible for the sham condition (-1 ± 5 %) compared with the exercise condition (37 ± 5%, $P < 0.05$).

Interstitial Tryptase Concentrations. Raw tryptase concentrations for sham (upright rest) and exercise conditions are shown in the top panel of **Figure 5.3**. Baseline tryptase did not differ between sham (2.7 ± 0.7 ng ml^{-1}) and exercise (2.8 ± 0.7 ng ml^{-1}, $P = 0.9$) conditions. Compared with pre-exercise, tryptase was elevated during exercise (8.1 ± 0.6 ng ml^{-1}, $P < 0.05$), but not upright rest (3.6 ± 0.6 ng ml^{-1}, $P = 0.1$). Compared with upright rest, tryptase was greater during exercise ($P < 0.05$).

Table 5.3. Study 2 Hemodynamics

	Time (min)			
Variable	Pre	30	60	90
Heart rate (beats min^{-1})				
Sham	55.1 ± 2.2	57.6 ± 2.3	57.5 ± 2.1	56.2 ± 2.1
Exercise	63.8 ± 1.5	69.8 ± 1.8*	67.0 ± 1.5	66.2 ± 1.6
Mean arterial pressure (mmHg)				
Sham	92.8 ± 1.3	87.0 ± 1.5*	91.3 ± 1.8	91.1 ± 1.8
Exercise	86.6 ± 1.4	85.9 ± 1.7	87.0 ± 1.7	86.3 ± 1.2
Femoral blood flow (ml min^{-1})				
Sham	237.6 ± 10.9	232.6 ± 15.2	237.6 ± 14.3	240.2 ± 11.9
Exercise	228.9 ± 16.1	353.1 ± 27.2*	379.8 ± 31.0*†	425.2 ± 27.9*†
Femoral Vascular Conductance (ml min^{-1} mmHg)				
Sham	2.5 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.1
Exercise	2.6 ± 0.1	4.0 ± 0.2*	4.3 ± 0.3*†	4.9 ± 0.3*†

Values are mean ± SEM. *P < 0.05 vs. pre-exercise. †P < 0.05 vs. sham within time period

Compared with pre-exercise, tryptase was elevated during the recovery from exercise (5.5 ± 0.6 ng ml^{-1}, $P < 0.05$), but not during the recovery from upright rest (3.3 ± 0.6 ng ml^{-1}, $P = 0.2$). Compared with the recovery from upright rest, tryptase was greater during the recovery from exercise ($P < 0.05$).

Corrected tryptase is expressed as the ratio of tryptase to total protein in the sample and is shown in **Figure 5.3** for sham and exercise conditions. Baseline tryptase did not differ between sham ($1.2x10^{-4} \pm 1.6x10^{-6}$) and exercise ($1.3x10^{-4} \pm 1.9x10^{-5}$, $P = 0.7$) conditions. Compared with pre-exercise, tryptase tended to be lower during exercise ($1.0x10^{-4} \pm 1.2x10^{-5}$, $P = 0.06$), but not upright rest ($1.1x10^{-4} \pm 9.6x10^{-6}$, $P = 0.6$). Compared with upright rest, tryptase did not differ during exercise ($P = 0.5$). Compared with pre-exercise, tryptase did not differ during the recovery from exercise ($1.1x10^{-4} \pm 1.1x10^{-5}$, $P = 0.2$) and during the recovery from upright rest ($1.4x10^{-4} \pm 1.1x10^{-5}$, $P = 0.1$). Compared with the recovery from upright rest, tryptase did not differ during the recovery from exercise ($P = 0.9$).

DISCUSSION

The purpose of this project was twofold. First, we wanted to quantitate interstitial histamine concentrations in skeletal muscle during and after exercise. Second, we wanted to delineate the histaminergic mechanism responsible for local histamine formation within previously active skeletal muscle. In agreement with our hypothesis for study 1, we documented that interstitial histamine is elevated during and following 1 hr unilateral dynamic knee extension exercise. Moreover, blockade of histidine decarboxylase, via the irreversible inhibitor α-FMH, reduced the rise in interstitial histamine formation.

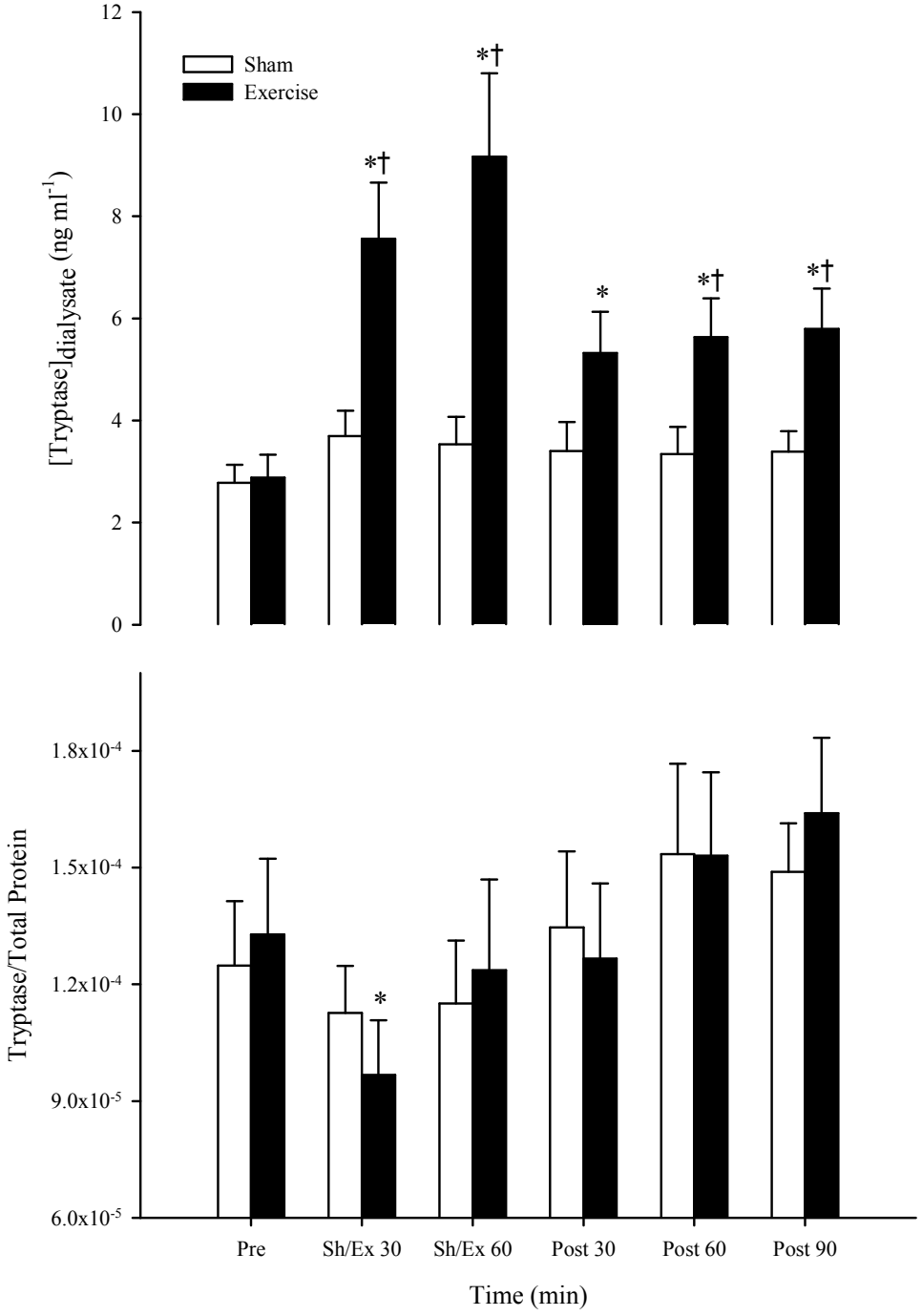

Figure 5.3. Interstitial Tryptase
The top panel shows raw interstitial tryptase concentrations throughout sham or exercise conditions. The bottom panel shows the ratio of tryptase to total protein throughout sham or exercise conditions. Open bars, sham condition; Closed bars, exercise condition. *P < 0.05 vs. pre-exercise. †P < 0.05 vs. sham within time period

In agreement with the secondary hypothesis for study 1, blockade of histidine decarboxylase tended to reduce the flux of interstitial fluid during exercise and completely inhibited it after exercise. This effect was similar in magnitude to that observed with local H_1/H_2 histamine receptor blockade. In opposition to our hypothesis in study 2, it appears that mast cells are activated during and following 1 hr unilateral dynamic knee extension exercise, as tryptase was elevated during and after exercise. Collectively, these studies demonstrate that histamine is the ligand activating H_1/H_2 receptors during the recovery from exercise. Induction of histidine decarboxylase is the primary histaminergic mechanisms augmenting local histamine formation in skeletal muscle along with a possible contribution from mast cell activation.

Histaminergic Mechanisms of Sustained Post-exercise Vasodilation

This study provides unique insight into the histaminergic mechanisms regulating sustained post-exercise vasodilation. Using skeletal muscle microdialysis, we attempted to pharmaco-dissect parallel histaminergic pathways that may respond to exercise. We documented that interstitial histamine concentrations are elevated during and after exercise, but can be reduced by infusing an irreversible inhibitor of histidine decarboxylase, the enzyme responsible for *de novo* histamine synthesis. This observation points towards the induction of histidine decarboxylase as a primary mechanism augmenting histamine locally in active skeletal muscle during exercise. It should be noted that irreversibly inhibiting histidine decarboxylase during exercise appears to partially reduce the rise in interstitial histamine. This inability to fully blunt the rise in histamine suggests that the drug may not fully inhibit histidine decarboxylase *in vivo*, or that another mechanism accounts for the histidine decarboxylase independent portion of

histamine formation. We infused α-FMH at a concentration of 200 μM, a dose extrapolated from studies using the drug to inhibit histidine decarboxylase in cell culture (181, 200). It is possible that this dose is lower than necessary to fully inhibit the large induction of histidine decarboxylase during exercise. Alternatively, the results in study 2 point toward mast cells as the secondary source of local histamine formation. Tryptase, a marker of mast cell degranulation, was elevated with exercise indicating that mast cells may degranulate in response to exercise. Histamine is released in addition to tryptase upon activation of mast cells and may therefore be responsible for the unaccounted for rise in histamine during exercise.

Interestingly, α-FMH fully inhibited the rise in histamine during the first 30 min of exercise recovery, an effect that tended to remain throughout the recovery from exercise. Likewise, the percent reduction in interstitial fluid flux was similar between H_1/H_2 blockade and α-FMH probes at the same time points. Together these observations suggest that the contribution of the parallel histaminergic pathways differs during and following exercise. Perhaps, both the induction of histidine decarboxylase and mast cell activation contribute to the rise in histamine during exercise, but *de novo* synthesis predominates following exercise.

Taken together, histidine decarboxylase induction is the primary mechanism augmenting interstitial histamine during and after exercise. However, mast cells may also contribute to the rise in interstitial histamine. If so, it is possible that these parallel histaminergic pathways cooperatively mediate sustained post-exercise vasodilation. Evidence in support of this has been provided by the work of Endo and colleagues (10, 55, 140, 201). Their elegant work over the last decade has led them to propose that the

induction of histidine decarboxylase replenishes the pool of mast cell histamine lost with degranulation during exercise. Our study provides evidence that a similar mechanism may occur in humans, but future research is needed to clarify if mast cells are activated with exercise and if they function synergistically with histidine decarboxylase.

Microdialysis Probe Recovery: To Correct or Not? If So, How?

Skeletal muscle microdialysis is a powerful yet complex technique that allows for the measurement of various target analyates in a fluid compartment not readily accessible. The recovery of substances from the interstitial space is dependent on: 1) perfusion rate; 2) probe length; 3) probe membrane characteristics; and, 4) the rate of diffusion in the interstitial space (108, 122, 139). The first three factors are fixed within an experiment and therefore do not affect recovery. However, the rate of diffusion is influenced by a number of factors and can vary greatly within a study thus influencing the recovery of a target analyte. The diffusion of substances from the interstitial space is dependent on: tortuosity, volume fraction, blood flow, and perfusate flow rate (86, 108, 122, 139). Experiments with dynamic conditions such as exercise can greatly alter the factors that influence diffusion. Thus, an *in vivo* calibration should be performed in such conditions in order to account for changes in probe recovery.

In study 1, we used the internal standard technique (179) to correct for probe recovery. The histamine concentrations when adjusting for probe recovery appear to follow the same trends during and after exercise when not correcting for probe recovery, but a clear picture does not emerge as the "waters have been muddied." The internal standard technique is easily performed but may have inherent limitations due to its simplistic approach. It assumes diffusion from the probe down a concentration gradient to

zero at an infinite distance from the probe. However, changes in perfusion (and washout of the analyte) or metabolism of the analyte impact the nature of the diffusion gradient such that this method may falsely correct the data. Perhaps, an endogenous "tracer" such as urea can be used to better account for changes in probe recovery, thus providing a more clear view and ultimately improving our ability to interpret the data.

Even though the correcting for probe recovery adds some inconsistency to the data, we believe our interpretation to be true. Multiple lines of evidence support our view that interstitial histamine is increased during and after exercise and is dependent on *de novo* synthesis via histidine decarboxylase. First, histamine concentrations were unaffected prior to exercise but were reduced during and after exercise, suggesting that the normal increase, irrespective of recovery, was inhibited by α-FMH. Moreover, this inhibition occurred at a time when we would expect the induction of histidine decarboxylase to increase. Second, given that the flux of interstitial fluid tended to be reduced during exercise and was inhibited during the recovery period with the infusion of α-FMH demonstrates a clear relationship between the histidine decarboxylase dependent synthesis of histamine and regulation of local microvasculature blood flow in the area of the probe.

In study 2, we attempted to correct for probe recovery by measuring the total protein in the sample. This approach has never been used in microdialysis studies nor has it been validated as an acceptable measure of probe recovery. However, we attempted to use this approach as we were limited by the volume of dialysate remaining after analyzing tryptase via ELISA. Therefore, it is very probable that the correction of probe recovery in this manner does not reflect the physiology *in vivo*.

These microdialysis studies have provided evidence regarding the histaminergic mechanisms regulating local histamine formation in skeletal muscle and sustained post-exercise vasodilation. However, we believe that additional evidence is needed to support our initial findings. Using imaging techniques such as immunohistochemistry, we plan to visualize mast cells and their activation in the skeletal muscle tissues collected from the study detailed in chapter VI. Likewise, we can visualize the localization (e.g. endothelial cells) of histidine decarboxylase and examine its gene expression in response to acute exercise. Data from these future analysis coupled with that of the current study should collectively provide a clear picture of histaminergic mechanisms mediating sustained post-exercise vasodilation.

Summary & Bridge

As highlighted in chapter in II and originally presented by John T. Shepard, a proposed mediator of hyperemia must meet the following criteria: 1) the substance(s) or its precursor(s) should be present in skeletal muscle; 2) the substance(s) should have access to the muscle resistance vessels; 3) the concentration in the interstitial fluid must be capable of causing and maintaining vasodilation, and there should be a close relationship between the interstitial fluid concentration and the blood flow; and, 4) the substance(s) should be capable of producing dilatation of arterioles on topical application. Our laboratory's prior findings coupled with those in this current investigation reveal that histamine satisfies each of the above criteria. Thus, histamine is indeed the ligand activating histamine receptors during the recovery from exercise thus mediating sustained post-exercise vasodilation. Moreover, histidine decarboxylase induction is the primary mechanism augmenting local histamine formation within the skeletal muscle. Mast cells

may also contribute, but future work is needed to determine their exact role in the formation of histamine in skeletal muscle

Chapters IV and V of this dissertation have focused on the upstream mechanisms mediating sustained post-exercise vasodilation. The final chapter focuses on some of the downstream adaptations associated with the activation of histamine receptor during the recovery from exercise, specifically exercise-induced angiogenesis.

CHAPTER VI

EFFECT OF HISTAMINE RECEPTOR BLOCKADE ON THE EXPRESSION OF PRO-ANGIOGENIC GROWTH FACTORS DURING THE RECOVERY FROM AEROBIC EXERCISE

INTRODUCTION

Acute aerobic exercise induces a prolonged vasodilation within the previously active skeletal muscle vasculature. This sustained post-exercise vasodilation lasts upwards of several hours and occurs following both whole-body exercise (78, 79, 154) and isolated small muscle-mass exercise (16). Histamine H_1 and H_2 receptor activation (119, 125, 126) and alterations in the sympathetic control of the peripheral vascular system (75, 78) mediate sustained post-exercise vasodilation in humans following large muscle mass exercise. In contrast, H_1 and H_2 receptor blockade abolishes sustained post-exercise vasodilation following small-muscle mass exercise (16).

The recovery from aerobic exercise is not simply a return to pre-exercise homeostasis, but is a critical period in which multiple physiological systems are primed for functional and structural adaptations. Much of these adaptations are due to transcriptional and translational changes that ultimately alter signaling pathways within skeletal muscle. Chronic activation of these cellular pathways leads to the well-known phenotypic changes that occur with aerobic exercise training.

The skeletal muscle vascular system adapts exquisitely to chronic aerobic exercise training via functional and structural changes (114). Functional changes encompass adaptations that improve vasomotor function/sensitivity. Structural adaptations include vascular remodeling of larger conduit blood vessels which is referred

to as arteriogenesis and angiogenesis which is the expansion of the capillary network. In general, the functional adaptations and arteriogenesis improve regional or "bulk" blood flow. On the other hand, angiogenesis is beneficial as it augments microvascular blood flow which is thought to occur from decreased red blood cell transit time, decreased diffusional path length, and increased capillary surface area for diffusion (70). Greater microvascular blood flow improves the delivery of nutrients and the removal of metabolic byproducts.

Angiogenesis in skeletal muscle has long been known to occur with chronic exercise training (27, 161) and is thought to result from the induction of pro- and anti-angiogenic growth factors via mechanical, metabolic, and hypoxic stimuli (50, 70). A number of pro- and anti-angiogenic growth factors respond to acute exercise (50, 70, 150); however the specific regulatory mechanism(s) that govern their release in humans remain uncertain.

What physiological purpose is subserved by sustained post-exercise histaminergic vasodilation? Recently our laboratory postulated that sustained post-exercise vasodilation may contribute to exercise-induced angiogenesis through upregulation of pro-angiogenic growth factors. In addition to its potent vasodilator effect, histamine has been implicated in angiogenesis since the late 1960's (202). Recent observations implicating histamine and its receptors (subtypes H_1 and/or H_2) as potent angiogenic stimuli in pathological and physiological angiogenesis (59, 64, 65, 100, 142, 184) have generated renewed interest in this biological amine.

Thus, we propose that there may be a distinct mechanistic link between sustained post-exercise vasodilation, histamine receptor activation, and the expression of pro- and

anti-angiogenic growth factors. Therefore, the purpose of this study was to determine if activation of histamine H_1 and H_2 receptors contributes to the expression of pro- and anti-angiogenic factors during the recovery from acute aerobic exercise. We tested the hypothesis that histamine H_1 and H_2 receptor blockade would blunt the expression of several pro-and anti-angiogenic growth factors during the recovery from 1 hr unilateral dynamic knee extension exercise.

METHODS

Subjects

Sixteen subjects (10 men and 6 women) were invited to participate in this study. 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 prior to the study. Additionally, subjects reported to the laboratory 2h post-prandial. 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 irrespective of their menstrual cycle phase. This study was approved by the Institutional Review Board at the University of Oregon and was performed in accordance to the principles outlined by the Declaration of Helsinki.

Screening Visit

The screening visit was used to determine peak power output during a unilateral dynamic knee extension exercise test performed to volitional fatigue. Dynamic knee-extension exercise during all 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 measures 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^{-1}.

Experimental Approach

Following the screening visit, subjects were randomly assigned to control or histamine receptor blockade conditions. All subjects performed 60 min of unilateral dynamic knee extension exercise at 60% of peak power and a cadence of 45 kicks min^{-1}. Power was ramped at the onset of exercise to 60% peak power over the first 5 min. Power output was recorded continuously throughout 60 min dynamic knee extension exercise. Skeletal muscle tissue was obtained via biopsy of the *vastus lateralis* before, immediately after, and 3 hours following dynamic knee extension exercise. All pre-exercise biopsies were performed in the non-exercised leg, whereas both of the post-exercise biopsies were performed in the previously exercise leg. Hemodynamic measurements were made prior to exercise and every 30 min throughout the 3 h recovery period.

Histamine Receptor Blockade. Histamine H_1 and H_2 receptors were blocked using 540 mg fexofenadine and 300 mg ranitidine. This combination of fexofenadine and ranitidine reduces sustained post-exercise vasodilation by ~86% following unilateral dynamic knee extension exercise (14). This dosage of oral fexofenadine has been shown to selectively

block H_1 receptors (time to peak concentration 1.15 h and half-life 12 h), while the dose of oral ranitidine has been shown to selectively block H_2 receptors (time to peak plasma concentration 2.2 h and 2.6 h half-life) (56, 156). Responses are 90% inhibited within 1 h and remain inhibited 6 h after administration (20, 56). Fexofenadine and ranitidine do not appear to cross into the central nervous system or possess sedative actions (20). Furthermore, these drugs do not have any direct cardiovascular effects in the absence of histamine receptor stimulation (i.e., when given under normal resting conditions, these drugs do not elicit any changes in heart rate, blood pressure, or smooth muscle tone) (114). Subjects ingested the histamine receptor antagonists with water at least 1 hr prior to exercise in all studies.

Skeletal Muscle Biopsy. All skeletal muscle biopsies were performed under sterile technique. The skin and underlying fascia were anesthetized using 1% lidocaine HCL (Hospira Worldwide, Lake Forest, IL, USA). Skeletal muscle was obtained using a 5 mm Bergström biopsy needle inserted through a small incision made in the skin and muscle fascia. Harvested skeletal muscle tissue was blotted, removed of any adipose tissue, and snap frozen in liquid nitrogen. When applicable, a small portion of the muscle sample was removed for future histological analysis. These tissue samples were snap frozen in isopentane cooled with liquid nitrogen and stored at -80°C until analysis.

Gene Expression

We utilized next-generation RNA sequencing to probe gene expression on a transcriptome-wide level. RNA sequencing provides a comprehensive index of all of the genes expressed in the transcriptome at the time when the host tissue and cells were obtained (191). Thus, RNA sequencing technology allows us to exhaustively and

precisely examine transcripts related to acute exercise, angiogenesis, and histamine receptor activation. Additionally, RNA sequencing will allows us to interrogate additional genes and pathways unrelated to angiogenesis but that may respond to acute exercise and histamine receptor activation so that we can generate novel hypothesis driven research questions. Gene expression analysis via RNA sequencing was performed at the University of Oregon Genomics Core Facility.

RNA Isolation. Approximately 15 mg of skeletal muscle tissue was homogenized in Eppendorph RNase-free tubes containing 1 ml TRI reagent. The sample was then separated using 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. The resulting RNA pellet was washed twice in 75% alcohol, dried, and subsequently dissolved in 1.5 µl 0.1 mM EDTA for each 1 mg of starting skeletal muscle tissue.

RNA Sequencing. Following RNA isolation, sequencing was carried using the following standardized workflow within the Genomics Core Facility: 1) RNA preparation; 2) cDNA library preparation; 3) sequencing; and, 4) bioinformatics analysis. Because mRNA is only 1 - 5% of total RNA, an isolation procedure is necessary to ensure that the sample is not contaminated with non-polyadenylated mRNA (e.g. ribosomal). Pure mRNA was isolated from the total RNA preparation using a commercially available kit (Dynabeads® mRNA Purification Kit, Life Technologies, Eugene, OR, USA). Input RNA yield and integrity was analyzed prior to library construction via automated capillary electrophoresis (Fragment Analyzer, Advanced Analytical Technologies, Inc., Ames, IA, USA) coupled with a High Sensitivity RNA Analysis Kit (DNF-491, Advanced Analytical Technologies, Inc., Ames, IA, USA).

The cDNA library preparation was performed using a KAPA Stranded RNA-Seq Library Preparation Kit (Kapa Biosystems, Boston, MA, USA), which is designed specifically for Illumina sequencers. Briefly, isolated mRNA was first fragmented by heating each sample to 94°C in the presence of magnesium. Double-stranded cDNA was then synthesized from the fragmented RNA using a two-step process which includes 1st strand cDNA synthesis via random priming and 2nd strand cDNA synthesis which converts the cDNA:RNA hybrid to double-stranded cDNA. Adapters were then ligated to the ends of the cDNA preceding library amplification using high-fidelity, low-bias polymerase chain reaction. Constructed libraries were validated using the High Sensitivity NGS Fragment Analysis Kit (DNF-486, Advanced Analytical Technologies, Inc., Ames, IA, USA) and quantitated using a Qubit 2.0 fluorometer coupled with a High Sensitivity DNA assay kit (Life Technologies, Eugene, OR, USA).

Subsequent to cDNA library preparation, flowcells were loaded with samples and clusters were formed and replicated for each oligo. Flowcells were then placed in the sequencer (Illumina HiSeq 2000, Illumina, San Diego CA, USA) and fluorescently tagged nucleotides were competitively attached to complimentary bases within each cluster. Single-end sequencing was performed at a length of 100 nucleotides.

Following the read process, adapters were removed and sequences were tiled and aligned to the reference human genome. At the time of this writing (9/17/2014) RNA sequencing is in the final stages of preparation and analysis. Thus, the bioinformatics component of data analysis is yet to be completed. The preliminary data presented herein were analyzed using standard real-time polymerase chain reaction (see appendix). Select genes of interest that are associated with histamine receptor activation, exercise, and

angiogenesis were identified *a priori* and are shown in **Table 6.1**. This hypothesis driven approach is in addition transcriptome wide approach that allows us to "cast a wide net" and investigate relationships not defined in our hypothesis.

Table 6.1. Select Genes of Interest

EntrezID	Symbol	Name
7422	VEGFA	Vascular endothelial growth factor A
3791	KDR	Kinase insert domain receptor
4846	NOS3	Nitric oxide synthase 3 (endothelial cell)
4313	MMP2	Matrix metallopeptidase 2
6347	CCL2	CCL22 chemokine (c-c motif) ligand 2
2247	FGF2	Fibroblast growth factor 2 (basic)
3164	NR4A1	Nuclear receptor subfamily 4, group a, member 1
7057	THBS1	Thrombospondin 1
10891	PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
3091	HIF1A	Hypoxia inducible factor 1, alpha subunit

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 all hemodynamic measurements. Room temperature remained thermoneutral (~23°C) throughout the study.

Heart Rate and Blood Pressure. Arterial blood pressure was measured in the right arm using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh, NC, USA). Heart rate was monitored using a three lead electrocardiograph (Tango+, SunTech

Medical, Raleigh, NC, USA). Heart rate and blood pressure were also measured during 60 min dynamic knee extension 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. Velocity measurements were made at an average depth of 1.77 ± 0.01 cm. Leg blood flow was calculated as cross-sectional area multiplied by femoral mean blood velocity and reported in ml per min. The average femoral artery diameter was 8.80 ± 0.07 mm. Femoral vascular conductance was calculated by dividing femoral blood flow by mean arterial pressures and expressed as ml per min per mmHg.

Statistics. Preliminary statistical analysis indicated that our primary hemodynamic outcome variables did not vary by sex. As such, all subsequent analyses were performed after grouping data for both men and women. Our primary hemodynamic outcome variables were analyzed using a two-way mixed model analysis of variance with repeated measures (JMP Pro 10; SAS Institute Inc. Cary, NC, USA). We used *a priori* contrasts to examine specific condition-time interactions. General interactions were examined using Tukey's post hoc procedure. Significance was set at $P < 0.05$. Data are reported as mean ± SEM unless stated otherwise (e.g., SD is used in **Table 2** to indicate the variability in the subject pool).

RESULTS

Subject Characteristics

Subject physical characteristics and data obtained during the screening visit are shown in **Table 6.2**. Subject characteristics are similar to those obtained previously in our laboratory in young healthy subjects and are consistent with recreationally active individuals.

Table 6.2. Subject Characteristics

	Control	H_1/H_2 blockade
n	8	8
Age (yrs)	23.0 ± 4.4	24.5 ± 5.4
Height (cm)	169 ± 9	175 ± 4
Weight (kg)	68.1 ± 10.1	75.9 ± 6.3
Body mass index (kg m^{-2})	23.7 ± 2.0	24.7 ± 1.9
Baecke sport index (arbitrary units)	3.4 ± 0.8	2.8 ± 0.9
Physical activity index (MET hr^{-1} $week^{-1}$)	49 ± 22	38 ± 25
Peak power output (W)	29.4 ± 7.1	38.4 ± 10.8

Values are mean ± SD. MET, metabolic equivalents.

Hemodynamics

Pre-exercise Hemodynamics. Pre-exercise heart rate and mean arterial blood pressure are shown in **Table 6.2**. Both heart rate ($P = 1.0$) and mean arterial pressure ($P = 0.6$) did not differ between control and H_1/H_2 blockade conditions. Pre-exercise femoral blood flow and femoral vascular conductance are shown in **Table 6.3**. Femoral blood flow did not differ ($P = 0.5$) between control and H_1/H_2 blockade conditions. Likewise, femoral vascular conductance did not differ between control and H_1/H_2 blockade conditions ($P = 0.7$).

Exercise Responses. Power output during steady state dynamic knee extension exercise was similar between control (17.1 ± 1.4 W) and H_1/H_2 blockade conditions (21.6 ± 2.9 W) ($P = 0.1$). Power output for each condition was within 1 W of the estimated 60% workload for the control condition (17.7 W) and the H_1/H_2 blockade condition (22.2 W). Mean arterial blood pressure did not differ between control (106.0 ± 2.9 mmHg) and H_1/H_2 blockade conditions (110.0 ± 3.8 mmHg) ($P = 0.4$). Heart rate did not differ between control (100.9 ± 3.5 beats min^{-1}) and H_1/H_2 blockade conditions (95.7 ± 3.5 beats min^{-1}) ($P = 0.3$).

Post-exercise Hemodynamics. Table 6.3 shows heart rate and mean arterial blood pressure during recovery from exercise. Heart rate was not different between control and H_1/H_2 blockade conditions ($P = 0.06$). Likewise, mean arterial pressure did not differ between control and H_1/H_2 blockade conditions ($P = 0.1$). **Figure 6.1** shows percent change from pre-exercise throughout the recovery from exercise for femoral blood flow and femoral vascular conductance. Absolute values for femoral blood flow and femoral vascular conductance are shown in **Table 6.4**.

Table 6.3. Central Hemodynamics

	Heart Rate (beats min^{-1})		Mean Arterial Pressure (mmHg)	
Time Point	Control	H_1/H_2 Blockade	Control	H_1/H_2 Blockade
Pre-exercise	63.6 ± 1.2	63.5 ± 1.7	82.5 ± 1.6	89.6 ± 1.2
Time post-exercise				
30 min	65.5 ± 1.2	59.0 ± 0.8	81.1 ± 1.5	88.1 ± 1.2
60 min	62.6 ± 2.0	60.8 ± 1.5	80.9 ± 1.7	89.0 ± 1.9
90 min	63.8 ± 1.8	60.6 ± 1.7	81.2 ± 2.3	89.1 ± 1.9
120 min	63.3 ± 1.6	60.3 ± 1.8	82.8 ± 1.5	86.6 ± 1.5
150 min	63.8 ± 1.5	64.0 ± 2.4	79.0 ± 1.8	89.4 ± 1.8
180 min	64.7 ± 1.5	64.0 ± 3.0	82.4 ± 1.2	88.2 ± 1.9

Values are mean ± SEM

Table 6.4. Absolute Femoral Blood Flow and Femoral Vascular Conductance

	Femoral Blood Flow (ml min^{-1})		Femoral Vascular Conductance (ml min^{-1} mmHg)	
Time Point	Control	H_1/H_2 Blockade	Control	H_1/H_2 Blockade
Pre-exercise	350 ± 21	394 ± 16	4.31 ± 0.3	4.39 ± 0.1
Time post-exercise				
30 min	467 ± 36*	403 ± 27	5.84 ± 0.5*	4.60 ± 0.3
60 min	428 ± 34*	379 ± 21	5.34 ± 0.4*	4.26 ± 0.2
90 min	465 ± 38*	403 ± 31	5.89 ± 0.5*	4.49 ± 0.2
120 min	461 ± 41*	422 ± 33	5.60 ± 0.5*	4.86 ± 0.3
150 min	480 ± 31*	409 ± 35	6.08 ± 0.3*	4.58 ± 0.3
180 min	450 ± 26*	426 ± 35	5.50 ± 0.3*	4.77 ± 0.3

Values are mean ± SEM. *$P < 0.05$ vs. pre-exercise

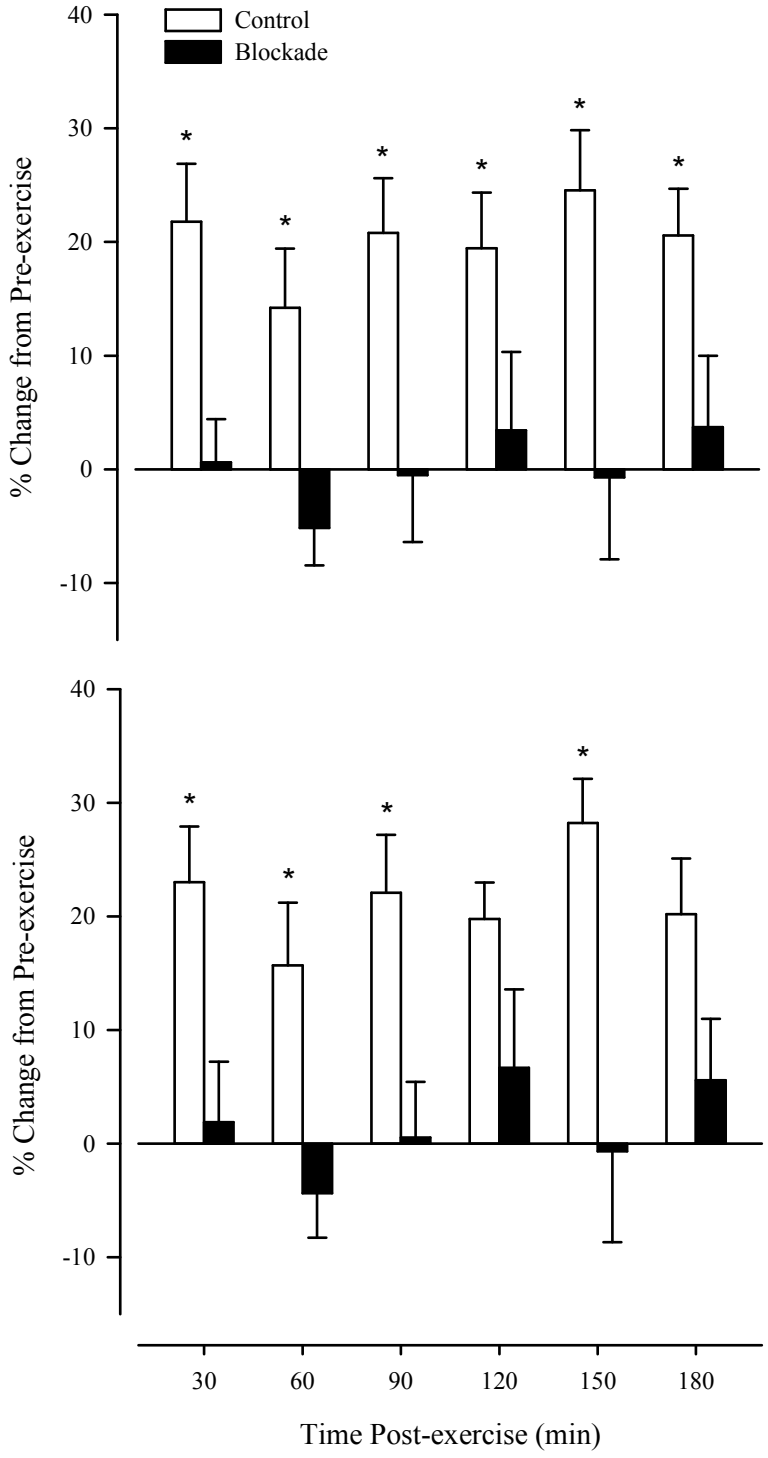

Figure 6.1. Femoral Blood Flow & Femoral Vascular Conductance
Top panel shows the percent change from pre-exercise of femoral blood flow throughout 180 min of exercise recovery. Bottom panel shows the percent change from pre-exercise of femoral vascular conductance. Open bars control group; Closed bars H_1/H_2 blockade group; * denotes $P < 0.05$ vs. blockade.

Differential Gene Expression

RNA sequencing is currently underway in the University of Oregon Genomics Core. I have therefore included preliminary gene expression data as determined by real-time polymerase chain reaction analysis for vascular endothelial growth factor A (VEGF), CCL2 chemokine (c-c motif) ligand 2 (CCL2), peroxisome proliferator-activated receptor gamma, coactivator 1 alpha, and fibroblast growth factor 2 (basic) in order to provide a preview of the expected gene expression levels of specific pro-angiogenic growth factors (**Figure 6.2**).

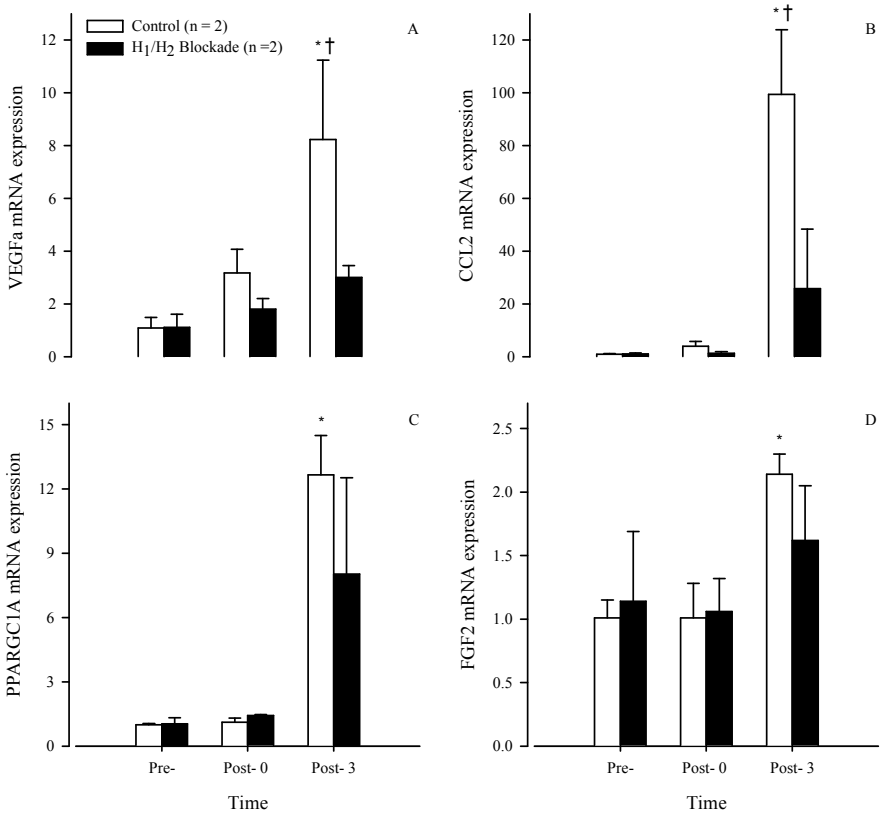

Figure 6.2. Pro-Angiogenic Growth Factor Gene Expression
Figure show mRNA expression for select genes prior to, immediately after, and 3 hr after exercise. Panel A - vascular endothelial growth factor A. Panel B - CCL22 chemokine (c-c motif) ligand 22. Panel C - peroxisome proliferator-activated receptor gamma, coactivator 1 alpha. Panel D - fibroblast growth factor 2 (basic); Open bars control group; Closed bars H_1/H_2 blockade group; *P < 0.05 vs. pre-exercise within condition. †P < 0.05 vs. H_1/H_2 Blockade within time point

DISCUSSION

The purpose of this study was to determine if activation of histamine H_1/H_2 receptors contributes to the expression of pro- and anti-angiogenic factors during the recovery from acute aerobic exercise. In agreement with our hypothesis, we documented that blockade of histamine H_1/H_2 receptors blunted the gene expression of VEGF and CCL2 3 hours into recovery from 1hr unilateral dynamic knee extension exercise. These preliminary results indicate that there is indeed a mechanistic link between sustained post-exercise vasodilation, histamine receptor activation, and the expression of pro-angiogenic growth factors. Moreover, these data provide novel evidence that histamine, a biological amine normally associated with pathological or anaphylactic conditions, may contribute beneficially to the normal changes that occur within skeletal muscle during the recovery from exercise and perhaps be an important mechanism contributing the expansion of the capillary network that accompanies chronic exercise training.

Histamine Receptor Activation and Angiogenesis

Histamine has long been linked with angiogenesis (202). Recent observations have provided detailed information regarding the specific mechanisms in which histamine and its receptors mediate the angiogenic response *in vivo*. These investigations have highlighted this biological amine as key mechanism contributing to both pathological and physiological angiogenesis. Histamine is synthesized *de novo* through decarboxylation of L-histidine by histidine decarboxylase and can function thereafter in a paracrine or endocrine fashion or can be taken up by mast cells or basophils. Much of the research regarding histamine formation/release, histamine receptor activation, and angiogenesis has focused on wound healing and cancer/tumor progression (59, 143, 149).

The notion that histamine and its receptors may play an important role in exercise-induced angiogenesis has only recently been investigated. To date, only one study has investigated the role of mast cell derived histamine in a model of compensatory muscle overload induced angiogenesis via synergist muscle ablation (45). In this study, Doyle and colleagues documented that muscle overload induced mast cell activation, but the chronic administration of the mast cell stabilizer, cromolyn, had no effect on the protein content of several pro-angiogeneic growth factors nor did it inhibit angiogenesis. These findings suggest that mast cell derived histamine is not a requisite for exercise-induced induced angiogenesis. However, it is unclear to what extent cromolyn stabilized mast cells during muscle overload as this was not reported. Additionally, the efficacy of cromolyn to stabilize mast cells has been questioned recently (194). Moreover, compensatory muscle overload via synergist muscle ablation does not replicate *in vivo* conditions encountered during aerobic exercise. While it is unclear to what extent the findings by Doyle and colleagues can be extended to humans, perhaps there is an alternative explanation. It remains possible that *de novo* histamine synthesis, and not mast cell activation, is the primary histaminergic mechanism contributing to exercise-induced angiogenesis. This is a reasonable explanation given the observation that both histidine decarboxylase activity and mRNA expression are elevated during acute aerobic exercise (10, 55, 140). This notion is further supported the findings documented in chapter V.

Potential Mechanism of Histamine Receptor Mediated Expression of Angiogenic Growth Factors

VEGF is arguably the most potent angiogenic stimulus that is upregulated with exercise and is also required for the full expression of skeletal muscle capillarity and

ultimately aerobic performance/capacity (152). In skeletal muscle, VEGF activates the kinase insert domain receptor/fetal liver kinase 1 receptor which activates a myriad of signaling pathways that contribute to angiogenesis by augmenting endothelial cell proliferation, migration, and survival (26, 70). Skeletal muscle VEGF is found in myocytes, endothelial cells, and pericytes, but skeletal muscle myocytes are thought to be the primary source of VEGF secretion during exercise (92, 93). Myocytes store VEGF in vesicles that translocate to the plasma membrane where it is then released into the interstitial space during and following exercise (93). Hoier and colleagues have proposed that augmented VEGF mRNA expression that occurs in the hours following acute aerobic exercise subserves the replenishment of the VEGF containing vesicles within skeletal muscle myocytes (93).

There are four known histamine receptors (H_1 - H_4), but it appears that subtypes H_1/H_2 mediate angiogenesis *in vivo* (65, 162, 184). Histamine H_1 and H_2 receptors are G-coupled protein receptors that when activated stimulate inositol triphosphate and adenylyl cyclase dependent signaling mechanisms that ultimately augment VEGF mRNA expression through transcriptional activation (65, 162). Thus, histamine receptor activation may contribute to exercise-induced angiogenesis by augmenting VEGF transcription and ultimately replenishing vesicular stores. Additionally, H_1/H_2 histamine receptor activation may indirectly increase VEGF expression through nitric oxide/shear-stress dependent mechanisms (50–52).

CCL2 chemokine (c-c motif) ligand 22, also known as monocyte chemotactic protein-1, is a pro-angiogenic growth factor (7, 174) that is upregulated following aerobic exercise (50, 117, 138). CCL2 induces angiogenesis by recruiting

monocyte/macrophages to the tissue where they can release growth factors such as fibroblast growth factor 2 (7). CCL2 can also stimulate directly endothelial cell chemotaxis (174). More recent evidence suggests that the angiogenic actions of CCL are mediated by activation of the novel transcription factor MCP-1 induced protein. It is unclear which mechanism/s are contribute to exercise-induced angiogenesis.

Fujikara et al. were the first to provide clear evidence linking histamine H_1 receptor activation to upregulation of CCL2 mRNA expression (60). Activation of H_1 histamine receptors stimulates inositol triphosphate signaling mechanisms that appear to upregulate CCL2 mRNA transcription through transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (113). Moreover, Marumo et al. have shown that VEGF induces CCL2 mRNA expression in endothelial cells (123). Thus, H_1/H_1 receptor activation may indirectly increase CCL2 mRNA expression by upregulating VEGF mRNA transcription. Taken together, multiple histaminergic pathways exist that may independently and/or cooperatively augment CCL2 mRNA expression during the recovery from exercise.

Considerations

The findings from this study indicate that histamine receptor activation increases the gene expression of pro-angiogenic growth factors during the recovery from aerobic exercise. It should be noted that our results provide evidence at only the transcriptional level that may not reflect true downstream changes at the protein level. For example, VEGF can undergo post-transcriptional and/or post-translation modification (6) thus preventing the formation of cellular VEGF. To our knowledge there have been no studies that have reported post-transcriptional and/or post-translation modifications of pro-

angiogenic growth factors during the recovery from exercise. Moreover, VEGF protein is elevated in both plasma and skeletal muscle hours into the recovery from acute aerobic exercise suggesting that the changes in gene expression reported in our study may translate into downstream changes in protein content. Additional analyses are necessary to determine if the changes observed at the transcriptional level indeed translate into changes at the protein level.

Perspectives

This study provides novel evidence that the activation histamine receptors during the recovery from exercise contributes to the full expression of pro-angiogenic growth factors during the recovery from exercise. Moreover, these data reveal that histamine receptors may contribute beneficially to the normal regulation of the vascular system and perhaps be an important mechanism contributing the expansion of the capillary network that accompanies chronic exercise training.

Can the chronic use of antihistamines blunt the angiogenic response that accompanies aerobic exercise training? Nearly one-half of Americans suffer from nasal allergy symptoms (e.g. rhinitis) that are attributable to seasonal allergies (137). Likewise, approximately 20% of Americans suffer from gastroesophageal reflux disease (53). Histamine H_1/H_2 receptor blockers (e.g. fexofenadine HCL and ranitidine HCL) are usually the first line of defense in the treatment of nasal symptoms associated with seasonal allergies and for acid reflux disease. Thus, it is possible that anyone taking Histamine H_1/H_2 receptor blockers for histamine mediated disorders may have a blunted angiogenic response if they participate an exercise training program.

Summary

We documented that H_1/H_2 histamine receptor blockade blunted the gene expression of VEGF and CCL2 mRNA 3 hours into recovery from 1hr unilateral dynamic knee extension exercise. These preliminary results indicate that there is indeed a mechanistic link between sustained post-exercise vasodilation, histamine receptor activation, and the expression of pro-angiogenic growth factors. Once RNA sequencing is complete, we expect to confirm these preliminary findings and expand in depth upon these findings. Future research is necessary to determine if the reduction in gene expression of pro-angiogenic growth factors by H_1/H_2 histamine receptor blockade observed in this study may blunt the angiogenic response associated with long term exercise training.

CHAPTER VII

CONCLUSIONS

MAIN FINDINGS

For over a decade our laboratory has studied cardiovascular regulation during the recovery from aerobic exercise. Our laboratory has focused its recent efforts on exploring the mechanisms and potential adaptations related to sustained post-exercise vasodilation. The studies detailed in this dissertation were designed to build upon these earlier studies regarding histaminergic mechanisms and adaptations of sustained post-exercise vasodilation.

In the study detailed in chapter VI, we examined if oxidative stress is the exercise-related factor mediating sustained post-exercise vasodilation. We intravenously infused the potent antioxidant ascorbate prior to and throughout exercise and examined its influence on sustained post-exercise vasodilation. We demonstrated that ascorbate blunted sustained post-exercise vasodilation and that this reduction was similar in magnitude to that observed with $H_1\backslash H_2$ blockade. However, in addition to its antioxidant effect, ascorbate can directly degrade histamine and may also blunt *de novo* synthesis via histidine decarboxylase. Therefore, we conducted a follow-up study to verify the findings in study 1. In this study we intravenous infused n-acetylcysteine, a potent antioxidant with no known histaminergic interactions. We demonstrated that n-acetylcysteine had no effect on sustained post-exercise vasodilation, indicating that exercise-induced oxidative stress is not the exercise-related factor mediating sustained post-exercise vasodilation and that the results in study 1 were due to ascorbates degradative effect on histamine.

In the study detailed in chapter V, we attempted to quantitate interstitial histamine in an effort to demonstrate that exercise induces the local formation of histamine in previously active skeletal muscle. Additionally, we wanted to determine if the source of histamine was from mast cell activation or *de novo* synthesis via histidine decarboxylase. Collectively, these studies would determine if histamine is the ligand activating histamine receptors during the recovery from exercise and determine its source. We found that histamine is increased in the interstitial fluid within skeletal muscle during and after exercise. Histidine decarboxylase appears to be the primary mechanism responsible for the local formation of histamine in active skeletal muscle, as irreversibly inhibiting this enzyme reduced the rise in histamine during and after exercise. Mast cells may also play an important role given the observation that inhibition of histidine decarboxylase did not fully blunt the rise in histamine during exercise and that tryptase was elevated during and after exercise.

Finally, in chapter VI, we attempted to determine if histamine receptor activation contributes to the expression of pro- and anti-angiogenic growth factors during the recovery from exercise. Our preliminary findings indicate that activation of histamine receptors may play a role in the expression of pro-angiogenic growth factors during the recovery from acute aerobic exercise. RNA sequencing analysis will provided a more thorough account of the effect of post-exercise histamine receptor activation on the expression of pro- and anti-angiogenic growth factors.

EXPANDING THE MODEL OF SUSTAINED POST-EXERCISE VASODILATION

Progress has been made recently in identifying the mechanisms that mediate

sustained post-exercise vasodilation. While both neural and local vascular mechanisms contribute to sustained post-exercise vasodilation following whole-body exercise, the local vascular mechanism (i.e. histamine receptor activation) predominates following small muscle mass exercise. By using the dynamic knee extension exercise model to isolate the histaminergic component of sustained post-exercise vasodilation, we are now able to better dissect the signaling mechanisms associated with the local control of sustained post-exercise vasodilation.

The studies detailed in this dissertation have provided clarity to our working model of the local vascular mechanisms of sustained post-exercise vasodilation. **Figure 7.1** reflects our current understanding of the mechanisms responsible and highlights some of the benefits associated sustained post-exercise histaminergic vasodilation. Our current understanding is as follows. An unknown exercise related factor increases local histamine formation through induction of histidine decarboxylase and possibly through the activation of mast cells. Subsequent formation of histamine locally within active skeletal muscle leads to activation of H_1 and H_2 histamine receptors which promotes vasodilation thus contributing to reductions in arterial blood pressure, increased glucose delivery, and to the expression of pro-angiogenic growth factors.

FUTURE DIRECTIONS

We now have a more comprehensive understanding of the histaminergic mechanisms and adaptations associated with sustained post-exercise vasodilation. However, several key questions remain unanswered. Future investigations will need to identify the exercise-related factor contributing to histamine formation that occurs with acute exercise.

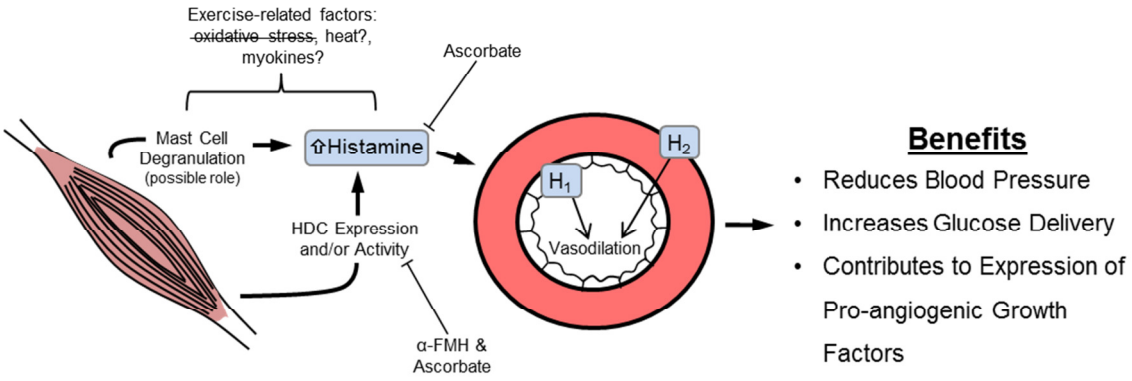

Figure 7.1. Updated Model of the Local Vascular Component of Sustained Post-exercise Vasodilation
Exercise-induced oxidative stress has been removed from the list and other candidates have been added as possible exercise related factors. The working model now reflects the possible role of mast cell activation and the induction histidine decarboxylase as the primary mediator of local histamine formation in active skeletal muscle. The model also reflects our current understanding of the benefits that are associated activation of H_1 and H_2 histamine receptors. Inhibitors of local histamine formation are also included. From (74).

Likewise, additional studies will be needed to determine if the acute changes in the expression of pro-angiogenic growth factors translate into long term changes in the microvascular phenotype. Data from the RNA sequencing analysis may open a "Pandora's Box" with respect to other beneficial adaptations associated with exercise and histamine receptor activation, but this is yet to be seen. Once we have developed a clear picture of all of the mechanisms and adaptations associated with sustained post-exercise histaminergic vasodilation, we can develop physiological and pharmacological interventions targeting these pathways in order to improve vascular health.

APPENDIX A

INFORMED CONSENT DOCUMENTS

Chapter IV: Study 1

TITLE: Exercise, histamine, receptors, and vascular function (Experiment 2)

INVESTIGATOR: Dr. J. R. Halliwill and Colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD: July 25, 2012

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 but we do not understand all the mechanisms that cause this increase in blood flow or why it happens. 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 Vitamin C, an antioxidant, reduces muscle blood flow responses after exercise. This topic is both clinically and scientific important. As such, this study is currently funded by The American Heart Association. You have been asked to participate in this study because you are a young healthy individual, who is recreationally active and free from any know cardiovascular disease.

What will happen in the study?

Initial Visit

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. 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. The questionnaire will take approximately 15 minutes to complete. You will need to wear a t-shirt and refrain from eating for two hours prior to arrival.
2. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the initial 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. During the initial 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.

4. 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 initial height and weight measurements, completion of the survey, placement of electrodes on your skin, warm-up, exercise, and cool-down) is approximately one hour.
5. 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.
6. This session will serve to familiarize you with the procedures to be used on the study days. 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 days.

Study Visits

1. You will then return to Dr. Halliwill's laboratory to participate in the three study visits, one of which will be between 7 and 10 days after the initial visit and the other will be between 7 and 10 days after that. These three testing session will take approximately 4 hours each.

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. 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. You will have one small flexible needle ("intravenous catheter") placed into a vein near your elbow before the exercise protocol. The skin will be sterilized before this procedure. This catheter will remain in your skin throughout the three hour study. After the study, we will remove the flexible needle in your vein and a bandage will be placed over that area of skin.
6. Before you exercise, we will administer a sterile solution containing Vitamin C through the intravenous catheter in two stages. First we will infuse a large dose over 20 minutes. Then, we will continue to infuse a smaller dose over 1 hour while you perform one-legged knee extension exercise at a moderate intensity. You will only receive Vitamin C on two of the study visits. On the other study visit, you will receive

a similar volume of sterile solution, but it will not contain Vitamin C. On each visit, we will obtain a small blood sample before and after the solutions are administered. The total amount of blood obtained for this experiment is less than three tablespoons per visit.

7. 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) in combination with the Vitamin C administration.
8. During each study visit, you will perform one-legged knee extension exercise while seated on an exercise machine that measures how hard you are working. During these exercise sessions you will be asked to maintain a selected kicking rate for 60 minutes.
9. 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.
10. Before and after the exercise session, a small probe (laser-Dopper probe) will be placed on the skin of your left and right thigh (quadriceps). This probe uses a laser beam to non-invasively determine skin blood flow.
11. 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 or the study visit. Some examples of discomfort include back pain, shortness of breath, light-headedness, and nausea.

How long will I be in the study?

You will be in the study for four days (initial visit and three study visits). The initial visit will last one hour. Each study visit will last four hours. You will need to refrain from eating for two hours prior to the initial visit and each study visit.

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. Vitamin C has been infused intravenously without any unfavorable side effects into young and older healthy subjects as well as patients with chronic cardiovascular diseases. However administration of concentrated Vitamin C may cause irritation to the area local to the infusion. In order to reduce this risk we will dilute the infusion of Vitamin C in a saline (sterile water) solution. There are no known risks of intravenous administration of Vitamin C at the doses proposed for the present study. There are no known risks associated with the oral administration of fexofenadine (brand name Allegra) or ranitidine hydrochloride (brand name Zantac) at this time. The only risk associated with the laser-Doppler probe is that you may have some slight skin irritation (redness) to the adhesive tape used to hold the probe on your skin. There are no major risks associated with this device.

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

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical

information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours prior to the day on which they might receive study drugs. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

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. 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. In this pilot study we are exploring whether Vitamin C, an antioxidant, reduces muscle blood flow responses after 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 $150 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 $10 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) 346-5425.

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

Office of Human Subjects Compliance
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.

The Office for Responsible Conduct of Research 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 24 months after your participation, whichever comes first. Other information may be stored by the researchers indefinitely.

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 Office for Protection of Human Subjects, 5237 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)

_____

(Printed Name of Participant) (Date)

_____

(Signature of Individual Obtaining Consent) (Date)

Chapter IV: Follow-up Study

TITLE: Exercise, histamine, receptors, and vascular function (Experiment 2 - n-acetylcysteine project)

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 but we do not understand all the mechanisms that cause this increase in blood flow or why it happens. 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 n-acetylcysteine (Acetadote), an antioxidant, reduces muscle blood flow responses after exercise. This topic is both clinically and scientific 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 recreationally active, free from any know cardiovascular disease, and has no history of an allergic or anaphylactic reaction to N-acetylcysteine.

What will happen in the study?

Initial Visit

7. 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. 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. The questionnaire will take approximately 15 minutes to complete. You will need to wear a t-shirt and refrain from eating for two hours prior to arrival.

8. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the initial 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.
9. 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.
10. During the initial 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.
11. 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 initial height and weight measurements, completion of the survey, placement of electrodes on your skin, warm-up, exercise, and cool-down) is approximately one hour.

12. 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.
13. This session will serve to familiarize you with the procedures to be used on the study days. 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 days.

Study Visits

12. You will then return to Dr. Halliwill's laboratory to participate in the two study visits, one of which will be between 7 and 10 days after the initial visit and the other will be between 7 and 10 days after that. These two testing session will take approximately 4 hours each.
13. 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.

14. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the study visits.
15. 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. 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.
16. You will have one small flexible needle ("intravenous catheter") placed into a vein near your elbow before the exercise protocol. The skin will be sterilized before this procedure. This catheter will remain in your skin throughout the three hour study. After the study, we will remove the flexible needle in your vein and a bandage will be placed over that area of skin.
17. Before you exercise, we will administer a sterile solution containing Acetadote through the intravenous catheter in two stages. First we will infuse a large dose over 20 minutes. Then, we will continue to infuse a smaller dose over 1 hour while you perform one-legged knee extension exercise at a moderate intensity. You will only receive Acetadote on one of the study visits. On the other study visit, you will receive a similar volume of sterile solution, but it will not contain Acetadote.
18. During each study visit, you will perform one-legged knee extension exercise while seated on an exercise machine that measures how hard you are working. During these

exercise sessions you will be asked to maintain a selected kicking rate for 60 minutes.

19. 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.
20. Before and after the exercise session, a small probe (laser-Doppler probe) will be placed on the skin of your left and right thigh (quadriceps). This probe uses a laser beam to non-invasively determine skin blood flow.
21. 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 or the study visit. Some examples of discomfort include back pain, shortness of breath, light-headedness, and nausea.

How long will I be in the study?

You will be in the study for three days (initial visit and two study visits). The initial visit will last one hour. Each study visit will last four hours. You will need to refrain from eating for two hours prior to the initial visit and each study visit.

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. Intravenous administration of Acetadote at the doses used for the present study

does not usually produce any side effects. However, an allergic (or anaphylactic) reaction to this drug is possible. Symptoms of an allergic reaction include rash, itching, swelling, severe dizziness, and trouble breathing. The only risk associated with the laser-Doppler probe is that you may have some slight skin irritation (redness) to the adhesive tape used to hold the probe on your skin. There are no major risks associated with this device.

Blood sampling for genotyping: In total, 10 ml of blood will be withdrawn, which is less than a tablespoon. The samples will be used for DNA analysis to determine which versions of specific genes you have related to the processing of histamine. There may exist potential risks of insurability, employability, and social discrimination if the results of individual DNA were to be made known to others. To safeguard against this risk, your DNA will be stored by an ID number only and your name or other personally identifiable information will not be included with any data shared with other investigators. We will not reveal any genetic data to your physicians, or any other health care provider. 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. You have the right to withdraw your samples from research in the future should you decide to do so. 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. Samples will be kept for five years. After the 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. 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.

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

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours prior to the day on which they might receive study drugs. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take

part in the study. There is no cost for the pregnancy test.

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. 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. In this pilot study we are exploring whether Acetadote, an antioxidant, reduces muscle blood flow responses after 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 $150 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) 346-5425.

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 24 months 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.

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 chose to abstain from the genetic testing portion of this study, please initial below. There will be no penalty if you chose 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

TITLE: Exercise, histamine receptors, and vascular function (Experiment 1)

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 but we do not understand all the mechanisms that cause this increase in blood flow or why it happens. The purpose of

this study is to provide more information on what causes blood flow increases to occur. This topic is both clinically and scientific 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 recreationally active and free from any know cardiovascular disease.

What will happen in the study?

Initial visit

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. 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. The questionnaire will take approximately 15 minutes to complete. You will need to wear a t-shirt and refrain from eating for two hours prior to arrival.
2) If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the initial 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.
4) During the initial 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.
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 initial height and weight measurements, completion of the survey, placement of electrodes on your skin, warm-up, exercise, and cool-down) is approximately one hour.

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 days. 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 one study visit. This testing session will take approximately five 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 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 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.
5) You will undergo the following procedures:

Microdialysis fibers in muscle: You will have 4 small probes (these are called "microdialysis fibers") placed into the vastus lateralus (outer thigh). First, the area of skin where the probes will enter and exit will be numbed with a local anesthetic (lidocaine/xylocaine with epinephrine). Then a small needle will be placed through the skin and through the muscle, exiting back out about 2-3 inches from where it entered your skin and muscle. The small probe will be passed through the needle, and then the needle will be withdrawn, leaving the small probe passing through your muscle. This process of inserting a small needle through the skin and muscle to place a probe will be repeated 4 times, once for each of the probes that are placed. The probes will be separated by about 1 inch. These 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 infusions through the fiber should not be painful, and there should only be minor swelling at the sites. We will put small doses of several drugs through the small probes in your muscle. The drugs you will receive include the following:

Microdialysis infusions: We will put small doses of several drugs through the small probes in your muscle. The drugs you may receive include the following:

a) Pyrilamine (a common ingredient in over the counter cold remedies). This drug blocks the effects of histamine and will prevent blood vessels in the

muscle from opening up if histamine is present. You should not feel anything when this drug infuses into your muscle.

b) Cimetidine (brand name Tagamet). This drug blocks the effects of histamine and will prevent blood vessels in the muscle from opening up if histamine is present. You should not feel anything when this drug infuses into your muscle.

c) Ethanol alcohol. This is used to determine how much blood flow muscle receives. You should not feel anything when this drug is infused into your muscle.

d) Alpha-fluoromethylhistidine. This drug prevents your body from producing histamine in the local area around the probe. You should not feel anything when this drug infuses into your muscle.

e) Histamine. This substance is naturally produced by your body and will cause blood vessels to open in the local area of muscles around the probe. You should not feel anything when this drugs is infused into your muscle.

f) Compound 48/80. This is a substance that releases histamine, which is naturally produced by your body, which will cause blood vessels in that local area of muscles to open. You should not feel anything when this drug infuses into your muscles.

g) Radioactive nuclide. Tritium is a radioactive form of hydrogen (known as a radioactive nuclide) that is produced naturally in the upper atmosphere or produced commercially for various uses. In this study, a small quantity of tritium will be infused through the microdialysis probe. This is being done to

assess the function of the microdialysis probes. You should not feel anything when this drug infuses into your muscle. Some of the tritium will be absorbed by your body and so you will be exposed to some radiation. This radiation exposure is not necessary for your medical care and is for research purposes only. The risk associated with this exposure is described below.

6) 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 microdialysis fibers will be in place while you exercise but should not cause any discomfort during exercise.
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 two hours after you exercise.
8) At the end of the study visit, the fibers 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 initial visit or the study visit. Some examples of discomfort include back pain, shortness of breath, light-headedness, and nausea.

How long will I be in the study?

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

What are the risks of the study?

1. Microdialysis: 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 infusions through the fiber should not be painful, and there should only be minor swelling at the sites. At the end of the study, the fibers 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.

2. Infusions: We will be infusing very small doses of each drug and only into a very small area of your muscle or skin. You will not have any systemic (whole body) effects of these drugs in the doses given in this study, unless you have an allergic reaction. There is a minimal risk that you are allergic to one of the drugs being infused during this study; however, an allergic reaction to the drug could include changes in blood pressure and difficulty breathing. Symptoms of an allergic reaction include: rash, itching, swelling, severe dizziness and trouble breathing.
3. Radioactive nuclide: This research study involves exposure to radiation from tritium, a radioactive label used to assess the function of the microdialysis probes. This radiation exposure is not necessary for your medical care and is for research purposes only. The total amount of radiation that you will receive in this study is equivalent to a uniform whole body exposure of less than 1 day of exposure to natural background radiation. For comparison, the amount of radiation you would receive from a chest X-ray is equivalent to 5 days of exposure and the amount of radiation you would receive from a dental X-ray is equivalent to 11 days of exposure. This use involves minimal risk and is necessary to obtain the research information desired.
4. Blood sampling for genotyping: In total, 10 ml of blood will be withdrawn, which is less than a tablespoon. The samples will be used for DNA analysis to determine which versions of specific genes you have related to the processing of histamine. There may exist potential risks of insurability, employability, and social discrimination if the results of individual DNA were to be made known to others. To safeguard against this risk, your DNA will be stored by an ID number only and your

name or other personally identifiable information will not be included with any data shared with other investigators. We will not reveal any genetic data to your physicians, or any other health care provider. 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. You have the right to withdraw your samples from research in the future should you decide to do so. 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. Samples will be kept for five years. After the 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. 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.

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

May I participate if I am pregnant or breast-feeding?

This study may be harmful to an unborn or breast-fed child. There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child in a woman who takes part in this study. Breast-feeding mothers are not able to take part in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours before taking part in the study visit. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

Are there benefits to taking part in this study?

This study will not make your health better. Fluid samples collected from microdialysis are not being collected for diagnostic purposes. The results will not be reviewed by a physician and no action will be taken if a laboratory result falls outside of the normal range. This study will help researchers understand the factors that control blood flow during recovery from 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 need to pay for any tests or procedures that are done just for this research study. You will get $105 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 of the study 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) 346-5425.

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 24 months 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 chose to abstain from the genetic testing portion of this study, please initial below. There will be no penalty if you chose 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

TITLE: Exercise, histamine, receptors, and vascular function (Experiment 6)

INVESTIGATOR: Dr. John Halliwill, Dr. Hans Dreyer, 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? The purpose of the current study is to examine the cellular changes that occur in skeletal muscle following aerobic exercise. In particular, we are interested in whether or not activation of histamine-receptor in response to exercise promotes the generation of certain cellular factors that eventually lead to the growth of new blood vessels within the skeletal muscle. Specifically, this research study is investigating the effects of a single bout of exercise on certain factors that are known to influence the growth of new blood vessels, and whether they are affected by drugs used to block histamine receptors. For this study, exercise will be performed with the thigh muscles of just one leg. We anticipate that 40 individuals will participate in this research study. This topic is both clinically and scientific 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 recreationally active and free from any know cardiovascular disease.

What will happen in the study?

<u>Initial Visit</u>

1. You will arrive at Dr. Dreyer's laboratory on the Second Floor of the Center for Medical Education and Research (CMER Building) for an initial visit. This initial visit will take approximately one hour.
2. 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. The questionnaire will take approximately 15 minutes to complete. You will need to refrain from consuming caffeine, medications or vitamins in the 12 hours prior to the study and refrain from exercise or alcohol for 24 hours prior to the study. You will need to wear a t-shirt, shorts, and refrain from eating at least two hours prior to arrival.
3. If you are a woman who can still become pregnant, you will be asked to undergo a pregnancy test during the initial 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.
5. During the initial 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 thresh or specific value that participants need to achieve; participants simply need to do their best. The total time for either test (including initial height and weight measurements, completion of the survey, placement of electrodes on your skin, warm-up, exercise, and cool-down) is approximately one hour.
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.
8. This session will serve to familiarize you with the procedures to be used on the study visit. 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 visit.

Study Visit

1. You will then return to Dr. Dreyer's laboratory to participate in the following protocol, which will be several days after the initial visit. This study visit will take approximately 5 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, Coke, RedBull) or medications (except oral contraceptives) for 24 hours prior to the study and will need to refrain from alcohol or exercise for 24 hours prior to the study. If you take oral contraceptives, you should take them when you normally would. If you are a women who can still become pregnant you will be asked to undergo a pregnancy test before the study.
3. During the study visits, your heart rate will be monitored by electrocardiogram electrodes placed on your skin. If you are a women, 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 cuff placed on your upper arm. The electrical activity of the muscle in your legs will also be monitored by electromyogram electrodes placed on the skin of the back and front of your legs. Before and after the exercise session, a small probe (Doppler ultrasound probe) will be held over an area of skin on your groin-hip intersection to image your femoral aretery. The ultrasound probe uses sound waves to meaure blood flow in your femoral artery. We will take periodic measurements before exercise and for the three hours of exercise recovery.
4. You will have one small flexible needle ("intravenous catheter") placed into a vein

near your elbow before the exercise protocol. The skin will be sterilized before this procedure. The catheter will remain in your skin throughout the three hour study. After the study, we will remove the flexible needle in your vein and a bandage will be placed over that area of skin. The catheter will be used to obtain samples of your blood. The amount that will be obtained is equal to about 6 tablespoons.

5. We will take one muscle biopsies from your non-dominant leg. This biopsy will be performed through a single incision and involves taking a small piece of muscle from your leg. First, a small patch of hair is removed (if needed) from your thigh and the skin is cleaned and sterilized. Next, the skin and tissue below are injected with a local anesthetic (numbing medicine) to eliminate pain. This medicine is similar to what dentists use to numb your mouth. After your leg is numb we will make a small incision about the size of this dash "____" at approximately mid-thigh. Through this incision a needle about the size of the letter "O" will be advanced into the muscle. A single piece of thigh muscle will then be removed with the needle, the skin closed with a single stitch and light dressing will be applied.
6. ☐ If this box is checked, 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) with a glass or water after the first biopsy. If the box is not checked, you will not receive these drugs.
7. You will be asked to perform one-legged knee extension exercise you're your dominant leg while seated on an exercise machine that measures how hard you are working. During these exercise sessions you will be asked maintain a selected kicking rate for 60 minutes.

8. Next, we will take two muscle biopsies from your dominant leg. Each biopsy will be performed through a single incision and involves taking a small piece of muscle from your leg. First, a small patch of hair is removed (if needed) from your thigh and the skin is cleaned and sterilized. Next, the skin and tissue below are injected with a local anesthetic (numbing medicine) to eliminate pain. This medicine is similar to what dentists use to numb your mouth. After your leg is numb we will make a small incision about the size of this dash "____" at approximately mid-thigh. Through this incision a needle about the size of the letter "O" will be advanced into the muscle. A single piece of thigh muscle will then be removed with the needle, the skin will be temporarily closed with a bandage between muscle biopsies and light dressing will be applied. One sample will be obtained immediately after you stop exercise (or the end of seated rest if you did not exercise). The other biopsy sample will be obtained 3 hours later from the same incision. Following the second biopsy from the same site we will close the incision site with a single stitch and dressing will be applied.
9. After the experiment you will go over things that you must do to help minimize the risk of infection. We will also provide a hardcopy of these instructions for you to take home. The handout is titled "Things to Remember After Muscle Biopsy," and must be taken seriously. We will also periodically contact you to see how the biopsy site is doing.
10. 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.

Follow-up Visits

We ask that you return to the Dreyer lab (for 20 minutes) 48 hours after the experiment to

allow us to inspect the biopsy site. You will return again at 7 days after the experiment to have your sutures removed and the biopsy site inspected. This will take about 20 minutes.

How long will I be in the study?

You may be in the study for up to 12 days. The initial visit will last for about 1 hour. The study visit will last 5 hours. Prior to the initial visit and study visit, you will need to refrain from eating for two hours, and refrain from consuming caffeine or medications for 12 hours and abstain from alcohol or exercise for 24 hours. You must also refrain from exercise 72 hours after the study visit. Following the study visit you will return two more times for follow-up visits. The first follow-up visit will be 48 hours after the study visit and the second follow-up visit will be 7 days after the study visit. Each of the follow-up visits will take approximately 20 minutes.

What are the risks of the study?

1. 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. There are no known risks associated with the oral administration of fexofenadine (brand name Allegra) or ranitidine (brand name Zantac) at this time.
2. The muscle biopsy carries the potential risk of pain, bleeding, bruising, infection, and scar formation at the site of the biopsy. Careful sterile technique should reduce the likelihood of any of these complications. The risk of bleeding is about 0.2%; the risk of bruising or a blue-and-black mark is 1.4%; and infection is so small that the precise number is unknown. After the study you have a 50% chance of experiencing soreness

at the site of biopsy for 24 to 48 hours. However, over the counter medications such as Motrin or Advil are sufficient to control such discomfort. Additionally, you may experience numbness around the area of the biopsy site (2x2 inches), which will likely go away with time (sensation returns) but in very rare instances may never return. The risk that you experience numbness is less than 0.5% and the risk that the numbness never goes away is much less. The scar will be approximately as long as the dash "____".

3. Blood sampling for genotyping: In total, 10 ml of blood will be withdrawn, which is less than a tablespoon. The samples will be used for DNA analysis to determine which versions of specific genes you have related to the processing of histamine. There may exist potential risks of insurability, employability, and social discrimination if the results of individual DNA were to be made known to others. To safeguard against this risk, your DNA will be stored by an ID number only and your name or other personally identifiable information will not be included with any data shared with other investigators. We will not reveal any genetic data to your physicians, or any other health care provider. 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. You have the right to withdraw your samples from research in the future should you decide to do so. 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. Samples will be kept for five years. After the 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. 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.

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

May I participate if I am pregnant or breast-feeding?

Women who are pregnant or breastfeeding may not participate in this study. Women who can still become pregnant must have a negative pregnancy test no more than 24 hours

prior to the day on which they might receive study drugs. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

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. 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.

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 $105 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. Hans Dreyer at any time about any questions you may have about this study. You may contact Dr. Dreyer by calling the Department of Human Physiology at (541) 346-5775. You can also reach Dr. Dreyer through email suing the following address hcdreyer@uoregon.edu. You may also 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) 346-5425.

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

Research Compliance Services
5237 University of Oregon
Eugene, OR 97403-5237

Eugene, OR 97403-1226 (541) 346-2510

(541) 346-3082

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 24 months 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 chose to abstain from the genetic testing portion of this study, please initial below. There will be no penalty if you chose 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)

APPENDIX B

ASSAY STANDARD OPERATING PROCEDURES

Tryptase ELISA

1. Determine wells for diluted calibrator, blank and sample. Prepare 7 wells for calibrator, 1 well for blank. Add 100 µL each of dilutions of calibrator (read Reagent Preparation), blank and samples (50ul) into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100 µL of Detection Reagent A working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.
4. Aspirate the solution and wash with 350 µL of 1X Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100 µL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for five times as conducted in step 4.
7. Add 90 µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15-25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

8. Add 50 µL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450 nm immediately

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).

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.

Ethanol Assay

1. Make EtOH Standards (use 2ml microtubes):
2. Make Sol'n A (NAD/EtOH Buffer) – light sensitive -> Falcon Tube wrapped in alum foil. Note - check pH of EtOH Buffer before each use – adjust with NaOH or HCl as appropriate. 50 µL NAD Sol'n per 10 mL EtOH Buffer (need 20 mL Sol'n A for entire plate).

3. Put 150 µL of Sol'n A into the appropriate wells (NUNC 96-well black plates, Fisher # 12-566-09).
4. Add 2 µL of Standards / Samples to wells in duplicate by pipetting just below the fluid surface of Sol'n A (duplicates in horizontal config, consecutive samples in vertical config).
5. Add 10 µL Solution B (ADH) to each well (use either multichannel or repeat pipette)
6. Cover with a plate sealer and incubate for 1 Hr in plate wrapped with foil.
7. Read on Biotek Plate Reader (Synergy HT) using "EtOH Protocol" Excitation = 360 Emission = 460

Quant-iT Total Protein Assay

1. Make working solution by diluting Quant-iT protein reagen 1:200 in Quant-iT protein Buffer
2. Load 200 µL of the working solution to each well of the microplate.
3. Add 10 µL of each BSA standard to separate wells and mix well.
4. Add 1 - 20 µL of each unknown protein sample to separate wells and mix well.
5. Measure fluorescence using a microplate reader (excitation 470, emission 570).

APPENDIX C

REAL-TIME POLYMERASE CHAIN REACTION

Subsequent to RNA isolation detailed in chapter VI, the concentration of RNA was measured via fluorometry (Qubit fluorometer, Invitrogen, Carlsbad CA, USA). RNA was reverse transcribed to cDNA from 1 μg RNA using a real-time polymerase chain reaction system (CFX96, BIO-RAD, Hercules, CA, USA) and a commercially available cDNA synthesis mix (iScript™, BIO-RAD, Hercules, CA, USA). All cDNA samples were then stored at -80°C until analysis. mRNA expression levels were examined by analyzing cDNA via SYBR Green fluorescence (IQ SYBR Green Supermix; BIO-RAD, Hercules, CA, USA). Beacon design software was used to design oligonucleotide primers specific for the Bio-Rad CFX96 real-time polymerase chain reaction detection system and to our genes of interest. Each polymerase chain reaction was measured in triplicate and contained the following:12.5 μl SYBR Green, 9.5 μl of Diethylpyrocarbonate - treated nuclease-free water, 0.5 μl of forward and reverse primers, and 2 ul cDNA template. cDNA was first denatured by an initial cycle at 95°C for 5 min. Thereafter, 50 cycles of denaturing at 95°C for 10 sec and 30 sec of primer annealing at the optimized primer pair-annealing temperature were carried out. All cycles were followed by a melt curve analysis. All genes of interest were normalized to the housekeeping gene GAPDH. mRNA expression was presented as a fold change relative to the housekeeping gene using the Livak method (116).

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