Histamine Metabolites as Biomarkers of Exercise Response: Implications for Vascular Function and Skeletal Muscle Adaptation

by

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DISSERTATION ABSTRACT

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As we unravel the complex integrative response to exercise, the physiological benefits of histamine are becoming clearer. Previous investigative efforts have characterized the role of histamine in post-exercise hemodynamics and have revealed its necessity in the adaptive response to training. Determining if histamine has a broader role that includes all types of exercise has been limited by our current detection methods. Thus, developing a novel means to determine its presence is an essential step toward a complete mechanistic understanding of histamine's production, release, and actions within the exercise response. Recent evidence suggests local histamine receptor activation coordinates cross-talk between the immune system and the recovering skeletal muscle tissues; however, our knowledge of histamine as a mediator of inflammation is largely limited to its actions following allergen exposure. In response to allergens, histamine is rapidly released to increase the permeability of the local tissue capillary network and facilitate the trafficking of immune cells toward target tissue sites. If similar steps are necessary for the recruitment of immune cells following exercise, histamine may be a necessary signal for the movement of immune cells between the circulation and skeletal muscle compartments. Additionally, this effect on capillary permeability may influence nutrient delivery and metabolite washout, altering the response during and following exercise. The results of this dissertation address these gaps in our knowledge of histamine in the response to exercise. Our results indicate that histamine is released in response to multiple forms of exercise, including steady-state aerobic and intermittent sets of heavy resistance exercise. We show that the production of its metabolites positively correlates with the increases in blood flow and vasodilation after exercise, supporting use of these metabolites as a novel means of detecting the histamine response across the exercise spectrum. Second, histamine's actions on vascular barrier function as a regulator of immune cell infiltration appear inconsequential. However, its actions on skeletal muscle oxygenation are significant and contribute to a change in the balance between

oxygen supply and demand during resistance exercise. We anticipate these findings will support efforts to characterize the histamine response in other research or applied settings. Further, these methods could potentially be used as a tool to manage overtraining progression. Our results provide evidence that histamine's unique actions on the exercise transcriptome is more likely imparted via actions on immune cells within the skeletal muscle tissue compartment in the acute phase of exercise recovery, rather than through regulation of cellular infiltration.

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CHAPTER I
ORIGINAL PROPOSAL

Across exercise type, distinct yet overlapping training responses may be mediated by histamine and its suggested influence on post-exercise hemodynamics, nutrient delivery, and exercise-adaptative gene expression [1–9]. Despite recent findings supporting histamine's actions on the long-term adaptive response to exercise training [8] there are still many gaps in understanding the histamine pathway within the context of exercise. Work from our lab has revealed two major processes (histamine formation and histamine release) contributing to exercise responses [10]. However, within the skeletal muscle microenvironment, it is unknown which cell types support these functions. Moreover, as neither process reflects the full magnitude of the histaminergic response to exercise, and because histamine has a notably short-half life in circulation, we lack strong tools to quantify the overall histamine response, thus, hindering the search for the exercise related signal(s) that promote histamine's actions. More complete explanations of histamine's actions as a vasodilator, regulator of endothelial permeability, and impact on immunological responses have been reported [11–15], however these processes, in the context of exercise, have yet to be characterized and the molecular signals responsible remain unresolved. To this point, advancements in our understanding of histamine's actions have been stifled by poor mechanistic clarity and an inability to easily quantify the entire histaminergic response to an exercising stimulus. Work that addresses these key gaps in literature are essential to developing novel therapeutic approaches through histamine's broad impact on the exercise responsome.

PURPOSE, SPECIFIC AIMS, AND HYPOTHESES

The purpose of this proposal is to clarify the physiological impact of an intact histaminergic response to exercise and to characterize the formation, release, and metabolism of histamine within the setting of exercising muscle. This work addresses key gaps in the literature by identifying cell types present within the skeletal muscle microenvironment participating in the histamine response, investigating novel biomarkers of histamine release, and to determine the

impact of intact histaminergic signaling mechanisms on the immunological responses to exercise.

1) Histological Identification (identifying cell types within resting skeletal muscle tissues responsible for histamine formation, release, and metabolism). Work from our lab has demonstrated skeletal muscle histamine concentrations increase during exercise through two major processes of formation and release, although our understanding of these processes is incomplete [10,16]. Further, the limited appearance of histamine in the systemic circulation suggests histamine's formation, release and metabolism are contained within the previously active tissues. To the best of our knowledge, mast cells are the primary site of histamine storage and release. However, evidence from other tissue types supports a role for non-mast cell formation and immediate release via histidine decarboxylase (HDC), commonly expressed on endothelial cells [17]. Within skeletal muscle tissue, the identification of cell types contributing to these processes are central to the development of future investigations targeting histamine's effect on exercise-induced health benefits. Through an immunohistological approach we will use the antibody for HDC (PA5-119194; Thermo Fischer Scientific) and histamine-N-methyltransferase (HNMT; 11874-1-AP; Thermo Fischer Scientific) in skeletal muscle tissue samples acquired from the vastus lateralis. Subsequent staining of the primary cell types found within skeletal muscle tissues samples (listed in Table 2) will be used to co-localize enzyme expression revealing cell type-specific capacity for histamine formation and/or metabolism. Hypothesis 1: Therefore, we hypothesize co-localization of HDC and HNMT with the cells present within skeletal muscle tissue samples will indicate cells participating in histamine formation and/or metabolism. Considering the strong possibility that these enzymes could co-localize with resident immune cell types, we will also use antibodies for mast cell tryptase (BSM-52533R; Thermo Fisher Scientific), leukocytes: macrophages (CD68; Abcam, Cambridge, MA), neutrophilic granulocytes (SPM250; Abcam, Cambridge, MA), and lymphocyte subtypes (CD3, CD4, CD8 and CD20; Abcam, Cambridge, MA) to determine immune cell type contributions to histamine formation and metabolism.

2) Biomarker Development (revealing novel biomarkers of the histaminergic response to exercise). Many studies have relied on measurements of post-exercise blood flow to assess histamine release [1,3,7,10,18,19], but this requires skill and equipment that is not readily available in some research settings. Clinically, tryptase and b-hexosaminidase have been used as biomarkers of mast cell degranulation but are unlikely to reflect the magnitude of histamine release when some portion of histamine comes from *de novo* formation. Two major pathways account for ~97% of histamine metabolism from the circulation [20] suggesting a biomarker based on the product of histamine breakdown, N-methylhistamine, may better represent histamine release in response to exercise. Hypothesis 2a: Therefore, we hypothesize a well-correlated response between histamine metabolite concentrations and the magnitude of histamine's well-established post-exercise blood flow response. Alternatively, in the absence of metabolite detection, measurement of known markers of mast cell degranulation (tryptase and b-hexosaminidase) still provide utility as indicators of a histamine response. Thus, measurement of mast cell degranulation markers, we hypothesize, will increase in concentration following exercise and correlate well with the changes in post-exercise blood flow. Furthermore, aerobic and resistance exercise elicit distinct yet overlapping training responses that we speculate are mediated by a histamine response to muscular work [1,2,5–9]. However, basic studies defining the magnitude of the histamine response between these exercise types are lacking. Presumably, high volume high load resistance training, due to the recruitment of more numerous muscle fibers and larger motor units, results in greater instances of metabolic stress within the active skeletal musculature that we speculate may elicit a larger, more robust histamine response that we will pursue in Aim 3. Hypothesis 2b: Thus, we hypothesize that the higher intensity contractions demanded by resistance exercise will result in greater histamine release relative to aerobic exercise. We also anticipate our findings will identify a robust novel biomarker of the histaminergic response to exercise and extend prior studies using aerobic exercise to resistance exercise. Alternatively, if aerobic exercise elicits a greater histaminergic response, then histamine's actions may be leveraged by energy substrate utilization via fat oxidation, or its subsequent breakdown products, providing guidance for future investigations.

3) Immunological Function (Exploring histamine's mediating role on the inflammatory recovery processes after exercise). Recently, poorer adaptive outcomes to intermittent high intensity interval training under histaminergic receptor blockade were displayed by Stede et. al., [8], a result, we speculate, may be mediated by the acute dysregulation of inflammatory processes during exercise recovery. Briefly, exercise initiates a profound non-specific immune response within previously active tissues and systemic circulation [21]. Clinically, histamine's contribution to allergic and inflammatory responses are viewed unfavorably in pathophysiological conditions (i.e., asthma, allergies, anaphylaxis, and tumor growth), whereas our understanding of its impact on exercise responses are only now being uncovered [22,23]. Histamine is amongst the first and most consistently released inflammatory mediators, facilitating activation, chemotaxis, and infiltration of inflammatory cell types within the previously active tissues [11–14] and directly modulating the expression of cytokines [15,24–30]. Hypothesis 3: Therefore, we hypothesize the use of histamine receptor (H1/H2) blockade will dysregulate the recruitment and infiltration of immune cell types, thus reducing the accumulation of leukocytes: macrophages (CD68; Abcam, Cambridge, MA), neutrophilic granulocytes (SPM250; Abcam, Cambridge, MA), and lymphocyte subtypes (CD3, CD4, CD8 and CD20; Abcam, Cambridge, MA) within previously active skeletal muscle tissue samples obtained from the vastus lateralis. Enzyme linked immunosorbent assays (ELISAs) will also be used to measure concentrations of exercise related cytokines: IL-6, IL-1 β, IL-8, IL-1ra, IL-10, IL-15, and tumor necrosis factor alpha (TNF-α), within the systemic circulation following exercise under histaminergic blockade. Together these findings, we speculate, will reveal a regulatory role of histamine on the acute inflammatory response to exercise and provide a mechanistic rationale for poorer adaptative responses to exercise training under histaminergic receptor blockade [8]. Alternatively, impairment of nutrient delivery and altered insulin sensitivity associated with histamine receptor antagonism, may support poorer adaptive outcomes following training (31-34). Protein synthesis pathways (mammalian target of rapamycin; mTOR) that are initiated by nutrient availability and insulin may be attenuated during the recovery period from exercise under histaminergic receptor blockade. Thus, western blot analysis of muscle samples acquired from the vastus lateralis we hypothesize will reveal reduced mTOR signaling protein

phosphorylation of protein kinase B (Akt), tumor sclerosis complex 2 (TSC2) and further downstream targets ribosomal s6 kinase 1 (p706sk) and ribosomal protein S6 (RPS6) [31–35] under histaminergic receptor blockade.

SIGNIFICANCE

Overall, this work advances our mechanistic understanding of histamine's role within the adaptive response to exercise. Previous work has established the presence of an unknown molecular signal(s) released within the previously active tissues that promotes histamine's actions. Results from aim 1 will extend previous findings and determine potential target cell types for the unknown molecular signal(s) thus, revealing cell types that express an enzymatic capacity for histamine production and metabolism that are potentially sensitive to the signal(s) coordinating histamine's actions. Findings from this work will therefore provide potential targets for future pharmacological interventions aimed toward revealing the identity of the molecular signal(s) responsible for histamine's exercise-induced health benefits. Under specific aim 2a, establishing biomarkers of histamine release will alleviate the need to measure changes in post-exercise blood flow as an indicator for histamine's presence following exercise, thus expanding our current methodology and providing others with an approachable means to determine histamine's presence within the context of exercise-related activity. Revealing histamine metabolite sensitivity to differences in contractile type and intensity (aim 2b) also provide an objective measurement of acute exercise training stress to be potentially used by athletes/coaches/training staff to mitigate development of overtraining symptoms and reduced the likelihood of overtraining related injury. Finally, work proposed in aim 3 will reveal an essential role for an intact histaminergic signaling pathway, indicating an essential histamine signal in the coordination of the immune response to an acute exercise session. Dysregulation of the acute immune responses accompanying exercise may account for long-term detriment to adaptive outcomes when training under histaminergic receptor blockade. Further, alternative findings from this work may reveal histamine's influence on pathways of protein synthesis that are well recognized as an integral signal of the adaptive response to exercise training.

CHAPTER II

NEW INSIGHTS

INTRODUCTION

Exercise promotes adaptation that serves as a first line non-pharmacological intervention [36,37] for reducing or delaying cardiovascular, musculoskeletal, and metabolic disease progression [38–42]. Specific adaptations share close association with exercise mode; however, it appears adaptive outcome(s) rely on an appropriate resolve of exercise-induced inflammation throughout the window of recovery [43–48]. In previous years, research efforts have focused on the role of cytokines as determinants of exercise performance, recovery, and adaptation often yielding complex or unclear results, however, a throughline of this exploration has been the necessary transition from pro-, to anti-inflammatory signaling necessary to complete the recovery process [45,46,48,49]. This suggests broad inflammatory mediators capable of casting a wide net of inflammatory influence may be an advantageous target for therapeutic intervention.
Although histamine's classic association with allergic and inflammatory responses have primarily been explored in pathophysiological conditions (i.e., asthma, allergies, anaphylaxis, and tumor growth), recent evidence has positioned histamine as an essential signal coordinating cross talk between the recovering skeletal muscle tissue and broader immune system in support of its well-recognized influence on the exercise response [6,8,50]. Thus, its actions as one of the first and most consistently released inflammatory mediators may extend to models of exercise-induced inflammation. However, investigations examining histamine's actions within this context are limited to date but are of primary interest to several research groups including our own.

PART I:

Targeting Histamine

Early observations of orthostatic intolerance in marathoners prompted a line of research implicating histamine as the primary vasodilator of the peripheral vasculature following

exercise[3,4,18]. Indeed, observations of depressed arterial pressures following muscular work were reported in some of the earliest literature examining exercise despite explanations for the phenomenon, termed post-exercise hypotension, remaining sparse for nearly a century[51–53]. Pharmacological blockade using over-the-counter antihistamine medications, inhibiting histamine receptors (H_1- and H_2), attenuates ~80% of the post exercise vasodilatory response following exercise, significantly impairing skeletal muscle blood flow[1], altering nutrient delivery dynamics[5], and reducing insulin sensitivity[2]. Further, histamine modifies the expression of >25% of nearly 3000 exercise-sensitive protein-coding genes[6] supporting histamine's involvement in various mechanisms of adaptive signaling in the response to exercise training[8,19].

Briefly, histamine is synthesized enzymatically via histidine decarboxylase (HDC)[22,23], and released into the cytosol where it is rapidly metabolized by two primary pathways (histamine n-methyltransferase, HNMT; monoamine oxidase-B, MAOB[20]), or preferentially conserved within granular storage sites of mast cells[20]. Current work from our lab aims to clarify the factor(s) promoting histamine release from mast cells, and/or its increase via enzymatic synthesis during exercise and exercise recovery [10,16]. We have learned the presence, and actions of histamine remain isolated within the active skeletal muscle tissues and some contribution from both mast cells and HDC, expressed on several cell types, contribute to rising histamine concentrations during exercise [10,18].

Accumulating evidence suggests that HDC may be expressed on several cells found within the skeletal muscle microenvironment and its function augmented across a physiological range of temperatures achieved during exercise[16,19]. However, recent evidence from Van der Stede et al., suggest that HDC is expressed primarily on mast cells within skeletal muscle and positions their production and release of histamine as essential for post-exercise adaptive responses to exercise[50]. Moreover, their results indicate that histamine receptor expression on immune cells appears to be a primary source of communication coordinating the transcriptomic responses to exercise and that differential activation or suppression of H_1- versus H_2-receptors may alter the recovering skeletal muscle environment. Interestingly, this work also indicates that the muscle-centric contribution in the adaptive response to exercise is limited, positioning immune cells, particularly those expressing histamine receptors, as essential to exercise-sensitive gene transcription.

In agreement, our own re-analysis of transcriptomic data from isolated human skeletal muscle fibers (Type I and Type IIa) and mononuclear cells from older, healthy participants[54] revealed the presence of mast cells, indicated by other granular contents besides histamine, tryptase (PSSAB1), and the uniquely expressed mas-related G-protein coupled receptor member X2 (MRGPRX2) within skeletal muscle fiber preparations. Though this finding suggests the deconvolution preparation utilized to separate skeletal muscle fibers from mononuclear cells may be incomplete, it confirms HDC expression was found within homogenized resting skeletal muscle samples of older individuals, and similar to younger participants from our previous work [6]. Additionally, re-analysis also indicates the presence of primary metabolizing enzymes, HNMT and MAOB, as well as histamine's receptors (H_1-, H_2-, and H_4-), that together with the findings from Stede et al.,[50] confirm resting skeletal muscle has the capacity to synthesize, degrade, and respond to the presence of histamine.

Additionally, metabolizing enzyme expression on fibro-adipogenic progenitor (FAP), and satellite cells indicate histamine may directly influence tissue repairing mechanisms carried out by these cells that have, to the best of our knowledge, not been directly investigated. Although we cannot speak directly to the enzymatic function, mast cell contents, or sensitivity of the receptors within the skeletal muscle environment between these two cohorts, it appears the functions of the resting skeletal muscle environment are well-preserved emphasizing histamine and its receptors as relevant targets for therapeutic intervention across the lifespan.

PART II:

Histamine: Broad Mediator of Inflammation

In response to one or several exercise-stress signal(s), histamine is released from granular storage compartments within nearby mast cells and/or synthesized via HDC[10] and is capable of binding to receptor targets that support immune cell recruitment. Activation of endothelial cell via H_1-receptors, promotes the release of cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor-a (TNF-a)[55–57] facilitating leukocyte recruitment toward areas of inflammation[58,59]. Similarly, cytokine release into circulation activates adhesive integrins (CD11b) on leukocyte cell surfaces that directly influences transit time resulting in slowed leukocyte rolling kinetics

and improving firm, shear-resistant adherence to surface selectins[60–63]. However, histamine activation on endothelial cells is not the sole source of circulating factors, as IL-6 and IL-8 release are influenced independently by muscle contraction[64–67], although exercise conditions supplemented with carbohydrate, to reduce energetic stress, reduce skeletal muscle IL-8 expression while circulating concentrations remain unaffected[68] and potentially highlight a histamine-mediated release of IL-8 from endothelial cells that functions independently from muscle contraction.

In agreement, many studies have implicated mast cells as essential for the initiation of inflammatory responses and effective recruitment of leukocytes to activation sites[69–71]. Chemically induced mast cell degranulation via compound 48/80, binding to the MRGPRX2 receptor, was accompanied by nearly instantaneous recruitment and rolling of leukocytes along endothelial surfaces[70]. The use of H_1-receptor antagonist, diphenhydramine, inhibits mast cell associated leukocyte responses[70] directly implicating histamine. Inconsistencies in this response have been reported[72] but are likely attributable to the differences in pharmacological blockade of H_1-receptors, via mepyramine opposed to diphenhydramine, as inhibition of other factors released from mast cells including leukotrienes, platelet-activating factor (PAF), or serotonin, did not alter the leukocyte response following mast cell degranulation and highlight the importance of histamine as an essential signal in this step of inflammatory initiation[70,73].

As leukocytes are attracted toward sites of inflammation, movement into the tissue compartment is influenced by establishment of weak and transient interactions between the circulating leukocyte and endothelial cell surfaces[74] and in response to histamine, endothelial cells rapidly express docking structures, termed selectins, on their vascular facing surfaces[75]. Leukocyte recruitment appears dependent, almost exclusively on P-selectin activation as antibodies against it completely inhibit adherence and rolling in some investigations[76]. An absence of histamine signaling does not appear to abolish this interaction completely, as activation of selectins can be driven by pro-inflammatory cytokines, like IL-1b and TNF-a[74,77], thus histamine, when present may act to enhance adhesive integrin expression and chemotactic signaling in this instance and potentially reduce the magnitude of pro-inflammatory cytokines necessary to elicit an effective immune response.

Consequently, the combined use of H_1- and H_2-receptor blockade may blunt the inflammatory responses to muscle damaging exercise as similar circulating TNF-a and IL-6

concentrations with and without blockade have been observed despite lower perceptions of muscle soreness, and greater instances of muscle damage measured via creatine kinase in the blockade condition[78]. This suggests that proliferative, and secondary damage effects caused by infiltrating immune cells, contributing to delayed onset muscle soreness (DOMS), may have been limited due to histamine receptor antagonism. Thus, histamine receptor antagonism, in this case, may impair immune cell infiltration diminishing their capacity to restore tissues following damage[79–81], and subsequently reduce adaptive signaling pathways through inflammatory resolve[82,83] and provide some context to the blunted adaptive response to training with H_1- and H_2-receptor blockade[8].

PART III:

Calling All Cells

Exercise type, time, and intensity influence the acute biphasic transition of immune cell populations within the systemic circulation as neutrophils are mobilized from the bone marrow toward sites of stressed, or previously active tissues [84,85]. Well documented are the acute leukocyte responses to high-force exercises, like resistance exercise, that quickly resolve and are replaced by a more prolonged period of immune cell activation referred to as delayed leukocytosis [86,87]. Importantly, delayed leukocytosis is characterized by a mobilization of immature, or non-segmented band neutrophils from the bone marrow that increase in concentration over the hours following exercise [88]. Although several synthesized factors have been postulated as the primary mediators of the acute neutrophilia in response to exercise (ie., IL-6, IL-8, or IL-1b) consistent patterns of increase measured in circulation are not correlated with the patterns of immune cell mobilization in all cases [88].

Suwa et al., [89] describe the biphasic relationship between exogenously introduced IL-6, and neutrophil mobilization of immediate neutrophil sources from marginated pools, and catalyzing an eventual recruitment of band neutrophils from bone marrow. Demargination of adherent neutrophils from the endothelium by IL-6 appears independently from hemodynamic changes, as IL-6 does not directly influence baseline hemodynamics [89] however, our understanding of IL-6's actions during exercise may be convoluted by the hemodynamic strain

associated with exercise across investigations [90]. Importantly, and independent of cardiovascular adjustments to exercise, IL-6 does reduce transit time of neutrophils from the bone marrow, and appears more intimately involved in the determination of neutrophil pools within the bone marrow, and may therefore have greater relevance within the hormetic response to exercise training as immune cell cycles are better realized [91].

The results of neutrophil migratory characteristics highlight the importance of an unknown exercise-related factor(s) contained within plasma samples obtained during exercise recovery [88]. Although limited changes to histamine concentrations are observed in circulation, histamine has been shown to alter the transcription of IL-6, and other cytokines in the skeletal muscle response to exercise over a 3h period of recovery [6]. Thus, the likelihood that histamine has a direct impact on the patterns of immune cell mobilization from bone marrow is unlikely, however histamine's direct impact on the peripheral vasculature, and its new role as a determinant of exercise adaptation suggest it is capable of altering the inflammatory environment that coordinates the immune response.

Ely et al., hypothesized attenuating histamines actions via pharmacological histamine-receptor blockade would attenuate inflammatory responses to a prolonged downhill running exercise protocol [78]. 60-minutes post exercise their results revealed no effect of histamine receptor antagonism on systemic markers of inflammation, cytokines tumor necrosis factor-a (TNF-a) or interleukin-6 (IL-6) [78]. Although downhill running induces modest muscle damage, evidenced by significantly elevated creatine kinase levels post-exercise in both conditions, the preserved inflammatory profile was surprising given histamine's previously established role as a mediator of inflammation [55,92,93]. Later investigations into the circulating immune cell responses would conflict with these findings as exercise with histamine receptor blockade increased peak leukocytes and neutrophil counts in the hours that followed eccentric skeletal muscle work [94], and clearly highlight histamine's influence in the immune response to exercise.

PART IV:

Regulation of Vascular Barrier Function

Possibly, histamine's actions as a vasodilator and regulator of vascular permeability may have been limited with histamine-receptor blockade in this investigation, leading to the higher counts of immune cells measured in circulation after exercise. In a closed system it is challenging to separate the effects of vascular barrier function and hemodynamic adjustments occurring with exercise (and presumably histamine release). Additionally, the mechanism of barrier function in response to inflammation remain undefined, are not uniform, and appear to respond differently to changes in activation site, the inducing signal, and the corresponding mediator(s). The vascular barrier is comprised of an endothelial cell monolayer, including the basement membrane and surface glycocalyx structure, and the surrounding pericytes[74,95], noted for regulatory function of solute, fluid, and leukocyte movement via trans- and paracellular mechanisms[96].

Micro-vessels found within the peripheral vasculature display greater quantities of adheren junctions providing the predominant barrier to cellular movements[97] and are directly impacted following histamine's release. H_1-receptors on endothelial cells stimulate phospholipase C-b family (PLCb) to activate second messengers, inositol 1,4,5-bisphosphate (IP3) and diacylglycerol (DAG), raising calcium (Ca^{2+}) within the cytoplasm, activating protein kinase C (PKC), resulting in impaired vascular barrier function[98]. Yet, this Gaq pathway only partially explains changes in vascular barrier function as histamine also stimulates the guanine nucleotide-exchange factor, Trio, downstream from Gaq which phosphorylates myosin light chain kinase through transforming protein RhoA, and serine-threonine kinase ROCK activation to increase tension of vascular-endothelial (VE-) cadherin complexes and results in the widening of adheren junctions between adjacent endothelial cells, (ie., increasing vascular permeability)[99].

The endothelial glycocalyx layer, in addition to its barrier function, imparts significant influence on the resistance to blood flow in the microvascular network[100] making it a unique structure. The endothelial glycocalyx is found along the luminal side of endothelial cells and is noted for its ability to transmit mechanical shear-stress signals to the underlying endothelial cells upregulating nitric oxide (NO) production[101]. Disruption of the glycocalyx structure attenuates

this function, but vasodilatory capacity of the peripheral vessels can be maintained by an intact histaminergic signaling mechanism on endothelial cells[101]. Further, the glycocalyx layer displays a remarkable capacity to modulate inflammatory signaling by attenuating circulating cytokine binding on endothelial surface receptors[102] and equally, to enhance leukocyte-endothelial cell interactions following degradation[103].

Systemic inflammatory conditions, like atherosclerosis, diabetes or obesity[104], can result in a long-term degradation of the endothelial glycocalyx structure through an increase of circulating reactive oxygen species (ROS), resulting in impaired regulation of NO synthesis[105]. It is unclear if degradation of the glycocalyx occurs following exercise, in fact it may expand[106] or be preserved[107] in some cases. However, we have established the exercise-induced production of ROS does not explain the presence of histamine[108] thus, our understanding of the interactions between vascular barrier function of the endothelial glycocalyx and the histamine signal remains incomplete. Mast cell degranulation results in the release of glycocalyx degrading molecules (heparinase), presumably in addition to the release of histamine, emphasizing the importance of NO signaling redundancies[101] to maintain endothelial function in the face of inflammatory perturbation.

PART V:

Conclusions and Remaining Gaps in Literature

To some degree, histamine's effect on immune cell recruitment, shear stress, adhesive interactions with endothelial cells, and/or mechanism regulating vascular barrier function and their impact on pathways of exercise-indued inflammation may be altered if histamine's actions are pharmacologically inhibited. This remains an area that is underexplored but may explain the decline in long-term benefits of exercise reported by Stede et al.,[8] as the acute inflammatory cascade is altered under H_1- and H_2-receptor antagonism. Importantly, it appears histamine may facilitate multiple steps in the extravasation of immune cells, and the broader inflammatory response to exercise, and if a single component of immune cell extravasation process (including factors influencing rolling, activation of selectins, and migration into tissue) is altered transmigratory effects may be reduced, but not completely abolished[109].

Investigation using HDC-knockout mice have displayed an aberrant inflammatory response following hind-limb injury that resulted in a greater magnitude of immune cell recruitment (CD11b+ cells; neutrophils, monocytes/macrophages, granulocytes, NK-, and T-cells [61,110,111]) measured in the peripheral blood and skeletal muscle tissues implicating chemoattractant signaling and endothelial-adhesive interactions that do not rely solely on an intact histamine signal [112]. However, HDC knockout mice also displayed delayed tissue regeneration mirrored by reduced anti-inflammatory cytokine expression within the damaged tissues, thus pathways supporting tissue regeneration and inflammatory resolve appear to be significantly influenced by histamine's presence [112], and support mechanisms of histaminergic crosstalk during recovery [50].

In response to one or several stress signal(s), histamine is released from granular storage compartments within nearby mast cells and/or synthesized via HDC and is capable of binding to receptor targets that support immune cell recruitment and infiltration into the previously active tissues [10]. The recent investigations conducted by us, and now others [50], have failed to examine the contribution of infiltrated immune cells and have instead captured a change in the phenotype of immune cells residing within the skeletal muscle tissue during recovery.

While measurements of sustained or attenuated vasodilation and blood flow responses after exercise are an interesting phenomenon, these measurements are a poor proxy for quantifying the magnitude of the histaminergic response and provide limited insight on the inflammatory responses to exercise. Similarly, an inability to easily assess histamine's effects on the microvasculature, independently from its effect on conduit vessels, via bulk blood flow measurements, is a considerable limitation in our understanding of histamine's actions on the structures that regulate vascular barrier function.

CHAPTER III
CHALLENGES AND ALTERNATIVE APPROACHES

OVERVIEW

During the completion of this dissertation several challenges were presented resulting in alteration to our project goals and timeline. The COVID-19 pandemic created a unique challenge for researchers worldwide and significantly stifled progress across our lab. As a result, the planned execution of these studies was altered following conception to accommodate factors related to this, and to incorporate findings from our collaborators within a niche research field.

SPECIFIC AIM CONSIDERATIONS

Aim 1 Considerations:

Histological Identification (identifying cell types within resting skeletal muscle tissues responsible for histamine formation, release, and metabolism), proposed serial immunohistochemical staining of skeletal muscle samples to determine the contribution of specific cell types that produce and release histamine during exercise. Although immunohistochemistry is a powerful technique to detect and identify structures and cell types within tissue sections, it is a slow and arduous process. Van der Stede et al.,[50] utilized paired single-cell and single-fiber transcriptomics to reliably detect the histamine-synthesizing enzyme histidine decarboxylase (HDC) within the skeletal muscle environment. Further, their work indicates that the expression of HDC is most prevalent in mast cells and, importantly, not expressed in other cell lines within skeletal muscle tissue. This observation, together with our previous work [10], collectively addressed the primary objective outlined by Aim 1.

Following the completion of data collection for Aim 2, a colleague brought to our attention the potential utility of measuring the terminal metabolite 1-methyl imidazole acetic acid (MIAA) over the proposed 1-methyhistamine as a urine biomarker of histamine production. The

polar nature of MIAA is a common property of substances that are readily excreted by the kidney as a final pathway for clearance from the system. To address this, we re-analyzed our biological samples (urine and plasma) collected during aim 2 to quantify histamine, 1-MH, and MIAA. This required the development of an updated assay and its validation by our collaborators at OHSU's Bioanalytical Shared Resource/Pharmacokinetics Core Facility. This effort resulted in a more thorough analysis of histamine metabolites, improving our research design. As presented in Chapter IV, this expanded analysis identified what appears to be a more robust biomarker of histamine release in response to exercise.

*Aim 3 Considerations**:***

Immunological Function (Exploring histamine's mediating role on the inflammatory recovery processes after exercise). Our initial proposal included monitoring the infiltration of monocyte populations within skeletal muscle tissue after exercise. Additionally, protein synthesis pathways (mammalian target of rapamycin; mTOR) that are initiated by nutrient availability and insulin may be attenuated during the recovery period from exercise under histaminergic receptor blockade and may not persist until 24- and 48-hour after exercise. Thus, a shorter time course for detection was employed to determine the acute disruption to immediate neutrophil infiltration and mTOR 4-hours after exercise with and without histamine receptor antagonists. Finally, we added the use of an essential amino acid supplement during the recovery from exercise to supply nutrients necessary to upregulate mTOR beyond exercise, alone. Unfortunately, our ability to capture changes in mTOR activation via western blot following exercise are incomplete but will remain a goal of our laboratory beyond this dissertation. Methods and available results are presented in Chapter V, but conclusions are not based on these results.

Finally, while Van der Stede et al., provide evidence of a histamine-mediated cross-talk among immune cells within skeletal muscle tissue in response to exercise, the methods they employed are unable to differentiate between responses imparted on resident immune cells or by immune cells trafficked to the skeletal muscle after exercise [6]. Ely et al., [94] highlight an increase in circulating immune cells following muscle-damaging exercise with H_1- and H_2-receptor antagonists[98], however, it is unknown if this increase is explained by poor extravasation or if the

stress imposed by poor skeletal muscle perfusion during blockade drives a greater inflammatory stimulus. Therefore, aim 3 was also adjusted to characterize histamine's role in the perfusion of skeletal muscle during resistance exercise. It was presumed, a greater decline in skeletal muscle oxygenation would be present during exercise with histamine receptor antagonists, lending support to histamine's actions in the balance between oxygen demand and supply during exercise.

UPDATED AIMS

Aim 1: Biomarker Development (revealing novel biomarkers of the histaminergic response to exercise).

Hypothesis 1a: We hypothesize a well-correlated response between histamine metabolite concentrations and the magnitude of histamine's well-established post-exercise blood flow response. Alternatively, in the absence of metabolite detection, measurement of known markers of mast cell degranulation (tryptase and b-hexosaminidase) still provide utility as indicators of a histamine response. Thus, measurement of mast cell degranulation markers, we hypothesize, will increase in concentration following exercise and correlate well with the changes in post-exercise blood flow. Hypothesis 2b: We hypothesize that the higher intensity contractions demanded by resistance exercise will result in greater histamine release relative to aerobic exercise. We also anticipate our findings will identify a robust novel biomarker of the histaminergic response to exercise and extend prior studies using aerobic exercise to resistance exercise. Alternatively, if aerobic exercise elicits a greater histaminergic response, then histamine's actions may be leveraged by energy substrate utilization via fat oxidation, or its subsequent breakdown products, providing guidance for future investigations.

Aim 2: Immunological Function (Exploring histamine's mediating role on the inflammatory recovery processes after exercise).

Hypothesis 2a: We hypothesize the use of histamine H_1- and H_2-receptor blockade will reduce the recruitment and infiltration of neutrophils 4-hours after exercise within previously

active skeletal muscle tissue samples obtained from the vastus lateralis. Hypothesis 2b: We hypothesized skeletal muscle oxygenation determined via near-infrared spectroscopy during resistance exercise would be lower during with H_1- and H_2-receptor antagonists, increasing the hypoxic stimulus within exercising tissue, and promote a superior blood flow response compared to the placebo-control condition.

CHAPTER IV
EVIDENCE OF HISTAMINE RELEASE IN RESPONSE TO BOTH AEROBIC AND RESISTANCE EXERCISE: HISTAMINE METABOLITES AS BIOMARKERS

INTRODUCTION

Histamine's canonical role in physiology includes functions as a neurotransmitter involved in the regulation of wakefulness, hunger, learning, and memory, and as an inflammatory mediator capable of altering microvasculature function (i.e., increasing vascular permeability), mobilizing and differentiating immune cells, and regulating cytokine expression [113–116]. Conventional wisdom highlights histamine's actions, particularly in response to allergens, as deleterious if left unresolved and has propelled the development and widespread use of antihistaminergic medications [56,117–119]. More recently, the prevalence of allergies within athletic populations has sparked a resurgence of histamine investigation, with alternative evidence shifting our view to support histamine's relevance during exercise as a determinant of performance, recovery, and adaptation [8]. Histamine is an essential signal of the vasodilatory response, reducing resistance to blood flow within the previously active skeletal muscle vascular beds (increased vascular conductance) supporting the elevated patterns of blood flow measured throughout exercise recovery [6–8,18,78,120].

Certainly, the presence of a sustained vasodilation and enhancements in blood flow after exercise are an interesting phenomenon however, sensitivity of these measurements may be a poor proxy for quantifying the complete magnitude of the histaminergic response [18]. Although we have established methods for detecting histamine's presence during exercise, histamine has a relatively short half-life, and its localization following release requires use of invasive techniques (i.e., intramuscular microdialysis or arteriovenous catheterization) to quantify its presence [1,10,19]. Furthermore, use of surrogate measures, like post-exercise blood flow, require skilled use of expensive equipment (i.e., vascular ultrasonography) not commonly found in most research or athletic settings [1,3,7,10,18,19,121]. And lastly, the bulk of our insights stem from investigations utilizing models of primarily aerobic exercise and limit the relevance of histamine's actions across the complete spectrum of exercise.

Notably, our work investigating the exercise-related production and release of histamine has revealed two separate pathways that accompany exercise [10]. These findings suggest multiple mechanisms contribute to rising histamine concentrations following exercise [8,122] while only two major pathways appear to account for ~97% of histamine metabolism, histamine-*n*-methyltransferase (HNMT) and diamine oxidase (DAO) [20], suggesting a biomarker based on the product of histamine's breakdown may better represent the magnitude of histamine release initiated by exercise. Our previous transcriptional analysis of exercised skeletal muscle has revealed little, or no contribution of DAO within the peripheral tissues after exercise and is therefore an unlikely contributor to histamine breakdown in this context [6].

We therefore suspect contribution of histamine-*n*-methyltransferase, HNMT, producing the primary breakdown products, 1-methylhistamine (1-MH), and its terminal metabolite 1-methyl imidazole acetic acid (MIAA), as shown in **Figure 4.1**, may yield a more suitable pathway for determining the fate of histamine released from skeletal muscle during exercise. We hypothesized exercise would promote a rise of these metabolite concentrations in circulation throughout exercise recovery, and measurements of their production rates in urine would reveal an acute sensitivity to exercise stress that positively correlates with the sustained increase in blood flow and vascular conductance supported by histamine following exercise.

Finally, histamine's role as a molecular transducer of exercise adaptation hinges on its constitutive presence across the complete spectrum of exercise, and includes sensitivities to exercise type, time and intensity. Although it is challenging to quantify the volume of exercise work completed between two modes of exercise, we anticipate evidence of histamine's metabolites following resistance exercise will extend our previous findings to include models of resistance exercise and support the utility of these metabolites to more easily quantify histamine's presence. Thus, a secondary aim of this investigation examined the response magnitude of histamine release by assessing the relationship of 1-MH and MIAA production and the sustained rise in blood flow and vascular conductance promoted by both aerobic and resistance exercise where we hypothesized the greater intensity and skeletal muscle mass innervated may yield a superior histamine response compared to aerobic exercise.

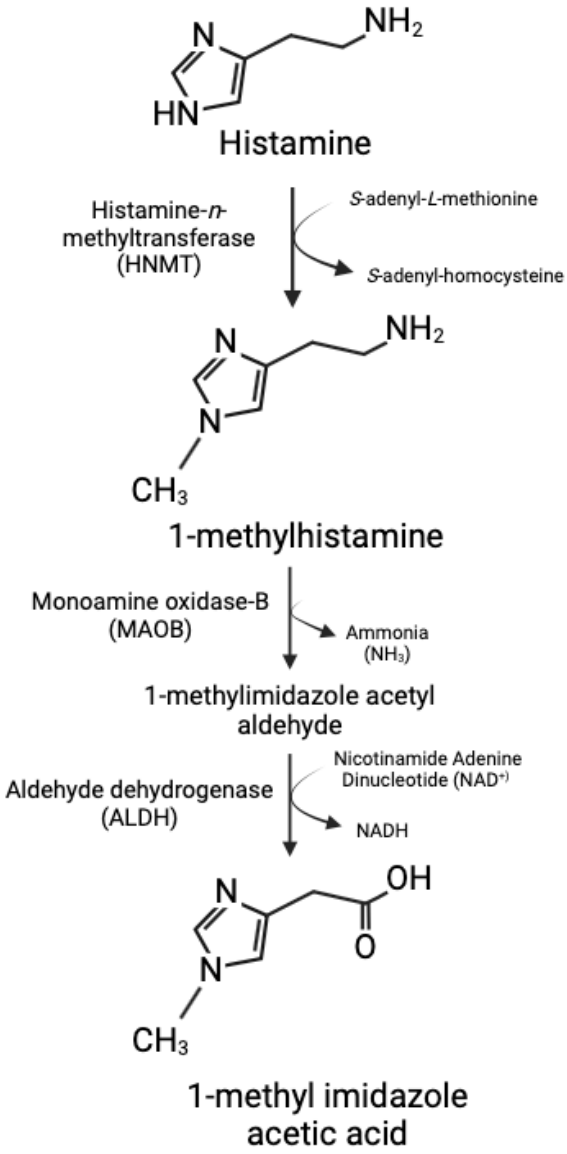

Figure 4.1 Fate of histamine released from skeletal muscle tissue. Histamine-n-methyltransferase (HNMT) utilizes *S*-adenosyl-*L*-methionine as a methyl donor to inactivate histamine in non-neuronal tissue and produces 1-methyhistamine (1-MH, also known as n-methylhistamine) as its primary metabolite [123]. 1-MH undergoes oxidation to 1-methyl imidazole acetyl aldehyde by monoamine oxidase-B (MOAB) before being further oxidized to its terminal metabolite 1-methyl imidazole acetic acid (MIAA) via aldehyde dehydrogenase (ALDH) [124,125].

MATERIALS AND METHODS

This study was approved by the Institutional Review Board at the University of Oregon. Written informed consent was obtained from all participants and the study conformed to the principles of the Declaration of Helsinki.

Participants

Twelve individuals (1F, 11M) between the ages of 18-35 participated in this investigation. All participants were healthy non-smokers. Inclusion criteria included being avid exercisers that satisfied the criteria for Tier 2 or Tier 3 as defined by McKay et al., [126], the ability to perform a traditional back squat 1-repitition maximum (1-RM) of between 1.25 and 3.0 times their body weight, and the ability to achieve a VO_{2peak} between 45 and 63 $mL \cdot min^{-1} \cdot kg^{-1}$ for males or between 32 and 46 $mL \cdot min^{-1} \cdot kg^{-1}$ for females (50-90th percentile of respective age and biological sex cohorts [127]). Exclusion of participants who were not considered moderate- to highly trained in one or both exercise modalities was used to minimize the impact of task novelty on outcomes. Participants self-reported as free of prescription medications, performance enhancing drugs, and dietary supplements with antioxidant or recovery altering properties.

Screening Visits

Participant characteristics including anthropometrics and exercise performance for peak aerobic capacity on a cycle ergometer ($VO2_{peak}$) and maximal strength of the traditional back squat (1-RM) were determined during two separate screening visits (one for each performance test), separated by a minimum of 72-hours (**Table 4.1**). Participants were asked to complete a three-minute warm-up on the cycle ergometer set at a resistance of 60 watts (W). Immediately following, the resistance was increased by 0.5 W per second (W/s) and continued until volitional exhaustion. The test was terminated when participants were no longer able to maintain a cadence greater than 60 revolutions per minute, despite strong verbal encouragement. Expired gases were analyzed breath-by-breath for determination of oxygen consumption (VO_2) (ParvoMedics, Salt

Lake City, UT, USA). A participant's VO_{2peak} was determined to have been reached if their respiratory exchange ratio exceeded 1.1, RPE of 17 or greater, or a plateau in heart rate or VO_2 was observed despite further increases in workload. Test termination criteria followed ACSM guidelines [128]. The 1-RM assessment was conducted per National Strength and Conditioning Association (NSCA) guidelines and was overseen by a well-trained member of the research team [129]. Briefly, participants were asked to complete a warm-up on a cycle ergometer at a self-selected intensity for 5 minutes, followed by 10-bodyweight squats, and 10-walking lunges. The research team then fit the participant to the weight rack, adjusting bar height and safety bars to a height that allowed for a full range of motion, but limit beyond the range of safety in the event of a failed attempt. Participants were asked to perform 3 warm-up sets of traditional back squat ranging from a light to moderate intensity (50-80% estimated 1-RM) and for no more than 10, 5 and 3 repetitions for each set. Participants then attempted their first 1-RM attempt and were allotted 3-5 minutes of rest time to recover prior to beginning the second attempt. Following each successful attempt, the load was increased, and the greatest load successfully completed by the participant, with appropriate form, and without assistance was deemed their 1-RM.

Table 4.1 Participant Characteristics

n	12 (1F, 11M)
Age (years)	22.4±2.6
Height (cm)	176.9±7.5
Weight (kg)	75.1±10.5
BMI ($kg \times m^{-2}$)	23.8±1.5
VO_{2peak} ($ml \times min^{-1} \times kg^{-1}$)	51.9±6.9
1-repetion maximum, 1-RM (kg)	119.8±26.9
Relative 1-RM ($kg \times bodyweight^{-1}$)	1.6±0.3

Data presented as mean±SD

Experimental Design

Figure 4.2 depicts the timeline of assessments during the two experiment days. Participants were asked to complete a randomized cross-over design with aerobic and resistance exercise visits separated by one-week. Upon arrival for exercise visits (Resistance or Aerobic), baseline blood samples were collected in a semi-recumbent seated position before beginning a supine rest to obtain baseline (BL) hemodynamic measurements including arterial blood pressure, heart rate, and common femoral artery blood flow. Following, participants were outfitted with a wireless heart rate monitor (Polar Electro Inc. Lake Success, NY, USA) and escorted to either the cycle ergometer (Lode BV, Groningen, Netherlands) or the strength lab to complete a brief standardized warm-up before being asked to complete the required exercise for that day. During the aerobic exercise visit this warm-up included a five-minute cycling period with beginning intensity set at 60 watts (W) and a gradual increase to working intensity achieved before the end of the final minute. During the resistance exercise visit, participants were allotted a five-minute cycling warm-up at a self-selected intensity, followed by 10-walking lunges, and 10-bodyweight squats. Participants were then asked to engage in the prescribed exercise for that day with intensity set based on their previously determined aerobic and resistance maximal values (VO_{2peak} and 1-RM). Following completion of the exercise session participants were escorted immediately to the resting table and placed in a supine position. Blood samples were acquired immediately post-exercise, and hemodynamic variables were assessed following successful intravenous catheter placement and blood draw. Participants were then asked to remain supine for the duration of a 120-minute exercise recovery period. Hemodynamic measurements and blood samples were acquired at each of the remaining 30-, 60-, 90-, and 120-minute time points. To determine concentrations of histamine's metabolite, 1-methyhistamine, captured in urine participants were asked to collect their urine for the 24-hours preceding their arrival for each exercise condition (PRE), again following the 120-minute recovery (2H), and separately for the 24-hours following end of exercise (24H). Participants were allotted a 1-week washout period between exercise sessions and asked to return to the lab having followed the required abstentions and repeat the protocol with the opposing exercise condition.

Rigor and Reproducibility

For assessments, participants were asked to arrive having abstained from food and drink (except water) for at least 2 h, caffeine for 10 h, vigorous and/or high intensity exercise, alcohol, and over-the-counter medications for 24 h. Women were studied irrespective of menstrual cycle phase and had a negative pregnancy test prior to all assessment days. Data collection start-time varied across participants due to availability; however, assessment start-time was consistent within a participant to minimize circadian differences across visits. Voluntary dietary logs were not collected; however, subjects were encouraged to monitor their activities and eat similarly preceding exercise sessions and asked to avoid foods well known for their histamine loading properties (i.e., fish). All protocol activities took place in a thermoneutral lab environment.

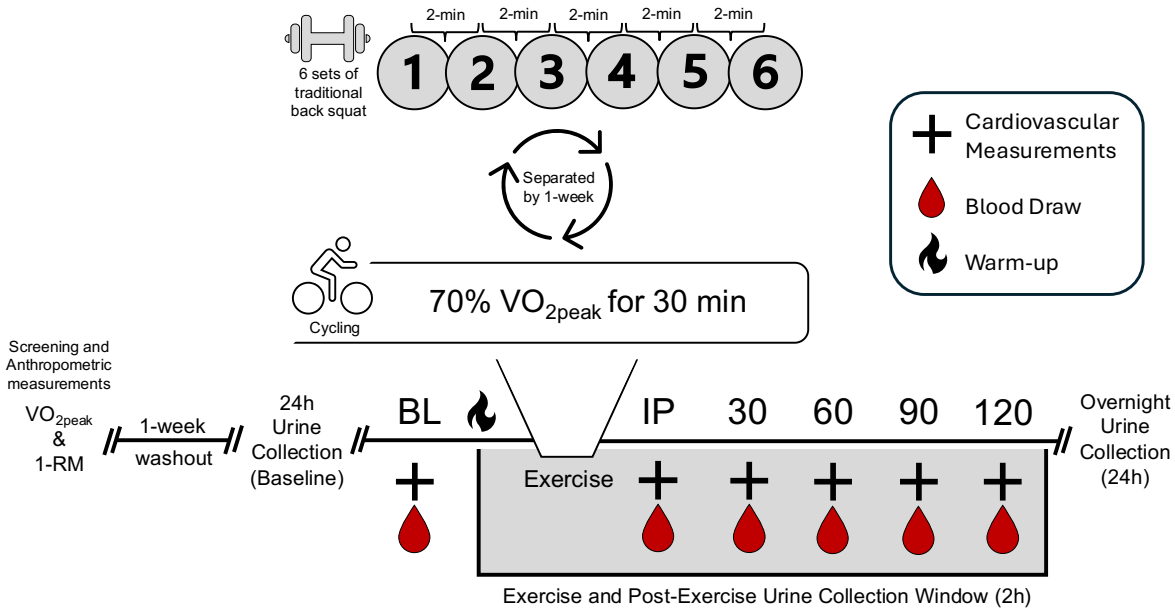

Figure 4.2 Timeline of assessments before and during the two data collection visits. Cardiovascular measurements (crosses), blood samples (red drops), were collected at baseline (BL), immediate postexercise (IP), and at 30-, 60-, 90-, and 120-min post-exercise. Urine was collected 24-h before the visit, during and up to 2h after exercise, and 24-h after exercise.

Exercise Visits (Aerobic and Resistance Exercise)

Following a brief 5-minute warm up at 60W, participants cycled for 25-minutes at 70% of their previously determined power at VO_{2peak}. Spot checks for VO_2 were assessed during the exercise session to ensure accurate exercise prescription for each participant. Participant RPE (Borg scale, 6-20), and heart rate were assessed throughout the exercise session. Average responses to aerobic exercise condition are displayed in Table 1. The resistance exercise session required participants to complete a 6 set by 10 repetition squat protocol set at 10-RM (based on the highest weight achieved during their 1-RM determination). Each set was separated by 2-minutes of seated recovery during which RPE, and perceived recovery status were collected for the preceding set(s) [130]. Before beginning he subsequent set(s) participants were asked to indicate their perceived recovery status assessed on a scale to determine perceived muscular fatigue [131]. To ensure participant safety, participants that indicated an inadequate recovery status (≤2, very poorly recovered) were allotted an additional 30 seconds of recovery time before being reassessed for their recovery status. An indication of inadequate recovery status more than twice (adding an additional 1-minute of rest) would have resulted in termination of the exercise session and the participant would have been excluded from further study activities. Fortunately, this did not occur. All repetitions were intended to be completed voluntarily and without assistance. In the event a repetition was assisted, remaining repetitions in the set were aided to complete the set and a new weight adjusted 10-RM reflected in the subsequent set by reducing the working load by a standard 10%. 98.3% of repetitions were completed unassisted in this study.

Arterial Blood Pressure

Arterial blood pressure was measured in the right arm at heart level while the participants remain supine using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh, N.C., USA). Arterial blood pressure will be measured in duplicate following supine rest to begin each visit and in accordance with guidelines set forth by the American Heart Association [132]. Mean arterial pressure was calculated as diastolic pressure plus 1/3 pulse pressure (systolic pressure – diastolic pressure) and reported in mmHg.

Femoral Blood Flow and Femoral Vascular Conductance

Blood flow velocity and vessel diameter of the common femoral artery were measured in the right leg, 2-3 cm proximal of the bifurcation of the superficial and deep branches of the femoral artery. Femoral blood velocity was estimated via duplex ultrasonography using a linear-array ultrasound transducer (L9-3 Probe, Philips iE33, Andover, MA) at an insonation angle of 60° and quantified by standard methods [121]. Briefly, velocity measures were made by recording the ante- and retrograde doppler frequencies with a custom audio recording software (DUC2). For this study, velocity measurements were made at an average depth of 1.20±0.25 cm and were thin-beam corrected, based on a known beam width of 2.80 mm at that depth, which resulted in an average correction factor of 0.781±0.009 [121]. Image recordings were then analyzed via custom software (Vascular Research Tools 5; Medical Imaging Applications LLC, Coralville, IA) to determine femoral mean diameter (mm). All diameter measurements were made during diastole and representative of average diameter across a 90-second recording. Femoral blood flow (FBF) was calculated using the equation below, where artery cross-sectional area is multiplied by femoral mean blood velocity (cm/s) and represented as milliliters per minute ($mL \cdot min^{-1}$). Femoral vascular conductance (FVC) was calculated using timepoint-matched mean arterial pressure value divided by the calculated femoral blood flow and expressed as $mL \cdot min^{-1} \cdot mmHg$.

Equation 4.1 Femoral Blood Flow

$$\textit{Femoral Blood Flow (mL/min)} = \pi\left(\frac{\text{Diameter}}{20}\right)^2 \text{ x mean blood velocity x } 60$$

Blood Sampling

Venous whole blood samples were collected into two Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), one was treated with potassium ethylenediaminetetraacetic acid (K_2EDTA) and one untreated for the collection of serum, containing no anti-clotting agents. K_2EDTA samples were centrifuged immediately, while serum samples rested for 30 minutes to allow for clotting at room temperature before centrifugation. All samples were centrifuged at 3000 RPM for 10 minutes, processed and aliquoted into separate microtubes and frozen at -80°C for subsequent analysis. Plasma samples were used to determine concentrations of histamine, 1-

methyhistamine, and 1-methyl imidazole acetic acid via hydrophilic interaction liquid chromatography (HILIC). Separately, serum samples were used to determine concentrations of serum tryptase assessed via ELISA.

Urine Collections

24h urine collections were initiated at the same time of day within each subject to account for diurnal fluctuations in urine concentration. All participant urine voids for 24h were collected into two light-resistant 3L containers (Medline Industries, Northfield, IL) until their visit the following day. Upon return to the lab the following day, participants were instructed to perform one final void into the 24h collection containers prior to beginning their experimental visit. Thoroughly mixed 24h urine samples were aliquoted into cryogenic tubes and frozen at -80°C until later analysis of urinary biomarkers of interest. Urine excretion ($l \times h^{-1}$) were calculated as the volume of urine produced during 24h collection periods (kg), multiplied by urine specific gravity (USG) to obtain a liter (l) equivalent, and then divided by the total collection time (h). Excretion rates were then used to determine the production rate ($mg \times h^{-1}$) during each exclusive collection window by multiplying concentrations of 1-MH, and MIAA ($ng \times mL^{-1}$) measured using hydrophilic interaction liquid chromatography (HILIC) and enzyme-linked immunosorbent assays (ELISA, when applicable). Collection times for exercise and exercise recovery were determined as time from the beginning of the warm-up to end of exercise within a condition.

Biochemical Analyses

Outsourced analysis. Plasma and urine histamine, 1-methylhistamine (1-MH, CAS Number 501-75-7), and 1-methyl-imidazole acetic acid (MIAA, CAS Number 2625-49-2) were quantified using hydrophilic interaction liquid chromatography (HILIC)-Tandem Mass Spectrometry (LC-MS/MS) using a custom assay, modified from Nelis et al., [133] and performed by the Bioanalytical Shared Resource/Pharmacokinetics Core at Oregon Health and Science University (Portland, OR). Concentrated stock solutions were prepared individually for urine and plasma histamine, 1-MH, and MIAA in dimethyl sulfoxide (DMSO; Fisher Scientific, Rockwood TN) at $10mg \times ml^{-1}$ and subsequently diluted to generate calibration curves. Urine and plasma

curve matrix were prepared by diluting control urine (UTAK, Valencia, CA) 1:100 and plasma (Innovative research, Novi, MI) 1:10 with water. Following spiking, a serial dilution was performed to generate acceptable calibration curves for urine (0,0.5,2.5,5.0,25,50,250 and 500ng×ml^{-1}) and plasma (0, 0.1,0.25,0.5,1.0,2.5,5.0 and 50ng×ml^{-1}). Internal standards solution with d4-histamine, d3-1-methylhistamine, and d5-Phenylalanine (CDN Isotopes, Quebec, Canada) were prepared in acetonitrile (Honeywell, Muskegon, WI) at 100ng×ml^{-1} except for 200ng×ml^{-1} for d5-PHE. Note that d5-Phenylalanine was used as internal standard for MIAA. All urine samples were diluted with water (1:10) before analysis. 50µl of internal standard solution was added to 50µl of unknown diluted urine sample, calibrator or diluted quality control urine sample, then 900µl of acetonitrile was added. Samples were vortexed for 1 minute, placed into -20°C freezer for one hour to complete precipitation. Samples were removed from freezer, centrifuged at 17000 x g for 5min at ambient temperature before supernatant was transferred to a 96-well plate. 5µl or 15µl was injected for analysis. Plasma samples were not diluted. 50µl of internal standard solution was added to 50µl of plasma sample, calibrator or quality control plasma, then 400µl of acetonitrile was added. Samples were then prepared as for urine and 15µl was injected for analysis. Histamine and its metabolites were then analyzed using a 5500 PLUS Q-TRAP hybrid/triple quadrupole linear ion trap mass spectrometer (MS; SCIEX, Framingham, MA) with electrospray ionization (ESI) in positive mode using HILIC chromatography. MS was interfaced to a SIL-20AC XR auto sampler followed by two LC-20AD XR LC pumps (Shimadzu, Columbia, MD). Data analysis was performed using MultiQuant software (SCIEX, Framingham, MA). Additional information about operating settings can be found in the supplemental materials.

In-house analysis. Serum concentrations of human mast cell tryptase were assessed via a commercially available enzyme-linked immunosorbent assay (ELISA) kit (KT-64891, Kamiya Biomedical Company, Seattle, WA). The mean intra-assay variance was 3.7%. Urine concentrations of 1-MH were assessed via a commercially available ELISA kit (EA208/96, Eagle Biosciences, Amherst, NH) with a mean intra-assay variance of 10.1%. This was done so we could compare the HILIC assay to a more widely available methodology. We were unable to find a suitable ELISA kit for MIAA for comparison to HILIC. ELISA was performed per manufacturer's instructions, using Epoch 2 Microplate spectrophotometer (BioTek, Winooski,

VT). To eliminate inter-assay variance, all samples were thawed once and analyzed in duplicate within the same assay and run by a single technician.

Statistics

Area under the curve (AUC) analyses using the trapezoidal method were performed to assess the response magnitude for variables of interest across the exercise recovery window lasting 2-h. Pearson correlations were used to explore the association between the AUC for the vasodilatory response and the urine production of metabolites. Bland-Altman analysis was used to explore the use of two methodologies for quantification 1-methyhistamine (ELISA and HILIC). Data are reported as mean with 95% confidence intervals. Alpha was set at 0.05 for all statistical inferences including familywise error rates. Inferences which were drawn from two-way mixed-effects models with pre-planned comparisons (Prism 10, GraphPad, Boston, MA, USA). Inferences regarding changes over time within each condition were drawn from Dunnett's multiple comparisons test versus baseline, with reported p-values and confidence intervals adjusted for multiplicity. Inferences regarding the differences between groups were drawn from Šidák's multiple comparisons test, applied to the change from baseline and restricted to comparisons at the same timepoint. Inferences regarding response magnitude between conditions (AUCs) were drawn from two-tailed paired samples t-tests.

RESULTS

Aerobic and Resistance Exercise

Aerobic exercise elicited a VO_2 of 72 [69, 75] % of VO_{2peak}. During resistance exercise, 59 [57, 61] repetitions were completed unassisted vs 1 [-1, 3] assisted, totaling an load of 4905 [4245, 5565] kg that was voluntarily accumulated. Heart rate and arterial pressures before and after exercise are shown in **Table 4.2**. A similar pattern of heart rate recovery was observed throughout 60-minutes of recovery following both conditions (both elevated relative to baseline, $p<0.01$), before returning toward resting values by 90-minutes (relative to baseline: aerobic, $p=0.20$; resistance $p=0.15$). Systolic blood pressure was elevated immediately following

resistance exercise only ($p<0.05$). Diastolic pressure was only depressed 30-minutes following aerobic exercise ($p=0.04$).

Femoral Artery Blood Flow

Figure 4.3 displays femoral blood flow measurements before exercise and every 30-minutes throughout the associated 2-hour post-exercise recovery period. Femoral blood flow was immediately elevated post-exercise in both conditions ($p<0.01$ vs baseline) and remained elevated at 30-minutes post-resistance exercise ($p<0.01$) but not for aerobic exercise ($p=0.09$). Blood flow was greater immediately post- ($p<0.01$) and at 30-minutes ($p=0.01$) following resistance exercise compared to aerobic exercise. AUC analysis (right panel) revealed resistance exercise elicited a greater average magnitude of femoral blood flow (12956 [8074, 17837] mL) delivered through the femoral artery during 2-hour of post exercise recovery compared to aerobic exercise (5150 [450, 9850] mL, $p=0.02$).

Femoral Vascular Conductance

Figure 4.3 displays femoral vascular conductance before exercise and every 30-minutes throughout the associated 2-hour post-exercise recovery period. Femoral vascular conductance was immediately elevated post-exercise in both conditions ($p<0.01$ vs baseline) and remained elevated at 30-minutes post-resistance exercise ($p<0.01$) but not for aerobic exercise ($p=0.18$). Vascular conductance was greater immediately post-exercise following resistance compared to aerobic exercise ($p<0.01$) but was not different at 30-minutes ($p=0.08$). AUC analysis (right panel) revealed resistance exercise elicited a greater average magnitude of vascular conductance (152 [96, 209] $mL \times mmHg^{-1}$) throughout the recovery period compared to aerobic exercise (73 [15, 131] $mL \times mmHg^{-1}$, $p<0.01$).

Table 4.2 Heart Rate, Arterial Blood Pressures, and Serum Tryptase Responses

Time Point	Exercise Condition	Heart Rate (bpm)	Systolic (mmHg)	Diastolic (mmHg)	Mean (mmHg)	Serum Tryptase (ng/mL)
Baseline (BL)	Aerobic	60 [54,67]	124 [117,130]	68 [64,73]	87 [84,89]	75 [56,94]
	Resistance	58 [53,63]	121 [116,126]	65 [61,70]	84 [81,87]	79 [59,98
Immediately Post Exercise (IP)	Aerobic	87 [82,92]*	121 [115,127]	69 [63,74]	86 [82,90]	76 [56,97]
	Resistance	89 [83,95]*	125 [118,131]*	64 [59,70]	85 [80,89]	75 [56,94]
30min Post Exercise (30)	Aerobic	75 [69,81]*	120 [114,126]	64 [59,68]*	83 [79,86]	77 [58,96]
	Resistance	76 [71,81]*	120 [114,127]	68 [62,74]	85 [80,90]	77 [57,96]
60min Post Exercise (60)	Aerobic	69 [63,74]*	119 [114,124]	65 [60,70]	83 [79,87]	79 [60,97]
	Resistance	67 [61,72]*	119 [113,125]	64 [58,70]	82 [78,87]	79 [59,98]
90min Post Exercise (90)	Aerobic	65 [59,70]	125 [119,131]	66 [60,71]	86 [81,90]	77 [59,94]
	Resistance	62 [55,69]	122 [117,126]	68 [62,73]	86 [81,90]	80 [61,99]
120min Post Exercise (120)	Aerobic	62 [56,68]	126 [119,132]	68 [64,73]	87 [84,91]	79 [61,96]
	Resistance	59 [52,66]	123 [118,128]	68 [62,75]	87 [83,91]	76 [59,93]

Data presented as mean with 95% CIs. * denotes $p < 0.05$ vs baseline.

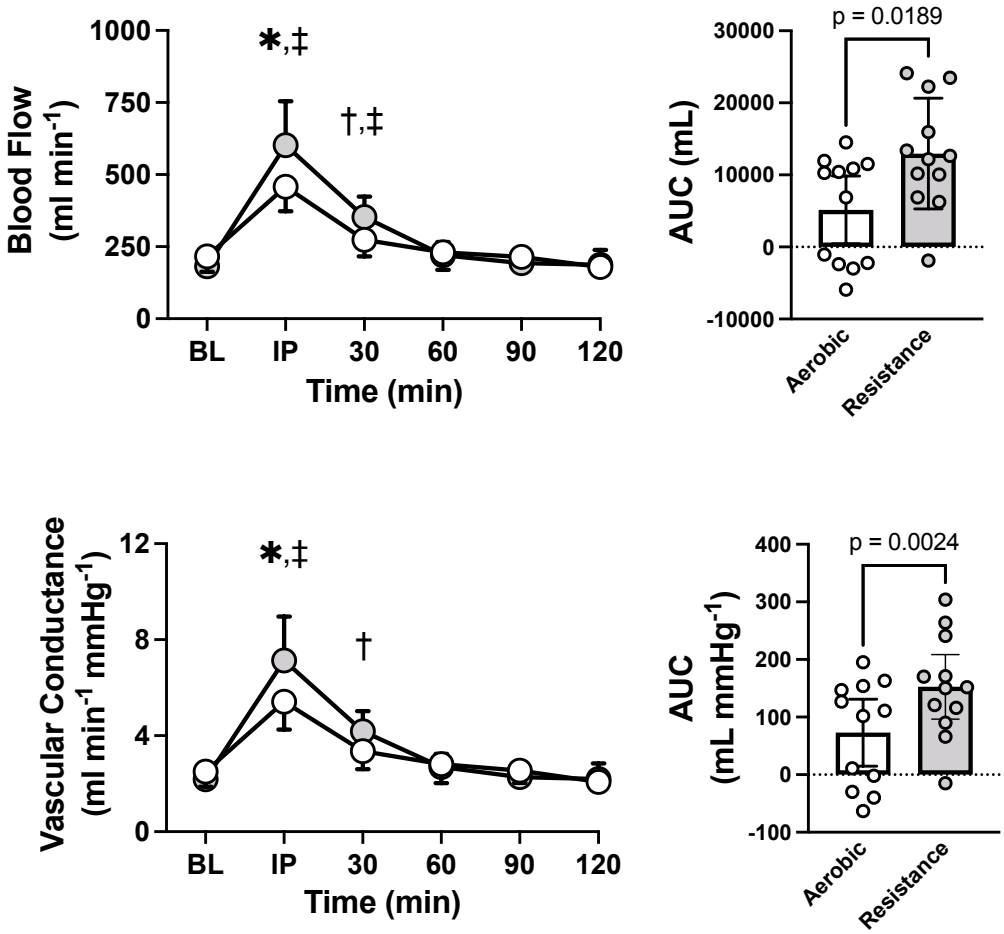

Figure 4.3 Femoral blood flow (top) and femoral vascular conductance (bottom) at baseline (BL), immediate postexercise (IP), and at 30-, 60-, 90-, and 120-min post-exercise (left panels). Right panels show area under the curve for each, including individual data. Values are means (95% confidence limits). Open symbols are aerobic exercise; filled are resistance exercise (n=12). * denotes $p < 0.05$ versus baseline for both exercise conditions; † denotes $p < 0.05$ resistance exercise vs baseline; ‡ denotes $p < 0.05$ resistance vs aerobic exercise.

Circulating Histamine and Histamine Metabolites

Figure 4.4 displays the concentrations of histamine and its primary and terminal metabolites, 1-methylhistamine and methyl imidazole acetic acid in plasma samples before

exercise began and throughout the 2-hour recovery period post exercise. Plasma concentration of histamine was transiently elevated immediately post-exercise for aerobic exercise ($p<0.01$) with a weak trend toward increased concentrations following resistance exercise ($p=0.18$). Plasma concentration of 1-methyhistamine was transiently elevated immediately post-exercise for both conditions ($p<0.01$). In contrast, plasma concentration of methyl imidazole acetic acid showed a delayed increase following exercise for both conditions ($p<0.01$).

Urine Production Rates of Histamine Metabolites

Table 4.3 displays the urine production rates determined via HILIC for histamine, and its metabolites, 1-MH and MIAA during the 24-hours before, throughout exercise and 2-h of exercise recovery, and throughout 24-hours following exercise. Production rates of urine histamine were not elevated at any timepoint. Urine 1-MH and MIAA production rates during the window of exercise plus 2-h exercise recovery were elevated in both conditions ($p<0.01$). **Table 4.3** also shows serum concentrations of tryptase at baseline and throughout exercise recovery. Serum tryptase remained unchanged at any timepoint following both aerobic and resistance exercise.

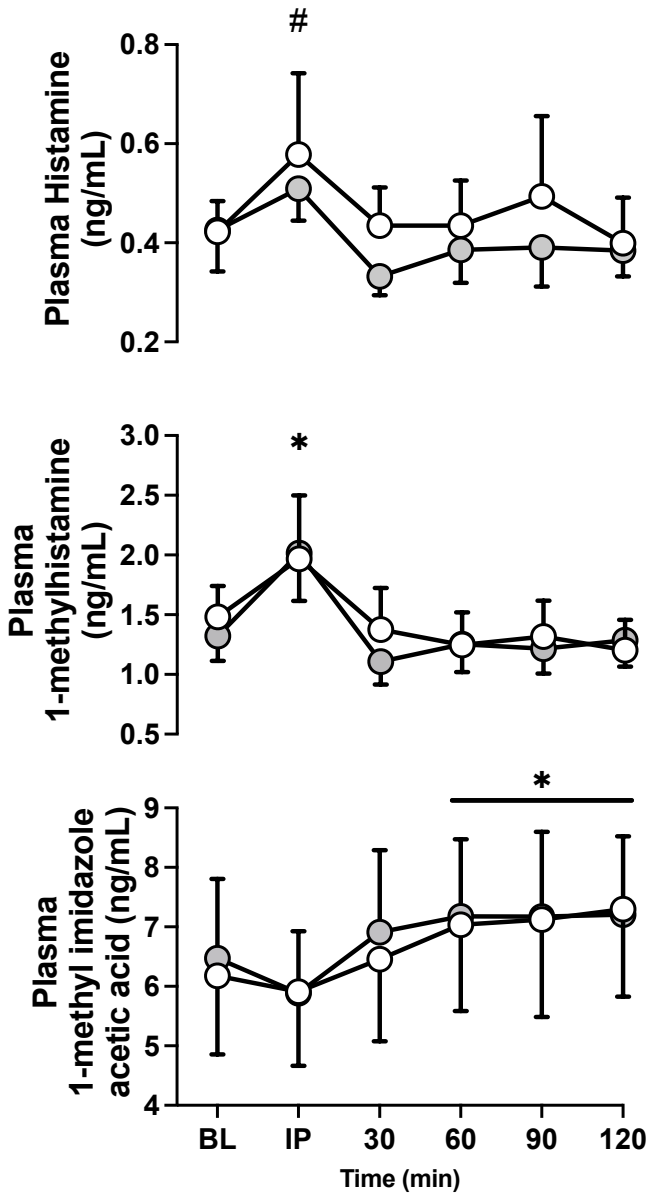

Figure 4.4. Circulating plasma concentrations of histamine (top), 1-methyhistamine (middle), 1-methyl imidazole acetic acid (bottom) at baseline (BL), immediate postexercise (IP), and at 30-, 60-, 90-, and 120-min post-exercise. Values are means (95% confidence limits). Open symbols are aerobic exercise; filled are resistance exercise (n=12). * denotes $p < 0.05$ versus baseline for both exercise conditions; # denotes $p < 0.05$ aerobic exercise vs baseline.

Table 4.3 Urine Production Rates of Histamine and Histamine Metabolites

Time Point	Exercise Condition	Histamine ($mg \times h^{-1}$)	1-methyhistamine (HILIC; $mg \times h^{-1}$)	1-methylhistamine (ELISA; $mg \times h^{-1}$)	1-methylimidazole acetic acid ($mg \times h^{-1}$)
Baseline (BL)	Aerobic	1.8 [1.3, 2.3]	9.3 [6.7, 12.0]	7.5 [6.2, 8.9]	148 [104, 193]
	Resistance	1.7 [1.3, 2.2]	8.5 [6.5, 10.6]	7.3 [5.7, 9.0]	130 [98, 162]
Exercise plus Exercise Recovery (2h)	Aerobic	2.4 [1.1, 3.6]	12.6 [8.8, 16.3]*	10.2 [7.9,12.4]*	209 [166, 253]*
	Resistance	2.3 [1.3, 3.3]	12.0 [10.0, 14.1]*	13.0 [9.8, 16.2]*‡	185 [147, 222]*
24h Post-exercise (24h)	Aerobic	1.7 [1.3, 2.0]	8.7 [6.6, 10.9]	7.2 [5.7, 8.6]	140 [109, 171]
	Resistance	1.4 [0.9, 1.9]	8.2 [6.3, 10.0]	7.0 [5.9, 8.1]	121 [97, 145]

Data presented as mean with 95% CIs. * denotes $p < 0.05$ vs baseline; ‡ denotes $p < 0.05$ aerobic vs resistance exercise

Relationship between Urine MIAA, Femoral Blood Flow, and Vascular Conductance

Figure 4.5 (left panels) displays the change in urine production rate of MIAA from baseline to 2-hours after exercise in both conditions and their relationship with the magnitude of the femoral blood flow (AUC) and the sustained increase of femoral vascular conductance (AUC). **Figure 4.5** (right panels) displays the change in urine production rate of MIAA from baseline to 2-hours after exercise in both condition and their relationship to the change in peak blood flow measure immediately following exercise (% from baseline). Correlation between MIAA and the magnitude of femoral blood flow (r=0.48, p=0.03), and the sustained rise of in vascular conductance (r=0.46, p=0.04) revealed a modest positive relationship. The strength of these relationships improved when urine MIAA production was correlated with the change from baseline to peak blood flow (r=0.64, $p<0.01$) and peak vascular conductance (r=0.62, $p<0.01$) measured immediately after exercise.

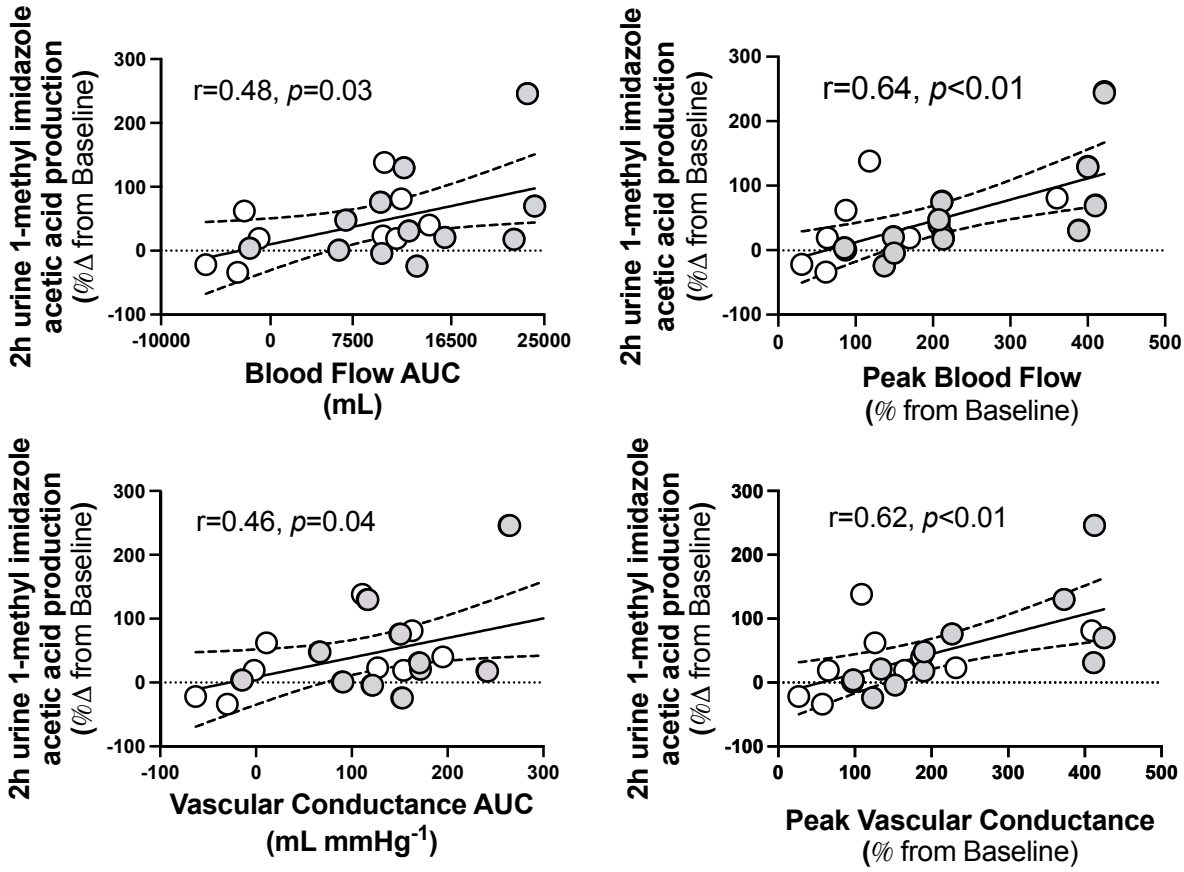

Figure 4.5 Pearson's correlation assessing the relationship between the change in urine production rates of 1-methyl imidazole acetic acid from baseline to 2-hours with the AUC for blood flow and vascular conductance (left panels), and peak blood flow and vascular conductance (right panels) measured immediately after exercise during both conditions (n=12). Open symbols are aerobic; filled are resistance.

Measurement Technique Comparisons (Bland-Altman)

As shown in **Figure 4.6** (top left panels), the change in urine production rates of 1-MH (based on HILIC) from baseline to 2-hours after exercise in both conditions was not as well correlated to the magnitude of femoral blood flow (AUC) (r=0.41, *p*=0.07) or vascular conductance (AUC) (r=0.38, p=0.09) compared to the terminal metabolite, MIAA, but the trend

was similar. As shown in **Figure 6** (top right panels), 2h urine production rates of 1-MH and peak blood flow (% from baseline) ($r=0.58$, $p<0.01$) and peak vascular conductance (% from baseline) ($r=0.55$, $p<0.01$) were well correlated.

Figure 4.6 (bottom) also compares 1-MH as measured by HILIC versus a commercially available ELISA kit. Based on Bland-Altman (left) assessment of bias and agreement, mean bias between the measurement techniques was 19.2 $ng \times mL^{-1}$ with a SD of 45.8 $ng \times mL^{-1}$. The limits of agreement, calculated as the mean bias±1.96xSD, ranged from -57.3 to 95.7 $ng \times mL^{-1}$. While the two measures were correlated (bottom; $r=0.83$, $p<0.01$), high bias and wide limits of agreement suggest the two measurements are related but not interchangeable.

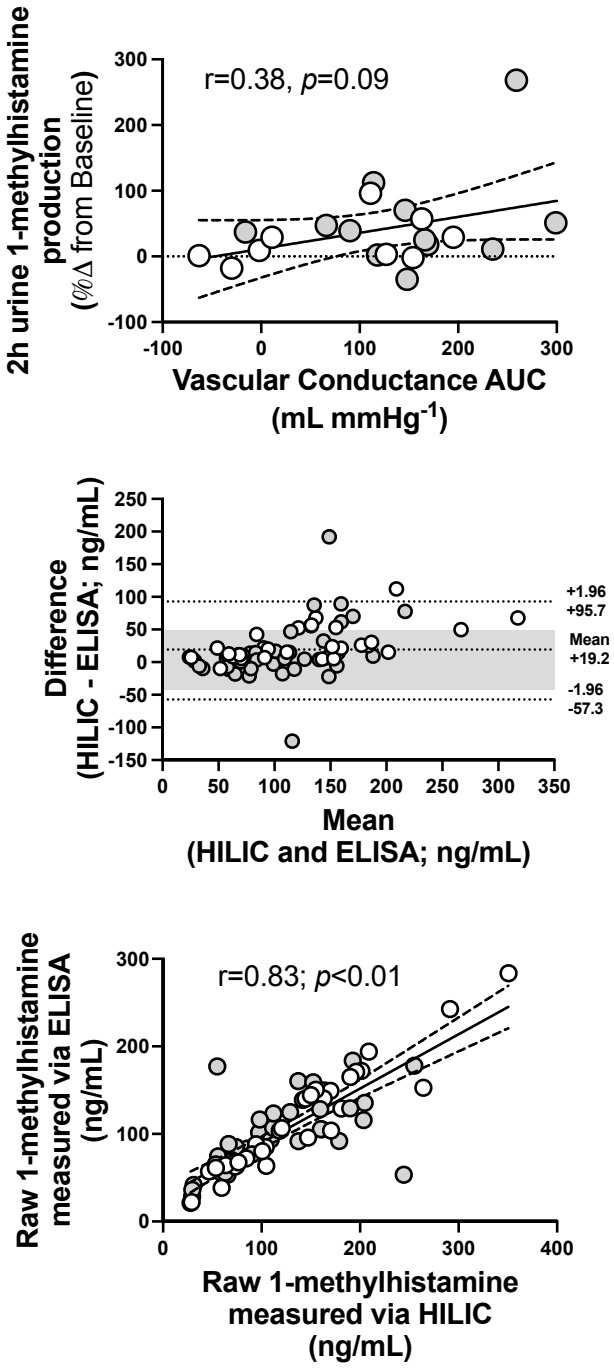

Figure 4.6. Pearson's correlation assessing the relationship between the change in urine production rates of 1-methylhistamine from baseline to 2-hours and the AUC for blood flow and vascular conductance (left panels), and peak blood flow and vascular conductance (right panels) (n=12). Bland-Altman (bottom left) assessment of agreement

between HILIC and ELISA urine concentrations for 1-methyhistamine at multiple time points for each participant (n=12). Mean values calculated for the measurement techniques are plotted against their difference at each time point for both aerobic and resistance exercise conditions. Correlation analysis (bottom right) represents the ability of the measurement techniques, HILIC and ELISA, to capture the same concentration value at each time point between both exercise conditions. Open symbols are aerobic; filled are resistance.

DISCUSSION

This investigation aimed to determine the utility of two of histamine's metabolites, 1-methylhistamine (1-MH) and methyl imidazole acetic acid (MIAA), as biomarkers of the histamine response to exercise. We hypothesized that these metabolites would increase in circulating plasma and urine following an acute exercise session. We also hypothesized that the production of these metabolites would correlate with sustained post-exercise vasodilation, a histamine-dependent response to exercise.

Our results indicate that the histamine response to both aerobic and resistance exercise is evident from the levels of histamine's metabolites in plasma and in urine. We observed transient elevations of plasma histamine and the primary metabolite 1-MH and a delayed elevation of the terminal metabolite MIAA following exercise. Of note, these changes were in the absence of any change in circulating tryptase, often used as a marker of mast cell degranulation syndromes. Urine production rates of both the metabolites (1-MH and MIAA) increased in response to a single bout of exercise and returned to baseline during the following 24 hours. The production rate of MIAA correlated most strongly with the post-exercise peak in blood flow ($r=0.64$, $p<0.01$) and vascular conductance ($r=0.62$, $p<0.01$) and also strongly correlated with the area under the curve representing the sustained post-exercise vasodilation. The production rate of 1-MH showed similar but somewhat weaker correlations.

These are novel observations of histamine metabolite production that reflect the physiological release of histamine during exercise. They appear to be generalizable in use as biomarkers of the exercise histamine response. In addition, our findings extend previous work on

the histamine response to aerobic exercise by demonstrating that resistance exercise also generates a substantial histamine signal, highlighting histamine's role as a molecular transducer of exercise adaptation across modalities.

Histamine Response to Exercise

The physiological role of histamine in response to exercise has been characterized extensively but often occurs without a detectable rise in circulating histamine concentrations [1,3,4]. Histamine release within skeletal muscle tissue during and after exercise promotes the dilation of arterioles, increasing vascular conductance, and enhancing blood flow but much of it is likely metabolized locally before accumulating in the circulation [10,19,108,134]. Invasive measurement techniques like intramuscular microdialysis have been able to document the local release of histamine during exercise [10], and while this approach has enabled significant progress toward a mechanistic understanding of histamine production and release within skeletal muscle during exercise, it is not approachable in many research labs or most athletic settings. Therefore, we have previously relied heavily on surrogate techniques based on measurements of blood flow, often in combination with pharmacological blockade of histamine H_1 and H_2 receptors, to explore the role of histamine in exercise responses.

From the more invasive methodologies in use, we know that histamine is released from degranulation of mast cells that reside within the skeletal muscle environment, based on the co-release of tryptase [10]. We also know that there is a contribution from *de novo* histamine production as the inhibition of histidine decarboxylase reduces the histamine response to exercise [10]. In addition, single-cell transcriptomics have confirmed that mast cells are the only common cell type within skeletal muscle tissue that express histidine decarboxylase [50]. While the exercise stimulus promoting histamine production and release remains elusive, one piece of the puzzle is that increasing skeletal muscle temperatures to match those achieved during aerobic exercise also elicits release of histamine [16].

Activation of histamine's receptors within the skeletal muscle tissue modifies the transcription of > 25% of the more than 3000 protein-coding genes that are exercise-response altering pathways related to cellular maintenance, inflammation, vascular remodeling, and

metabolism [6]. The broad footprint on the exercise transcriptome appears to translate into significant adaptations to aerobic exercise training which are attenuated when histamine receptors are blocked, demonstrating that histamine release during exercise transduces many of the adaptive benefits to exercise [8]. This appears to be the result of a coordinated crosstalk between non-muscle cells in the skeletal muscle environment that is driven by histamine receptor activation [50].

Measurement Techniques and Challenges

Pathways for histamine synthesis and metabolism are highly conserved. Histidine decarboxylase is the only enzyme that produces histamine, but there are two independent pathways to deactivate and metabolize histamine, histamine-n-methyltransferase (HNMT) and diamine oxidase (DAO) [20], as noted in the introduction. DAO is noted for its ability to metabolize extracellular histamine in tissues of the small intestine, colon, placenta, and kidneys [135,136], providing the main barrier against exogenous histamine within the intestines gaining passage into the systemic circulation [135,137]. Unlike DAO, HNMT is extensively expressed in skeletal muscle and other peripheral tissues [6,125] and is the most probable pathway for metabolism of histamine released within skeletal muscle, although there is some evidence for metabolism to imidazole acetic acid by either DAO or alcohol dehydrogenase in skeletal muscle [50]. Conversion of histamine by HNMT to 1-methylhistamine (1-MH) effectively deactivates its biological activity, and the subsequent conversion to its terminal metabolite 1-methyl imidazole acetic makes it readily excretable by the kidney.

We note that the kidney has an intrinsic histamine pathway [125], however it appears to function under pathophysiological conditions, and we are not aware of any evidence that it is activated during exercise [138]. Unlike skeletal muscle, which shows a sustained post-exercise vasodilation that is histamine dependent, there is no comparable post-exercise vasodilation in the renal vasculature [139] suggesting the intrinsic renal histamine pathway is quiescent during exercise and not contributing to the urine production of histamine or its metabolites.

Of the available measures that would appear to be valid biomarkers of the histamine response to exercise, the transient or negligible responses of plasma histamine, 1-MH, and

tryptase do not support their general use. Plasma MIAA, reaching stable values long after the end of exercise, shows considerable promise but not all labs will have the resources for measuring it. The same would be true for urine production of MIAA, although that marker is perhaps the most sensitive of them all. On the other hand, with the availability of commercial ELISA kits for quantifying urine 1-MH, urine production of 1-MH is the most readily available biomarker at this time, if not the most sensitive of the biomarkers.

Potential Applications

Having a validated biomarker for the histamine response to exercise may have utility beyond its use in foundational investigations into the role of histamine. One such application may be in providing insight into stress and adaptation by quantifying the overall exercise stress load. The field has failed to identify a quantifiable variable that can accurately determine the acute strain following an exercise session and this has grossly limited our ability to prevent overreaching from becoming overtraining.

Overtraining, characterized by sustained fatigue and a decline in athletic performance, can take weeks or months to recover from and can be detrimental to the long-term health of athletes [46]. While increased exercise volume and intensity are necessary stressors to promote adaptation, excessive increases in either variable, particularly when paired with inadequate rest [140], can tip the scale toward overreaching [141]. Symptoms of overreaching include a decline of exercise performance and altered skeletal muscle function [142,143], incomplete resolution of exercise-induced inflammation [45], and reductions in cardiac output limited by stroke volume, independent from reduced blood volume [144].

With histamine being identified as a molecular transducer of exercise adaptation [8] and coordinator of crosstalk between non-muscle cells in the skeletal muscle environment [50] , this raises the possibility that a biomarker such as urine production of MIAA might have potential applications in monitoring the exercise stress load, potentially foretelling overtraining. The current work reveals our ability to quantify histamine production during exercise, through measurement of its metabolites, capable of capturing patterns of blood flow elicited by an exercise session, and a response that bears close relationship with exercise intensity [145]. Although follow-up investigation will be necessary to determine the sensitivity of histamine's metabolites

to acute changes in exercise volume and intensity for use as a diagnostic tool to assess athlete training and competition preparedness (suggested utility described in **Figure 4.7**), we think this is a concept with great promise.

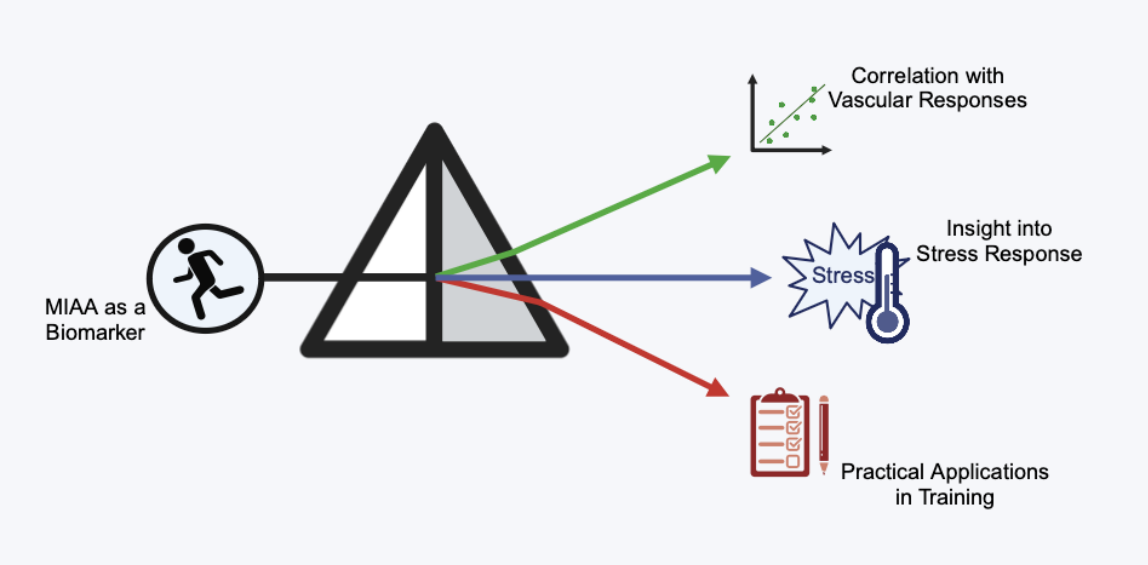

Figure 4.7. Potential application of 1-methyl imidazole acetic acid (MIAA) as a biomarker of the stress response to exercise. Changes in MIAA production correlate with intensity-dependent vascular responses following exercise and act as a non-invasive measurement of acute exercise-induced stress. Monitoring the change in its production rates following exercise and throughout recovery might provide useful insight for more accurate prescription of exercise training or to monitor overload to mitigate against overtraining progression. Image created with BioRender.com

CONCLUSION

Histamine is a critical signaling molecule to exercise influencing vascular function and eliciting transcriptome level stimulus that crosses systems and impacts inflammation,

metabolism, cellular maintenance, and adaptation to exercise training [6]. The current study points toward the future by demonstrating the utility of multiple biomarkers of histamine, based on known metabolic pathways and renal excretion of histamine metabolites. Some are more limited by currently available analysis methods, some by the inherent physiology, but collectively they provide multiple means to monitor the histamine response to exercise. The current study also shows that the histamine response to exercise is not limited to aerobic or endurance modalities of exercise, but also responds to traditional resistance or strength training modalities, extending the relevance of histamine as a molecular transducer of exercise adaptation across the exercise spectrum.

CHAPTER V
HISTAMINE MODULATES SKELETAL MUSCLE BLOOD FLOW AND OXYGENATION BUT DOES NOT DETERMINE NEUTROPHIL INFILTRATION FOLLOWING RESISTANCE EXERCISE

INTRODUCTION

Histamine is classically associated with allergic and inflammatory responses in pathophysiological conditions (i.e., asthma, allergies, anaphylaxis, and tumor growth), where it acts as one of the first and most consistently released inflammatory mediators [146]. Similarly, a highly stereotyped immune response to exercise is initiated in response to exercise-induced stress and is necessary for adaptation [45,46,48,49]. Recent work from Van der Stede et al. indicates crosstalk between the skeletal muscle tissue and the immune system is mediated by histamine receptor activation, and provides novel insight for histamine's role in the recovery of skeletal muscle [50.] However, their study methodologies are unable to disentangle this from histamine's effects on peripheral blood flow [7], vasodilation [147], vascular permeability [99], and cell adhesion dynamics [148] making it challenging to determine where histamine signaling is most critical in the post-exercise inflammatory cascade.

As part of the early inflammatory response to exercise, neutrophils are mobilized from marginal pools toward sites of stressed tissues [84,85]. Following high-force exercises such as resistance exercise, this initial phase quickly resolves before giving way to a more prolonged leukocytosis [86,87] as bone marrow-derived immune cell concentrations increase in the hours that follow [88]. Ely et al., captured this response following muscle-damaging exercise and revealed that an elevated peak concentration of neutrophils is present in circulation when histamine receptors are inhibited [94]. Yet, it remains unknown if the attenuation of histamine signaling could impair immune cell movement into the skeletal muscle after exercise. Alternatively, histamine's well-characterized effect on skeletal muscle blood flow is potentially blunted during exercise, with histamine receptor antagonists reducing skeletal muscle perfusion and limiting oxygen delivery that could exacerbate the inflammatory response observed by Ely et al., [94].

Therefore, this investigation sought to determine the role of histamine in neutrophil extravasation during an acute inflammatory window of recovery following resistance exercise.

We hypothesized that blocking histamine receptors would reduce neutrophils that gain access to the skeletal muscle tissue. In addition, we sought to determine the role of histamine on skeletal muscle perfusion during and following resistance exercise. We hypothesized that exercise with histamine receptor antagonists would reduce skeletal muscle oxygenation during exercise, reducing exercise performance, and also reduce the delivery of nutrients during recovery and diminishing the phosphorylation of proteins necessary for muscle protein synthesis (i.e., mammalian target of rapamycin, mTOR).

Although the recent work by Van der Stede et al., are insightful, their omics-based approaches are unable to separate the impact of histamine receptor antagonism on resident immune cells, or if the altered transcription within bulk muscle samples is a product of poor immune cell extravasation [50]. Thus, we anticipate the primary result of this study will yield evidence to support a role for histamine in the movement of immune cells between compartments, or alternatively, highlight histamine receptor activation that may alter the transcription of local resident immune cells during the recovery from exercise.

We anticipate the results of this study will clarify the unique histamine paradox displayed by Ely et al., [120] where lower values of skeletal muscle pH were achieved during exercise despite an elevated pattern of bulk skeletal muscle blood flow in the femoral artery (a conduit artery) during graded aerobic exercise with histamine receptor antagonists. We hypothesize that skeletal muscle blood flow following resistance exercise will be higher when measured in the femoral artery in the histamine blockade condition in response to lower values of skeletal muscle oxygenation, indicating poor perfusion within the microcirculation of skeletal muscle despite the increased conduit artery flow.

Finally, most of our understanding of histamine stems from investigation utilizing models of aerobic and dynamic exercise. These studies [1,2,5–8] have generated substantial data on skeletal muscle responses, in both the acute and chronic setting, including impacts of histamine receptor blockade on skeletal muscle perfusion and nutrient delivery. However, Barrett O'keefe et al., [18] suggest the role for histamine signaling during resistance exercise may not be relevant in this way. We suspect these conclusions were made based on a poor model of resistance exercise that did not promote a sustained post-exercise vasodilation and thus, may not have been strenuous enough to promote the release of histamine [18]. We anticipate the difference in physiological response to a sufficient intensive model of resistance exercise with-, and without histamine

receptor antagonism, will extend previous results to include a model of resistance exercise and extend histamine's role across the spectrum of exercise.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board at the University of Oregon. Written informed consent was obtained from all participants, and the study conformed to the principles of the Declaration of Helsinki.

Participants

Ten individuals (4F, 6M) between the ages of 18-35 (22.4±2.6y) participated in this double-blind investigation. All participants were considered healthy, non-smokers, and were avid exercisers. Participant characteristics, age, anthropometrics, and exercise performance for maximal strength of the traditional back squat (1-repetiton maximum, 1-RM) were obtained during a screening visit, and separated by a minimum of 1-week from subsequent visits. Participants were required to achieve a 1-RM of at least 1.15x-3.0x (for females) or 1.25x-3.0x (for males) their body weight (BW) to minimize the potential impact of naïvity on the exercise task. Participants were free from current use of antihistamine medications or use of prescription medications that modulate immune function. Additionally, participants were free from performance enhancing drugs, and/or dietary supplements that have antioxidant or recovery altering properties. We did not track female participants' menstrual cycle status.

Screening Visit

Participant characteristics including anthropometrics and exercise performance for maximal strength of the traditional back squat (1-RM) were determined during a single screening visit (**Table 5.1**). The 1-RM assessment was conducted per National Strength and Conditioning Association (NSCA) guidelines and was overseen by a well-trained member of the research team [129]. Details for the determination of the 1-RM are noted below.

Table 5.1. Participant Characteristics

n	10 (4F, 6M)
Age (years)	22.0±2.9
Height (cm)	173.9±8.8
Weight (kg)	74.2±17.2
BMI ($kg \times m^{-2}$)	24.3±3.6
1-repetion maximum, 1-RM (kg)	113.2±43.9
Relative 1-RM ($kg \times bodyweight^{-1}$)	1.49±0.3

Data presented as mean±SD

Experimental Design

Each participant completed both resistance exercise visits with and without histamine receptor antagonists in a double-blind, randomized order and separated by at least one week. Three total muscle biopsies were acquired from the vastus lateralis. The first biopsy was taken from the left leg during a baseline (BL) to establish rested characteristics. This was done at least 1 week prior to the two exercise sessions. The other two biopsies were taken following the two exercise sessions (Placebo vs Blockade). Each exercise visit and subsequent biopsy were separated by one-week and biopsy samples were acquired from the opposing limb from the preceding visit (ex. BL, left; Placebo, right; Blockade, left).

During the two exercise visits, participants were provided with over-the-counter histamine H_1-and H_2-receptor antagonists (540mg of fexofenadine, a selective H_1-receptor antagonist, and 40mg of famotidine, a selective H_2-receptor antagonist) or placebo (Zeebo Effect LLC, Huntington, Vermont, United States). This dosage of histamine receptor antagonists is consistent with previous investigation [8] and results in more than 90% inhibition of H_1- and H_2-receptors with a half-life of ~6h. Following administration of antihistamines or placebo, participants rested for one hour to allow time for absorption [149,150]. During this time, participants were asked to rest supine. Then, baseline (BL) hemodynamic measurements, including arterial blood pressure, heart rate, blood flow (ultrasound), and skeletal muscle tissue oxygenation (NIRS), were obtained in the resting supine position.

Participants were then asked to engage in the resistance exercise protocol (outlined in detail below). Immediately following completion of the exercise session, participants were escorted to the resting table and placed in a supine position for immediate hemodynamic measurements that repeated at 30-, 60-, 90-, and 120-minutes post-exercise. Participants remained supine for the duration of a 120-minute exercise recovery period. 30-minutes after exercise, participants were provided with an essential amino acid supplement (contents listed below) to support the anabolic effect of resistance exercise [151]. Participants were asked to remain in the laboratory until 4h after exercise recovery was reached at which time the post-exercise biopsy was obtained.

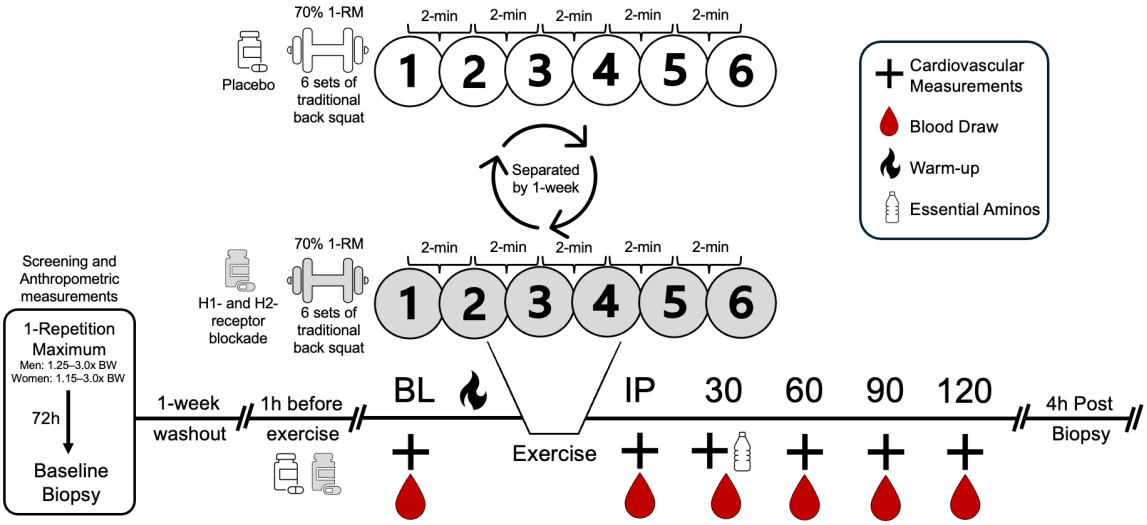

Figure 5.1 depicts the timeline of assessment days that were used for muscle biopsy collections, peak exercise tests, hemodynamic and vascular function tests, blood volume determination, and body composition assessment as detailed below.

Rigor and Reproducibility

Prior to baseline and experimental visits, participants were asked to abstain from food for 12 hours (overnight fast), consuming caffeine for at least 12 hours, nutritional supplements for

24 hours, alcohol consumption for 48 hours, and moderate or vigorous exercise for 48 hours. Women were studied irrespective of menstrual cycle phase and had a negative pregnancy test prior to all assessment days. Data collection start-time varied across participants due to availability; however, assessment start-time was consistent within a participant to minimize the influence of circadian rhythm across visits. Dietary logs were collected to maintain similar eating patterns between visits. All protocol activities took place in a thermoneutral lab environment.

1-Repetition Maximum Assessment (1-RM)

The 1-RM assessment was conducted per National Strength and Conditioning Association (NSCA) guidelines and was overseen by a well-trained member of the research team [129]. Briefly, participants were asked to complete a warm-up on a cycle ergometer at a self-selected intensity for 5 minutes, followed by 10-bodyweight squats, and 10-walking lunges. The research team then fit the participant to the weight rack, adjusting bar height and safety bars to a height that allowed for a full range of motion, but limit beyond the range of safety in the event of a failed attempt. Participants were asked to perform 3 warm-up sets of traditional back squat ranging from a light to moderate intensity (50-80% estimated 1-RM) and for no more than 10, 5 and 3 repetitions for each set. Participants then attempted their first 1-RM attempt and were allotted 3-5 minutes of rest time to recover prior to beginning the second attempt. Following each successful attempt, the load was increased, and the greatest load successfully completed by the participant, with appropriate form, and without assistance was deemed their 1-RM.

Skeletal Muscle Oxygenation (%SmO_2)

Skeletal muscle tissue oxygenation (%SmO_2) was estimated using the near-infrared spectroscopy (NIRS, MOXY Monitors, Fortiori Design, LLC, Hutchinson, MN). Two small non-invasive NIRS devices were placed on the skin surface of the vastus lateralis at the proximal border of the mid-point from the hip to the lateral femoral condyle.

Arterial Blood Pressure

At each timepoint arterial blood pressure was measured using an automated sphygmomanometer (Tango+, SunTech Medical, Raleigh, N.C., USA) in the right arm at heart level while the participant remained supine. Mean arterial pressure was calculated as diastolic pressure plus 1/3 pulse pressure (systolic pressure – diastolic pressure) and reported in mmHg.

Femoral Blood Flow and Femoral Vascular Conductance

Blood flow velocity and vessel diameter of the common femoral artery were measured in the right leg, 2-3 cm proximal of the bifurcation of the superficial and deep branches of the femoral artery. Femoral blood velocity was estimated via duplex ultrasonography using a linear-array ultrasound transducer (L9-3 Probe, Philips iE33, Andover, MA) at an insonation angle of 60° and quantified by standard methods [121]. Briefly, velocity measures were made by recording the ante- and retrograde doppler frequencies with a custom audio recording software (DUC2). For this study, velocity measurements were made at an average depth of 1.56±0.28 cm and were thin-beam corrected, based on a known beam width of 2.80 mm at that depth, which resulted in an average correction factor of 0.774±0.010 [121]. Image recordings were then analyzed via custom software (Vascular Research Tools 5; Medical Imaging Applications LLC, Coralville, IA) to determine femoral mean diameter (mm). All diameter measurements were made during diastole and representative of average diameter across a 90-second recording. Femoral blood flow (FBF) was calculated using the equation below, where artery cross-sectional area is multiplied by femoral mean blood velocity (cm/s) and represented as milliliters per minute ($mL \cdot min^{-1}$).

Equation 5.1 Femoral Blood Flow

$$\textit{Femoral Blood Flow (mL/min)} = \pi\left(\frac{\text{Diameter}}{20}\right)^2 \text{ x mean blood velocity x } 60$$

Oral Essential Amino Acid (EAA) Supplementation

EAAs were supplemented 30-minutes after resistance exercise in both conditions. Ingestion was recorded and verified by research personnel. Supplements were prepared by

Northwest Compounders with use of amino acids purchased from Ajinomoto U.S.A. EAAs distributed in pre-defined proportions and were pre-mixed with a calorie free mango-flavored beverage Supplement composition for the EAAs was: histidine, 1.8 g (9% of total); isoleucine, 1.8 g (9%); leucine, 5.8 g (29%); lysine, 2.8 g (14%); methionine, 0.6 g (3%); phenylalanine, 2.8 g (14%); threonine, 2.4 g (12%); and valine, 2.0 g (10%).. The goal of this supplementation was to maximize the anabolic effect of exercise and EAAs [151].

Skeletal Muscle Biopsy

All skeletal muscle biopsies were taken from the mid-portion of the vastus lateralis approximately 18 cm proximal to the patella, approximating the midline of the quadriceps muscle group. Subsequent biopsies acquired from the same leg were performed at a distance of 2-4 cm proximal or distal (randomly determined) to the baseline biopsy site. Biopsies were performed using a sterile technique as previously described [152]. The skin and underlying fascia were anesthetized using 1% lidocaine hydrochloride (Hospira Worldwide, Lake Forest, IL, USA). Participant's perception of pain was verbally obtained to ensure the target area was effectively anesthetized. Once verbal confirmation of local anesthetic effectiveness was obtained, a small incision was made in the skin and underlying fascia before introduction of a 5mm Bergstrom biopsy (Micrins Surgical, Lake Forest, Illinois) needle to the vastus lateralis muscle. A 20-cc syringe and tubing were attached to the aspiration port on the Bergstrom needle to apply negative pressure to assist the biopsy using applied suction, and a muscle sample approximately 100-175mg was obtained from each biopsy. After the vastus lateralis muscle sample is collected, the tissue sample was initially blotted with gauze, and any visible adipose tissue was removed. The sample was then covered in medium (Optimal Cutting Temperature Compound (OCT)) and immediately flash frozen in melting isopentane cooled with liquid nitrogen and stored at -80°C until later analysis.

Cryostat Sectioning

Tissue sections were cut at 7mm thickness in a cryostat maintained at -25°C, and mounted on Fisherbrand Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania). 6 cuts from each timepoint (BL, 4H Placebo or Blockade) within a single participant were placed on a single slide. Each cut of tissue included on the same slide was carefully placed to avoid any overlap between cuts. Additionally, tissue from both conditions were placed onto a single slide, however tissue samples were coded to maintain investigator blind.

Immunohistochemistry

For determination of the number of neutrophils, sections were first fixed in ice cold acetone (-20°C) for three minutes. After being washed 3x in Phosphate Buffered Saline (PBS), endogenous peroxidases were then blocked with 3% hydrogen peroxide (H_2O_2) diluted in PBS for seven minutes at room temperature. Following a second wash in PBS, all sections were blocked using 2.5% Normal Goat Serum (NGS) and 2.5% Normal Donkey Serum (NDS; both Jackson Immunoresearch, West Grove, PA) pre-mixed with 1% glycine and left at room temperature for one hour. Sections were then incubated for 1 hour at room temperature with primary antibody cocktail diluted in 2.5% NGS and 2.5% NDS (Jackson Immunoresearch, West Grove, PA) and PBS. Primary antibody cocktail contained an antibody for neutrophils (myeloperoxidase, MPO; Invitrogen MA1-34067, 1:500), capillary (UEA Rhodamine 1:500, labelled with Ulex Europaeus Agglutinin, Vector Labs, RL-1062), and pericentriolar material 1, PCM1 (anti-PCM1 Polyclonal, 1:1000, HPA023370-100UL, Human Protein Atlas Number HPA023370). Sections were then incubated overnight at 4°C. Following overnight incubation sections were twice washed and treated with 3% H_2O_2 diluted in PBS for seven minutes at room temperature. Sections were washed again before adding secondary antibodies to a cocktail containing 2.5% NGS and 2.5% NDS (Jackson Immunoresearch, West Grove, PA) and PBS. Secondary antibody cocktail containing Goat (Gt) anti-mouse (Ms) IgG1, Alexa Fluor 488 (1:500) Invitrogen, A21121) and Gt anti-rabbit IgG Alexa Fuor 647 (1:500, Invitrogen A21245) was added and sections were left to incubate for one-hour at room temperature. Sections were

then post-fixed with methanol for 5 minutes at room temperature and washed in PBS. Finally, sections were mounted with DAPI (SlowFade Diamond Antifade with DAPI; Invitrogen, S36964) stored at -20°C.

Imagining and Analysis

Tissue sections were imaged using a Leica Flourescence Micropscope (DM4000B) equipped with a Leica Camera (DFC 360FX). Imaging was performed at 20x magnification unless otherwise noted. Multiple images of each section were taken systematically to avoid imaging the same region of the section twice. Sections were coded to ensure that the researcher performing the imaging was blinded to treatment group.

Western Blotting

Homogenized skeletal muscle samples (20–50 μg) were separated by electrophoresis on 4–20% SDS polyacrylamide separating gels (Life Technologies, Grand Island, NY) and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were Ponceau-stained to assess transfer across each gel. Membranes were incubated for 1 h in Intercept Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and then incubated overnight at 4°C in blocking buffer containing primary antibodies (p70 S6 Kinase, and Phosph-p70 S6 Kinase, Thr389; mTOR, and Phospho-mTOR, Ser2448; 4E-BP1, and Phospho-4EBP1, Ser65, Cell Signaling Technology, Danvers, Massachusetts, USA). Membranes were then washed and incubated with the appropriate secondary antibodies (LI-COR Biosciences) for 1 h at room temperature. The fluorescent bands were digitized using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences). Digitized images were quantified using LI-COR Image Studio™ software. Antibodies were stripped using NewBlot™ Nitro Stripping Buffer (LI-COR Biosciences) in between probing for primary antibodies. Initial antibodies did not appear to function. We are troubleshooting, so no results will be displayed from western blot analyses.

Statistics

Data are reported as mean with 95% confidence intervals. Alpha was set at 0.05 for all statistical inferences including familywise error rates. Inferences which were drawn from two-way mixed-effects models with pre-planned comparisons (Prism 10, GraphPad, Boston, MA, USA). Inferences regarding changes over time within each condition were drawn from Dunnett's multiple comparisons test versus baseline, with reported p-values and confidence intervals adjusted for multiplicity. Inferences regarding the differences between conditions were drawn from Šidák's multiple comparisons test, applied to the change from baseline and restricted to comparisons at the same timepoint.

RESULTS

Exercise Performance

During the placebo condition, 54.5 [47.4, 61.6] repetitions were completed unassisted. This was similar (p=0.40) to the blockade condition, where 56.4 [53.0, 60.0] repetitions were completed unassisted. On average, the recovery between sets was also not different between placebo (130.5 [122.4,138.6] s) and blockade (129.6 [120.0, 139.2] s; p=0.76). **Figure 5.2B** displays the accumulation of voluntary volume achieved during each exercise condition. There were no differences between the total voluntary volume accumulated during placebo (4140 [3160, 5121] kg) and blockade (4348 [3351,5345] kg; p=0.44).

Heart Rate Response

Figure 5.2A displays the heart rate response during exercise and throughout exercise recovery in both conditions. Exercise heart rates were elevated above baseline in both conditions following set 1 (all p<0.01). During blockade, end-set heart rates were elevated above placebo at several times, and intra-set recovery heart rates were elevated above placebo on one occasion, as depicted in the figure. Specifically, higher end-set heart rates were achieved following sets 1 (p<0.01), 2 (p=0.05), 4 (p<0.01), and 5 (p=0.04) in the blockade condition. Heart rates remained elevated above baseline in both conditions throughout 2h recovery (all p<0.01).

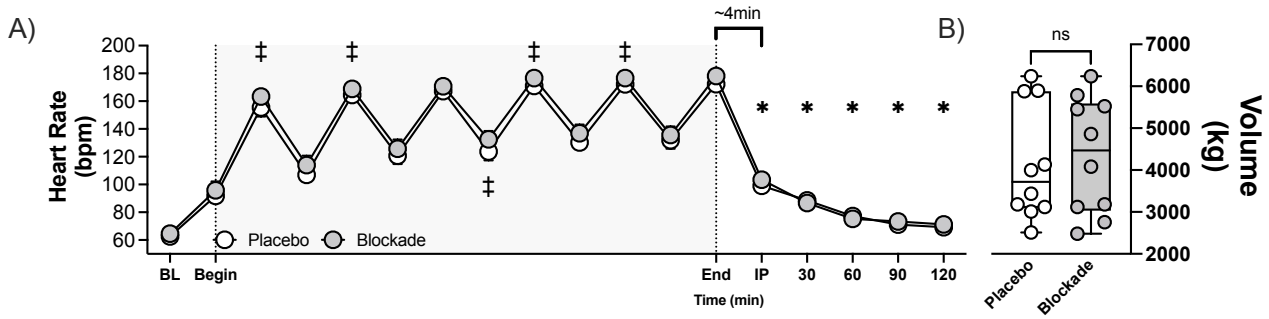

Figure 5.2 A) Heart rate response during exercise and exercise recovery for both conditions. B) Voluntary volume accumulation during exercise for both conditions. Values are means (95% confidence limits). Open symbols are placebo-control; filled are blockade (n=10). * denotes $p < 0.05$ versus baseline for both exercise conditions; ‡ denotes $p < 0.05$ placebo-control versus blockade.

Skeletal Muscle Oxygenation

Figures 5.3A, 5.3B, and 5.3C display measurements of skeletal muscle oxygenation (%SmO_2) measured via near-infrared spectroscopy during both exercise conditions. Pre-set %SmO_2 (5.3A) was increased following set 2 in both conditions (both $p<0.05$) compared to before set 1, but was not different between exercise conditions at any time point. End-set %SmO_2 (5.3B) remained similar between sets, aside from an increase following set 6, seen only in the placebo condition ($p=0.04$). Blockade was lower than placebo following several of the sets, as depicted in the figure. Specifically, sets 1 ($p=0.04$), 4 ($p<0.01$), 5 ($p<0.01$), and 6 ($p<0.01$) were lower with blockade. Desaturation represents the difference between pre- and end-set %SmO_2 (Δ%SmO_2) and was greater following some of the later sets in blockade compared to set one and placebo, as depicted in the figure. Specifically, sets 4 ($p=0.01$), 5 ($p=0.09$), and 6 ($p<0.01$) were greater in blockade.

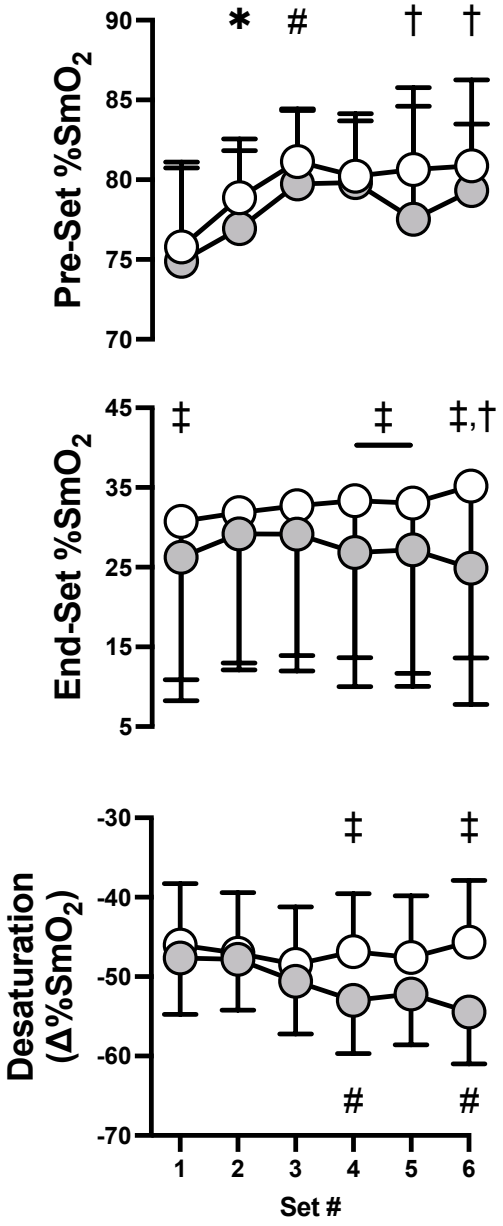

Figure 5.3. Skeletal muscle oxygenation values achieved between the conditions throughout the resistance exercise protocol. A) displays pre-set skeletal muscle oxygenation (%SmO_2); B) End-set %SmO_2; and C) Desaturation (Δ%SmO_2) in each set or the difference between pre- and end-set %SmO_2. Values are means (95% confidence limits). Open symbols are placebo-control; filled are blockade (n=9). ‡ denotes $p < 0.05$ placebo-control versus blockade; † denotes $p < 0.05$ from set one, placebo-control only. # denotes $p < 0.05$ from set one, blockade only.

Skeletal Muscle Reoxygenation (between sets)

Figures 5.4A, 5.4B, 5.4C, and 5.4D display the rate of skeletal muscle reoxygenation expressed as $\%SmO_2 \cdot s^{-1}$, the time to reach half-maximum skeletal muscle oxygenation ($D\%SmO_{2\ 1/2RT}$), time to peak $\%SmO_2$ (measured in s), and the total inter-set recovery time. The differences observed were during inter-set recovery following set 3 were blockade recovery time was greater than following set 1 (p=0.03) and following set 5 compared to set 1 in placebo-control (p<0.01); however, $\%SmO_2 \cdot s^{-1}$ following set 4 also tended to be greater in blockade compared to the control condition (p=0.07). Values following set 6 were not analyzed as they were influenced by the setup for post-exercise recovery measurements.

Femoral Blood Flow

Figure 5.5A displays the change in femoral blood flow from baseline during exercise recovery in both conditions. Blood flow was increased above baseline immediately post-exercise in both conditions (both p<0.01). However, blood flow immediately post-exercise was lower in blockade (255 [191, 320] $mL \cdot min^{-1}$) than in placebo (414 [336, 492] $mL \cdot min^{-1}$; p<0.01). Blood flow in both conditions remained above baseline until 90-min post-exercise. At 120-min, blockade blood flow was no longer above resting levels (p=0.48), but the placebo blood flow remained elevated (p=0.07).

Femoral Vascular Conductance

Figure 5.5B displays the change in femoral vascular conductance from baseline during exercise recovery in both conditions. Vascular conductance was increased above baseline immediately post-exercise in both conditions (both p<0.01). However, conductance immediately post-exercise was lower in blockade (274 [207, 340] $mL \cdot min^{-1} \cdot mmHg$) than in placebo (468 [367, 570] $mL \cdot min^{-1} \cdot mmHg$; p<0.01). Vascular conductance in both conditions remained above baseline until 90-min post-exercise. At that time, blockade conductance was no longer above resting (p=0.19), but the placebo conductance remained elevated (p<0.01).

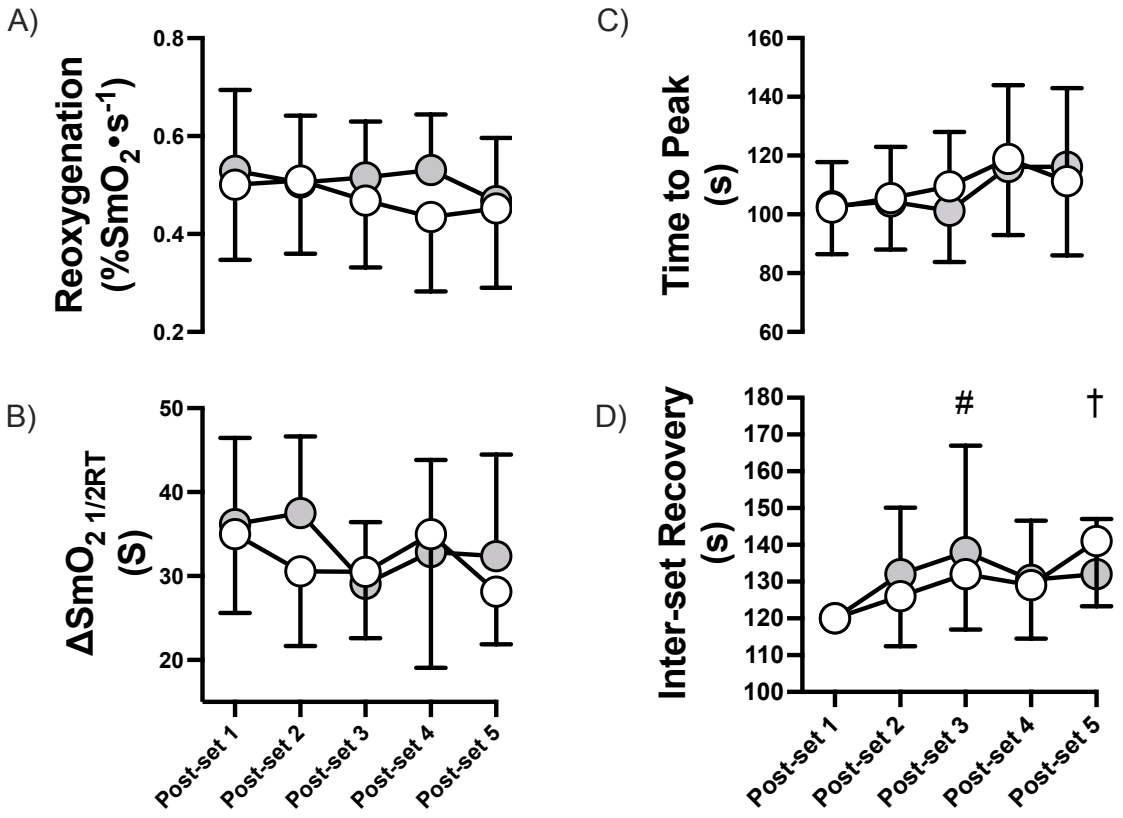

Figure 5.4. Skeletal muscle reoxygenation kinetics during inter-set recovery periods. A) rate of skeletal muscle reoxygenation expressed as $\%SmO_2 \cdot s^{-1}$, B) the time to reach half-maximum skeletal muscle oxygenation ($\Delta\%SmO_{2\ 1/2RT}$), C) the time to peak $\%SmO_2$ and Δ) total inter-set recovery time, (measured in s). Values are means (95% confidence limits). Open symbols are placebo-control; filled are blockade (n=9). † denotes $p < 0.05$ from set one, placebo-control only. # denotes $p < 0.05$ from set one, blockade only.

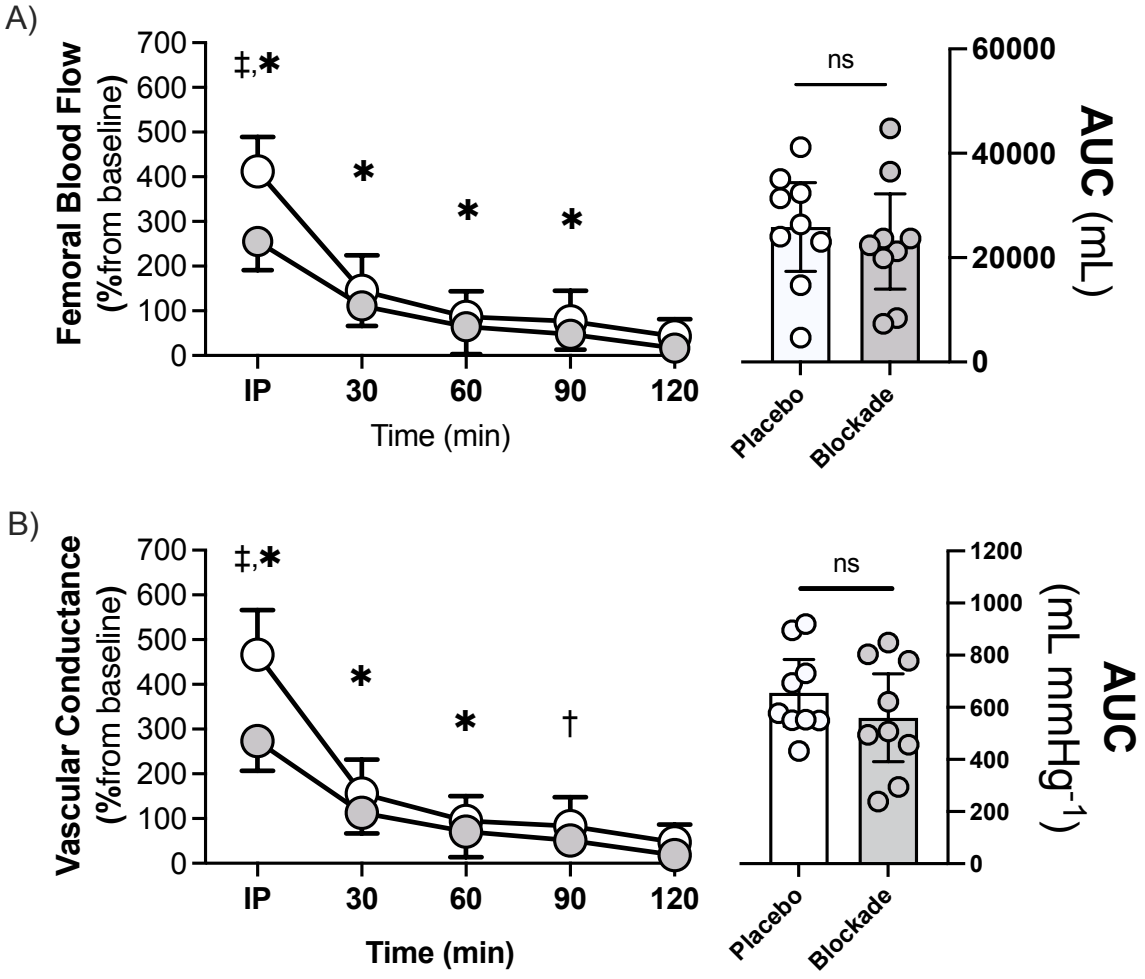

Figure 5.5 Change in femoral blood flow and femoral vascular conductance from baseline immediately postexercise (IP), 30-, 60-, 90-, and 120-min post-exercise (left panels). Right panels show area under the curve for each, including individual data. Values are means (95% confidence limits). Open symbols are placebo-control; filled are blockade (n=10). * denotes $p < 0.05$ versus baseline for both exercise conditions; ‡ denotes $p < 0.05$ placebo-control versus blockade; † denotes $p < 0.05$ placebo-control versus baseline.

Arterial Blood Pressure

Figures 5.6A, 5.6B, and 5.6C display the changes in systolic (5.6A), diastolic (5.6B), and mean arterial pressure (5.6C) from baseline in response to both conditions. There were no notable changes to systolic pressures following either condition. Diastolic pressure was reduced immediately following both conditions (both $p<0.01$) before returning toward baseline by 30-min (both $p>0.05$). These reductions were not different between conditions ($p=0.31$). Mean arterial pressures were reduced immediately following the placebo condition ($p<0.01$) and remained reduced until 30-min ($p=0.04$) before returning toward baseline at 60-min ($p=0.08$). Following blockade, there was a tendency for mean arterial pressures to be reduced immediately following exercise ($p=0.07$) and again at 60-min ($p=0.06$).

Neutrophil Response

Figure 5.7 displays the neutrophil infiltration response to exercise with and without histamine receptor blockade following 4h of recovery. There was a similar increase in neutrophils per tissue area (left panel, both conditions $p<0.05$) and the percent change from baseline (right panel, both conditions $p<0.05$). No differences were observed between the conditions ($p=0.85$).

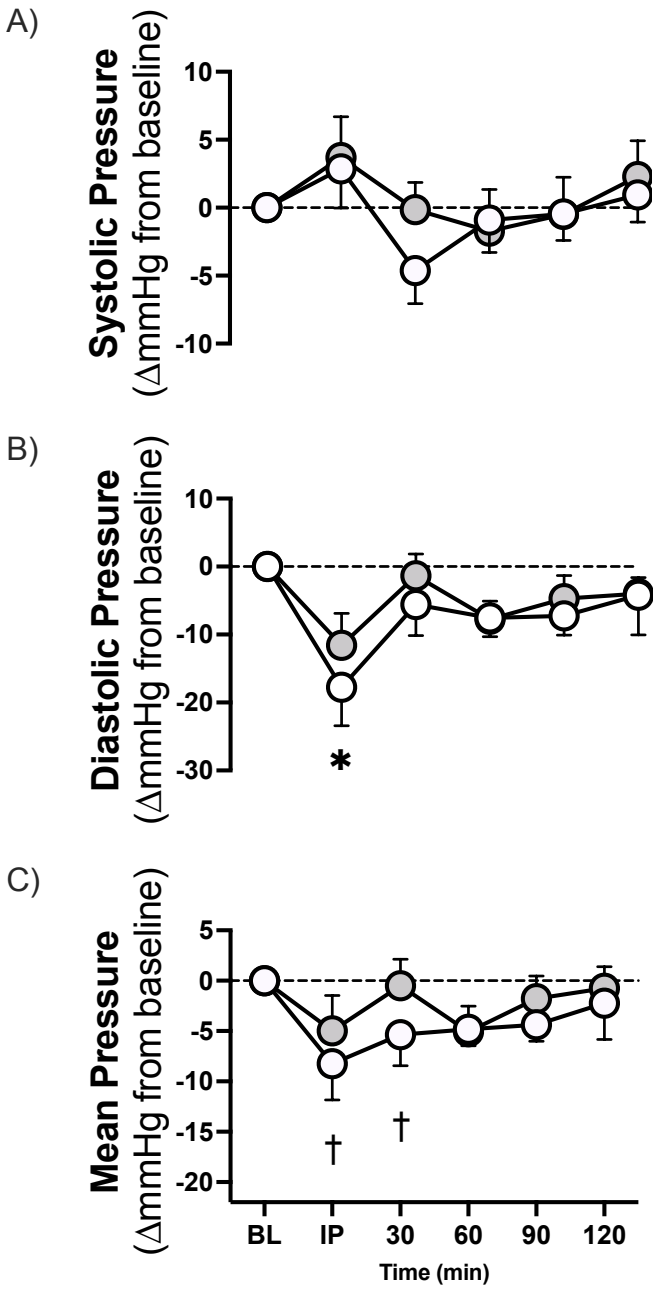

Figure 5.6. Change in arterial blood pressure following resistance exercise. A) Systolic, B) Diastolic, and C) Mean arterial pressure. Values are means (95% confidence limits). Open symbols are placebo-control; filled are blockade (n=10). * denotes $p < 0.05$ versus baseline for both exercise conditions; † denotes $p < 0.05$ placebo-control versus baseline.

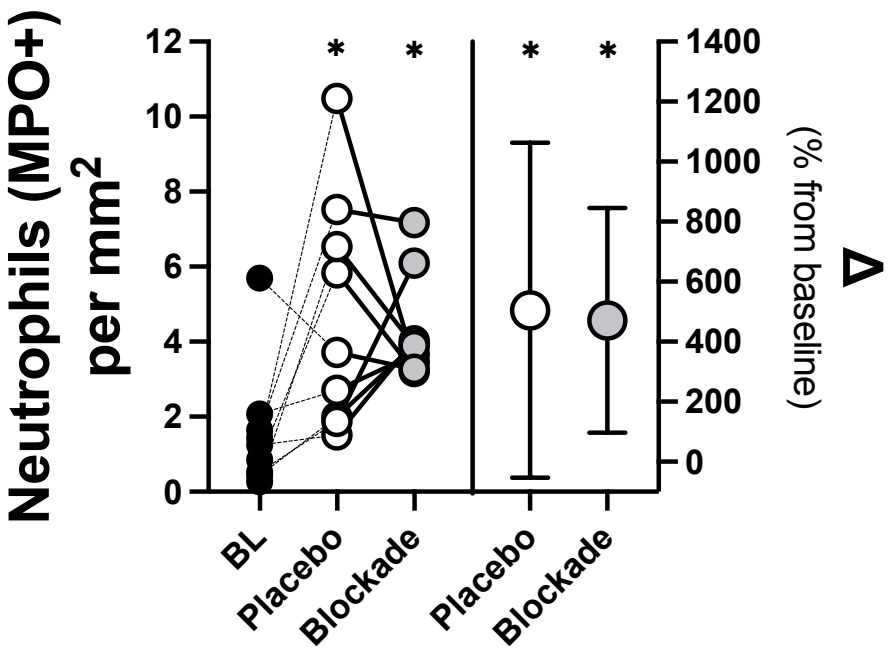

Figure 5.7. Neutrophil response to resistance exercise from baseline (BL) to 4h recovery. Individual responses (right panel) and average change from baseline (Δ; right panel). Values are means (95% confidence limits). Open symbols are placebo-control; filled are blockade (n=9). * denotes $p < 0.05$ versus baseline for both exercise conditions.

DISCUSSION

In this investigation, we sought to determine the impact of histamine signaling on vascular permeability and neutrophil infiltration. We hypothesized that when histamine signaling was attenuated, fewer neutrophils would migrate from the circulation into the skeletal muscle tissue during exercise recovery. In contrast, the results of our study indicate that neutrophil infiltration into skeletal muscle is not histamine dependent. This suggests that other mechanisms of neutrophil movement between the circulation and skeletal muscle may persist during the recovery after exercise.

We also hypothesized a decline in skeletal muscle oxygenation during exercise with histamine receptor antagonists and that this would induce a greater blood flow response to exercise. While we did find skeletal muscle oxygenation was reduced during exercise with

histamine receptor antagonists, however, this was associated with a reduced skeletal muscle blood flow measured immediately after exercise.

We anticipated poor skeletal muscle perfusion would continue throughout exercise recovery following exercise with histamine receptor antagonism, diminishing nutrient delivery and reducing the phosphorylation of mTOR proteins. Unfortunately, we could not assess the impact of this poor perfusion on mTOR protein phosphorylation status due to technical issues noted above.

Finally, the results of our study confirm that histamine does play a role in the response to resistance exercise, a finding that was suggested by the study presented in Chapter IV and stands in contrast to earlier work using much more limited muscle mass [18].

Neutrophil Response

Generally, exercise-induced inflammation is initiated by some combination of exercise-related stressors: thermal strain, damage-associated molecular patterns (DAMPs) from damaged and/or dead cells, tissue and/or cellular stresses, like hypoxia, or energetic stress[74]. These stressors promote a measurable rise in circulating white blood cells, or leukocyte concentrations, followed by an eventual decline toward pre-exercise values[92,153,154]. The acute leukocyte responses to high-force exercises, like resistance exercise, are well documented [86,87]. While multiple inflammatory signal molecules (i.e., Interluekin-(IL-) 6, IL-8, or IL-1b) can be measured in the circulation following exercise, none of these directly correlate with the patterns of immune cell mobilization [88]. Because histamine concentrations are seldom altered in circulation following exercise [50], histamine also seems an unlikely candidate for the direct mobilization of leukocytes after exercise, yet, Ely et al. found some modulation of the magnitude of the circulating immune cells response following exercise [94]. The authors concluded that histamine could be exerting a local influence within the skeletal muscle that impacts the systemic responses to exercise (rather than a direct role of histamine in driving leukocytosis).

Ely's conclusion was based on observations by Romero et al.,[6] , that histamine regulates the transcription of inflammatory cytokines within the skeletal muscle environment following exercise [6], and other work has shown us that histamine receptor activation on endothelial cells can promote their release [55–57] to facilitate neutrophil recruitment toward areas of inflammation

[58,59]. Neutrophils, once mobilized into circulation, are attracted toward sites of inflammation, and the establishment of weak and transient interactions with endothelial cell surfaces influences their movement into the tissue compartment[74]. Histamine-receptor activation on endothelial cells promotes expression of docking structures, termed selectins, on their vascular facing surfaces to facilitate this process [75] and promotes widening of gap junctions (increasing permeability) between adjacent endothelial cells [99,155]; however, an absence of histamine signaling does not abolish the former completely, as activation of selectins can be driven by pro-inflammatory cytokines, like IL-1b and TNF-a[74,77]. Hence, it may be that histamine generally promotes infiltration of immune cells into skeletal muscle tissue following exercise, but it does not appear to be necessary for the process.

One of the challenges in interpreting the prior transcriptomic work by Romero et al..,[6] and Van der Stede et al., [50] were the inability to disentangle the contribution from resident versus infiltrating immune cells in the observed responses to exercise. The differential expression of transcripts [6] within bulk muscle following exercise may be sensitive to a change in phenotype of infiltrating cells. However, given that the current study shows no overall impact of histamine receptor blockade on the entry of neutrophils into skeletal muscle, this indirectly supports the interpretation that the differential transcriptome responses observed previously with histamine receptor blockade are indeed due to histamine signaling within skeletal muscle tissue and not a matter of changing cell populations. Hence, Van der Stede et al.,[50] They were right to conclude that there is a unique histamine-receptor axis that functions between mast cells and other histamine-receptor-expressing cells, including vascular endothelial cells and other mononuclear cells that reside within skeletal muscle tissue. Of note, mast cells are the only cell line within human skeletal muscle tissue that express histidine decarboxylase, the enzyme that is essential to producing histamine. Many cell types within skeletal muscle tissue express either H_1 or H_2 receptors or both, and are hence responsive to intramuscular histamine from mast cells. [2146–50].

Van der Stede et al., also posited a histamine-mediated regulatory role in neutrophils as a possible contributor of glycogen resynthesis via IL-1-mediated translocation of the glucose receptor, GLUT4, from neutrophils. Again, our results suggest there is no impact on neutrophil infiltration following exercise with and without histamine receptor antagonism; however, this may support a change in neutrophil phenotypic function, releasing IL-1[156] and reducing glucose

uptake and glycogen resynthesis. Alternatively, no difference in neutrophil infiltration may emphasize previous findings indicating histamine's modulatory role of histamine on blood flow and skeletal muscle perfusion supports glucose uptake and the resynthesis of skeletal muscle glycogen [5,50].

Muscle Oxygen Supply and Demand

Although the body of literature examining skeletal muscle oxygenation during resistance exercise is sparse when compared to aerobic exercise, resistance exercise poses a considerable demand on the cardiovascular system to deliver oxygenated blood to working tissues, especially during recovery between and after sets. Muscle oximetry based on NIRS provides real-time insight into oxy-hemoglobin saturation of the vascular bed of exercising tissues [157]. Considering that capillaries contribute to > 90% of the total blood volume within skeletal muscle [158], under normoxic breathing conditions in young healthy individuals, the changes in oxygenation of hemoglobin and myoglobin detected by NIRS mostly reflect reductions in capillary and myocellular oxygen levels during exercise [157].

Resistance exercise primarily relies on the phosphocreatine (PCr) system, however, the link between PCr pool depletion and resynthesis as a function of mitochondrial energy production directly relates it to skeletal muscle oxygenation [159,160]. Thus, resistance exercise paired with short inter-set rest may push the limits of oxygen delivery within a short time course that does not allow for complete PCr resynthesis and could potentially diminish exercise performance across repeated efforts. In this study, however, we did not observe a reduction in the voluntary volume accumulated between conditions, despite lower values of skeletal muscle oxygenation achieved during the blockade condition. Similarly, the decline in reoxygenation kinetics between subsequent sets does not appear to be compromised by histamine receptor antagonism. Thus, it is not surprising that task completion was similar in both conditions.

Preceding each set, participants were asked to report their perceived recovery status [161] and participants were allowed to rest until a perceived recovery status (value indicated >2 out of 10) was reached before a subsequent set could begin. This resulted in some variation in recovery times between sets, but there was no difference between conditions. However, it is plausible that the accumulation of voluntary volume may have been influenced by allowing so much rest.. The

average time to reach peak skeletal muscle oxygenation between sets did not exceed 120-s for any set, and did not differ between conditions, suggesting that the perceived recovery (generally longer than time to peak reoxygenation) does not align with the recovery of skeletal muscle tissue oxygenation between each set. Within the constraints of the current study, tissue oxygenation often did not align with perception. However, following the first exercise set, participants' perceived exertion was greater in blockade compared to placebo and this did align with a lower end-set skeletal muscle oxygenation in blockade.

Thus, the regulation of skeletal muscle blood flow during heavy resistance exercise may be impaired when histamine receptors are attenuated, potentially setting the stage for a reduced exercise performance if recovery between sets is inadequate. The current data suggests that by set 4, blockade consistently achieved lower end-set skeletal muscle oxygenation values, indicating histamine modulates skeletal muscle oxygenation during exercise at least in this specific model. However, reoxygenation between sets may have remained sufficient to restore PCr prior to the next set, maintaining performance[57].

Histamine Response to Resistance Exercise

Over the past 20 years, it has been well documented that aerobic exercise models elicit the release of histamine within skeletal muscle and that this impacts blood flow and vascular conductance for up to two hours following cycling exercise [7]. In contrast, only one prior study had explored the potential release of histamine with resistance exercise. In that study, Barrett-O'Keefe et al., [18] did not observe histamine-mediated effects on blood flow. Our current observations differ in that we did observe histamine-mediated dilation immediately after resistance exercise. An important distinction between the current study and the prior one is that Barrett-O'Keefe et al., [18] used a unilateral knee-extension model incorporating three sets of eight repetitions at 80% of peak torque.

By comparison, that was a small exercise volume, and below what would be expected to generate an adaptive response within the context of strength training (i.e., not likely to improve muscle strength or size [162]). It was likely an insufficient exercise signal for histamine release, whereas the current study used a substantial exercise volume and did appear to be sufficient to signal histamine release[23]. Of note, the exercise-related signal (or signals) for histamine release is

still unknown, although there is some evidence that muscle temperature may be one of the stimuli involved. One might look to commonality between aerobic and resistance exercise for what could trigger histamine release within skeletal muscle.

In the current study, we have captured a role of histamine signaling in the immediate post-exercise hyperemic response that was short-lived compared to the sustained post-exercise vasodilation seen after aerobic exercise. Hence, the signals that drive the histamine response are likely dependent upon the duration of exercise, the exercise type, and the exercise intensity [147]. What we have observed following this model of resistance exercise is similar to what was observed in classic investigations of H_1-receptor antagonists on hind-limb blood flow following electrically induced skeletal muscle contraction. Those studies found that the histamine contribution to vascular conductance was short-lived [163], as did the current study[60–63].

It seems clear that high force production *per se* is not associated with whatever signals trigger histamine release, as cycling at high powers such as 75% of peak aerobic capacity elicits only about 38% of maximal dynamic muscle force [164]. If it were about high force production, then one would expect the current protocol to generate a larger histamine response than observed in aerobic exercise studies, and this is not the case. The answer could lie in the metabolic flux, energy demand, or thermal loads associated with both types of exercise. One could speculate that some component of fatigue elicited by a high number of total contractions during aerobic exercise, which also appears to be a determinant of resistance exercise adaptation [162] may be a necessary prerequisite for histamine release. However, other data indicate histamine release occurs early during aerobic exercise [10], before notable fatigue.

With both aerobic and resistance exercise being capable of generating a histamine response, this suggests that the transcriptomic response to both resistance and aerobic exercise may be similarly influenced by histamine signaling. This might inform some of the common adaptations observed between both exercise types [9], but we also consider the possibility that histamine signaling may interact with the major transcriptional programs that are sensitive to each exercise type.

CONCLUSION

To our knowledge, this is the first study to use H_1 and H_2-receptor antagonism during large muscle mass resistance exercise. We demonstrate that resistance exercise incorporating large muscle mass and short inter-set rest generates a histamine response. We are learning there is potentially a threshold of exercise variables (i.e., intensity, duration, volume, etc) that must be met to elicit histamine release, not unlike what is necessary to elicit adaptive responses to exercise. Thus, the current results suggest that histamine is a molecular signal present in exercise in a broad sense, capable of altering the transcriptomic response [6] necessary for adaptation [8]. Our findings indicate that there is no impact of histamine on neutrophil movement between the circulation and skeletal muscle at 4 h after resistance exercise. However, in light of other investigations [8], it is likely that histamine modulates the activity of immune cells within skeletal muscle and is necessary [6]for the coordinated effort between the skeletal muscle and the immune system to complete the adaptive remodeling process imitated by exercise [50] .

CHAPTER VI
TAKE AWAYS

REVIEW OF FINDINGS

Our findings indicate that there is indeed a histamine response initiated by multiple forms of exercise and that its presence is detectable using measurements based on its breakdown products. We have also revealed the role of histamine signaling on the blood flow response to and skeletal muscle oxygenation during resistance exercise. Although more research will need to be conducted to characterize the sensitivity of histamine's metabolites to variations in exercise intensity and exercise duration, we have shown a coherence of histamine's metabolites using multiple methods to capture the histaminergic response to exercise. Together, these results extend histamine's relevance as a molecular transducer of exercise adaptation across the exercise spectrum.

Potential Application

In the time after exercise, histamine's influence as a vasodilator of the peripheral vasculature has been the most easily observed of its effects [1,3,4,122,165–167]. More recently, histamine's actions on post-exercise gene transcription[6] have become recognized and appear necessary for the complete adaptive benefits of exercise to be realized [8]However, our ability to quantify the magnitude of histamine released during exercise has been challenging. Therefore, the current work fills a critical gap in our ability to determine histamine production during multiple forms of exercise through measurement of its metabolites. This potentially enables its novel use as a biomarker of acute exercise strain to limit overtraining progression.

Increasing exercise volume and intensity are necessary to meet overloading principles of adaptation. However, excessive increases in either variable, particularly when paired with inadequate rest [140], can tip the scale toward overreaching [141]. The integrative nature of overtraining symptoms (including a decline of exercise performance and altered skeletal muscle function [142,143], incomplete resolution of exercise-induced inflammation [45], reductions in cardiac output limited by stroke volume independently from reduced blood volume [144], and increased

resting arterial stiffness) emphasizes the need for a measurement that is sensitive to the broad stress imposed during multiple forms of exercise. Such a measure might enable researchers and athletic training teams to limit the progression of overtraining more effectively.

We speculate that the next phase in this work might be to explore whether an elevated histamine profile in the morning (i.e., increased MIAA) may indicate poor or incomplete recovery status. The current findings indicate acute urinary MIAA production rates increase following exercise (2h) before returning toward baseline 24-h later. Thus, prolonged exercise, or training regimens that elicit elevated baseline production rates of MIAA, or those that are not reduced following 24-h after exercise may indicate poor or incomplete recovery. Importantly, the current data support the utility of a urine-based biomarker capable of capturing patterns of blood flow elicited by an exercise session, a response that bears close relationship with exercise intensity [145]. Follow-up investigation will be necessary to determine the sensitivity of histamine and its metabolites to acute changes in exercise volume and intensity for use as a diagnostic tool assessing athlete training and competition preparedness (suggested utility described in Chapter IV, Figure 7).

Another interesting application of histamine metabolic analysis might be in response to non-exercise sources of histamine. Swanevelder et al., [168] and du Toit et al., [169] have shown that having a documented history of allergies in athletes is well correlated with a greater likelihood of gradual onset injury (i.e., overtraining injuries). Thus, an elevated basal release of histamine may be present before exercise and link to poor outcomes with exercise training. In this scenario, detecting elevated histamine metabolites may indicate a need to reduce exercise volume or intensity to limit the progression of overtraining or reduce injury risk during periods of greater allergen exposures. Alternatively, the findings from Swanevelder et al., [168] and du Toit et al., [169] may highlight the impairment of recovery and adaptive processes if these allergy symptoms are treated via over-the-counter antihistamine medications (H_1- and H_2-receptor antagonism). In most cases, the work from our lab, and now others [8], would suggest that treating allergic symptoms with over-the-counter antihistamine medication may be deleterious to exercise performance and adaptation; however, we do not know how untreated histamine symptoms, originating from non-exercising sources such as environmental allergens, may perturb the system beyond its impact on reducing lung function in asthmatic patients [170].

Role of Histamine and its Receptors During Exercise Recovery

Van der Stede et al., [50] have clarified a complex communication network between skeletal muscle and the immune system, specifically showing that the expression of exercise-responsive genes in bulk muscle is greater than in muscle fibers alone and results from responses in multiple lines of mononuclear cells. We know that blockade of histamine H_1- and H_2-receptors induces divergent transcriptional responses following exercise [6]. Van der Stede et al., extended this to include disparate actions between receptors on transcriptional responses to exercise with either H_1- or H_2-receptor antagonism [50].

Histamine receptor expression ratios on circulating macrophages favor H_2- to H_1-receptors (>200:1) and shift to favor H_1-receptors once having entered target tissue compartments [171,172]. Following this pattern, selective H_2-receptor antagonism elicits a stimulatory effect on circulating immune cells, augmenting pro-inflammatory signaling considered deleterious [173] and H_1-receptor activation on T-helper (Th-) cells enhances interferon-g (IFN-g) activating M1- (pro-inflammatory) macrophages while inhibiting IL-4 release, and delaying progression toward the anti-inflammatory M2-macrophage phenotype [114,174]. Thus, selective inhibition of H_1- versus H_2-receptors has the capacity to alter the inflammatory environment within the skeletal muscle tissue, as captured by Van der Stede et al., [50].

Importantly, the current work indicates that the differential expression of exercise-sensitive transcripts that has been observed with histamine receptor antagonism in bulk muscle [6,50] is not likely in response to alterations in immune cell infiltration. Our findings suggest neutrophils are capable of extravasating without histamine's actions to promote the release of localized recruitment signals (i.e., cytokines, IL-6, IL-8) from endothelial cells via H_1-receptor activation [148,175], nor is neutrophil infiltration limited by abolishing histamine's actions on vascular permeability [99]. However, it is plausible that greater strain imposed on neutrophils, or other immune cells, as they pass through a less permeable barrier in response to histamine receptor antagonism may alter inflammatory signaling capacity [6,50,176], thus contributing to altered expression of adaptive signaling within the skeletal muscle tissue [6,50].

Histamine Modulates Hemodynamic Responses Across Exercise Types

An important goal of this dissertation was to define the presence and actions of histamine following resistance exercise. Importantly, we report that histamine production and release are present following resistance exercise. Not only have we observed an accumulation of histamine's breakdown products in the response to resistance exercise, but we have also revealed a role for histamine in the immediate blood flow response detected following exercise. As a collective, these results suggest that histamine influences the response to resistance exercise, modulating skeletal muscle perfusion and supporting exercising skeletal muscle tissue oxygenation.

Previous work conducted by Barrett O'Keefe et al., [18] concluded through the use of small-muscle mass resistance exercise that histamine does not contribute to post-exercise blood flow responses to resistance exercise. The current work employed a more relevant model of resistance exercise designed to elicit fatigue through incorporation of relatively high exercise intensity and short inter-set rest, and found that histamine receptor activation augments skeletal muscle blood flow by ~150% measured immediately following exercise. Beyond this time point, it does not appear that histamine is the sole contributor to blood flow to the skeletal muscle, as placebo control and H_1- and H_2-receptor blockade elicited similar increases beyond baseline.

However, vascular conductance in the placebo condition remains elevated above baseline at 90 min post-exercise. These responses are impressive when you consider the duration of resistance exercise relative to the extent that blood flow was increased. In completing six sets of ten repetitions, the mechanical work may have lasted for approximately six minutes, likely less, and generated a greater blood flow response compared to sustained aerobic exercise. Furthermore, this short duration of work led to substantial histamine production, similar to 30 minutes of continuous aerobic exercise. Taken together, this highlights the lasting impact of large muscle mass resistance exercise on the vascular system.

CONCLUSIONS

In conclusion, histamine likely mediates similar mechanisms of adaptation between aerobic and resistance exercise [9]. However, more specific responses may be influenced by

histamine signaling but are not dependent upon it. These findings extend previous work examining histamine's role in response to aerobic and dynamic exercise to include models of resistance exercise. Further work should manipulate the variables of resistance exercise training, as this might reveal more mechanistic insight into the production and release of histamine from skeletal muscle during exercise. Finally, this work positions histamine metabolites as a potential target for a biomarker of exercise-related stress for monitoring exercise load, preventing overtraining, and mitigating injury development.

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