Histamine in Action: Exploring Its Release and Role in Exercising Skeletal Muscle

by

Kieran Shay Struebin Abbotts

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Dissertation Committee:

Dr. Brad W. Wilkins, Chair

Dr. John R. Halliwill, Core Member

Dr. Christopher T. Minson, Core Member

Dr. J. Josh Snodgrass, Institutional Representative

University of Oregon

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DISSERTATION ABSTRACT

Kieran Shay Struebin Abbotts

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Title: Histamine in Action: Exploring Its Release and Role in Exercising Skeletal Muscle

Histamine is released in exercising skeletal muscle, where it has a pronounced effect on sustained post-exercise vasodilation and post-exercise hypotension. The actions of histamine in skeletal muscle further include facilitating cellular crosstalk and transcriptional responses following exercise, and blocking its actions impairs functional adaptations to training that are important for both health and exercise performance. However, little is known about the upstream mechanisms that cause histamine release during exercise or about histamine's influence on exercise performance. The overarching goal of this project was to investigate the mechanisms responsible for exercise-induced histamine release in skeletal muscle and to evaluate the role of histamine in exercise performance. Aim 1 employed local passive heating to evaluate whether changes in muscle temperature stimulate *de novo* histamine formation and whether this was responsible for histamine release in skeletal muscle. Local passive heating did not increase histamine concentrations in skeletal muscle dialysate, indicating that changes in temperature alone are an insufficient stimulus to evoke appreciable histamine release in skeletal muscle. Aim 2 sought to improve upon a previously used mast cell bioassay and evaluate the contributions of a specific receptor pathway to exercise-induced degranulation. In doing so, it enabled the use of a different mast cell line, identified an effective receptor antagonist, and provided evidence of a receptor pathway that may contribute to exercise-induced degranulation, all of which lay the foundation for future mast cell assays. Aim 3 investigated the effects of histamine receptor blockade on oxygen supply-demand balance and performance during cycling exercise. Histamine receptor blockade did not affect the oxygen supply-demand balance or the time to task failure in the severe exercise domain. Taken together, these findings provide insight into how histamine, a potentially important exerkine, fits within the complex and multifaceted exercise responses within skeletal muscle.

This dissertation includes previously published co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Kieran Shay Struebin Abbotts

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, OR
Colorado State University, Fort Collins, CO
Gonzaga University, Spokane, WA

DEGREES AWARDED:

Doctor of Philosophy, Human Physiology, 2026, University of Oregon
Master of Science, Health and Exercise Science, 2022, Colorado State University
Bachelor of Arts, Sociology, 2016, Gonzaga University

AREAS OF SPECIAL INTEREST:

Exercise Physiology
Exercise Metabolism
Stress Adaptation Responses

PROFESSIONAL EXPERIENCE:

Graduate Employee, University of Oregon, 2022-2026

Graduate Assistant, Colorado State University, 2020-2022

GRANTS, AWARDS, AND HONORS:

O'Day Fellowship, The Impact of Antihistamines on Oxygen Uptake Kinetics During Cycling, University of Oregon, 2025

Eugene and Clarissa Evonuk Memorial Graduate Fellowship, Impact of Antihistamines on Muscle Oxygenation and Exercise Performance, University of Oregon, 2024

National Master's Scholar Award, American Kinesiology Association, 2022

Student Grant, Caffeine Augments the Lactate and Interleukin-6 Response to Exercise: Potential Favorable Implications for those with Cachexia, American College of Sports Medicine Rocky Mountain Chapter, 2020

PUBLICATIONS:

Gibson, B.M.; **Abbotts, K.S.S.**; Cursetjee, H. F.; Wiedenfeld Needham, K.; Minson, C.T.; Halliwill, J.R. Evidence of histamine release in response to both aerobic and resistance exercise: histamine metabolites as biomarkers. *Journal of Applied Physiology*. Online ahead of print, 2026. DOI: 10.1152/japplphysiol.01166.2025.

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[*]Authors contributed equally to this work.

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CHAPTER I

ORIGINAL PROPOSAL

Project Summary/Abstract

Regular exercise results in functional adaptations yielding health benefits and improving health outcomes. Exercise is often prescribed as a first-line non-pharmacological intervention for the prevention and treatment of many diseases. The release of exerkines, which are molecules that are altered or changed in response to acute or chronic exercise, appears to trigger many of the functional adaptations to exercise. Histamine, traditionally studied in the context of inflammation, allergic reactions, and muscle damage, is one such exerkine. During exercise, histamine is released in exercising skeletal muscle and is critical to regulating blood flow during and following exercise. Intramuscular histamine production occurs via two pathways: *de novo* synthesis of histamine and mast cell degranulation. Exercise results in numerous changes to the muscle environment that may stimulate these pathways, but which changes cause histamine release remain poorly understood. Upon release, histamine exerts its actions by binding with H_1 and H_2 receptors in various locations within skeletal muscle. Blocking the actions of histamine with H_1 and H_2 receptor antagonists reduces blood flow to exercising skeletal muscle during and following exercise. Additionally, histamine appears important in obtaining the functional adaptations to exercise and exercise performance. H_1 and H_2 receptor blockade attenuates the health benefits of exercise training and impairs exercise performance. Despite these tangible outcomes, little is known surrounding the mechanisms triggering the release and subsequent effect of histamine during exercise. This represents a *critical gap in knowledge* surrounding an exerkine with an important role during and following exercise. This investigation plans to fill that essential gap in knowledge by investigating the upstream mechanisms responsible for exercise-induced intramuscular histamine release and the subsequent actions of histamine during exercise. Regarding the mechanistic causes of histamine release, previous investigations have generated data to support two synergistic hypotheses: increased intramuscular temperature stimulates ***de novo* histamine formation** and **exercise results in the release of a soluble factor causing mast cell degranulation**. We plan to test these hypotheses to illuminate the upstream causes of histamine release in skeletal muscle. Further insight into this understudied exerkine comes from understanding its actions during exercise. To that end, we will investigate **the impact of histamine receptor antagonism on muscle oxygenation and exercise performance**

in trained cyclists. We hypothesize that histamine receptor antagonism will result in altered muscle oxygenation and impaired performance. The successful completion of this research moves the field forward by characterizing the impact of a poorly understood exerkine. In doing so, we will fill in critical gaps surrounding the causes of histamine release and identify mechanisms that may be targeted to mimic or enhance some of the beneficial adaptations to exercise. Additionally, we will develop a robust protocol that can later be used in future studies to test the impacts of antihistamines and allergies on athletic performance and the functional adaptations to exercise. Fundamentally, we hope to change the current dogma on histamine being viewed as a pathophysiological response and illustrate its important role during exercise.

Specific Aims

Regular exercise is associated with many health benefits, including a lower risk of cardiovascular disease, a lower risk of type 2 diabetes, and a lower risk of all-cause mortality [1]. However, the mechanisms that transduce these adaptations are poorly understood. Histamine is released within skeletal muscle during exercise [2,3] and is important in regulating blood flow to exercising skeletal muscle during and following exercise [4–6]. Blocking histamine's actions also reduces exercise-induced differential expression of protein-coding genes impacting various cellular functions, including inflammation, metabolism, and vascular function [7]. This may explain the attenuated adaptations to exercise training [8] and worsened exercise performance [9] following histamine receptor antagonism. Histamine has a significant role in the physiological responses to exercise but the causes of histamine release and its subsequent effect in exercising skeletal muscle remain unclear. **Aims 1 and 2** directly address the mechanisms causing exercise-induced intramuscular histamine release.

Increases in intramuscular histamine concentration occur via two pathways: *de novo* histamine formation via the actions of histidine decarboxylase (HDC) and mast cell degranulation [2]. Exercise induces a variety of changes to the intramuscular environment that may precipitate these pathways, one of which is a rise in temperature. Increasing intramuscular temperature increases intramuscular histamine concentrations [10]. The rise in temperature may stimulate HDC activity and *de novo* histamine formation, but this hypothesis has not been confirmed. Following HDC inhibition intramuscular histamine concentrations still increase [2], suggesting that mast cell degranulation also contributes to intramuscular histamine release. Mast cells degranulate when exposed to external stimuli. We have generated preliminary data suggesting exercise results in the release of a soluble factor that triggers mast cell degranulation, but the identity of that factor remains elusive.

Given that histamine receptor antagonism has a detrimental effect on exercise performance, it is important to understand its effects on exercising skeletal muscle. **Aim 3** investigates the impact of histamine receptor antagonism on muscle oxygenation in conjunction with exercise performance. Muscle oxygenation represents the balance of muscle oxygen supply and demand. Dysregulation of this balance leads to the inability to match oxygen supply and demand and, ultimately, the inability to continue exercising at that intensity. Histamine regulates the microvasculature, and blocking its actions may result in alterations in the balance of muscle

oxygen supply and demand. We will address this question by examining the effects of histamine receptor antagonism on muscle oxygenation in conjunction with exercise performance to provide insight into the causes of worsened exercise performance with histamine blockade.

Aim 1: Identify whether the rise in muscle temperature increases *de novo* histamine production. We hypothesize that the rise in muscle temperature will increase *de novo* histamine production in skeletal muscle. To test this hypothesis, we will analyze histamine concentrations in skeletal muscle interstitial fluid during and following local heating independent of exercise, in a control condition and a condition where the actions of HDC are inhibited.

Aim 2: Identify soluble factors released in exercising skeletal muscle that cause mast cell degranulation. We hypothesize that the presence of a soluble factor induces mast cell degranulation. To test this hypothesis, we will employ untargeted proteomic analysis to identify differentially expressed proteins in dialysate collected from resting and exercising skeletal muscle. We will use this information to perform a mast cell bioassay, isolating the effects of the identified factors on mast cell degranulation.

Aim 3: Evaluate the effects of histamine receptor antagonism on muscle oxygenation, critical power, and exercise performance. We hypothesize that administering antihistamines will alter the balance of muscle oxygen supply and demand and thus critical power, and result in impaired exercise performance. To test this hypothesis, we will compare muscle oxygenation to different exercise intensities in conjunction with cycling time to exhaustion following administration of H_1 and H_2 receptor antagonists or placebo.

Successful completion of this research *advances the field* by characterizing the impact of a poorly understood exerkine. Findings from this investigation may be used to identify future interventions to mimic or enhance the beneficial adaptations to exercise. Further, our results may serve to guide clinicians, athletes, and coaches regarding the management and treatment of allergies with antihistamines. Fundamentally, we hope to change the current dogma surrounding histamine as only associated with pathophysiological responses and muscle damage.

Research Strategy

Significance

Exercise is a physiological stressor to the human body resulting in functional adaptations that can improve health outcomes. One example lies in its effects on cardiovascular disease (CVD), which is the leading cause of death in the United States and incurs an economic cost of $407.3 billion [11,12]. Individuals at a higher risk for developing CVD frequently present with diminished vascular function and perfusion of skeletal muscle [13–16]. Exercise improves cardiovascular function and is associated with lower CVD risk and mortality [17–19], which has led to exercise being prescribed as the first line of non-pharmacological intervention in preventing and treating CVD. Additionally, regular exercise is associated with improved cognition, reduced anxiety and risk of depression, lower risk of type 2 diabetes, and lower all-cause mortality [1]. Despite the well-known health benefits of exercise, the signals and mechanisms that trigger these functional adaptations are poorly understood. The National Institutes of Health have highlighted this as a *critical gap in knowledge* by establishing the Common Fund Program on "Molecular Transducers of Physical Activity in Humans".

Many beneficial adaptations of exercise are mediated by the release of exerkines, which are molecules that are altered in response to acute and chronic exercise [20]. Exerkines are released from multiple tissues, including adipose tissue, heart tissue, the nervous system, and skeletal muscle. They include peptides, nucleic acids, lipids, and miRNA species that act in endocrine, autocrine, and paracrine fashions to facilitate exercise-induced adaptation [20,21]. Particular interest has been paid to skeletal muscle exerkines (also termed myokines), as they are large in number and appear important in exercise performance and improvements in health [22–25]. Histamine is an exerkine affecting the skeletal muscle microenvironment and transcriptional responses to exercise, culminating in a wide-ranging impact on the functional adaptations to exercise training and exercise performance. Despite histamine's tangible impact, mechanisms surrounding intramuscular histamine release and subsequent action during exercise are not well understood. The proposed study addresses this topic and *advances the field* by directly studying a poorly understood exerkine. Our findings will better characterize the exerkine response and may be used in identifying novel therapeutic targets and improving care and recommendations for recreational exercisers and competitive athletes.

During exercise, histamine is released within exercising skeletal muscle, where it can act in an autocrine and paracrine fashion by binding to histamine H_1 and H_2 receptors [2,3,26,27]. These receptors are widely distributed, but important locations include endothelial cells, vascular smooth muscle cells, and nociceptive afferent neurons [27]. The actions of histamine appear important to regulating blood flow to exercising skeletal muscle, evidenced by reductions in blood flow and vascular conductance following H_1 and H_2 receptor antagonism [4–6,28]. Histamine also appears integral to exercise-driven differential expression of numerous protein-coding genes, impacting numerous cellular functions, including inflammation, metabolism, and vascular function [7]. This may explain why histamine receptor blockade attenuates several of the systemic health benefits obtained from exercise training, including improvements in vascular function, muscle capillarization, and aerobic capacity [8]. These observations indicate that histamine is an essential transducer of the beneficial adaptations to exercise.

Increases in intramuscular histamine occur via two pathways: *de novo* synthesis from L-histidine by the enzyme HDC and mast cell degranulation. Exercise results in changes to the intramuscular environment, including increased temperature, decreased pH, and increased oxidative stress, that may precipitate these pathways. We have previously ruled out oxidative stress as a cause of histamine release in skeletal muscle [29], but other upstream causes merit investigation. It is well known that exercise results in a marked increase in muscle temperature to well over 38° C [10,30–32]. Our lab found that passively heating skeletal muscle to temperatures seen during exercise increases intramuscular histamine concentrations [10]. Increases in temperature do not induce mast cell degranulation *in vitro*, suggesting that the temperature changes do not impact histamine release from mast cells [10]. Increasing temperature increases HDC activity *in vitro* [33,34] indicating that increases in intramuscular temperature stimulate *de novo* histamine formation, resulting in increased intramuscular histamine following heating *in vivo*. However, **this hypothesis has yet to be confirmed**. We plan to directly test this hypothesis by examining the effect of raising intramuscular temperature via passive heating on *de novo* histamine formation in healthy adults.

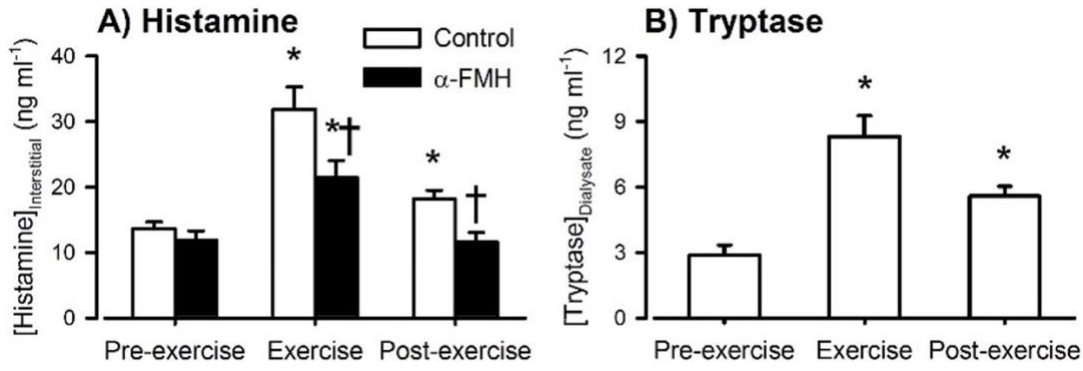

Figure 1: Dialysate samples were obtained by intramuscular microdialysis during one hour of dynamic knee extension. (A) Histamine concentrations increase during exercise, and this is only partially inhibited by the administration of a-FMH. (B) Tryptase, a biomarker of mast cell degranulation, increases during exercise. Values are means ± SE. *$p<0.05$ vs Pre-exercise. †$p<0.05$ vs Control. From[2].

During exercise, interstitial histamine concentrations still increase following administration of the potent HDC inhibitor α-fluoromethylhistidine (α-FMH), indicating that mast cell degranulation also contributes to intramuscular histamine release during exercise (Figure 1) [2]. Exposure to an external trigger induces mast cell degranulation and histamine release. The binding of IgE to the FcεRI receptor on the mast cell surface is the most robust stimulus triggering mast cell activation, but numerous other substances have been shown to induce degranulation [35,36]. We have generated preliminary data showing that exposing mast cells to dialysate from exercising muscle *in vitro* results in appreciably more degranulation than exposing mast cells to dialysate collected from non-exercising skeletal muscle. This implies the existence of a substance released by skeletal muscle that is a potent trigger of mast cell degranulation. However, **the substance in question remains unidentified**. We plan to use untargeted proteomic analysis to identify potential triggers and test their effect on mast cell activation in a mast cell degranulation bioassay.

Histamine is important to regulating exercising skeletal muscle blood flow. Originally, it was thought to regulate resistance vessels that determine overall blood flow to exercising skeletal muscle [4] and the distribution of that blood flow within exercising skeletal muscle [2]. This was challenged by work showing that histamine receptor antagonism increased femoral blood flow during unilateral knee extension exercise [37]. However, this same study found greater decreases in intramuscular pH with blockade [37] suggesting there may be alterations in muscle perfusion impacting metabolite washout that may be independent of "bulk" blood flow measure in conduit arteries. Given histamine's role as an intrinsic regulator of the microvasculature and its ability to

increase capillary recruitment and permeability in skeletal muscle [38,39] , histamine receptor antagonism may cause dysregulation of the microvasculature, altering nutrient delivery and metabolite washout in exercising skeletal muscle. This is supported by the observation of reductions in post-exercise interstitial glucose concentrations in previously active skeletal muscle following histamine receptor antagonism [28]. However, evidence for histamine's regulatory role on the microvasculature during exercise remains sparse.

Understanding the role of histamine receptor antagonism during exercise is important to athletes and recreational exercisers alike. Seasonal allergic rhinitis (AR) is prevalent, with over 25% of adults [40] and 18% of children [41] in the United States reporting seasonal AR. This prevalence increases in athletes, with up to 56% of athletes experiencing seasonal AR [42]. Seasonal AR is generally treated with antihistamines, the most common of which are histamine H_1 receptor antagonists to relieve allergy symptoms and H_2 receptor antagonists to reduce stomach acid [42]. This may provide insight into the observations that a history of allergies is an independent risk factor for injury in runners [43,44] and cyclists [45] and that athletes suffering from seasonal AR lower quality of life [46] and impaired performance [47]. Histamine receptor antagonism slows cycling 10-km time trial performance by roughly 1% [9]. This may seem trivial, but in competition, the difference from first to second can be 0.52% and 2% from first to fourth [48]. Further, the decreased performance is comparable to the benefit seen from well-known ergogenic aids, for example, caffeine, which has been shown to improve cycling 10-km time trial performance by ~3% [49]. Despite these tangible outcomes, little is known about the physiological effect of histamine receptor blockade during exercise and how this may influence performance. We plan to characterize the effect of histamine receptor antagonism in exercising skeletal muscle in conjunction with exercise performance.

The proposed research hopes to change the current dogma, that histamine is only associated with pathophysiological responses and muscle damage. In doing so, we will add to the growing literature characterizing exerkines, their effects during exercise, and their role as transducers of functional adaptations that improve health. Expected outcomes that *move the field forward* include demonstrating that rises in muscle temperature during exercise increases *de novo* histamine production (Aim 1); demonstrating that a soluble factor released in exercising skeletal muscle causes mast cell degranulation (Aim 2); demonstrating that histamine receptor antagonism alters skeletal muscle oxygenation during exercise, in conjunction with worsened

performance (Aim 3). The scientific rationale for these hypotheses will be expanded on in the approach. The findings of this study will provide insight into the mechanistic actions of histamine during exercise, generate hypotheses as to the role of histamine during exercise, and inform antihistamine use and prescription among athletes and recreational exercisers.

Innovation

Traditionally, histamine has been studied in the context of inflammation, allergic responses, and muscle damage. However, there is evidence of an influential role of histamine in exercise performance and obtaining the functional adaptations to exercise training, indicating histamine may be a transducer of the adaptations to exercise. **The proposed research is innovative as it employs novel models to explore the role of a poorly understood exerkine.** Previous work has shown that histamine is released in exercising skeletal muscle [2], regulates skeletal muscle blood flow [2,5,6,28], and is important in exercise performance [9] and functional adaptations to exercise [7,8]. We will examine the upstream mechanisms causing histamine release and the effect of histamine receptor antagonism on the exercising skeletal muscle environment. Fundamentally, we hope to change the current discourse on histamine being viewed as a pathophysiological response and illustrate its important role during exercise.

A source of innovation comes from using a relatively novel model to explore a new niche. We are one of the few labs that study histamine release in skeletal muscle. We can use intramuscular microdialysis (MMD) to isolate the interstitial fluid of skeletal muscle and use analytical chemistry to test the histamine concentrations. Additionally, we have developed a physiologically relevant bioassay to measure mast cell degranulation. This project is innovative as it uses untargeted proteomic analysis to isolate potential factors causing mast cell degranulation. Findings from the proteomic analysis will guide the manipulation of the bioassay to isolate the factors responsible for mast cell degranulation.

Further innovation comes from using a physiologically relevant measure in conjunction with exercise performance. Adequate balance of oxygen supply and demand in exercising muscle results in steady-state oxygen uptake ($\dot{V}O_2$), blood lactate, and muscle metabolite responses (such as pH, pCr, and Pi) and the ability to exercise at that intensity for an extended period of time [50–53]. The highest external work rate at which this occurs is referred to as the maximal metabolic steady state and can be derived from the critical power (CP), which is the

hyperbolic relationship between power output and the duration for which that power output can be sustained [54]. Exercise below CP results in a balance of muscle oxygen supply and demand, resulting in steady-state kinetics, whereas exercise above CP results in a mismatch of oxygen supply and demand, accumulation of metabolites, and eventual achievement of maximal O_2 uptake and limit of exercise tolerance [51,52,55,56]. Near Infrared Spectroscopy (NIRS) can be used to measure muscle oxygen saturation (%SmO_2) profiles during whole-body exercise. The slope of %SmO_2 at a given intensity over time can be used to identify and determine CP and, thus, the maximal sustainable metabolic steady state [55,57]. Exercise over CP results in a negative %SmO_2 slope whereas exercise at or below CP results in a zero %SmO_2 slope [55]. Previous work has shown that histamine receptor blockade impairs cycling performance [9]. However, the mechanism by which this occurs is unknown. We will address this gap in knowledge by using the slope method to examine the effect of histamine receptor antagonism on the balance of muscle oxygen supply and demand and CP in conjunction with exercise performance. We have developed a protocol that will allow us to address these questions and serve as a benchmark for future studies. To our knowledge, **no group** has investigated the effect of antihistamine use on muscle oxygenation and CP in conjunction with performance. We will employ a cycling exercise protocol to assess if blockade impacts the balance of oxygen supply and demand and alters the highest sustainable steady state during exercise. This study can then be used as a benchmark for future studies examining the role of antihistamines or other exogenous supplementation on exercise performance.

Approach

The overarching goal of this proposal is to increase the knowledge surrounding histamine, a poorly understood exerkine. We will address this topic by investigating two main lines of research, the upstream mechanisms causing intramuscular histamine release and the effect of histamine in exercising skeletal muscle. To address the first two aims we will conduct a series of protocols in cohorts of healthy adult males and females who are recreationally active. To address the third aim we will recruit trained male and female cyclists. Regarding **sex as a biological variable,** these protocols are not intended to test for sex differences, and based on our research, there is no evidence for sex-based differences in the histamine response to exercise. Additionally, previous research indicates that the menstrual cycle has little effect on post-

exercise hemodynamics [58–61] and exercise performance [62–64]. As such, to increase ecological validity, we will record but not control for menstrual cycle phase and oral contraceptive use during the proposed research.

Relevant Literature and Scientific Rationale

Exercise-induced increases in intramuscular histamine concentration occur via both *de novo* histamine synthesis and mast cell degranulation [2]. Increases in muscle temperature, up to levels seen during exercise, increase intramuscular histamine concentrations but do not stimulate mast cell degranulation in vitro [10], suggesting the increased temperature stimulates HDC activity and *de novo* histamine synthesis. The first aim of the proposed research is to directly test this hypothesis.

Greater mast cell degranulation is a second source of increased intramuscular histamine. Mast cells are immune cells that develop in the bone marrow and then are released into the bloodstream where they subsequently migrate to peripheral tissues, including skeletal muscle [65]. They are activated by the binding of a specific trigger, initiating a second messaging system and resulting in degranulation. Degranulation is a process characterized by the exocytosis of a cytoplasmic granule into the extracellular space resulting in the release of inflammatory mediators such as histamine, serotonin, tumor necrosis factors (TNF), kinins, and proteases [36,65]. Exposing mast cells to dialysate collected from exercising skeletal muscle results in a substantial increase in degranulation *in vitro* (Figure 2)[66]. This observation, combined with the rise in intramuscular histamine following HDC inhibition, indicates the presence of an exercise-induced trigger in the skeletal muscle causing mast cell degranulation. The second aim of the proposed research will help to identify the exercise-induced factor causing mast cell degranulation.

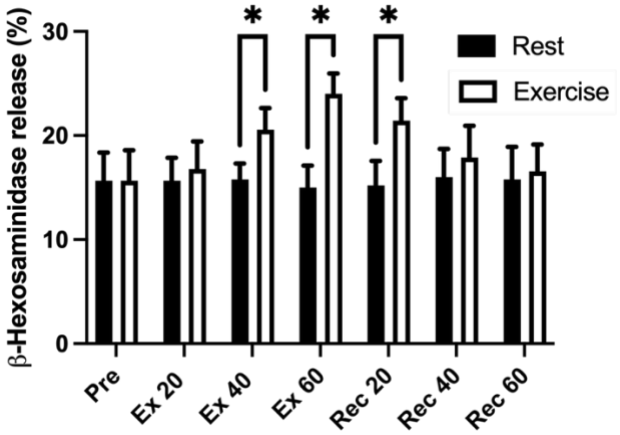

Figure 2: Exposure of mast cells to dialysate from exercising skeletal muscle results in greater degranulation (quantified by β-hexosaminidase release) than exposure to dialysate from resting skeletal muscle. Means and 95% CI. *p< 0.01 vs Rest. From [66].

Once released in skeletal muscle, histamine binds to histamine H_1 and H_2 receptors and helps to regulate blood flow to exercising skeletal muscle [4]. Antagonism of the H_1 and H_2 receptors using over-the-counter antihistamines impairs cycling performance [9]. Curiously, a follow-up study found the same histamine receptor antagonism increased femoral blood flow but greater decreases in intramuscular pH during single-leg knee extension exercise [37]. This suggests that there may be alterations in muscle perfusion resulting in altered nutrient delivery and metabolite washout that may be independent of blood flow measures in conduit arteries. As histamine is an intrinsic regulator of the microvasculature [38,39], histamine receptor antagonism may impact the skeletal muscle microenvironment and/or muscle oxygenation. Muscle oxygenation represents the balance of muscle oxygen supply and demand and is important to sustain high levels of exercise, delineate training intensities, and understand the physiological strain of an exercise bout [55,57,67]. If histamine receptor antagonism results in dysregulation of the balance of muscle oxygen supply and demand, that may explain its negative effects on exercise performance. To our knowledge, **only one study** using single-leg knee extension directly tested the impact of histamine receptor antagonism on muscle oxygenation. Single-leg knee extension provides an insightful model, but there are very few real-world instances when an athlete engages in prolonged isolated single-leg knee extension. The third aim of the proposal addresses this **gap in knowledge** in a more ecologically valid manner of exercise to generate more applicable findings.

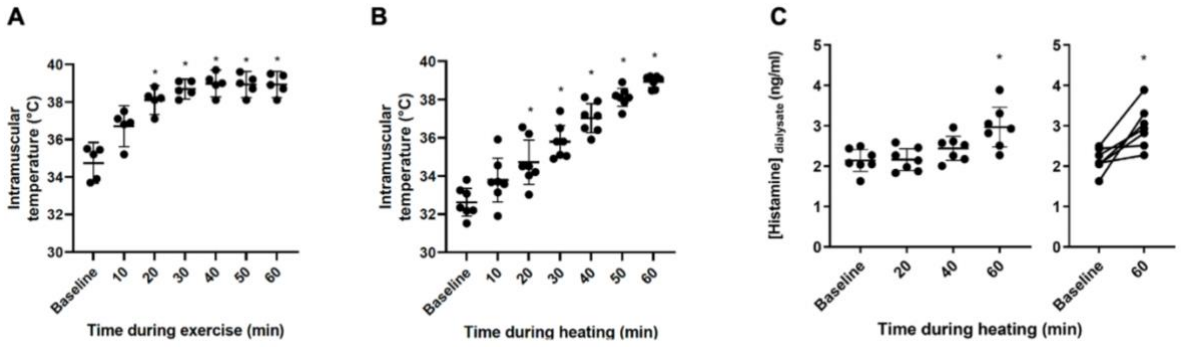

Figure 3: A) Intramuscular temperature in the vastus lateralis during knee extension exercise (n=5). Temperature increased to 39.2 °C. B) Intramuscular temperature in the vastus lateralis increased during 60 minutes of heating using pulsed short-wave diathermy (n=7). C) Histamine concentrations in skeletal muscle dialysate increased during 60 minutes of heating the vastus lateralis by pulsed short-wave diathermy (n=7). Means and 95% CI. *p< 0.05 vs baseline. From [10].

Experience and Previous Work

We have previously used MMD to identify a rise in intramuscular histamine following passive heating [10] and exercise [2]. During one hour of unilateral knee extension exercise, we found that intramuscular temperature of the *vastus lateralis* increases to a steady state of 39.2 °C. Using pulsed short-wave diathermy (PSWD) we were able to successfully heat the *vastus lateralis* to this temperature while using intramuscular MMD to isolate the interstitial fluid (Figure 3) [10]. We have also developed a mast cell bioassay allowing us to quantify degranulation following exposure of mast cells to dialysate. As an exercise physiology lab, we have extensive experience with exercise testing. We previously administered antihistamines prior to exercise with no adverse events reported. We expect that participants will be able to safely exercise following both placebo and antihistamine administration.

Research Design and Rigor

Aims 1 and 2 employ similar techniques in a similar population and thus undergo similar screening processes. Following screening, which consists of a medical history, measurements of height, weight, and blood pressure, and an exercise test to determine peak power for unilateral knee-extension exercise [2,4], participants will undergo MMD to collect dialysate under two conditions, heating, and exercise. **Aim 1 (heating)**: We will insert an intramuscular thermocouple and two MMD probes into the participant's *vastus lateralis*. One probe will be used to administer α-FMH and the second will serve as a control. Using PSWD we will heat the

vastus lateralis to 39.2 °C. Previous analysis has shown an inflection point of 38.4 °C for rises in intramuscular histamine, indicating this may be the activation threshold for HDC [10]. Thus, the temperature to which we will heat the muscle should increase intramuscular histamine concentrations. We will quantify histamine concentrations in the dialysate from before, every 20 minutes during heating, and post-heating via enzyme-linked immunosorbent assay (ELISA) as previously described [2,10]. **Aim 2 (exercise):** Participants will have one MMD fiber placed in the *vastus lateralis* on each of their left (control) and right (exercise) legs. They will then perform one hour of unilateral knee extension exercise at 60% of peak power. Dialysate from each probe will be collected before, every 20 minutes during, and post-exercise will be partitioned into separate containers. To generate a hypothesis about the identity of the exercise-released substance that induces mast cell degranulation a portion of the dialysate will be submitted for untargeted proteomic analysis to the Oregon Health Sciences University Proteomics Shared Resource facility. The remaining dialysate will be used in a mast cell degranulation bioassay. The results of the proteomic analysis will guide us in the manipulation of the bioassay to assess the contribution of the identified substances to mast cell degranulation.

Aim 3: We will employ a randomized, double-blind, placebo-controlled, repeated measures, crossover design to address the impact of histamine receptor antagonism on muscle oxygenation and exercise performance. Participants will be male and female cyclists classified as well-trained (performance level 4 or higher) based on previously defined categories surrounding peak oxygen uptake (VO_{2peak}) [68,69]. We chose trained cyclists as they have experience completing difficult exercise and are similar to the populations previously studied in conjunction with the use of antihistamines and exercise performance [9]. Following an initial screening, consisting of a medical and training history and an exercise test to assess cycling VO_{2peak}, participants will return to the lab on two separate occasions, separated by at least one week. Participants will ingest antihistamines (540 mg fexofenadine, an H_1 receptor antagonist, and 40 mg famotidine, an H_2 receptor antagonist) or a placebo and sit for one hour to facilitate absorption. Following this hour, participants will engage in cycling exercise for approximately one hour. Exercise will consist of a warm-up followed by six stages. The first five stages will last four minutes, separated by four minutes recovery of near load-less cycling and increase in intensity with the final stage being a time-to-exhaustion trial. Intensities will be determined from the peak test and

based on previously outlined intensity zones [55,70,71]. This protocol was developed to mimic the demands of a race or training session while allowing us to assess maximal metabolic steady state.

Throughout the exercise, muscle oxygenation will be measured via NIRS portable devices (Moxy Monitor, Fortiori Design LLC, Hutchinson, MN) placed on the *vastus lateralis* of each leg. These devices were chosen due to their specificity for muscle tissue, wireless capability, lack of participant interference during exercise, and their high reproducibility, repeatability, dynamic range, and face validity of venous oxygen [72–74]. The NIRS devices employed determine the ratio of the oxy-hemoglobin concentration to the total hemoglobin concentration and output the value as a percentage via the equation:

$$\%SmO_2 = \left(\frac{oxygenated\ hemoblogin + myoglobin}{total\ amount\ of\ hemoglobin + myoglobin}\right) \times 100$$

NIRS has emerged as a reliable tool for delineating exercise intensity zones, identifying the highest sustainable metabolic rate during exercise, and assessing the recovery status from bouts, when compared to previous methodologies (e.g., lactate, oxygen uptake, etc.) [55,57,67,75], and the portable devices are used by competitive athletes, increasing the ecological validity of the methodology. We will then quantify the balance of muscle oxygen supply and demand using the slope method, as previously described [55,57] at the different exercise intensities. This method allows us to assess whether the use of antihistamines altered the balance of oxygen supply and demand in exercising muscle and the highest sustainable steady state. The time to exhaustion will allow us to assess exercise performance.

Analysis and Expected Outcomes

For each aim, primary outcome variables will be measured within subjects across conditions and assessed using a two-way or mixed-model repeated-measures design with predefined contrasts. Based on our prospective power estimates performed with our biostatistician consultants Dr. Jodi Lapidus and her Biostatistics & Design Program team at Oregon Health Sciences University, we will enroll 24 participants for Aim 1 and 20 participants for Aim 2. For Aim 3, a repeated-measures ANOVA analysis, selecting conventional α (0.05) and β (0.20) parameters indicated that 12 participants would provide adequate power to detect a

1% difference in performance. A 1% difference was selected because previous analysis has shown that antihistamine blockade decreased performance by ~1% [9].

For **Aim 1**, as α-FMH inhibits the actions of HDC, and heat does not induce mast cell degranulation, we expect to find that 60 minutes of heating increases histamine only in the dialysate collected from the control probe. For **Aim 2**, we expect that dialysate collected from exercising skeletal muscle will induce mast cell degranulation and the untargeted proteomic analysis will generate information as to the identity of the factor responsible. For **Aim 3**, we expect the administration of antihistamines will alter the balance of muscle oxygen supply and demand at higher intensities resulting in a lower CP and reduced time to exhaustion when compared to placebo. The balance of muscle oxygen supply and demand will be analyzed using an analysis of the slope of $\%SmO_2$ similar to validated methods used in the zero-slope protocol [55,57]. Time to exhaustion will be measured by the time the participants can maintain a cadence above 60 revolutions per minute in the final stage of exercise.

Potential Problems and Alternative Strategies

Concerns may exist surrounding the ability to heat the muscle to 39.2 °C. We and others have previously shown that 60 minutes of PSWD can heat the muscle beyond that temperature without incurring heat-related discomfort [10,76,77]. For the first two aims, we will use MMD to sample the interstitial fluid from skeletal muscle during heating and exercise. For Aim 1 MMD probes will have a 20-kDa molecular weight cutoff, allowing histamine to diffuse into the probe, but not the enzymes histamine N-methyltransferase (molecular weight 30-kDa) and diamine oxidase (molecular weight 91-kDa). For Aim 2, the probes have a 100-kDa molecular mass cutoff, allowing factors with molecular weights under 100-kDa to pass through. A potential problem lies in the proteomics analysis identifying multiple differentially expressed soluble factors capable of inducing mast cell degranulation. If that occurs, we will isolate the most likely factor or factors and manipulate those in the bioassay. In doing so, we will be able to determine the effect of these factors on mast cell degranulation, which will inform future studies. For Aim 3, there is a chance that the intensities may be high enough such that individuals will not be able to complete the full exercise trial. To combat this potential issue, we are recruiting trained and well-trained cyclists [68,69] who regularly engage in intense physical activity and are used to exercise-associated discomfort.

Benchmarks for Success & Future Directions

Documentation of the contribution of increased muscle temperature to *de novo* histamine release will be considered success for Aim 1 and documentation of the soluble factors and their ability to trigger mast cell degranulation will be considered success for Aim 2. Should we find that neither heat nor a soluble factor trigger histamine release, we would broaden our search to include alternative triggers, such as larger molecules (> 100kD) and other physical stimuli (e.g., shear stress, vibration, and muscle compression). For Aim 3, if we see a lower critical power as derived from the slope of muscle oxygenation with antihistamines, it will confirm our understanding that histamine receptor antagonism reduces perfusion to exercising skeletal muscle, resulting in impaired performance with antihistamines. These results will allow for future investigations to isolate the impacts of allergies, partial blockade of histamine receptors (i.e., H_1 antagonism only, H_2 antagonism only), and different dosing schemes allowing investigation of novel antihistamine regimens for athletes with allergies. Successful completion of all three aims lays the groundwork for future studies by characterizing the causes of histamine release and subsequent action of histamine during exercise.

CHAPTER II
NEW INSIGHTS

Allergies can evoke symptoms that make exercise difficult and undesirable. The International Olympic Committee guidance for athletes dealing with allergies recommends trigger avoidance when possible, followed by symptom management when that is unattainable [78]. However, allergen exposure may be more than just an annoyance, as a history of allergies is an independent risk factor for injury in runners [43,44] and cyclists [45]. Our group has wondered whether this is partly due to their treatment. Some allergy symptoms, such as urticaria and rhinitis, are mediated by histamine release, making antihistamines a common treatment for allergy management [42]. Histamine is also released in exercising muscle [2], and is important in facilitating exercise responses [27,79] and adaptations to training [8,80]. Despite the seemingly important role of histamine signaling, little is known about the mechanisms causing its release and its effects during exercise. This fundamental gap in knowledge makes it difficult to begin evaluating the scope of histamine's influence during exercise and recovery. This work seeks to help fill that gap and, in doing so, contribute to the growing literature surrounding histamine, exercise, and skeletal muscle.

Since the submission of the dissertation proposal, advances have been made relevant to the work in this dissertation. These advances pertain to the methods employed in the following investigations and in the understanding and interpretation of the results in the broader context of exercise physiology. The following are advances that have come to light since the submission of the proposal, specifically pertaining to and influencing the completion and interpretation of the previously proposed aims.

Histamine, mast cells, and cellular signaling

Following exercise, substances released in skeletal muscle initiate a cellular crosstalk that drives recovery and remodeling. These processes begin with transcriptomic and translational changes, resulting in the differential expression of protein-coding genes and, subsequently, proteins – the fundamental building blocks underlying training-induced adaptation. Structural remodeling and systemic adjustments emerge from this communication network and are

necessary for systemic changes that ultimately manifest as functional improvements, health, and performance.

Exercise elicits the differential expression of approximately 3,000 protein-coding genes in skeletal muscle, and histamine receptor blockade alters this by roughly 25% [7]. This original finding was in bulk tissue obtained by muscle biopsy. The use of bulk tissue makes it difficult to discern the relative alterations amongst the different populations within that tissue. In 2025, Van der Stede et al. employed transcriptomic profiling of individual myofibers and found that following high-intensity interval exercise, the transcriptome changes in individual myofibers were only a small portion of the changes observed in bulk muscle tissue [81]. By integrating single-cell data, they identified distinct mononuclear non-muscle cells within the bulk tissue that contribute substantially to the post-exercise transcriptome. These cell populations likely comprise resident and infiltrating cells, both of which can influence the skeletal muscle response to exercise. The transcriptomic response among these cells was altered with H_1 and H_2 receptor blockade, implicating histamine in cellular communication following exercise.

That same study found that histamine is contained in mast cells, pockets of which reside within skeletal muscle, and that histidine decarboxylase (HDC) expression appears to be unique to those mast cells [81]. This indicates that these mast cells are the source of *de novo* histamine synthesis and may represent the exclusive histamine-producing cells in skeletal muscle. In addition, myeloid cells express both H_1 and H_2 receptors, further supporting the presence of a histamine-mediated communication network within skeletal muscle. Through histamine signaling this network appears to facilitate many of the exercise-induced transcriptional response. When considered alongside previous evidence demonstrating the influence of histamine receptor antagonism on the exercise transcriptome [7], this cellular communication pathway appears central to exercise-induced transcriptional changes. Moreover, disruption of these responses appears to negatively affect training-induced improvements in aerobic capacity, vascular function, and insulin sensitivity [8,80]. These findings have clear implications for the present work, as they reshape current thinking regarding the sources of histamine in exercising skeletal muscle, HDC activity and *de novo* histamine formation, and histamine's role as a cellular communicator that underlies transcriptomic responses to exercise.

Histamine receptor antagonism and resistance exercise

To this point, many studies on exercise-induced histamine release have used aerobic exercise, high-intensity interval training, or models amenable to invasive studies (e.g., single-leg knee extension). Initial work indicated that histamine blockade attenuated the blood flow and vascular conductance cycling [6,58,82] and dynamic knee extension [4]. Single-leg resistance exercise (knee-extension, two sets of five reps at 80% max torque) did not elicit an appreciable post-exercise hyperemic response, limiting the ability to detect an effect of histamine receptor blockade [4]. Van de Loock et al., evaluated the effect of histamine receptor antagonism on the blood flow response to a more strenuous single-leg resistance exercise bout. Participants completed four sets to failure of each single-leg press, leg extension, and leg curl, at their 12-repetition maximum [83]. They found that the single-leg exercise induced a significant post-exercise hyperemic response, but this response was unaffected by a conventional dose of an H_1 receptor antagonist (180 mg fexofenadine hydrochloride) or by complete H_1 and H_2 receptor blockade (540 mg and 40 mg famotidine)[8383]. However, these findings are not ubiquitous. Our lab found that immediately following a barbell squatting protocol (6 sets of 10 repetitions at 70% 1-reptition maximum), blood flow and vascular conductance were reduced with H_1 and H_2 receptor blockade [84].

Given the potential effects of histamine receptor blockade on cellular signaling and exercise adaptation, Van de Loock et al., then evaluated the impact of H_1-receptor antagonism on responses to a resistance program in sedentary to moderately active individuals. Participants completed full-body resistance training three times per week for ten weeks. Prior to each session, participants ingested either 180 mg fexofenadine hydrochloride or a placebo (lactose). Increases in muscle size, muscle strength (as measured by one repetition maximum), and fat-free mass were not different between groups, although the H_1-antagonist group had a modest increase in fat mass not seen in the control. It is worth noting that in sedentary or untrained individuals, many of the initial improvements in strength are mediated primarily by neurological adaptations (e.g., motor unit recruitment, firing patterns, etc.) [85,86]. This context is important when interpreting the lack of differences in strength gains between the two groups, as it suggests that H_1 receptor antagonism is unlikely to affect neurological adaptations that influence initial strength gains.

H_1 receptor antagonism did alter training-induced changes to the muscle proteome, including those in pathways related to mitochondrial translation, adaptive immunity, and fatty acid metabolism [83]. When considered in conjunction with previous findings showing H_1 and H_2

receptor blockade modify the exercise transcriptome [7], it is plausible that blocking histamine's actions impacts translational and subsequent proteomic responses to exercise. It may be that, with sufficient duration, the differences in transcriptomic and proteomic responses to resistance exercise with histamine receptor antagonism may manifest as differences in functional outcomes (i.e., strength, hypertrophy), although at this point this is purely speculative.

MRGPRX2 receptor pathway

Mast cells are tissue-resident immune cells best known for their roles in allergic and inflammatory diseases, including food allergy, rhinitis, itch, urticaria, and anaphylaxis. In humans, mast cell progenitors migrate to peripheral tissues where they diversify under the influence of the local environment [87–89]. Despite their diverse phenotypic profiles, in humans, mast cells are sometimes broadly divided into two types based on the contents of their granules. Those cells that only express tryptase are known as MC_T, which are expressed mainly in the lung and gastrointestinal tract, and those cells with granules containing tryptase, chymase, and carboxypeptase, known as MC_{TC}, are often found in connective tissue close to blood vessels [90,91]. Mast cells can be activated when a 'trigger' binds to a cell surface receptor, initiating a signaling cascade resulting in the release of their granule contents to the external environment via exocytosis in a process termed degranulation [36,88,92]. Mast cells can also undergo 'selective release' whereby activation results in the secretion of specific mast cell contents without complete degranulation [93]. The contents of mast cell granules consist of preformed and newly synthesized vasoactive and immunomodulatory mediators, such as cytokines, chemokines, peptides, and biologic amines, one of which is histamine [88,89]. In host defense reactions, activation occurs via IgE-bound antigens binding to FcεRI receptors, which are a potent mediator of degranulation [92]. Both subtypes of mast cells are activated via the aggregation of FcεRI, but the MC_{TC} are also activated by other substances, including endogenous peptides, indicating they not only express other receptors outside of FcεRI, but these receptors are important to their functions in the immune response [36,90]. Identifying, understanding, and targeting these receptors has become of great interest in understanding autoimmune regulation and developing therapeutic interventions.

Mas-related G protein-coupled receptors are G protein-coupled receptors expressed on sensory neurons and innate immune cells in humans [94,95]. Of these, the X2 subtype (MRGPRX2) is expressed on the surface of mast cells, sensory neurons, in the dermis of skin and gut, and immune cells [94,96]. MRGRPX2 is highly expressed in human MC_{TC}, although the expression levels vary between subjects [97]. It is activated by numerous endogenous substances, including neuropeptides, such as neurokinins and substance P [90,94,98]. When activated, MRGPRX2 stimulates the IP_3 pathway inducing calcium influx and degranulation of mast cells [94]. MRGPRX2-mediated degranulation has been implicated in wheal-and-flare reactions in skin, asthma exacerbations in the lungs, and general inflammatory responses [90,98]. Importantly, MRGPRX2 induces degranulation in an IgE-independent manner, meaning that it does not occur through cross-linking of FcεRI receptors or solely in response to exogenous stimuli. As such, reactions via the MRGPRX2 receptor are often referred to as "pseudo-allergic" or "anaphylactoid" reactions, distinguishing them from true allergies [99].

Given its role in IgE-independent activation, the variation in its expression among individuals, and the receptor's promiscuity for endogenous peptides, MRGRPX2 inhibition has become a therapeutic target for mast cell-mediated diseases. In 2019, Ogasawara et al. identified two compounds capable of inhibiting MRGRPX2-mediated mast cell degranulation [100]. These compounds worked as inverse receptor agonists capable of selectively inhibiting MRGPRX2-mediated activation and calcium mobilization. Since then, these compounds have been shown to inhibit degranulation induced by MRGRPX2-specific activators such as substance P, Rocuronium, and Icatibant both *in vitro* and in rodents [101–103]. While there have been no human trials, these studies indicate that these compounds can be successfully used in *in vitro* experiments to inhibit MRGRPX2-mediated degranulation.

As it pertains to the aims of this dissertation, an investigation into MRGRPX2-induced degranulation or selective release may provide insight into the factors causing exercise-induced histamine release in skeletal muscle. The localization and scale of histamine release during exercise does not mirror that of allergy-induced degranulation [2,7,81] suggesting exercise-induced mast cell degranulation likely occurs via non-IgE-mediated pathways. Unpublished data from our lab shows greater beta-hexosaminidase release (an indicator of mast cell degranulation or selective release) from mast cells exposed to dialysate collected from exercising skeletal muscle, compared with dialysate from non-exercising skeletal muscle [66]. These *in vitro* findings suggest

that there is an endogenous factor, released during exercise, capable of activating mast cells in skeletal muscle. Given its widespread expression and affinity for numerous endogenous peptides, MRGPRX2 is a likely candidate to mediate degranulation via an exercise-induced soluble factor. Thus, it is worth exploring MRGRPX2 represents a candidate as a mast cell receptor mediating exercise-induced degranulation. The presence of local mast cells in human skeletal muscle has been confirmed [81] , but the mast cell type (MC_T vs MC_{TC}) has not. The presence of chymase has been confirmed in cultured rat muscle [104] and within human skeletal muscle [7] , making it safe to suspect that the mast cells in human skeletal muscle are MC_{TC} type mast cells and express MRGPRX2.

It is worth exploring whether the MRGPRX2 receptor is responsible for exercise-induced mast cell degranulation. As of the writing of this dissertation, the inverse antagonists outlined above are not approved for use in humans, meaning that isolation of MRGPRX2-specific degranulation will need to occur *ex vivo*. To do so involves characterizing the expression of MRGRPRX2 on mast cell lines and the suitability of those to be used in degranulation assays. Investigations into MRGPRX2 inverse agonists have commonly employed either the Laboratory of Allergic Diseases (LAD2) or rat basophilic leukemia (RBL) cells expressing MRGPRX2 [101–103]. Investigations surrounding exercise-mediated mast cell degranulation in skeletal muscle have utilized the Laboratory University of Virginia (LUVA) mast cell line [10,66]. This line, while developed in 2011, is much less commonly used and less well-characterized than LAD2 or RBL-MRGPRX2 [105] making it difficult to compare findings to previous mast cell investigations.

Validity and reliability of the zero-slope protocol

The metabolic demands of a given workload define the exercise intensity domain in which it falls [52,54,70] and the ability to sustain that workload depends on whether the energetic demands of maintaining it can be met. The highest metabolic rate at which energetic demand can be met through oxidative metabolism is the maximal metabolic steady state [52,54]. At intensities above maximal metabolic steady state, energetic demands exceed the rate at which they can be met by oxidative metabolism, resulting in non-steady-state oxygen uptake, metabolite buildup, and eventual task failure [50]. The external workload eliciting the maximal metabolic steady state is termed critical power or critical speed and demarcates the boundary between sustainable and unsustainable exercise.

Identifying this boundary, or the external workload that elicits maximal metabolic steady state, has substantial implications for exercise training and performance. Precise quantification of this transition is difficult, and many methods of determining critical power or speed require multiple exhaustive trials over multiple days or a single 3-minute all-out effort [106,107]. The requirements of these methodologies provide logistical challenges for researchers, increase participant or athlete burden, and can lead to overestimations of critical power/speed [106,108,109]. Recently, near-infrared spectroscopy has emerged as a viable method to evaluate the balance of oxygen supply and identify the maximal metabolic steady state [55,57,110]. Portable near-infrared spectroscopy devices have been shown to accurately identify this transition in runners [55], cyclists [57,110,111], and cross-country skiers [112] and have been validated against traditional markers delineating intensity (i.e., oxygen uptake)[57,67,110–113]. This method, termed by some the zero-slope protocol, involves using near-infrared spectroscopy to measure $\%S_mO_2$ in exercising and non-exercising muscle during a series of sub-maximal repetitions to identify the highest external workload that elicits a $\Delta\%S_mO_2/\Delta$time of zero [55,57,110,111]. In this way, investigators can evaluate the physiology underlying the sustainability of a given workload in real time and in field settings (e.g., cross-country skiing, road cycling, trail running, etc.) where traditional measurements (i.e., lactate, oxygen uptake, etc.) may be difficult or unfeasible [112]. Further, using single submaximal exercise trials the zero-slope protocol lessens the time burden for athletes and researchers and has implications for exercise testing in populations where maximal exercise is poorly tolerated. At the time of the proposal, this methodology had only been employed in two published investigations [55,57], but was commonly being used in practice with competitive athletes. Since the proposal, five more investigations employing this, or a similar, methodology have been published [110–112,112,114] , including one evaluating the reliability and validity of the protocol for identifying the power at maximal metabolic steady state [115].

This reliability paper on the zero-slope protocol found a low measurement error (standard error measurement <2%) and high reliability and repeatability (intraclass correlation coefficient >0.9) [115]. Further, the ability to identify the maximal metabolic steady state via the zero-slope protocol does not appear to be sex-dependent,[110,111] confirming the utility of this protocol in mixed cohorts. However, while the zero-slope protocol appears to be reliable for determining the maximal metabolic steady state in both sexes, there are considerations necessary surrounding its application and interpretation. First, the minimal difference to detect real change

has been shown to be 3.4% [115] , which should guide researchers' interpretation of changes and day-to-day variability. Additionally, there are various near-infrared spectroscopy devices (i.e., Moxy Monitor, Artinis, etc.) which employ different proprietary algorithms for determining muscle oxygenation [73] , making it inadvisable to directly compare $\%S_mO_2$ between devices. Until these comparisons between devices are established, it is advised that researchers select a single near-infrared spectroscopy device for their zero-slope determination. Finally, differences between muscles and minor aerobic leg asymmetries may contribute to different $\%S_mO_2$ readings a different zero-slope identification. For consistency, researchers should investigate a large, highly used muscle (such as the rectus femoris in running or cycling, or the vastus lateralis in cycling, as used in Aim 3) and average the zero-slope power between legs. Although this reliability paper was published after Aim 3 had been completed, personal communication with the paper's authors assisted with protocol development and the interpretation of the results.

CHAPTER III
CHALLENGES AND ALTERNATIVE APPROACHES

During this dissertation, challenges arose that necessitated alternative approaches to and alterations of the originally proposed aims. When determining how to address these challenges, care was taken to ensure any changes made did not compromise the investigation and still satisfied the criteria for a rigorous and thorough dissertation. The challenges, considerations, alternative approaches, and updated aims and hypotheses are outlined below. Importantly, the changes to the aims do not substantially alter the scope of the investigation and still fit within the project's overarching goal of exploring the histaminergic response to exercise. Thus, we can confidently say that this dissertation expands the understanding of exercise-induced histamine release and of histamine as a transducer of exercise adaptation.

Aim 1 Considerations

Pulsed short-wave diathermy uses high-frequency electromagnetic waves to deliver a continuous or pulsed heat stimulus capable of reaching a depth of 3-4 cm, and effectively heating deeper lying tissue [116–118]. In this manner, pulsed short-wave diathermy safely delivers a controlled passive heating stimulus to a localized area, making it a useful tool in physical therapy. Its localized heating area and the ability to effectively control temperature allow for safe and effective heating of various durations, making it uniquely suited for investigations into the mechanistic effects of passive heating in skeletal muscle. For example, recent investigations have employed pulsed short-wave diathermy for up to two hours daily with no reported adverse events [76,77,119,120]. Further, these investigations found that repeated passive heating with pulsed short-wave diathermy resulted in positive mitochondrial adaptations, and attenuated atrophy and declines in vascular function following limb immobilization [76,77,119,120] indicating that not only is this form of heating safe and well-tolerated, but can elicit physiological adaptations.

Originally, in Aim 1, we planned to compare interstitial histamine concentrations during 60 minutes of passive heating via pulsed short-wave diathermy between a control site and a site perfusing α-fluoromethyhistidine, a suicide inhibitor of histidine decarboxylase, allowing us to evaluate the contribution of *de novo* synthesis to heating-induced increases in interstitial histamine concentrations. We chose 60 minutes because pulsed short-wave diathermy can

increase muscle temperature to over ~39°C, similar to temperatures achieved during exercise [10,77,120,121] , and elicited histamine release in the previous study [10]. Given that the previous investigation identified a suggested histamine threshold of 38.4°C [10], the 60 minutes should have been sufficient to reach temperatures capable of increasing *de novo* histamine synthesis and appreciably increasing the histamine concentrations in skeletal muscle.

In the first round of data collection comprising 10 young, healthy adults (4 males, 6 females), 60 minutes of heating did not increase histamine in either site despite reaching temperatures above 38.4°C. Critical reflection and consultation with collaborators experienced in the use of pulsed short-wave diathermy, specifically Megapulse II (Accelerated Care Plus-LLC, Topeka, KS, USA) provided us with two considerations. First, the center of the diathermy drum may generate slightly more heat than the periphery, suggesting that our original assumption of uniform heating beneath the drum may have been incorrect. While these heating differences are likely small, we wanted to reduce the possibility that the thermocouple reading did not represent the temperature at the probes. To address this concern, we altered the orientation of the probes and the thermocouple, ensuring they were positioned in a triangular formation, equidistant from one another, and centrally under the drum. Second, there may be a temporal component, that is time above a threshold temperature, which influences histamine release. To address this concern, the heating protocol was extended to 100 minutes. These changes were implemented in a second cohort of nine individuals, allowing us to ensure that we were not missing something that had been present previously. Further, we reanalyzed the dialysate collected from the initial cohort for histamine and ethanol (our surrogate indicator of local blood flow) to account for improvements in workflow, methodology, or analytical skills.

Aim 2 Considerations

Mast cells are the primary source of histamine in skeletal muscle [2,122]. When activated by an external trigger, mast cells release their contents into the external environment [88,92]. Although mast cell activation is classically associated with IgE-mediated signaling, numerous endogenous stimuli, as well as alterations in the local skeletal muscle microenvironment, can also induce degranulation or selective release [36,92]. It is likely that during exercise, endogenous substances or changes in the intramuscular environment mediate mast cell degranulation in skeletal muscle.

Investigating the endogenous triggers and pathways underlying degranulation is imperative to understanding exercise-induced histamine release in skeletal muscle.

Exercise-induced mast cell degranulation does not appear to be caused by reactive oxygen species [29] or heating alone [10]. During exercise, various cytokines, neuropeptides, and other soluble factors are released within the exercising muscle [21,123,124] , many of which can bind and activate mast cells [36,93]. Preliminary work from our lab found that exposing mast cells to dialysate collected from exercising tissue induces greater degranulation than dialysate from non-exercising tissue ex vivo, indicating that a soluble factor released during exercise can induce mast cell degranulation [6666]. However, to date, the identity of the signal-response pathways that cause exercise-induced mast cell degranulation remains unconfirmed.

The original goal of Aim 2 was to employ untargeted proteomics in a hypothesis-generating approach to compare protein expression in dialysate collected from exercising and non-exercising skeletal muscle. Identifying differentially expressed proteins provides a list of potential exercise-induced triggers of mast cell degranulation. From there, we planned to identify a factor of interest and evaluate its influence on mast cell degranulation *in vitro* via mast cell degranulation assays. In this manner, we could work down the list of possible targets and ultimately identify the soluble factor(s) released during exercise that may trigger the smoking gun of exercise-induced mast cell degranulation.

Initial proteomic analysis identified larger proteins, many of which were related to the trauma response of insertion. While that finding is interesting and has led to subsequent investigation regarding the proteomic responses to muscle microdialysis, it is outside the scope of this dissertation. After several meetings with the Oregon Health & Science University Proteomics Shared Resource Core, they modified the peptide identification method to potentially capture smaller peptides that were unable to be identified in the initial proteomic analysis. Unfortunately, this method meant that the dialysate samples originally slated for proteomics, which had been treated with detergent, were no longer usable for quantification. We selected samples with expected differences in proteomic responses and used them for further analysis, but this meant that a portion of our dialysate was now unusable in the mast cell assay. Further, this method of analysis did not appear sufficiently targeted to capture the peptide responses we were investigating.

Given the lack of definitive proteomic findings, we decided to pivot and explore potential receptor pathways involved in exercise-induced degranulation. By evaluating non-IgE-mediated receptor pathways, we can narrow the list of potential to better characterize exercise-induced mast cell degranulation. In evaluating potential targets, the mas-related G-protein-coupled receptor member X2 (MRPGRX2) stood out. Recently, MRGPRX2 has garnered substantial interest and investigation regarding its potential as a therapeutic target in treating mast cell-mediated diseases [94,125–127]. Of note, MRGPRX2 receptor is stimulated by neuropeptides and has a high affinity for neurokinin and substance P [100,102,126,127], which are released at the neuromuscular junction and have greater release during exercise [124] supporting the notion that MRGPRX2 is important in facilitating exercise-induced mast cell degranulation. We planned to evaluate the importance of MRGPRX2 in exercise-induced degranulation *in vitro*, by employing MRGPRX2-specific antagonist, Compound C9-6 [101,102,128] , and comparing mast cell degranulation when exposed to dialysate from exercise and control legs, in the presence and absence of the antagonist. The first step in the process was to confirm the blockade of MRGRPX2 via the Compound C9-6 using a known MRGPRX2 agonist substance P [102,128,129]. However, during this first round of trials, instead of confirming the blockade, we found that substance P did not induce degranulation in the mast cell line we were using (Table 1).

Table 1: Initial mast cell degranulation assay outcomes

Treatment	Degranulation (%)
1 mg/ml Compound 48/80 (n=3)	47 ± 1
0.3 μM Substance P (n=3)	4 ± 3
1 μM Substance P (n=3)	4 ± 3
3 μM Substance P (n=3)	3 ± 2
5 μM Substance P (n=2)	3 ± 1

Degranulation is expressed as a percent of β-hexosaminidase release. Percent calculated as β-hexosaminidase in supernatant divided by the total β-hexosaminidase (supernatant plus pellet). Data are mean ± SD.

A brief literature review found that many mast cell degranulation studies utilized the LAD2 cell line [101,102,130]. Our lab's previous investigations, and the current pilot trials, were using the LUVA cell line. The LUVA cell line was first characterized in by groups at Harvard

University and the University of Virginia (LUVA stands for Laboratory at the University of Virginia), in 2011 and has similar IgE-mediated degranulation to the LAD2 line [131]. However, few studies have used the LUVA cell line in degranulation assays, meaning its capacity for degranulation and utility are poorly characterized in the literature [105]. At the time of this dissertation, only four studies have employed LUVA cells *in vitro* to quantify degranulation via β-hexosaminidase release [10,131–133]. In those studies, the only non-IgE-mediated degranulation occurred through Compound 48/80. Based on the literature and our preliminary trials, we could confirm that LUVA cells were capable of β-hexosaminidase release, but we could not confirm that the LUVA cells expressed the MRGPRX2, or that they were capable of degranulation (as opposed to selective or partial release). Determination of MRGPRX2 expression via qPCr and flow cytometry would incur substantial time and resource costs not justified under the scope of the current investigation. Accordingly, the aim shifted to characterizing MRGPRX2 expression in LUVA cells via protein analysis (Western blot) and in LAD2 cells, assessing the utility of LAD2 cells in our mast cell assay, evaluating the efficacy of compound C9-6, and beginning to characterize MRGPRX2's potential role in exercise-induced degranulation. This revised Aim 2 enables comparison with other mast cell assays using LAD2 cells, refines the protocol for future mast cell bioassays, and provides the opportunity to subculture and then cryopreserve LAD2 cells for future investigations.

Aim 3 Considerations

Between the proposal and the study's execution, some pilot work was conducted to fine-tune the exercise protocol. Aside from those minor changes, Aim 3 was conducted as originally proposed, without substantial unanticipated challenges or the need for alternative approaches. It has since been published [114].

Updated Aims and Hypotheses

Aim 1: Evaluating the effect of local heating on histamine release and *de novo* histamine synthesis in skeletal muscle and investigating a potential temporal component (i.e., time with increased temperature) on histamine release. We hypothesize that local heating will increase histamine concentrations in skeletal muscle dialysate from a control condition, and this increase will be higher than a condition when histidine decarboxylase is inhibited. We also hypothesize

that this increase will be augmented by longer heating duration, providing evidence of a temporal component to heating-induced histamine release. These aims will expand upon previous findings indicating that local passive heating increases histamine concentration in skeletal muscle dialysate.

Aim 2: Exploratory evaluation of the LAD2 cell line for use in our mast cell assay. We will assess MRGPRX2-mediated degranulation and the efficacy of the MRGPRX2 antagonist Compound C9-6 in the LAD2 cell line. At the same time, we will determine the presence or absence of the MRGPRX2 protein by Western blot in both the LUVA and LAD2 cell lines. Finally, we will evaluate the effect of dialysate taken from exercising skeletal muscle on mast cell degranulation in the LAD2 cell line, in the presence or absence of the MRGPRX2 antagonist Compound C9-6. This exploratory work will help characterize the LUVA cell line, assess the utility of the LAD2 cell line in our assay, evaluate the efficacy of Compound C9-6, and begin to characterize MRGPRX2's potential role in exercise-induced degranulation. Ultimately, this aim functions to refine our *in vitro* mast cell degranulation protocol, serving as a reference for future investigations using mast cell bioassays.

Aim 3: Evaluate the effects of histamine receptor antagonism on muscle oxygenation, critical power, and exercise performance. We hypothesize that administering antihistamines will alter the balance between muscle oxygen supply and demand, thereby affecting critical power and resulting in impaired exercise performance. To test this hypothesis, we will compare muscle oxygenation to different exercise intensities in conjunction with cycling time to exhaustion following administration of H_1 and H_2 receptor antagonists or a placebo. Aim 3 is unchanged from the original proposal.

CHAPTER IV

LOCAL PASSIVE HEATING DOES NOT INCREASE HISTAMINE CONCENTRATIONS IN SKELETAL MUSCLE

Introduction

Histamine concentrations increase in exercising skeletal muscle [2,122]. Within the intramuscular environment, histamine binds to H_1 and H_2 receptors located on numerous cell types, including myeloid cells, vascular smooth muscle cells, and the vascular endothelium [26,27]. It's actions initiate a signaling cascade important in facilitating cardiovascular [4–6], inflammatory [134,135], and transcriptional [7] responses to exercise. Further, histamine appears important in obtaining adaptations to and health benefits of exercise training [8,80], pointing toward histamine as an important transducer of the benefits of physical activity. However, although histamine appears to be an important component of exercise response, the upstream factors responsible for initiating its increase in skeletal muscle remain unknown.

Exercise-induced histamine release occurs via two distinct pathways: mast cell degranulation and *de novo* histamine synthesis. Within skeletal muscle tissue, there reside small populations of mast cells, which appear responsible for exercise-induced increases in skeletal muscle histamine concentrations [122]. Inside these mast cells are granules that contain cytokines, chemokines, and other substances, including histamine and tryptase. When stimulated, mast cells release the contents of their granules to the external environment via exocytosis in a process termed degranulation [65,136]. Exercise elicits this degranulation, as evidenced by increasing concentrations of intramuscular tryptase, which is co-released with histamine during degranulation [2].

The second pathway that contributes to increased histamine is *de novo* histamine synthesis. This process is catalyzed by the enzyme histidine decarboxylase (HDC), which decarboxylates the amino acid L-histidine to form histamine [137]. Initial investigations indicated the expression of HDC in bulk muscle tissue from humans[7], and recent single-cell transcriptomic techniques have expanded upon those findings, finding that HDC expression may be specific to small populations of mast cells within skeletal muscle [122]. Evidence in rodents indicates that the enzymatic activity of HDC is increased during exercise [138,139]. In humans, increases in intramuscular histamine during exercise are reduced when HDC is pharmacologically inhibited by alpha-fluoromethylhistidine [2] , evidencing a contribution from *de novo* histamine synthesis to

the exercise-induced histamine concentrations in skeletal muscle. *In vitro* HDC activity can be regulated by pH [33,34], hypoxia [140], and temperature [34], all of which are known to change in skeletal muscle during exercise. It is unclear how any of these factors modulate histamine synthesis in exercising skeletal muscle, but investigation into these intramuscular environmental changes warrants consideration.

During exercise, increased metabolic heat production results in a rise in intramuscular temperature [10,30–32]. In a small cohort of individuals, 60 minutes of passive heating elicited a modest increase in interstitial histamine concentrations [10]. That same study found that *in vitro* heating did not induce mast cell degranulation, indicating that heat-induced increases in intramuscular histamine concentrations likely occur via accelerated *de novo* histamine synthesis. As heat stimulates rat HDC activity *in vitro* [33,34], this hypothesis presents a promising avenue for understanding the exercise-induced mechanisms underlying increases in skeletal muscle histamine concentrations. Linear regression identified a suspected intramuscular temperature threshold of 38.4 °C necessary to elicit increases in histamine within human skeletal muscle [10]. This was achieved roughly 40 minutes into heating, meaning that the individuals spent ~20 minutes above this threshold. However, it is unclear if histamine release in skeletal muscle is only temperature-driven or if there is a temporal component (i.e., time above 38.4° C) necessary to evoke appreciable histamine release.

This investigation sought to expand upon previous findings that increased intramuscular temperature increases intramuscular histamine concentrations. The primary purpose of this investigation was to evaluate the effect of passive heating on *de novo* histamine synthesis in human skeletal muscle. We hypothesized that passive heating would increase interstitial histamine concentrations and that these concentrations would be greater under control conditions than when HDC is inhibited. A secondary purpose of this investigation was to explore the potential temporal component to heating-induced histamine release. We hypothesized that longer heating periods would increase histamine concentrations in skeletal muscle.

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

This investigation consisted of two studies. 16 (5 male, 11 female) volunteers participated in the study one, and nine (3 males, 6 females) volunteers participated in study two. One participant took part in both studies and their visits were treated as two independent sets of data. Additionally, their participation in each study was separated by more than six months, allowing for sufficient washout for any residual effects of the initial investigation. Participant characteristics for each study are outlined in Table 2. Inclusion criteria consisted of age 18-40 years and activity levels tiers 0 (sedentary), 1 (recreationally active), or 2 (trained/developmental) as defined by McKay et al.[141]. Exclusion criteria consisted of body mass index <18 or > 28 kg/m^2, diagnosis of cardiovascular, metabolic, or pulmonary disease, autonomic disorders, ongoing use of antihistamines, sensitivity to high temperatures, elevated blood pressure (systolic ≥ 120, diastolic ≥ 80 mmHg), ongoing medical therapy, implanted electrical devices, and contraindications to muscle microdialysis placement (i.e., insufficient muscle (≤ 2 cm muscle depth), excessive subcutaneous adipose, abnormal vasculature of the muscle as identified by ultrasound).

Table 2: Participant Characteristics

Variable	Study 1	Study 2
Age (yr)	23 ± 3	30 ± 6
Height (cm)	170 ± 12	168 ± 11
Weight (kg)	64.4 ± 11.3	69.5 ± 11.0
BMI (kg/m^2)	22.3 ± 1.6	24.5 ± 1.5
Systolic Blood Pressure (mmHg)	111 ± 6	111 ± 7
Diastolic Blood Pressure (mmHg)	66 ± 5	67 ± 3
Mean Arterial Pressure (mmHg)	81 ± 4	82 ± 3
n	16 (11F)	9 (6F)

Data are mean ± SD.

Experimental Design

Study one experimental approach

The goal of this study was to evaluate whether *de novo* histamine formation was the cause of previously seen heat-induced histamine increases in skeletal muscle. After confirmation of abstentions and baseline measurements of heart rate and blood pressure were obtained, an intramuscular thermocouple and two microdialysis probes were inserted into the vastus lateralis under sterile conditions. The microdialysis probes were perfused continuously at a rate of 5 µl/min (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA, USA) from insertion until the end of heating. One probe was perfused with a 0.9% saline and 5 mM ethanol solution (Control). The other probe was perfused with a 0.9% saline, 5 mM ethanol, and α-fluromethylhistidine (α-FMH; 200 µM, Santa Cruz, Biotechnology, Dallas TX) solution. α-fluromethylhistidine was used to irreversibly inhibit HDC activity [142]. This dose was extrapolated from studies using α-fluromethylhistidine to inhibit HDC in cell culture [143,144] and previously used to inhibit HDC in muscle microdialysis during exercise [2]. Dialysate was collected in passivated microcentrifuge tubes every 20 minutes during the post-insertion recovery period and heating. The final collection (i.e., from minutes 80-100 post-insertion) was considered the pre-heating time point for subsequent analysis.

Following insertion, participants rested for 100 minutes to allow resolution of local muscle trauma from the probe insertion procedure, before undergoing passive heating via pulsed-shortwave diathermy for 60 minutes. Intramuscular temperature was recorded prior to the start of heating and every ten minutes during heating.

Study two experimental approach

As the findings in Study 1 differed from previous investigations [2110], Study 2 set out to address concerns that Study 1 might have missed something in the heat-histamine response in skeletal muscle that had been observed previously. In doing so, Study 2 also aimed to explore the possibility of a temporal component to the effect of local passive heating on intramuscular histamine release. The preparation and procedure up until heating were identical to those of study one, with one small exception. To avoid administering more than 50 µg α-FMH (what had been previously approved in the FDA-IND application) α-FMH probe perfused saline and

ethanol control solution for the first 20-minute period. Following this initial 20 minutes, the perfusate was switched to the α-FMH solution, which was perfused for the remainder of the study. The estimated dead space of the microdialysis probe (63 MD Catheter, MDialysis, Stockholm, Sweden) is 10-20 µl. At a perfusion rate of 5 µl/min, the probe would be cleared of the control solution after five minutes, meaning the final 75 minutes of recovery were perfusing the α-FMH solution. As only the final time point (i.e., minutes 80-100 post-insertion) was used as the pre-heating time point in subsequent analysis, collections were not offset to account for dead space transitions.

Heating lasted 100 minutes. As the diathermy unit heats in 20-minute intervals, intramuscular temperature was recorded every 20 minutes prior to the start of a new interval. Additionally, discussions with other researchers using the same diathermy unit model as in this study indicated that skeletal muscle temperature increases to a marginally greater extent directly beneath the center of the unit. To limit the possibility of the thermocouple reading a higher temperature than what is reflected around microdialysis probes, the probes and thermocouple were placed approximately one centimeter apart in a triangular pattern, allowing them to be directly beneath the center of the unit.

Rigor and Reproducibility

Participants were required to abstain from food for two hours, consuming caffeine for 10 hours, and moderate or vigorous exercise, alcohol or recreational drugs, or vitamins, supplements, over-the-counter medications, and prescription drugs for 24 hours prior to arrival for the heating visit. Previous research indicates that the menstrual cycle has little effect on histamine release or receptor activation in humans [59–61,145], and as such, the menstrual cycle was recorded but not controlled for.

Intramuscular Temperature and Microdialysis

An intramuscular thermocouple was placed into the right *vastus lateralis* using sterile technique. The skin was initially anesthetized using prilocaine hydrochloride (4%) (Citanest®, Septodont, Lancaster, PA, US), followed by a lidocaine (2%) and epinephrine (1:1000,000) solution (Lingospan®, Septodont, Lancaster, PA, US) applied down to the underlying fascia. Care was taken to ensure that prilocaine and lidocaine were not injected into the skeletal muscle.

An 18-gauge introducer needle (Becton, Dickinson and Company, East Rutherford, NJ, US) was inserted into the right *vastus lateralis* and the thermocouple was threaded through the inner portion of the needle. Once the thermocouple was in place within the skeletal muscle, the introducer was removed, leaving the wire in place, which was then secured with steri-strips. The thermocouple was used to record intramuscular temperature throughout the experiment.

Following the placement of the thermocouple, two intramuscular microdialysis probes were placed in the right *vastus* lateralis using sterile technique analogous to the placement of the intramuscular thermocouple. The thermocouple and the microdialysis probes were placed approximately one centimeter apart in a triangular formation. After insertion, the thermocouple was held in place by covering the entry site with a sterile transparent medical dressing (Tegaderm, 3M, St. Paul, MN, USA). The microdialysis probes had a 20-kDa molecular mass cutoff with a 30-mm polyarylethersulphone membrane (63 MD Catheter, MDialysis, Stockholm, Sweden).

Dialysate histamine concentrations were measured using an enzyme-linked immunosorbent assay (Histamine ELISA, BA E-1000R, Rocky Mountain Diagnostics, Colorado Springs, Co, USA) performed in accordance with the manufacturer's instructions. Regarding missing data points, in Study 1 two individuals were removed from the histamine analysis due to consistently high variation (CV > 20%) across multiple samples and multiple assays, so the data reflect 14 individuals. Local blood flow responses were assessed by ethanol washout (ethanol outflow-to-inflow ratio) from the interstitial space. Ethanol in the perfusate and dialysate was measured using a modified alcohol dehydrogenase enzymatic assay [146–148] , which has been previously used in our lab [145] and others [149,150].

Local Heating via Pulsed Short-Wave Diathermy

Pulsed short-wave diathermy (OmniSWD, Accelerated Care Plus-LLC, Reno, NV, USA) was used to passively increase the temperature of skeletal muscle without inducing systemic thermoregulatory responses. The pulsed electromagnetic energy was applied at 27.12 MHz (800 bursts/s; 400-psecond burst duration) averaging 48 W for both the 60 and 100 minute trials, with a 200 cm^2 induction drum positioned 3.5 cm above the tissue so that there was no direct contact with the skin surface. The drum was positioned so that the microdialysis probes and thermocouples were centered under the drum. Because of electrical interference with the

machinery used to read the thermocouple, temperature readings were obtained by pausing the heating momentarily (<5 seconds) to measure and record intramuscular temperature.

Statistics

Participant characteristics are reported as means ± SD. All other data are reported as means and 95% confidence intervals. Alpha was set at 0.05 for all statistical inferences including familywise error rates. Shapiro-Wilks tests were used to test for normality. Inferences which were drawn from two-way mixed-effects models (for measurements across time and between treatments) 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 the group-time interaction in the mixed-effects model, restricted to Pre and Post measurements by group. Inferences regarding the differences between groups were drawn from Šidák's Multiple Comparisons Test, restricted to comparisons at the same timepoint between groups. 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 between groups.

Results

Skeletal muscle temperature

Changes in intramuscular temperatures over time are presented in Figure 4. In Study 1 baseline muscle temperature was 33.5 [32.9, 34.0] °C. During heating, intramuscular temperature was elevated from minute 10 until the end of heating *(p*<0.05), reaching 38.9 [38.2, 39.7] °C by the end of heating.

In Study 2 baseline temperature was 35.8 [35.4, 36.2] °C. During heating, intramuscular temperature was elevated from minute 20 until the end of heating *(p*<0.01), reaching 39.1 [38.8, 39.0] °C by the end of heating. Intramuscular temperature increased to 38.5 [37.9, 39.4] °C by minute 40 and remained elevated to the end of heating.

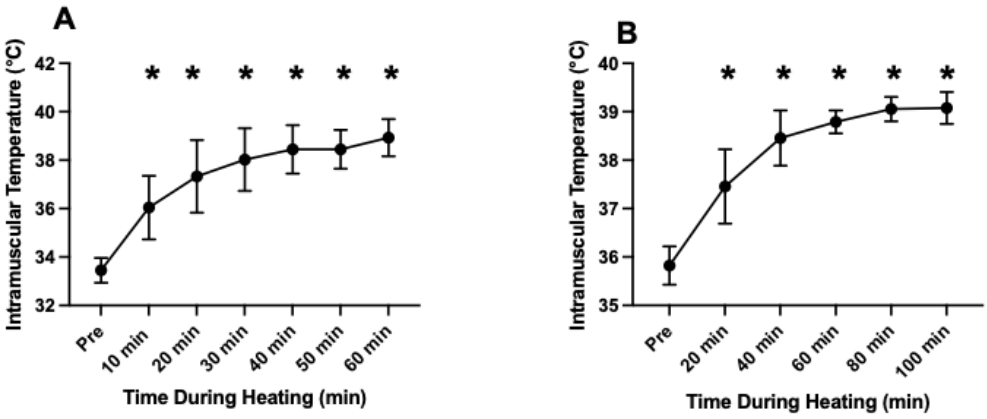

Figure 4: In Study 1 (A, n=16), intramuscular temperature increased during heating, reaching 38.9 °C after 60 minutes. In Study 2 (B, n=9), intramuscular temperature increased during heating, reaching 39.1 °C after 100 minutes. * Indicates different from baseline *(p*<0.05). Data are means ± 95% CI.

Interstitial histamine concentrations

Interstitial histamine concentrations are presented in Figure 5. In Study 1, there was no difference in histamine concentrations between conditions *(p*=0.52) and no change during heating *(p*=0.28). Histamine concentrations in dialysate were 1.17 [0.65, 1.70] ng/ml and 1.08 [0.68, 1.48] ng/ml at baseline for the Control and α-FMH probes, respectively. At the end of heating, histamine concentrations were 1.10 [0.63, 1.56] ng/ml in Control and 1.20 [0.60, 1.80] ng/ml in α-FMH.

In Study 2, baseline histamine concentrations were 1.62 [1.00, 2.24] ng/ml and 2.17 [1.49, 2.85] ng/ml in the Control and alpha FMH probes, respectively. Histamine concentrations were higher in the α-FMH probe across the heating period *(p*<0.01), with differences observed at 20 *(p*=0.04), 40 *(p*<0.01), and 60 *(p*=0.03) minutes. Interstitial histamine concentrations at the end of heating were 1.16 [0.60, 1.71] ng/ml in Control and 1.71 [1.10, 2.33] ng/ml in α-FMH.

Ethanol outflow/inflow ratio

Changes in the ethanol outflow/inflow ratio during heating are presented in Figure 5. Local blood flow was estimated via the ethanol washout technique, measuring the outflow/inflow ratio. Ethanol outflow/inflow was assessed in a subset of participants (n=10) in Study 1. Ethanol outflow/inflow ratio was lower in the CTRL than α-FMH *(p*<0.01) indicating

higher local blood flow. Ethanol outflow/inflow ratio did not change during heating *(p*=0.48) in either condition, indicating no change in local blood flow.

In Study 2, the ethanol outflow/inflow ratio was not different between control and α-FMH *(p*=0.22) and did not change during heating *(p*=0.59), indicating no difference between conditions or change during heating in local blood flow.

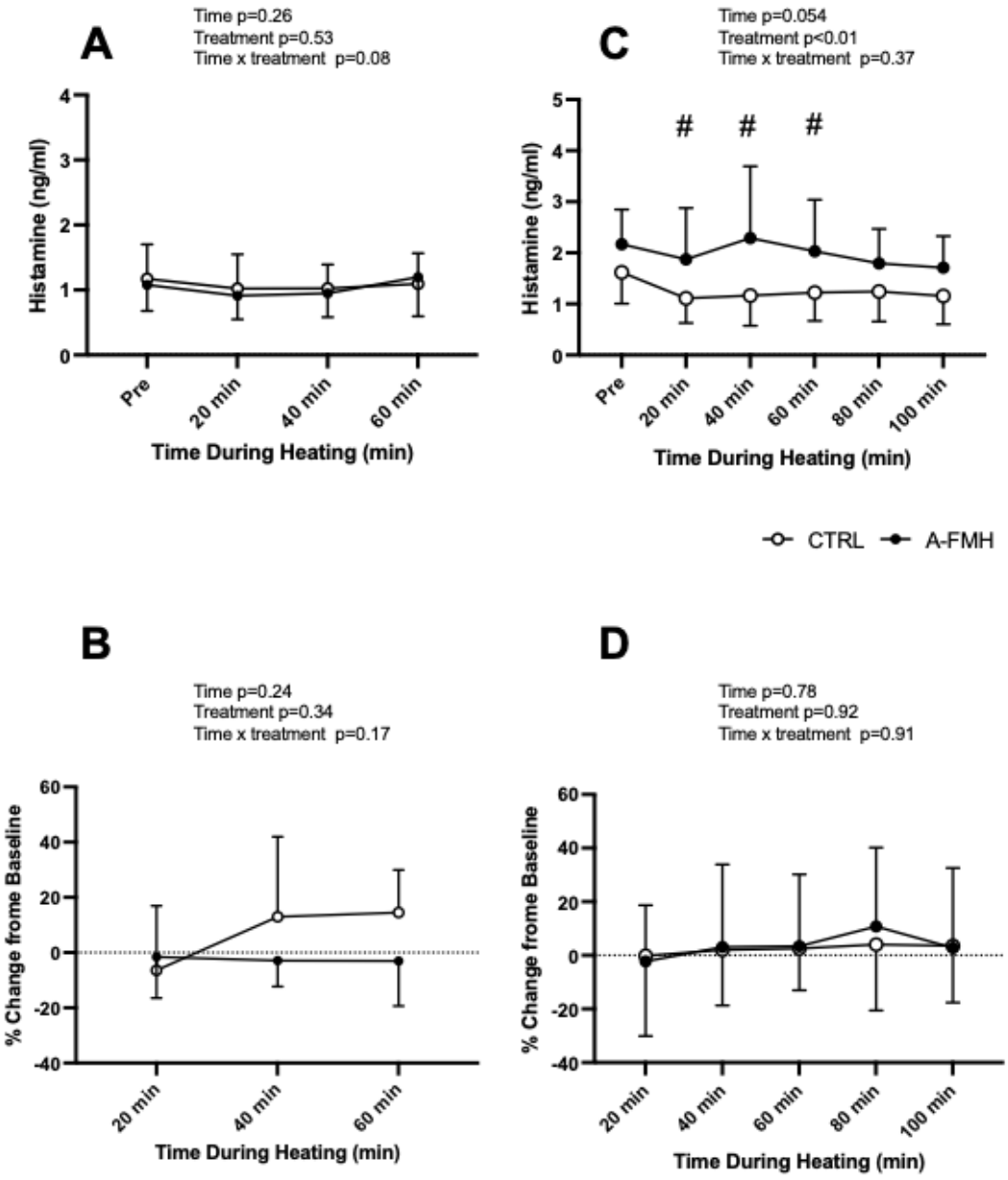

Figure 5: Study 1 intramuscular histamine concentrations (A, n=14) and changes in local blood flow (B, n=10) during heating. Study 2 intramuscular histamine concentrations (C, n=9) and changes in local blood flow (D, n=9) during heating. # indicates different from control at designated time point *(p*<0.05). Data are means ± 95% CI.

Discussion

This project set out to evaluate the effect of passive heating on *de novo* histamine synthesis in skeletal muscle. In doing so, it would confirm that the rise in muscle temperature during exercise stimulates *de novo* histamine synthesis, providing a mechanism by which changes in skeletal muscle temperature are a trigger of histamine release in skeletal muscle. Experimentally, this was done using intramuscular microdialysis and local passive heating to measure histamine concentrations in dialysate collected from skeletal muscle under control conditions and after pharmacologic inhibition of HDC. By employing pulsed short-wave diathermy, this investigation evaluated changes in muscle temperature, independent of muscle contraction or metabolic heat production, in a manner directly comparable to other investigations which found local passive heating increases histamine concentrations in skeletal muscle [10].

In Study 1, heating increased intramuscular temperature to 38.9 °C, which is similar to temperatures achieved during exercise[10,32,151] and what has been previously shown to increase histamine concentrations in skeletal muscle [10]. However, in Study 1, 60 minutes of heating did not increase histamine concentrations in dialysate collected from skeletal muscle. This was true in both a control condition and a condition where HDC was pharmacologically inhibited. Ethanol outflow/inflow ratio (a surrogate for local blood flow) did not change during heating and was not different in either condition.

Study 2 set out to reinforce that the investigation was not missing a previously observed component necessary for histamine release. As the increase in temperature with diathermy was slower than that observed during exercise, something observed by others [10], Study 2 increased the duration of heating from 60 to 100 minutes. By increasing the heating duration, Study 2 also investigated the potential of a temporal component (i.e., time at an elevated temperature) in heat-induced histamine release. Passive heating increased intramuscular temperature to 39.1 °C. Histamine concentrations were unchanged during heating in either condition in Study 2. Ethanol outflow/inflow ratio was not different in either condition and did not change during heating.

Heating and HDC Activity

The rise in skeletal muscle temperature during exercise can increase enzymatic activity[152]. The greater activity of some enzymes at higher temperatures then increases the

production of end products of those reactions. *In vitro* HDC activity is increased at higher temperatures [33,34], suggesting that an increase in intramuscular temperature during exercise may stimulate the histamine appearance in skeletal muscle via *de novo* histamine synthesis. The use of local passive heating and intramuscular microdialysis provides an opportunity to evaluate the effect of increased muscle temperature *in vivo*, independent of muscular contraction or other exercise-induced alterations in the intramuscular environment. By pharmacologically inhibiting HDC and comparing to a control condition, we can evaluate the contribution of *de novo* histamine synthesis heat-induced histamine release *in vivo*. However, because passive heating did not change histamine concentrations in the dialysate under either condition, we cannot assess the contribution of *de novo* histamine synthesis in the present study. However, while changes in intramuscular temperature may not evoke appreciable histamine release, they may stimulate HDC activity, helping replenish mast cell histamine stores. Dialysate collected from skeletal muscle is unlikely to provide insight into changes in HDC mRNA expression or activity within mast cells. Resolving questions surrounding heat-induced modulation of HDC activity in mast cells would require examination of HDC expression using mast cell resolved approaches (e.g., integrated single cell approaches in combination with spatial methods) to isolate the effect of heating on HDC activity within skeletal muscle.

Temperature and mast cells in skeletal muscle

Single-cell transcriptomic data indicate that HDC is expressed exclusively in mast cells within skeletal muscle[2122], suggesting they are the only cells capable of histamine synthesis in skeletal muscle[122]. If an increase in temperature were to stimulate HDC activity in these mast cells, it could increase or replenish the histamine pool in their granules, but this does not explain the rise in histamine in the skeletal muscle. For that, histamine within mast cells must be released into the skeletal muscle environment, primarily through mast cell activation and degranulation. Exposure to mast cells at temperatures from 34 to 40 °C did not induce degranulation of isolated human mast cells *in vitro* [10]. Thus, it is unclear how temperature changes elicit histamine release. One option is that, during exercise, changes in temperature act synergistically with other stimuli to produce sufficient calcium influx into mast cells to trigger degranulation. Mast cells express numerous surface receptors, including transient receptor potential vanilloid (TRPV) channels 1-4, which are activated in response to heat [153]. These receptors can also be stimulated by

reductions in pH, osmotic swelling, and proinflammatory cytokines [153], all of which change in the skeletal muscle environment during exercise. This alone or in combination with other triggered mast cell surface receptors may be enough to evoke degranulation and histamine release during exercise. Thus, while it is unlikely that temperature changes are a primary driver of exercise-induced histamine release, they cannot be ruled out as a contributing factor.

As changes in temperature alone are insufficient to induce histamine release, it remains unclear how local passive heating previously evoked histamine release [10]. Mast cells also exhibit selective release of contents without full degranulation [93]. It may be that increases in temperature lead to a slower "leak" of histamine as opposed to what is seen in degranulation. Histamine has a short half-life and is rapidly broken down in skeletal muscle by histamine-N-methyltransferase [154] , and these small increases may not be easily captured in dialysate. As such, what may have appeared as a robust heat-induced histamine response [10] may be more subdued and difficult to replicate.

It is also worth a brief discussion of mast cell heterogeneity and phenotypic plasticity. Mast cells mature and differentiate under the influence of the local environment [87–89] , leading to substantial heterogeneity and phenotypic plasticity amongst mature mast cells [155]. This heterogeneity can influence the types and abundances of mast cell receptors, the mediators they release, and, presumably, the extent to which they are stimulated by activators. As much of mast cell research has focused on their role in host defenses, mast cells from the bronchioles, skin, and gastric mucosa have been most commonly investigated in the literature [155]. It is unclear whether, or how, skeletal muscle mast cells would differ from mast cells in more commonly studied locations or from the mast cell lines used *in vitro*. However, it is possible that these differences are substantial enough to allow for differential responses to similar stimuli. It also raises questions surrounding the heterogeneity of mast cells between humans. It is certainly possible that differences among humans (e.g., genetics, physical activity) may influence the local environment in which mast cells mature and contribute to phenotypic plasticity among mast cells across humans. Understanding this diversity may explain mast cell-mediated responses to exercise, and given the role of mast cell mediators (e.g., histamine) in functional adaptations to exercise [8,80], exercise responses as a whole.

A potential critical temperature for histamine release

We elected to employ pulsed short-wave diathermy as the passive heating modality, rather than using another modality for heating skeletal muscle, such as warm-water limb immersion. This avoided potential confounding variables (e.g., hydrostatic influence of water on skeletal muscle) and avoided the challenges of maintaining a sterile field probe insertion site with warm water immersion. Additionally, it allowed for a more direct comparison the previous investigation of heat and histamine release which also employed pulsed short-wave diathermy as their passive heating modality [10]. Consistent with that investigation, we were able to heat the muscle to 38.9 °C and 39.1°C in Study 1 and Study 2, respectively, temperatures comparable to those achieved during exercise [10,30–32]. These temperatures are sufficient to increase HDC activity *in vitro* [34] and similar to the 38.9 °C previously shown to elicit histamine release [10].

Mangum et al., performed an inflection point analysis and identified a suspected temperature threshold for histamine release of 38.4 °C [10]. In Study 1, the average muscle temperature reached 38.4 °C at 40 minutes into heating and remained above the suggested threshold until heating ended, spending 20 minutes above the previously suspected threshold. Of note, five participants did not reach 38.4 °C during heating. The reason for this is not readily apparent, but the histamine response for those individuals did not differ appreciably from that of those who reached and surpassed the suspected threshold. In Study 2, average muscle temperature reached 38.4 °C 40 minutes into heating and remained elevated until the end of heating, spending 60 minutes above the suspected threshold. All participants in Study 2 exceeded a muscle temperature of 38.4 °C. In both Study 1 and Study 2, histamine concentrations did not increase during heating, and in Study 2, histamine concentrations were trending toward being lower at the end of heating. Taken together, the findings of Studies 1 and 2 suggest that 38.4 °C is not an obligatory threshold for histamine release.

No changes in blood flow during heating

A curious finding in the current investigation was that the ethanol outflow/inflow ratio did not change during heating in either study. The ethanol outflow/inflow ratio is a surrogate for local blood flow. Perfusing a known concentration of ethanol and measuring the collected ethanol enables estimation of local blood flow via ethanol diffusion. Greater local blood flow results in greater ethanol diffusion and a lower ethanol concentration in the effusate, and thus a

lower ratio. This methodology has been evaluated against ^{133}xenon clearance and direct measurement [146,156–158] and has previously been used in our lab [58] and others [149,150]. The lack of change in ethanol outflow/inflow ratio suggests that local blood flow was unchanged during heating in both Study 1 and Study 2.

Changes in muscle temperature during exercise appear to increase skeletal muscle blood flow [30,31]. However, the effects of local passive heating appear to be mixed, with some studies finding that acute passive heating increases local blood flow and others finding it is unchanged [159]. Some of these discrepancies may have to do with the technique of measuring muscle blood flow as some techniques measuring bulk blood flow to heated areas may also be picking up increases in skin blood flow as well. The use of intramuscular microdialysis in combination with pulsed short-wave diathermy allows us to evaluate changes in local muscle blood flow of the heated area, independent of skin blood flow. To our knowledge, only one study has employed pulsed short-wave diathermy and the ethanol washout technique [150]. They found blood flow doubled during 90 minutes of heating to temperatures similar to the current study (~39°C). While no direct explanation for the disparate findings is readily available, it may be that consistent changes in skeletal muscle blood flow are not fully captured using intramuscular microdialysis.

Conclusion and Perspectives

At the onset of exercise, skeletal muscle temperature increases. This is important for stimulating enzyme activity and meeting the metabolic demands of exercise [152]. Histamine is released in exercising skeletal muscle, and its actions are important in post-exercise responses and facilitating the beneficial adaptations to exercise [80,122]. Part of the histamine release is suggested to result from *de novo* formation by HDC [2]. While it is plausible that increases in temperature stimulate HDC, it is not readily apparent whether this translates into greater histamine release. The mechanisms by which histamine is released from mast cells into skeletal muscle during heating are not readily apparent. It is possible that increases in temperature acts synergistically with other environmental changes during exercise to evoke degranulation or augment selective release. It also may be that the skeletal muscle environment in which mast cells mature influences their reactivity to changes in temperature and how that may facilitate histamine release during exercise. Those ideas, while speculative at this point, may provide

insight into previous findings that heating evoked histamine release in skeletal muscle[10]. However, the current investigation, and the lack of mast cell degranulation in response to heating *in vitro* [10] indicate that changes in temperature alone appears not to be the driving stimulus behind exercise-induced histamine release in skeletal muscle.

CHAPTER V

CHARACTERIZING THE EXPRESSION OF MRGPRX2 IN A MAST CELL LINE

Introduction

To understand the mechanisms of histamine release during exercise, one must characterize the sources of histamine in skeletal muscle and outline their biology and physiological roles. Histamine is primarily stored in the granules of mast cells, which are tissue-resident effector cells important in both the innate [160] and adaptive [155] immune responses. Derived from hematopoietic stem cells in bone marrow, mast cell progenitors are released into the bloodstream and migrate to peripheral tissues, where they mature and differentiate under the influence of factors within the local environment [65,87,89]. The substantial influence of the local environment on mast cell maturation results in extensive phenotypic plasticity among mature mast cells [155]. In humans, mast cells are widely distributed throughout highly vascularized tissues and tend to reside within epithelia and in close proximity to blood vessels, nerves, smooth muscle cells, mucus-producing glands, and hair follicles [155]. Mast cells are most prominent in areas exposed to the external environment, such as mucosal or gastrointestinal tissue [161] , but are also found in other tissues, such as skeletal muscle [122]. This variance in tissue type and location results in substantial heterogeneity in the morphology of mature mast cells [87] and can influence mast cell activators and which surface receptors they stimulate.

The relative abundance and expression of mast cell surface receptors largely determine how, and to what, they respond. Mast cells are activated when cell-surface receptors bind to or are stimulated by external triggers. This initiates a second-messenger signaling cascade leading to calcium influx and triggering the release of pre-formed vasoactive and immunomodulatory mediators into the external environment and *de novo* synthesis of new mediators [36,65,92,93]. These chemical messengers are released via degranulation, a process by which mast cells extrude the contents of their granules via exocytosis [88,89] or selective release, in which only certain mast cell contents are released into the environment [36,93]. The molecular mechanisms underpinning degranulation and selective release have been thoroughly described elsewhere [162,163], but the extent to which mast cells are activated and mediators are released is generally determined by the type and magnitude of receptor binding and activation of signaling pathways [164]. In this way,

mast cell-mediated responses are coordinated and regulated to match the stimulation via their triggers.

The high abundance of mast cells in tissues exposed to the external environment (i.e., mucosal and epithelial tissues) underscores their important role in responses to external pathogens and implicates them as a central in facilitating allergic and irritant reactions. Consequently, traditional research has largely emphasized antigen-mediated mechanisms of activation. Mast cells highly express the FcεRI receptor, which has a high affinity for the antibody immunoglobulin E (IgE) [165]. IgE-bound particles attach to the binding site on the FcεRI receptor and trigger mast cell degranulation via antigen-dependent cross-linking of antigen-specific IgE molecules binding to FcεRI [65,92]. In this way, reactions to foreign pathogens are potent stimuli for mast cell degranulation. However, stimulation of other receptors causes mast cell activation independently of IgE-mediated pathways [36,92] , expanding the list of potential activators to include endogenously produced peptides, cytokines, and chemokines [36,161,166,167]. Non-IgE-mediated degranulation has been implicated in mast cell-related diseases such as mastocytosis, chronic urticaria, and rosacea, and has subsequently generated substantial therapeutic interest in characterizing prominent receptors beyond FcεRI, one of interest being mas-related G protein-coupled receptor X2 (MRGPRX2).

MRGPRX2 is a member of the MRGPR family of receptors, of which there are nine subtypes [168] . MRGPRX2 is a surface receptor expressed in mast cells in the skin, as well as other granulocytes throughout the body [96,125,168,169]. There appears to be substantial heterogeneity in the level of MRGPRX2 expression on mast cells between humans [97]. This may be due in part to MRGPRX2 contributing to the migration and position of mast cell progenitors within tissue [129], although the details require further investigation. MRGPRX2 is selectively activated, responding to numerous secretagogues, including chemokines, cytokines, and neuropeptides [126]. In mature mast cells, MRGPRX2 activation leads to degranulation and the release of mast cell mediators, including histamine [94,126,169,170]. The neuropeptide substance P is the most well-characterized agonist in humans [129], but MRGPRX2 can be activated by a variety of cationic compounds, including other peptides and small molecules [126,168]. This broad set of potential stimulators has implicated MRGPRX2 in inappropriate activation of mast cells and has positioned it as a target for pharmacological treatment of mast cell-mediated disorders. However,

these same features also make the receptor a potential mediator of exercise-induced mast cell degranulation.

Exercise alters the intramuscular concentrations of numerous endogenously produced substances, including acetylcholine, vasoactive intestinal peptide, substance P, and adenosine, which can act on mast cell receptors [36,164]. Coupled with the numerous other changes in the interstitial environment during exercise (e.g., temperature, pH, etc.) it is difficult to assess the triggers of mast cell degranulation *in vivo*. It then becomes necessary to employ model systems to isolate and identify the signals responsible for exercise-induced mast cell degranulation. *In vitro* models allow for isolation and stimulation of specific receptor pathways, such as MRGPRX2, to evaluate their specific effect on degranulation.

Traditional degranulation assays have employed the Laboratory of Allergic Diseases 2 (LAD2) cell line to investigate mast cell degranulation in human-derived mast cells [98,101,102,129]. This cell line was established from bone marrow aspirates of a 44-year-old male donor with mast cell sarcoma/leukemia [171]. In 2011, a new human mast cell line LUVA (Laboratory of the University of Virginia) was derived and characterized from peripheral blood $CD34^+$ cells of a donor with aspirin-exacerbated respiratory disease [131]. This showed similar responsiveness to IgE-mediated activation as LAD2 cells, which investigators pointed to as functioning in a similar manner [131]. Yet, the LUVA cell line is not commonly used and thus is poorly characterized in the literature. Our lab employed LUVA cells in previous *in vitro* degranulation assays and found they are not activated in response to heat [10] but that they are activated when exposed to dialysate collected from exercising tissue [66]. However, LUVA cells may not release histamine, and their degranulation characteristics or receptor expression are less well defined (per vendor product information[172] and personal communication[173]). MRGPRX2 expression is highly heterogeneous between human mast cell types and amongst human donors [97,129], leading to differing activation in response to peptides across cell lines isolated from humans [97,174]. Previous *in vitro* investigations of MRGPRX2-mediated degranulation employed LAD2 cell line, and found that it responded to showed a strong response to the high-affinity MRGPRX2 agonist substance P [90,95,130]. Pilot trials indicated that LUVA cells did not respond to varying concentrations of substance P. Thus, the purpose of this project was to explore and develop an appropriate *in vitro* model system to support future investigations involving mast cell degranulation. The first aim was to generate preliminary data as to whether LAD2 cells respond to substance P, and whether

this was MRPGRX2-mediated. The second aim was to determine the expression of MRGPRX2 in LUVA cells and compare it to LAD2 cells. Finally, the third aim was to evaluate LAD2-mediated degranulation when exposed to dialysate collected from exercising tissue and investigate the potential role of MRPGRX2. Completing these three aims compares and contrasts the two cell lines, provides context for previous degranulation assays, and establishes a base model which can be built upon in future investigations.

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.

Table 3: Participant Characteristics

Variable	
Age (yr)	24 ± 4
Height (cm)	171 ± 8
Weight (kg)	69.7 ± 13.6
BMI (kg/m^2)	23.8 ± 2.9
Systolic Blood Pressure (mmHg)	110 ± 8
Diastolic Blood Pressure (mmHg)	65 ± 9
Peak Power (watts)	31.8 ± 7.1
60% Peak Power (watts)	19.1 ± 4.2
n	8 (6F)

Data are mean ± SD.

Human Participants

Eight volunteers (2 male / 6 female) completed a screening visit to determine eligibility, a test of peak power using dynamic knee extension, and a study visit involving the placement of intramuscular microdialysis probes and 60-minutes of dynamic knee extension exercise. Inclusion criteria consisted of age 18-40 years and activity levels tiers 0-2 (sedentary to recreationally active) [141]. Exclusion criteria consisted of body mass index <18 or > 28 kg/m^2,

diagnosis of cardiovascular, metabolic, or pulmonary disease, autonomic disorders, ongoing use of antihistamines, sensitivity to high temperatures, elevated blood pressure (systolic ≥ 120, diastolic ≥ 80 mmHg), ongoing medical therapy, implanted electrical devices, and contraindications to muscle microdialysis placement (i.e., insufficient muscle (≤ 2 cm muscle depth), excessive subcutaneous adipose, abnormal vasculature of the muscle as identified by ultrasound). Participant characteristics are in Table 2.

Dynamic unilateral knee-extension exercise

During the test of peak power, participants underwent a dynamic knee extension test to volitional fatigue to determine the work rate (power output) for the following experiment. During the study visit, participants performed 60-minutes of dynamic unilateral knee extension exercise at 60% of peak power, as determined by the previous test. Exercise was performed using a custom-built knee extension ergometer based on a computer-controlled step motor that generated resistance against the participant's lower leg [2,4,175]. Participant seat and knee position, as well as the specifics of the peak power test and 60 minutes, were in accordance with previous investigations [10]. Heart rate (3 lead electrocardiogram) and rating of perceived exertion (Borg Scale) were recorded at baseline and every minute during the peak test and at baseline and every ten minutes during the 60-minutes of exercise.

Intramuscular Microdialysis

An intramuscular microdialysis probe was placed into the vastus lateralis of the exercising (right) and non-exercising (left) leg. The microdialysis probes had a 100-kDa molecular mass cutoff with a 30-mm polyarylethersulphone membrane (61 MD Catheter, MDialysis, Stockholm, Sweden). The skin of each leg was initially anesthetized using prilocaine hydrochloride (4%) (Citanest®, Septodont, Lancaster, PA, US), followed by a lidocaine (2%) and epinephrine (1:1000,000) solution (Lingospan®, Septodont, Lancaster, PA, US) applied down to the underlying fascia. Care was taken to ensure that prilocaine and lidocaine were not injected into the skeletal muscle. Probes were placed in each leg using sterile technique, and instrumented analogous to previous investigations [2,10].

Following probe insertion, participants then rested in a supine position for 100 minutes before being transferred from the bed to the ergometer and completing 60 minutes of dynamic

knee extension exercise. Following the exercise, participants were carefully transferred to the bed, where they remained supine for 60 minutes, after which the probes were removed. The microdialysis probes were perfused continuously at a rate of 5 µl/min (CMA 400 Microdialysis pump, CMA, North Chelmsford, MA, USA) from insertion until one hour following exercise (approximately 3.5 hours) with 0.9% sterile saline. Dialysate was collected in passivated microcentrifuge tubes, and tubes were changed every 20 minutes.

Mast Cell Lines

LAD2 mast cells were obtained from Applied Biological Materials (Richmond, British Columbia, Canada). The LAD2 cell line originates from bone marrow aspirates of a 44-year-old male donor with mast cell sarcoma/leukemia [171]. Cells were thawed and then grown in PriGrow XSeries Medium (TM8157) + 100 ng/ml human Stem Cell Factor (Z100815) + 2 mM L-glutamine (G275) + 1% Penicillin/Streptomycin Solution. Cells were incubated at 37.0 °C, 5% CO_2, and hemidepletion of the media was performed twice per week per the manufacturer's instructions. This cell line is commonly used in degranulation assays and has been previously employed to evaluate MRGPRX2-induced degranulation [102,103,128,176].

LUVA cells are an immortalized cell line from a male donor (EG1701-FP, Kerafast, Boston, MA). Cells were grown in StemPro-34 SFC Medium + 2 mM L-glutamine (G275) + 1% Penicillin/Streptomycin Solution + Primocin 100/ml (Invitrogen, Carlsbad, CA, USA) provided 50% fresh medium weekly and incubated at 37.0 °C, 5% CO_2 per manufacturer's instructions and as previously described [131].

Mast Cell Degranulation Assay

Mast cell degranulation was quantified by the release of the granule marker β-hexosaminidase in a standard bioassay [177]. LAD2 cells were cultured and plated following the vendor's instructions and standard methods [177,178] on a 96-well plate (2.5x 10^5 cells/well) in 50-µL imaging buffer (142 mM NaCl, 5mM $NaHCO_3$, 10 mM HEPES, 16 mM glucose, 2 mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 0.1% BSA, pH to 7.3 with NaOH). Cells were pre-incubated in the absence or presence of compound C9-6, a MRGPRX2-specific antagonist [101,102], for 10 minutes at 37.0 °C. Imaging buffer (100 µL) was added to blank wells. As a positive control, 2 mg/mL compound 48/80- (Sigma-Aldrich, St. Louis, MO) in 50-µL imaging buffer was added to a

subset of plated cells, yielding a final well concentration of 1 mg/mL to activate mast cell degranulation. Compound 48/80 serves as a cell viability check because it stimulates non-IgE-mediated mast cell degranulation via multiple receptor pathways, including MRGPRX2. An additional 50-µL of imaging buffer (vehicle) was added to the remaining wells. 50 µL of skeletal muscle dialysate was added to their respective wells (Aim 3 only), then incubated at 37.0 °C for 30 minutes, after which the samples were centrifuged and the supernatants were removed.

To determine total β-hexosaminidase activity, 0.06% Triton X-100 (Sigma-Aldrich, St. Louis, MO) was added to the pelleted cells to lyse them. Supernatants, cell lysates, and blank wells were reacted with 2 mM 4-nitrophenyl N-atetyl-β—D-Glucosamide (Sigma-Aldrich, St. Louis, MO), the substrate for β-hexosaminidase, at 37.0 °C for 2 hours. Then, 0.5 M Tris(hydroxymethyl)aminomethane (Sigma-Aldrich, St. Louis, MO), pH 9.0, was used to stop the reaction, and the absorbance was read at 405 nm. Degranulation was expressed as a percentage of β-hexosaminidase activity in the supernatant divided by the total (supernatant plus pellet) activity after correcting for the background readings of the cells containing only imaging buffer and substrate.

Protein Quantification and Western Blots

Cells were lysed in 25 ul per 10^6 cells of RIPA (Thermo Fisher, Waltham, MA, USA) buffer enhanced with 5% sodium dodecyl sulfate (SDS) EDTA and 10 ul/mL protease inhibitor cocktail. Cells were sonicated at 10% amplitude for 6 x 1-second pulses and incubated in lysis buffer for 2 hours at room temperature. Cell debris was pelleted, and the soluble protein extract was stored at -80 °C until the time of analysis.

Extracts were quantified against a BSA standard using the Bio-Rad BCA quantification kit (Bio-Rad, Hercules, CA, USA). Protein was loaded in quantities of 50 and 25 ug per lane in LICOR 4x load buffer and separated on a Bio-Rad Criterion Mini 4-12% acrylamide gradient gel at 200 volts in tris-glycine buffer with 0.1% SDS. Proteins were transferred onto nitrocellulose in Tris-Glycine buffer with 20% methanol. Protein loading and transfer efficiency were assessed with Ponceau staining. Blots were blocked in SuperBlock Blocking Buffer (ThermoFisher, Waltham, MA, USA) for 15 minutes before incubating in 1:300 rabbit anti-MRPGPRX2 (ThermoFisher, Waltham, MA, USA) for an hour at room temperature. Blots were subsequently transferred to the refrigerator to incubate at 4 °C overnight. The following morning, membranes

were washed in PBST before incubating for an hour at room temperature in 1:15,000 goat anti-rabbit-680 (LICOR) before imaging on a LICOR Odyssey Classic infrared imaging system. Bands were analyzed in Image Studio 2.0 and normalized to total protein ponceau staining.

Statistical Analysis

Participant characteristics are presented as mean ± SD, all other data are reported as mean with 95% confidence intervals where appropriate. Alpha was set at 0.05 for all statistical inferences, including familywise error rates. Shapiro-Wilks tests were used to test for normality. As this was a preliminary investigation, statistical analysis was only performed on the exercise responses. In that situation, a one-way analysis of variance was used to assess changes in heart rate and rating of perceived exertion over time and in degranulation from baseline to exercise (Prism 10, GraphPad, Boston, MA, USA).

Results

Phase 1 degranulation

Results from the degranulation are presented in Figure 6. Viability was demonstrated by degranulation with compound 48/80, which occurred in the absence and presence of compound C9-6. Substance P caused degranulation at concentrations ranging from 0.3 µM to 5 µM but did not show a dose-response relationship. Substance P-induced degranulation was abolished by the addition of Compound C9-6.

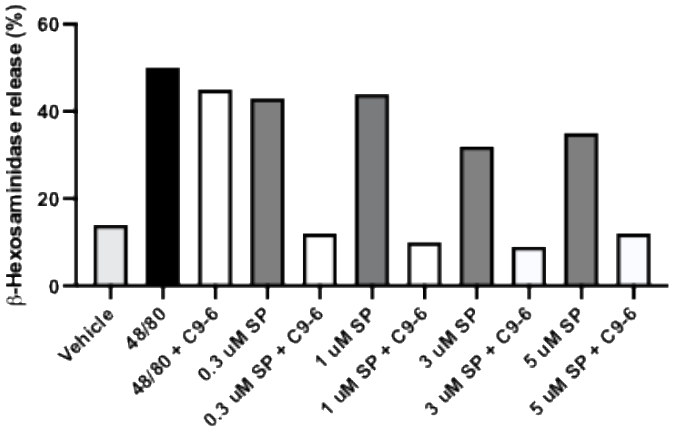

Figure 6: Results of the initial degranulation assay using the LAD2 cells. Each condition was run in triplicate. Labels represent what was used in each condition. Vehicle; 0.5% dimethyl sulfoxide. 48/80; 1 mg/ml Compound 48/80. SP; Substance P. C9-6; Compound C9-6.

Western blot analysis

Human umbilical vein endothelial cells (HUVEC) were used as a control. Only one of the blots (1:300 dilution) provided an image that could be analyzed (Figure 7). In this image, the relative abundance of the MGRPRX2 protein was highest in HUVECs, followed by LUVA and LAD2. As MRGPRX2 expression is restricted to mast cells and is not detected in endothelial cells, HUVECs should not contain the MRGPRX2 protein. As the HUVECs had the highest protein-binding abundance, this was taken as evidence of protein saturation rather than specific binding. The blots were incubated in lower concentrations of the antibody (1:1000, 1:500), but those concentrations did not produce images that could be confidently analyzed (i.e., no well-defined protein bands).

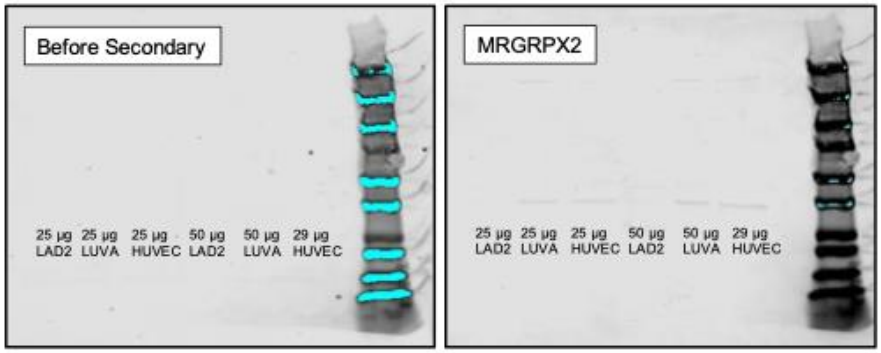

Figure 7: Image of the western blot analysis. This was performed at a 1:300 dilution for the MRGPRX2 antibody. Lanes 1-3 used 50 µg of protein, except for HUVEC, which had a low protein content and required only 29 µg. Lanes 4-6 used 25 µg protein to normalize for all cell types.

Mast cells and dialysate

All participants completed 60-minutes of dynamic knee extension exercise. Heart rate and rating of perceived exertion increased at the onset of exercise and remained elevated throughout the 60 minutes ($p<0.01$) with heart rate reaching a maximum of 113 [97, 128] beats per minute and rating of perceived exertion reaching 16 [14, 18] on the Borg Scale. On average, there was no difference in degranulation between cells exposed to dialysate at rest and during exercise ($p=0.76$), and there was no effect of C9-6 on the degranulation of cells exposed to dialysate collected at rest or during exercise (Figure 8). However, the exercise response was more variable than the baseline response. Further, it appears that in two participants, exercising dialysate induced substantially greater degranulation than baseline. In one of these participants, this response was attenuated by MRGPRX2 inhibition. The effect of MRGPRX2 inhibition was

unable to be determined in the other, as one sample had absorbance outside of the quantifiable limit. This may have been due to erythrocyte fragments in the dialysate altering the absorbance.

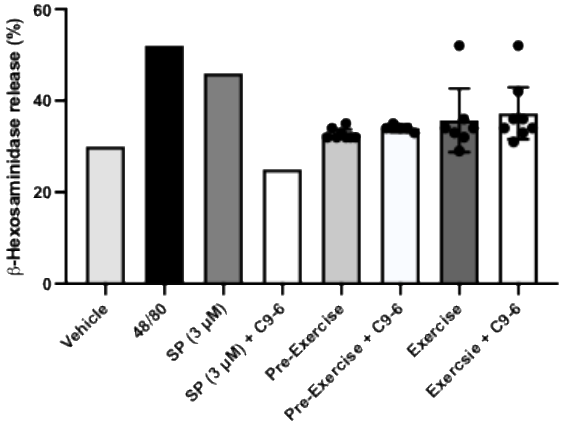

Figure 8: Findings from the degranulation assay when using skeletal muscle dialysate. Compound 48/80 indicates cell viability. Substance P indicates MRGPRX2-induced degranulation and blockade with C9-6. Conditions without multiple values confidence intervals indicate a single point. Data for the dialysate-induced degranulation are means and 95% CI.

Discussion

The purpose of this study was to explore and develop an appropriate *in vitro* model system to support future investigations involving mast cell degranulation. The initial assay found substance P induced degranulation in LAD2 cells at concentrations ranging from 0.3 μM to 5 μM and this was abolished by the addition of Compound C9-6. In fact, in cells treated with Compound C9-6, degranulation was similar to that observed with the vehicle alone, indicating that C9-6 completely antagonized the MRGPRX2 receptor. Importantly, cell viability was evidenced by degranulation seen with compound 48/80, which was still present with compound C9-6, although degranulation was slightly attenuated due to MRGPRX2 inhibition. These findings indicate that LAD2 cells express MRGPRX2, that substance P can induce degranulation, though not necessarily in a dose-dependent manner, and confirm that Compound C9-6 effectively antagonizes MRGPRX2. This phase established that the LAD2 cell line can be used to test the contributions of the MRGPRX2 pathway, a necessary step for the next phase, determining the involvement of this pathway in exercise responses.

We did not observe appreciable differences in degranulation between mast cells exposed to dialysate collected during exercise and at rest. These findings are in contrast to previous work

showing that mast cell degranulation is greater when exposed to dialysate collected from exercising skeletal muscle [66]. The reasons for these divergent findings are not readily apparent, but comparisons are difficult given that the investigations employed different mast cell lines. Compound C9-6 also did not appear to impact degranulation following exposure to dialysate from both resting and exercising muscle. As Compound C9-6 abolished degranulation induced by substance P, this may be taken to mean that any degranulation of LAD2 mast cells via dialysate does not occur through MRGRPX2.

It should be noted that in this assay, the vehicle (0.5% DMSO and image buffer) did induce 30% degranulation. The reasons for this level of degranulation are unknown; in previous assays, the same vehicle induced 10% or less degranulation. It is unclear whether this masked any degranulation observed in other conditions or was unique to the vehicle condition. Compound 48/80 (an internal control for cell viability) consistently induces approximately 50% or greater degranulation, and in this assay, it induced 52% degranulation. As compound 48/80 is a reliable and robust mast cell degranulator, it is curious that over half of its degranulation is attributed to the vehicle solution. Further, if the vehicle induces a certain amount of degranulation, that baseline degranulation would be expected across all the measured conditions. However, the substance P and C9-6 condition showed 25% degranulation, and one of the exercise wells showing 29% degranulation. It may be that these are within the error of measurement, or it may be that the vehicle's only condition had greater degranulation than what was elicited by the vehicle in other conditions. Nonetheless, we cannot rule out the possibility that the vehicle may have masked any degranulation observed upon exposure to the dialysate. To be transparent, we have included the vehicle in the figures showing degranulation.

Western blot analysis showed evidence of protein saturation rather than specific protein binding. Evidence for this came from HUVECs showing the highest protein quantification, despite MRGPRX2 not being known to be expressed in endothelial cells. As such, we were unable to confirm whether LUVA cells expressed MRGPRX2. Evidence from preliminary investigations shows they did not degranulate in response to substance P, suggesting they do not. However, this cannot be confirmed without first confirming protein expression in the cell line. Confirming the presence of the MRGPRX2 protein provides context for the previous insight into the characteristics of the LUVA cell line. Specifically, how the LUVA cell line might release β-hexosaminidase, what may stimulate it, and if it is appropriate for use in mast cell degranulation

assays going forward. This context is important for other investigations using the LUVA cell line to evaluate mast cell stimulation/degranulation/activation.

Understanding mast cells in exercise

Mast cells in skeletal muscle degranulate during exercise, releasing their contents into the skeletal muscle environment [2,122]. These contents contain, amongst other things, inflammatory mediators, which exert autocrine and paracrine actions important in facilitating exercise responses. One notable example is histamine, which has been shown to be important in acute postexercise vasodilatory [4–6], inflammatory [134,135], and transcriptomic [7] responses. Further, histamine release from mast cells appears important in the intracellular communication network following exercise [122], which when blocked, alters the responses to aerobic [8,80] and resistance [83] exercise training. However, not much is known about the triggers that cause mast cells to release histamine in exercising skeletal muscle. Increases in temperature [10] and reactive oxygen species [29] do not appear to mediate degranulation. It is likely that a soluble factor, released within skeletal muscle, mediates mast cell degranulation.

During exercise, neuropeptides are released into the intramuscular environment. These neuropeptides act in an autocrine and paracrine manner to induce vascular, immune, and metabolic responses in skeletal muscle. Of these, substance P is a neuropeptide of particular interest. Substance P is released by group IV afferents, whose endings are located at small blood vessels in skeletal muscle [179], and its concentrations in skeletal muscle increase during exercise [124,180]. *In vitro* substance P is a potent trigger of degranulation via MRGPRX2 [90,94,130], making it an intriguing potential mediator of mast cell degranulation. However, to date, isolating its effects in exercising skeletal muscle remains difficult.

In humans, MRGPRX2 is primarily activated by a seemingly ever-increasing number of cationic ligands, including neuropeptides, antimicrobial/host defense peptides, and some opioid-related peptides [130]. Some of these, as well as dynorphins and chemokines, increase during exercise [124,180–182] and may act synergistically to stimulate MRGPRX2, making it difficult to determine whether a single factor or a combination of factors within the interstitial milieu is responsible. Ex vivo models enable tightly controlled experiments evaluating its role in degranulation. However, those models cannot accurately recreate the conditions occurring within skeletal muscle during exercise. As such, *in vivo* investigations are necessary. Intramuscular

microdialysis is a promising avenue for evaluating changes in the interstitial environment during exercise. That technique, plus pharmacological inhibition, provided the groundwork for much of the insight into the role of histamine following exercise. Something similar may be achieved to evaluate the pathways involved in exercise-induced degranulation, although at the time of this writing, MRGPRX2 antagonists are not currently approved for use in humans.

Mast Cell Phenotypes

Examining mast cells in exercise is a far different context than the ones in which mast cells are traditionally studied. As much of the research has focused on their role in host defenses and allergic reactions, much of the current understanding of mast cells comes from studying populations in areas exposed to the external environment. These include mast cell populations in the skin, airways, lungs, and gastrointestinal tract. Indeed, if one does a quick search, they might think that those are the only locations of mast cells in the human body. However, mast cells located in skeletal muscle play an important role, just not in the responses traditionally associated with mast cells.

The influence of the tissue environment on mast cell maturation leads to substantial heterogeneity in mast cell phenotypes [87,88,155] and receptor expression [97], and may contribute to the differences in mast cell responses between humans [97]. To date, the understanding of mast cell phenotypes in skeletal muscle remains limited, opening the door to a promising line of inquiry. Mast cells degranulate during exercise [2,122], releasing their granule contents, which are important in facilitating post-exercise responses [27] and beneficial adaptation to exercise [8,80,83]. Improving the knowledge surrounding skeletal muscle mast cells has important implications in biomedical and exercise training research. A better understanding of this unique cell type provides important insight into cellular communication that underpins exercise adaptation. It also raises exciting avenues surrounding the development of therapeutic strategies that may, through mast cells, enhance the benefits of exercise adaptation. However, those investigations are, at this point, purely speculative and necessitate the isolation and characterization of mast cell populations within skeletal muscle. Furthermore, they will require robust model systems to characterize those populations and responses. This study is but a piece that hopefully adds to the puzzle that is the mediators of exercise-induced mast cell degranulation.

Employing model systems in human physiology

From cells to systems, human physiological function is a complex network of interwoven processes. When stress is imposed, such as during exercise, these systems mount a coordinated and integrated response to ensure the organism's survival. While one can appreciate the beauty of this response and subsequent adaptation, its complex nature, complete with levels of redundancy, makes it difficult to investigate in a scientifically systematic and rigorous manner. As a result, systems have been developed to model singular components of complex physiological systems. From animal models to cell-based work, these methods have enabled scientists to eliminate confounding variables or extraneous influences and assess direct responses. From pharmaceutical development to the treatment of disease to the understanding of physiological processes, much of what we know is based on the use of omics systems. These methods establish a basic science foundation that can then be built upon and integrated into the whole.

Developing a model system enables a more robust comparison to previous findings. However, the challenge lies in adapting the model to investigate different questions. The same rigidity that makes models useful presents issues when applying them across different contexts. The majority of mast cell research has focused on their roles in allergic reactions, anaphylaxis, and mast cell-mediated disorders. These investigations often employ *in vitro* models to evaluate the efficacy of potential therapeutics or to determine the effects of agonists on degranulation. Of course, insights from this work can be applied to better understand the role of mast cells in exercise. However, given the differing goals and varying context of the investigations, those methodologies may need refinement to be best utilized in the context of exercise. Identifying an antagonist for a certain receptor is a great start, but its solubility and mode of delivery can present unexpected challenges.

In the current investigation, the antagonist Compound C9-6 had to be dissolved dimethyl-sulfoxide to go into solution. Dimethyl-sulfoxide can be deadly to cells in too high of a dose. Pilot work indicated that a 0.5% dimethyl-sulfoxide solution was largely non-toxic to mast cells and elicited minimal degranulation. However, the vehicle control showed 30% degranulation. Whether this was true of all the conditions (i.e., that the vehicle was largely responsible for the degranulation seen) or something unique to the vehicle wells is not readily apparent. It is also not apparent whether there would be an additive effect or whether the vehicle masked some degranulation that would otherwise have been observed when cells were exposed to dialysate.

This may not have been much of a concern when evaluating the efficacy of C9-6 as a MRGPRX2 antagonist, as substance P induces robust degranulation, and its absence is readily apparent [102]. However, this becomes more of an issue when dealing with smaller differences change, such as the 5-10% differences seen between exposure to resting and exercising dialysate [66], or trying to parse apart the magnitudes of degranulation. Consideration and care must be taken in the difficult process of adapting models to different contexts, as what may be useful in one scenario may be limiting in another.

Conclusion and Perspectives

In the words of George E.P. Box, "all models are wrong, some models are useful." Given the complexity of physiology and the heterogeneity amongst humans, that statement seems apropos regarding human physiology research. This investigation is not intended to develop a final "one size fits all" model system. Instead, it aids in developing more useful models that can be employed and built upon for future investigations. These findings show that MRGRPX2-induced degranulation is robust in LAD2 mast cells and is inhibited by Compound C9-6. They also indicate that the use of dialysate to induce degranulation *in vitro* may not be as robust a model as originally thought. While these are not definitive endpoints, they advance the conversation and provide insight and adjustment necessary to improve the models going forward. This is by no means an endpoint, but rather a stepping stone for future investigations and an effort to develop more useful models to evaluate mast cells and their physiological function.

CHAPTER VI

HISTAMINE-RECEPTOR BLOCKADE DOES NOT INFLUENCE THE HEAVY-SEVERE DOMAIN BOUNDARY AND TIME TO TASK FAILURE IN THE SEVERE DOMAIN

This work was published as Abbotts, K.S.S.; Hudgins, J.H.; Viveros, I.S.; Minson, C.T.; Wilkins, B.W.; Halliwill, J.R. Histamine-receptor blockade does not influence the heavy-severe domain boundary and time to task failure in the severe domain during cycling exercise in adults. *Physiological Reports* 13 (19): e70587, 2025. DOI: 10.14814/phy2.70587.

Introduction

During intense exercise, ventilation can reach 200 L/min [183], increasing exposure to allergens in the air, which can induce pathophysiological responses. This may be why 56% of athletes report seasonal allergic rhinitis [42]. Allergies are usually treated with antihistamines, which alleviate allergy symptoms [42]. The most commonly used antihistamines block the actions of histamine via histamine receptor antagonism. In addition to being involved in allergic responses, histamine plays a crucial role as a mediator of exercise responses. Blocking its actions reduces sustained post-exercise vasodilation [4–6], alters the transcriptional [7] and inflammatory response to exercise [81], and attenuates functional adaptations to exercise training [8,80]. Athletes experiencing allergic rhinitis report lower quality of life [46] and impaired performance [47]. Additionally, a history of allergies is an independent risk factor for injury in runners [43,44] and cyclists [45]. Since histamine mediates critical physiological responses to exercise, athletes' use of antihistamines to manage allergy symptoms may inadvertently lead to these or other undesirable outcomes.

Histamine is released in exercising skeletal muscle, where it can act in an autocrine and paracrine fashion by binding to H_1 and H_2 receptors located in immune and vascular cells within the muscle environment [2,3,26,27,81]. Histamine is the primary mediator of sustained post-exercise vasodilation, evidenced by H_1 and H_2 receptor blockade abolishing post-exercise increases in vascular conductance and reducing post-exercise hypotension [5,6,184]. Histamine also appears to be integral to the adaptive responses to exercise. H_1 and H_2 receptor blockade blunts exercise-induced activation of the p38 mitogen-activated protein kinase pathway, [81] alters the exercise-induced differential expression of numerous protein-coding genes [7], and attenuates important systemic health benefits obtained from exercise training, including improvements in vascular

function, insulin sensitivity, and aerobic capacity [8,80]. These findings highlight histamine as an important component of post-exercise signaling and crucial to obtaining the full benefits of physical activity.

In addition to its influential role in exercise adaptation, histamine appears to play a role during exercise. In trained cyclists, histamine receptor antagonism slows 10-km time trial performance by roughly 1% [9]. For perspective, this degree of impaired performance is enough to negate the ergogenic benefit of caffeine [49] and can be the difference between first-place and off-the-podium [48]. It could be inferred that histamine receptor antagonism worsened performance by reducing blood flow to exercising skeletal muscle [4]. However, histamine receptor blockade paradoxically increases femoral blood flow during unilateral knee extension exercise while also resulting in greater decreases in intramuscular pH [37]. As an intrinsic regulator of the microvasculature, it is possible that histamine receptor blockade alters muscle perfusion, impacting nutrient delivery and metabolite washout, independent of "bulk" blood flow measures in conduit arteries. This may also affect the balance of oxygen supply and demand in exercising skeletal muscle, as well as the ability to sustain a given workload. Balancing oxygen supply and demand is important for sustaining exercise and is predictive of exercise performance [55]. Alterations in microvascular recruitment, as hypothesized to be elicited by histamine receptor antagonism, may alter exercise intensity domains and lower the highest sustainable workload.

Exercise intensities can be categorized into the moderate, heavy, and severe domains based on metabolic profiles in response to external workloads [52,54,70]. These domains inform the sustainability of exercise, where exercise in the severe domain and above is unsustainable. The transition between the moderate and heavy domains can be identified by the gas exchange threshold (GET), whereas the transition between heavy and severe domains can be identified by the maximal metabolic steady state [54], which is the highest intensity where oxygen supply and demand are balanced [55]. The external workload associated with this intensity is known as critical power and is the boundary between sustainable and unsustainable exercise work rates [54]. Determination of critical power traditionally requires multiple days [107] and/or exhaustive trials [106,185], presenting challenges for athletes, habitual exercisers, and researchers. For example, the need for multiple trials increases participant time burden and makes it difficult to design studies evaluating the effects of interventions (pharmacological, nutritional, etc.) on critical power. Further, exhaustive exercise trials may prove undesirable or unfeasible, particularly in clinical

populations. Yet, using critical power to determine exercise intensity domains leads to more accurate exercise prescriptions in healthy [186] and clinical [187] populations than traditional metrics (i.e., relative to VO_{2max}). Thus, a single submaximal exercise session to determine critical power would be ideal for exploring the impact of an intervention, such as histamine receptor blockade, on maximal metabolic steady state/critical power. Such a protocol could also be used to evaluate critical power in populations where exhaustive exercise is undesirable and/or unattainable.

Traditional methods to identify critical power may lead to inaccuracies when employing interventional or repeated measures study designs with critical power as a primary outcome variable [71,108–110,188]. As an example, critical power decreases with prior exercise in the heavy domain [189–191], making previously sustainable exercise unsustainable. Portable near-infrared spectroscopy (NIRS) devices have emerged as a reliable tool for determining muscle oxygenation ($\%SmO_2$) which is indicative of the oxygen supply-demand balance during exercise. Recent studies examining the change in muscle oxygen saturation over time ($\%SmO_2$ slope) suggest that a positive $\%SmO_2$ slope indicates O_2 supply exceeds demand, whereas a negative $\%SmO_2$ slope demonstrates that demand exceeds O_2 supply. A $\%SmO_2$ slope of zero indicates a matched O_2 supply with demand, representing the maximal metabolic steady state [55,57,110]. $\%SmO_2$ slope has been used to successfully identify work rates above versus below the heavy-severe domain boundary, predict sustainable exercise intensities, and explore potential modifiers of critical power [55,57,67]. To our knowledge, only one study using single-leg knee extension tested the impact of histamine receptor antagonism on $\%SmO_2$ using NIRS; however, the investigators did not assess the $\%SmO_2$ slope as it pertains to predicting the maximal metabolic steady state. It is unknown if the maximal metabolic steady state is altered by histamine receptor blockade [37]. The primary aim of this study was to investigate the impact of histamine receptor blockade on work rate associated with balanced muscle oxygen supply and demand (i.e., maximal metabolic steady state) or critical power. We hypothesized that combined H_1 and H_2 receptor blockade would lower the power associated with the heavy-severe domain boundary when compared to placebo. In addition, we aimed to investigate the impact of histamine receptor blockade on cycling performance as measured by time to task failure in the severe domain. We hypothesized that blockade would decrease the time to task failure in the severe domain when compared to placebo. Finally, evaluating dynamic changes in $\%SmO_2$ enables the reliable assessment of intensity domains and determination of critical power in a single submaximal exercise session

[55,57,110,115], thereby facilitating the evaluation of interventions on critical power. A notable aspect of the study was to demonstrate the efficacy of the $\%SmO_2$ zero-slope approach in assessing the physiological adjustments to a pharmacological intervention.

Materials and Methods

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

Participants

Seventeen healthy, non-smoking individuals (8 males, 9 females, self-reported) participated in this study. Individuals were between the ages of 18 and 60 who were experienced in cycling and classified as "trained" or "well-trained" [68,69]. Participant characteristics are outlined in Table 4.

Table 4: Participant Characteristics

Variable	All	Male	Female	P-Value
Age (yr)	29 ± 8	30 ± 10	29 ± 5	0.706
Height (cm)	172 ± 9	178 ± 9	166 ± 5	0.004
Weight (kg)	66.0 ± 8.2	72.8 ± 6.6	60.0 ± 3.2	<0.001
BMI (kg/m^2)	22.4 ± 1.6	23.0 ± 1.4	21.8 ± 1.6	0.112
VO_{2peak} (L/min)	3.98 ± 0.80	4.70 ± 0.45	3.33 ± 0.37	<0.001
VO_{2peak} (ml/kg/min)	60.0 ± 7.5	64.8 ± 5.7	55.7 ± 6.4	0.008
Peak Power (W)	333 ± 51	378 ± 28	292 ± 26	<0.001
Relative Peak Power (W/kg)	5.03 ± 0.41	5.21 ± 0.33	4.88 ± 0.44	0.106
n	17	8	9	

Peak power is the highest 30-sec power during the graded exercise test. The p-value represents differences between males and females. Data are mean ± SD.

Experimental Design

The study consisted of four laboratory visits, including screening, familiarization, and two experimental testing sessions. The screening visit consisted of obtaining informed consent,

measuring demographic and anthropometric information, and conducting a graded exercise test to determine peak oxygen uptake (VO_{2peak}). Provided they met the inclusion criteria, participants then reported to the laboratory on three separate occasions: one familiarization visit and two double-blind, placebo-controlled testing visits. The testing visits (i.e., blockade or placebo) occurred in a randomized order and were separated by a minimum of one week. A schematic of the testing visits is presented in Figure 9. Primary outcomes were the power associated with the heavy-severe domain boundary, as determined by the zero-slope method, and the time to task failure in the severe domain. Secondary outcomes were $\%SmO_2$, heart rate, rating of perceived exertion, and expired gas measures. An additional goal of the study was to apply a recently established methodology to assess the impact of an intervention (in this case, antihistamines) on critical power. As the submaximal zero-slope protocol allows for the assessment of critical power in conjunction with a measure of exercise performance, we aimed to apply it, as intended by its developers, to explore both the underlying physiology and the impact of an acute intervention on performance, thereby demonstrating its utility.

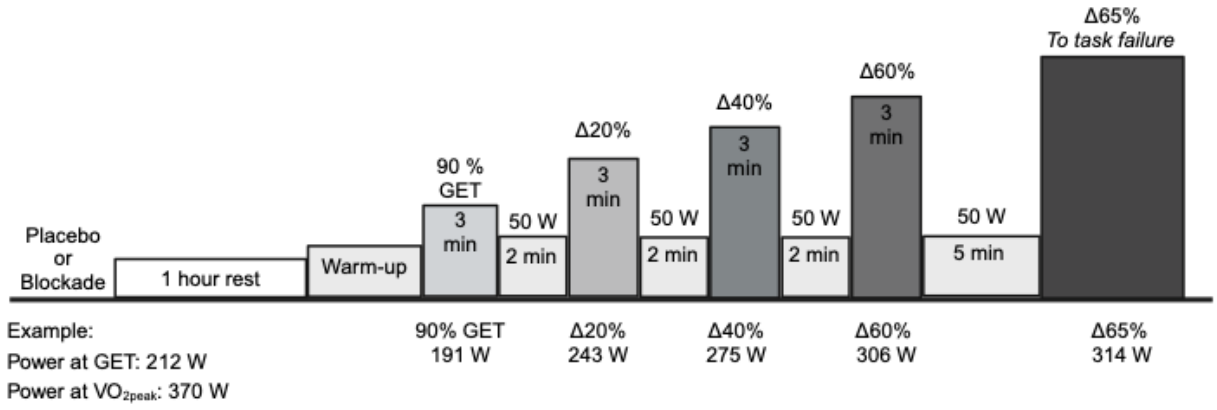

Figure 9: Schematic of the testing visits. Participants ingested placebo or antihistamines 1 h before exercise. Participants completed a 10-min self-paced warm-up and then were instrumented with a mouthpiece for the collection of expired gases. Participants then completed a 10-min standardized warm-up immediately followed by the first stage (90% GET, gas exchange threshold). Example power outputs are based on data from a single participant. Created in BioRender.

Measurements

The graded exercise test and all subsequent exercise sessions were conducted using an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) and took place in a temperature-controlled laboratory (~20°C). The ergometer seat and handlebars were adjusted to the cyclist's specifications and replicated for all exercise sessions. Heart rate was measured via telemetry (Model H10, Polar Electro, Kempele, Finland) throughout all

exercise bouts. Oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were measured by indirect calorimetry using a mixing-chamber system (Parvo Medics, TrueOne 2400 Sandy, UT). Expired gas values are averages from the final minute of the stage unless otherwise noted. During the familiarization and testing visits, an index of muscle tissue saturation ($\%SmO_2$) was recorded continuously via NIRS (Moxy Monitors, Fortiori Design LLC, Hutchinson, MN) based on the ratio of oxy-hemoglobin and myoglobin concentration to the total hemoglobin and myoglobin concentration in the tissue and expressed as a percentage. When used as specified, $\%SmO_2$ is an index of tissue oxygenation at the microvascular level and is weighted to largely reflect the skeletal muscle under the sensor but with some contribution from other tissues [72,192–194]. NIRS has been used previously to delineate intensity domains, identify the highest sustainable steady state during exercise, and assess recovery from an exercise bout, and its use in this capacity compares favorably to more time-intensive approaches [55,57,67,75,113]. To omit any light interference, sensors were enclosed in a black light-blocking case provided by the manufacturer and affixed with adhesive tape. For consistent sensor placement across all trials, sensors were placed on both the right and left vastus lateralis, 2/3^{rds} the distance on the line from the anterior superior iliac spine to the lateral side of the patella, consistent with previous investigations [67,113]. Reported tissue oxygenation is the average over the final minute of the stage unless otherwise noted.

VO_{2peak} Test

The ramp-style protocol began with three minutes of cycling at 60 watts before the resistance was increased at a fixed rate until volitional exhaustion. The ramp rate (1 watt every 2 seconds or 1 watt every 2.4 seconds) was determined such that all participants would reach exhaustion within 8-12 minutes. The test was terminated when the subjects were unable to maintain a cadence ≥ 60 RPM, despite strong verbal encouragement. Following the test, participants recovered for 10 minutes before performing a supramaximal verification trial to ensure no further increases in peak oxygen consumption at a greater power output than that which elicited a plateau in oxygen consumption [195]. The verification trial was performed at a constant power output of 110% of the peak power achieved during the ramp until volitional exhaustion (unable to maintain a cadence ≥ 60 rpm). VO_{2peak} was determined as the highest 30-s average during the protocol and GET was estimated using the V-slope method [196]. When

identifying the power output at VO_{2peak} and at GET, the lag in VO_2 was accounted for by deducting 2/3 the incremental ramp rate from the power output that was time aligned with VO_{2peak} and GET [71,197,198].

Determination of Stage Exercise Intensities

For each participant, the intensities for the staged exercise test were determined based on the results of the VO_{2peak} test and spanned the moderate, heavy, and severe domains. The power output corresponding to GET was determined as the transition between the moderate and heavy domains, as previously established [50,70,71]. To derive exercise intensities for the heavy and severe domains, the relative difference (%Δ) between the power output corresponding to GET and the power output corresponding to VO_{2peak} was determined and added to the power output corresponding to GET for each participant. This method has previously been used to accurately define moderate, heavy, and severe domain exercise [55,70,71,199]. Since the goal was to span the heavy and severe domains, the power output corresponding to Δ50% was used to approximate the boundary between the heavy and severe domains [57,70,71], and intensities were selected that would be appreciably above and below this 'transition.'

The intensities for the staged exercise test were as follows: Stage one was at the power output corresponding 90% of the intensity at GET (moderate domain). Stage two was at the power output corresponding to Δ20% (heavy domain), stage three was at the power output corresponding to Δ40% (heavy domain), and stage four was at the power output corresponding to Δ60% (severe domain). The time to task failure trial was completed at a power output corresponding to Δ65% (severe domain).

Familiarization visit

Participants performed a familiarization visit to acquaint them with the exercise and laboratory instrumentation. The visit and instrumentation were the same as the experimental testing visits apart from the ingestion of placebo or antihistamines and subsequent hour wait. The familiarization exercise test protocol was similar to the experimental visits' protocol, except the final time to task failure trial was omitted (i.e., cool-down began after stage four). Following the visit, participants were given a 24-hour dietary recall and instructed to document their diet 24 hours prior to their first testing visit.

Experimental testing visits

Following the familiarization visit, participants returned to the laboratory on two separate occasions, separated by at least one week. Each of these visits started with a confirmation of adherence to the abstention criteria and collection of the dietary recall, then body mass was obtained and participants ingested either antihistamines (540 mg fexofenadine, a selective H_1-receptor antagonist and 40 mg famotidine, a selective H_2-receptor antagonist) or placebo (Zeebo Effect LLC, Huntington, Vermont, United States). This dosage of histamine receptor antagonists results in more than 90% inhibition of histamine H_1 and H_2 receptors lasting for six hours after administration and is consistent with previous histamine receptor blockade studies [8,200,201]. Care was taken to ensure that participants were unaware of which treatment they were receiving. Following administration of antihistamines or placebo, participants rested for one hour to facilitate absorption [8,200,201]. During this hour, participants were instrumented with a heart rate monitor and NIRS-Moxy sensors. After approximately one hour post-ingestion, participants completed a self-selected warm-up (recorded and replicated in the subsequent exercise trial). Participants were then instrumented with a nose clip and mouthpiece to collect expired gases. Participants began a 10-minute standardized warm-up, consisting of 30 seconds of cycling at each stage's intensity, separated by 30 seconds of cycling at 50 watts, followed by five minutes of cycling at 50 watts. Stage one of the staged exercise test began immediately following the standardized warm-up.

The staged exercise test consisted of four three-minute-long stages of increasing intensity. Each stage was separated by two minutes of active recovery, during which participants cycled at 50 watts. Following stage four, participants cycled for five minutes at 50 watts before completing a final stage until task failure. Task failure was defined as either volitional exhaustion or when the participant could no longer maintain a cadence above 60 RPM despite verbal encouragement. During the final stage, participants were provided with verbal encouragement but no information regarding the time elapsed. Heart rate was continuously monitored and recorded during the last 30 seconds of each stage. Rating of perceived exertion (Borg 6-20) was recorded during the last 30 seconds of each stage. During the final stage, the heart rate was recorded as the highest (usually in the last 30 seconds), and rating of perceived exertion was taken immediately following task failure.

Determination of %SmO_2 Zero-Slope

Data from the NIRS-Moxy sensors were sampled at two Hz and imported into a Microsoft Excel file for analysis. For each exercise stage, %SmO_2 was plotted against time for each stage for both the right and left legs. Previous findings [55] observed a rapid transient onset phase resulting in an initial nadir in %SmO_2 signal. To account for this transient response, the initial nadir was identified and omitted from the stage-slope determination. The stage %SmO_2 slope was determined from the linear regression of %SmO_2 and time (in minutes) following the nadir (~60 seconds into the stage) until the end of the stage. If a portion of the stage contained erratic transient changes in %SmO_2, (i.e., noise or artifacts) or loss of signal, stage slope was taken from the longest uninterrupted segment of %SmO_2 data lasting at least 90 seconds (half the stage duration). For the time to task failure trial, the slope was determined from the regression of %SmO_2 and time (in minutes) following the nadir (~60 seconds into the stage) for the longest reliable time (at least 90 seconds) following the nadir.

To determine the power output that elicited a zero-slope, the previously calculated slopes from each stage were plotted on the y-axis against power on the x-axis for both legs. Then, a linear regression model was performed for each leg to identify the x-intercept—the power at which the slope equaled zero [57,110]. Stages were omitted from the linear regression for determining the power at zero-slope if they did not contain reliable data. Zero-slope power for the trial was determined by averaging the zero-slope power of both legs. If one leg had a linear regression that returned an R^2 value of less than 0.85 or had less than three stages included in the regression (i.e., two or more stages omitted), it was not used to determine zero-slope power. If the power from one leg was omitted, the zero-slope power from the leg included was used for the visit instead of the average of both legs. The slopes of each stage and the power that elicited a zero slope were determined by two experienced, independent reviewers.

Rigor and Reproducibility

None of the participants were using any over-the-counter or prescription medications at the time of the study, except for oral contraceptives. Women were studied irrespective of menstrual cycle phase and had a negative pregnancy test prior to all assessment days. The menstrual cycle was not controlled for in female participants as female athletes generally train

and compete throughout all phases of the menstrual cycle, and previous research indicates that the menstrual cycle has little effect on exercise performance [62–64] or other exercise responses that are dependent on histamine receptor activation, such as post-exercise hemodynamics [58–61]. Prior to all assessments (i.e., all visits but the screening), participants were asked to abstain from alcohol, strenuous exercise, over-the-counter medication for 24 hours, and food and beverages (except water), and caffeine for two hours. Data collection start-time varied across participants due to scheduling conflicts; however, exercise testing visits for each participant were conducted at the same time of day (±1 hour) to reduce the potential influence of circadian rhythms on muscle blood flow and exercise performance. Participants were asked to maintain a similar diet prior to each of their testing visits (i.e., blockade or placebo), which was confirmed via collection of a 24-hour dietary recall at each visit. All protocol activities took place in a thermoneutral lab environment. Blinding and randomization were done by an individual not involved in data collection or analysis. Investigators, including those involved in data entry and analysis, and participants were blind to the condition and order of administration.

Statistics

Participant characteristics are presented as mean ± SD. Given that the study was not intended to examine sex differences, only differences in characteristics between males and females were compared, and these were compared using multiple independent t-tests. All other data are reported as mean with 95% confidence intervals. Alpha was set at 0.05 for all statistical inferences including familywise error rates. Shapiro-Wilks tests were used to test for normality. Inferences regarding time to task failure and estimated power at zero-slope with blockade or placebo were drawn using a paired t-test (Prism 10, GraphPad, Boston, MA, USA). Inferences surrounding variables of interest over time were drawn from two-way mixed-effects models (for measurements across time and between conditions) with pre-planned comparisons. 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, restricted to comparisons at the same timepoint between groups. 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 between groups.

Results

Participant Characteristics

Table 4 shows participant characteristics, including anthropometric data and results from the graded exercise test. All participants were experienced with cycling, had high levels of aerobic fitness, and identified as triathletes, runners, or cyclists. Since the intent of the study was to identify the impact of blockade on $\%SmO_2$ zero-slope and time to task failure across populations, and not as a function of biological sex, all data for male and female participants are combined.

Time to task failure and estimated zero-slope power

The estimated power at zero slope and time to task failure are shown in Figure 10**.** Estimated zero-slope was not different (p=0.41) between placebo (213 [191, 234] W) and blockade (216 [195, 236] W) conditions. During the final stage, the time to task failure was not different (p=0.95) between placebo (473 [380, 566] s) and blockade (474 [377, 572] s) conditions. The final stage elicited a negative $\%SmO_2$ slope in both placebo (-1.7 [-1.2, -2.2] %) and blockade (-1.4 [-0.9, -2.0] %) conditions, but there was no difference between the conditions (p=0.83).

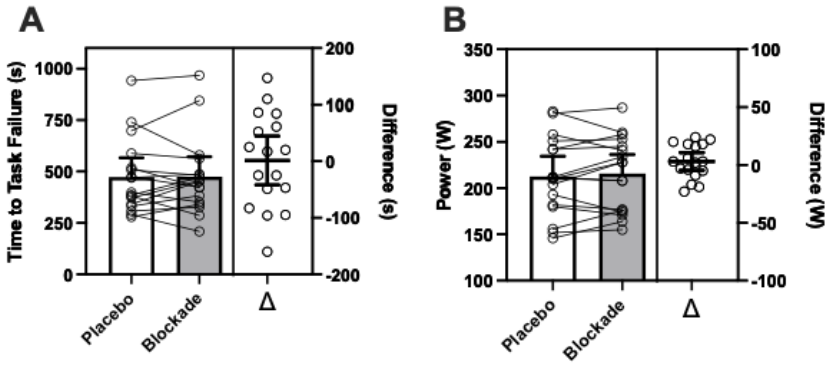

Figure 10: Estimation plots showing time to task failure in the severe domain (A) and estimated zero slope power (B) in placebo (open bars) and antihistamine (closed bars) conditions. Left panel shows group averages and individual points, and right panel shows the difference between conditions (Δ, blockade minus placebo). (○) indicate females and (□) indicate males. Data are means and 95% CI, n = 17. There were no differences between conditions (blockade vs. placebo).

Tissue oxygenation and SmO_2 slope

$\%SmO_2$ slopes and tissue oxygenation throughout the stages are displayed in Figure 11**.** $\%SmO_2$ slope decreased (became more negative) as the intensity increased (main effect of stage; $p<0.05$), indicating the selected intensities spanned the moderate, heavy, and severe domains. Stage slopes were positive in the predicted moderate domain, approached zero in the predicted heavy domain, and were increasingly negative in the predicted severe domain. However, $\%SmO_2$ slopes for each stage were not different between conditions ($p=0.43$). Tissue oxygenation showed a main effect of stage (i.e., lower with higher intensities; $p<0.05$) but was not different between placebo and blockade ($p=0.46$) (**Figure 3).** Tissue oxygenation was lowest in the time to task failure trial but was not different between placebo and blockade.

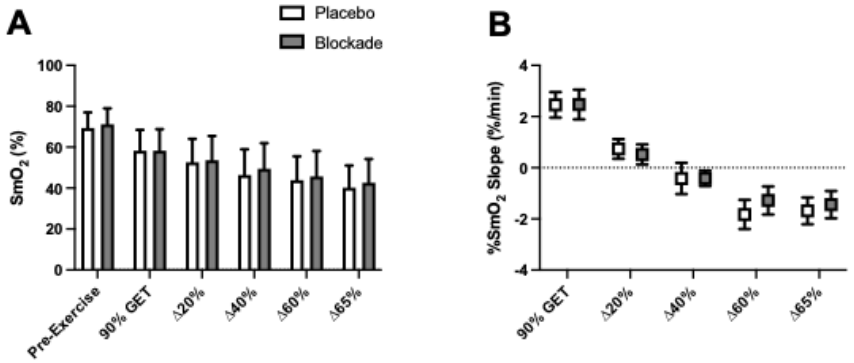

Figure 11: Tissue oxygenation (a) and slopes for each stage (b) in placebo (open bars) and blockade (closed bars) conditions. Tissue oxygenation was the average across the final minute of the stage. Both measurements showed a main effect of stage ($p<0.05$), but there was no difference between conditions (blockade vs. placebo). GET, gas exchange threshold. Data are mean and 95% CI, $n = 17$.

Gas Exchange, Heart Rate, Rating of Perceived Exertion

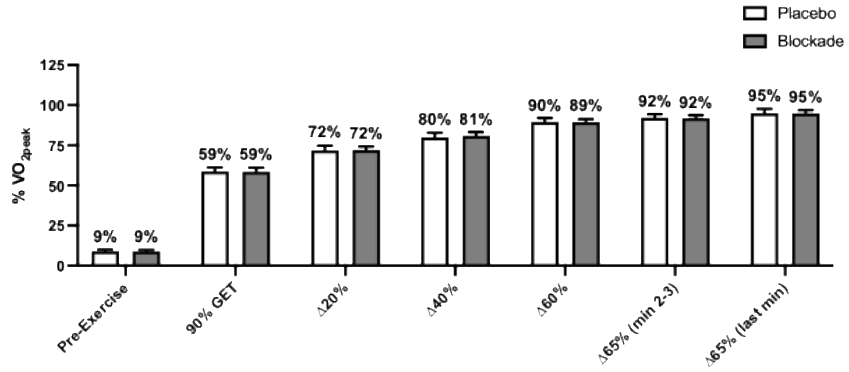

Figure 12: VO_2 as a percentage of VO_{2peak} in placebo (open bars) and blockade (closed bars) conditions. VO_2 as a percentage of VO_{2peak} showed a main effect of the stage ($p < 0.05$), but there was no difference between conditions (blockade vs. placebo). GET, gas exchange threshold. Data are means and 95% CI, $n = 17$.

During the staged exercise tests, VO_2, VCO_2, respiratory exchange ratio, heart rate, and rating of perceived exertion all showed a main effect of stage (p<0.01) but were not different between conditions (Table 5). VO_2 as a percentage of VO_{2peak} increased throughout the stages (p<0.05, Figure 12**)** but was not different between conditions (p=0.95). During the final minute of the time to task failure trial, VO_2 was 3.77 [3.38, 4.17] L/min and 3.77 [3.37, 4.16], VCO_2 was 3.65 [3.26, 4.03] L/min and 3.64 [3.25, 4.03] L/min, respiratory exchange ratio was 0.97 [0.95, 0.99] and 0.97 [0.94, 1.00] in placebo and blockade, respectively.

Table 5: Cardiorespiratory responses and perceived exertion during protocol

		Pre-Exercise	**90% GET**	**Δ20%**	**Δ40%**	**Δ60%**
VO_2 (L/min)	Placebo	0.36 [0.31, 0.40]	2.32 [2.11, 2.53]*	2.84 [2.57, 3.11]*	3.16 [2.86, 3.47]*	3.55 [3.20, 3.91]*
	Blockade	0.35 [0.31, 0.39]	2.31 [2.11, 2.51]*	2.84 [2.59, 3.09]*	3.20 [2.90, 3.50]*	3.54 [3.20, 3.89]*
VCO_2 (L/min)	Placebo	0.34 [0.28, 0.39]	1.85 [1.64, 2.07]*	2.56 [2.32, 2.81]*	2.96 [2.68, 3.23]*	3.48 [3.13, 3.84]*
	Blockade	0.32 [0.29, 0.35]	1.93 [1.77, 2.10]*	2.55 [2.33, 2.78]*	3.02 [2.72, 3.31]*	3.48 [3.14, 3.83]*
Respiratory exchange ratio	Placebo	0.93 [0.88, 0.99]	0.84 [0.81, 0.87]*	0.90 [0.87, 0.93]*	0.94 [0.91, 0.96]*	0.98 [0.95, 1.01]*
	Blockade	0.93 [0.89, 0.97]	0.84 [0.82, 0.86]*	0.90 [0.88, 0.93]*	0.94 [0.92, 0.97]*	0.98 [0.96, 1.01]*
Heart rate (beats/min)	Placebo	76 [69, 84]	136 [128, 145]*	152 [144, 160]*	162 [154, 169]*	171 [164, 178]*
	Blockade	75 [66, 85]	138 [129, 147]*	154 [146, 162]*	164 [157, 172]*	172 [166, 179]*
Rating of perceived exertion	Placebo	6 [6, 6]	9 [8, 10]*	12 [11, 12]*	13 [12, 14]*	15 [14, 16]*
	Blockade	6 [6, 6]	9 [8, 10]*	12 [11, 12]*	13 [12, 14]*	16 [14, 17]*

VO_2, VCO_2, and respiratory exchange ratio are averaged over the final minute of each stage. Heart rate and rating of perceived exertion were recorded during the last 30 seconds of each stage. Data are mean [95% CI]. There were no differences between conditions for any timepoint. * p <0.001 versus pre-exercise.

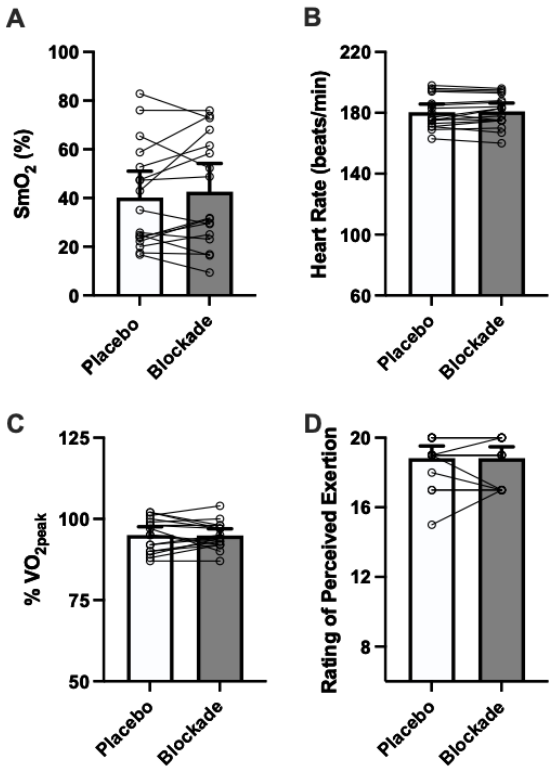

Figure 13: Tissue oxygenation (A), peak heart rate (B), VO_2 as $\%VO_{2peak}$ (C), rating of perceived exertion (D) during the time to task failure trial in placebo (open bars) and blockade (closed bars) conditions. $\%SmO_2$ and $\%VO_{2peak}$ are averaged over the final minute. Heart rate is the highest heart rate achieved during the time to task failure trial, and a rating of perceived exertion was taken at task failure. (○) indicates females and (□) indicates males. There were no differences between blockade and placebo. Data are means and 95% CI, $n = 17$.

Tissue oxygenation, heart rate, rating of perceived exertion, and the percentage of VO_{2peak} during the final minute of the time to task failure trial are presented in Figure 13**.** During the final stage, VO_2 increased from 92 [90, 95]% and 92 [90, 94]% VO_{2peak} during minute three, to 95 [92, 98]% and 95 [97, 93]% VO_{2peak} during the final minute, following placebo (p=0.04) and blockade (p=0.02), respectively. Heart rate was highest during the final stage and was 180 [175, 186] bpm and 181 [176, 186] bpm for placebo and blockade conditions. These corresponded with 99% [98, 100] (placebo) and 100% [98, 101] (blockade) of the peak heart rate achieved during the graded exercise test. Rating of perceived exertion was the highest at the end of the final stage (task failure) and was 19 [18, 20] with placebo and 19 [18, 19] with blockade.

Discussion

Histamine receptor blockade does not appear to alter oxygen supply-demand balance, or the power associated with the maximal metabolic steady state (i.e., critical power). Contrary to previous investigations reporting that antihistamines impair 10-km cycling time trial performance [9], blockade did not impact time to task failure in the severe domain. This investigation further established that, along with a performance task, the use of a %SmO_2 zero-slope protocol can be leveraged for assessing potential adjustments in O_2 supply–demand balance with pharmacological interventions. This demonstrates its efficacy for investigations examining acute (or chronic) interventions on maximal metabolic steady state / critical power.

Antihistamines and oxygen supply-demand balance

Previous investigations found that histamine receptor blockade slows 10-km time trial performance to a meaningful degree in trained cyclists [9]. While it is plausible that blockade impacted performance by reducing blood flow to exercising skeletal muscle, a subsequent investigation found that histamine blockade increases femoral blood flow and vascular conductance during dynamic knee extension while also decreasing intramuscular pH [37]. Therefore, there is a 'histamine paradox' such that blocking histamine receptors appears to increase bulk blood flow in conduit vessels, but may impair muscle perfusion (i.e., greater muscle tissue acidity and decreased performance). Skeletal muscle is a heterogeneous and complex tissue, and blood flow distribution within exercising tissue is not uniform. Within skeletal muscle, tissue metabolism and vascular smooth muscle constitute a local control system tightly coupling blood flow to tissue metabolic requirements [202]. Histamine acts on vascular smooth muscle, and its blockade may dysregulate the coupling of local perfusion and tissue metabolic requirements, thereby lowering critical power and potentially explaining the reduction in 10-km time trial performance.

Evaluating dynamic changes in muscle oxygenation through %SmO_2 slope provides insight into the effect of histamine receptor blockade on the matching of metabolism and perfusion within exercising tissue. A positive or zero-slope indicates that oxygen supply is greater than or equal to demand, whereas a negative slope indicates perfusion is inadequately meeting the metabolic demand. The highest external work rate at which perfusion can meet demand (i.e., zero-slope) is known as critical power and the boundary between sustainable and unsustainable work rate [55,57,110]. At intensities above critical power, there is an inability of

perfusion to meet metabolic demand, leading to an increase in and the accumulation of metabolites, necessitating either a reduction in work rate or the cessation of exercise [54,57,107]. Interventions that lower critical power would result in reduced work capacity, potentially explaining the previously observed reduced performance with histamine receptor antagonism. The present results suggest that histamine receptor antagonism does not impact the ability to balance oxygen supply and demand or the highest external work rate at which this can be achieved. Thus, it is unlikely that the previously reported ergolytic effect of histamine receptor blockade [9] is due to an inability to match oxygen supply and demand or a reduction in critical power.

Antihistamines and performance

Contrary to previous findings, we found no effect of blockade on our performance metric, time to task failure in the severe domain. The previous investigation used a 10-km cycling time trial as its metric and found that blockade slowed completion by approximately one percent [9]. While an exact reason for the disparate findings is not apparent, several ideas concerning both the physiological effects of blockade and methodological considerations may have contributed, including the duration of the performance test, nutrient delivery and metabolite washout, the presence of preceding exercise, and self-pacing.

Duration

There is an appreciable difference in duration between the time taken to complete the 10-km time trial in the previous investigation (~1000 s) and time to task failure (~475 s) in the present investigation. Blood lactate concentrations from the previous investigation [9] and the VO_2 and $\%SmO_2$ slope responses in the present investigation suggest both tasks consisted of significant work performed in the severe domain. However, given the longer duration of the time trial, it stands to reason that those individuals were closer to their critical power or were only in the severe domain during the final effort of their time trial. Given the relatively similar fitness levels and training histories of the populations, it is likely that the constant work rate trial in the present investigation was performed at an intensity that was further above critical power (i.e., more in the severe domain). It is possible that during shorter, more intense exercise, the effect of histamine receptor antagonism is less pronounced. Support for this comes from investigations

showing that H_1 receptor antagonism does not impact muscle strength [203] or high-intensity exercise performance [204]. Although these observations come from exercise modalities that differ from those employed in the current investigation, the results suggest that as the duration of intense exercise increases, the effect of antihistamines becomes more pronounced. Thus, antihistamines may have a more pronounced effect in events lasting > 12 minutes (e.g., 5 km running and above) than in shorter events, such as those employed in the current investigation.

Nutrient delivery and metabolite washout

It has been hypothesized that the use of antihistamines may impact nutrient delivery or metabolite washout, potentially explaining the decreased pH during knee extension with antihistamines [37]. Rodent studies show that histamine receptor blockade decreases intramuscular glycogen following a walking test [138]. While the effects of blockade on muscle glycogen content in humans have not been studied at this time, histamine receptor antagonism reduces post-exercise interstitial glucose concentrations [58], insulin sensitivity [28], and glycogen resynthesis [81]. Importantly, these effects do not appear to be mediated by direct effects on GLUT-4 translocation or glycogen synthase activity [81] suggesting these reductions are due to impaired glucose delivery. Blockade does not appear to reduce circulating glucose concentrations [9], which are often well-maintained during exercise. But given histamine's role in microvascular recruitment [38] and vascular permeability [39], it may be that blood glucose is maintained, but there is reduced interstitial glucose and therefore less is functionally available to the muscle. In this case, blockade could lead to a greater reliance on muscle glycogen, as seen in mice [138], and potentially greater generation of metabolites, as suggested by decreased pH during dynamic knee extension in humans [37]. While we would not expect glycogen depletion to occur during exercise of these durations, it may be that reductions in muscle glycogen led to afferent feedback that causes the individual to slow down or inhibits the final "kick" towards the end of a race or event. Such an effect would not have been evident in the current study, given the short duration of the time to task failure trial but may have contributed to the slower 10-km performance reported previously [9].

Preceding Exercise

In the previous investigation, the 10-km time trials were preceded by either two hours of rest or two hours of moderate intensity cycling [9]. When it was preceded by exercise, pacing during the 10-km time trial was slower [9]. Prolonged heavy domain [189–191] and high-intensity interval [205] cycling is known to lower critical power. In addition, prolonged exercise in the moderate domain lowers the power associated with the moderate-heavy domain boundary and increases heart rate at that power [206]. Moreover, the preceding exercise most likely altered the metabolic profile of exercise. The oxygen cost of exercising at the same power is likely to increase over time due to increased fat utilization [207]. The fat oxidation rate increases following prolonged moderate [206] and heavy [191] exercise. While the previous study found no differences in respiratory exchange ratio between trials [9], it cannot be ruled out that histamine receptor blockade may have an impact on the delivery and availability of free fatty acids to exercising muscle. This would likely lead to a higher reliance on glycogen earlier in exercise, which may decrease exercise performance after prolonged moderate-intensity exercise. Thus, the impact of the preceding exercise on the determinants of performance is notable and might be exacerbated by antihistamine administration.

Pacing

The present study employed a constant work rate until task failure in the severe domain, whereas the previous investigation employed a 10-km time trial. Participants in the present investigation pedaled at a fixed power output until task failure. Hence, as participants' fatigue and their cadence decreased, the torque required on the pedals increased to maintain the constant power output. Thus, participants could not pace as they would in other performance tests, such as time trials. The previous investigation saw a distinct pacing strategy in the 10-km time trials, where the first kilometer was the slowest, followed by similar times for the next eight kilometers, and finally a decrease (i.e., fastest) final kilometer [9]. This pacing strategy is common in cycling time trials [208], including those of shorter [209] and longer [210] durations. While it is likely not the sole cause of the differing observations between this investigation and previous work, it cannot be ruled out that the inability to pace may have contributed to the disparate findings regarding the effects of antihistamines on exercise performance.

Decisions surrounding pacing combine numerous physiological, psychological, and environmental factors, and an in-depth discussion of pacing is beyond the scope of this

discussion (for more in-depth see [211]). However, it is possible that antihistamines contribute to the perception of effort and decisions surrounding pacing strategy. The previous investigation did not find a difference between blockade and placebo regarding pacing; however, a difference in heart rate was observed (lower with blockade), suggesting that there may be some uncoupling of perception of effort and power output with blockade. It is possible that differences in perception influence pacing strategy in a variable-intensity event (i.e., a time trial), which may ultimately impact performance. Many events of similar durations (e.g., 5-km or 10-km running) follow a similar pacing strategy, with a slower start, a steady middle, and a faster finish [211]. A decoupling of effort perception and physiological strain may alter pacing strategy or the agility to finish fast.

A utilitarian protocol for understanding the impact of interventions on athletic performance

The traditional methodologies for determining the heavy-severe domain boundary require multiple testing days [107]. This diminishes their utility in evaluating the impact of acute interventions on critical power, as employing repeated testing days increases the burden on participants (e.g., more lab visits) and creates experimental challenges (e.g., repeating dosing across multiple study visits when studying supplement or drug interventions). The three-minute all-out test is an alternative method that relies on a single exhaustive trial, rather than multiple trials and visits [106,185]. However, it requires an all-out, exhaustive effort form the participant, which may not be suitable or attainable for many populations (e.g., untrained individuals, the elderly, those with illnesses). Critically important, participants must resist the natural tendency to pace their effort; failing to avoid pacing will result in an overestimation of critical power with this methodology. Indeed, previous investigations have found that the three-minute all-out test may overestimate power in both trained [108,109] and untrained [110,188] individuals. One must appreciate that a particular skill and familiarity are needed for participants to provide the necessary effort during a three-minute all-out test for determining critical power.

This investigation demonstrated the utility of the zero-slope protocol, a newly established methodology for determining power at the maximal metabolic steady state, in addressing an interventional question. The zero-slope protocol uses NIRS to assess changes in $\%SmO_2$ over time (i.e., $\%SmO_2$ slope), which is an indicator of the oxygen supply-demand balance in working tissue. This methodology accurately identifies exercise intensity domains above and below the

maximal metabolic steady state [55,57,110], aligns with traditional indicators of exercise intensity (e.g., lactate [57,67] and oxygen uptake [55,57]), and has excellent reliability (ICC>0.9) and low measurement error (<2%) for predicting power at the heavy-severe domain boundary for both men and women [115]. As the methodology relies on intermittent submaximal exercise, it broadens the populations in which it can be employed (i.e., those that may struggle with exhaustive exercise can complete this test). It also lessens the need for familiarization with the test. While we did not aim to assess the validity of this methodology, as it has been evaluated previously [55,57], the current study provides further evidence that utilizing NIRS to evaluate dynamic %SmO_2 is a reliable and accurate metric for assessing exercise intensity domains. It compares well to traditional indicators but has greater utility for acute intervention studies and/or investigations involving clinical or nonathletic populations.

Conclusion and Perspectives

The primary and secondary aims of this project were to evaluate the impact of histamine receptor blockade on exercise intensity domains relevant to performance and time to task failure in the severe domain. Compared to placebo, blockade did not alter exercise intensity domains or impact time to task failure in the severe domain. Thus, histamine receptor blockade does not appear to critically alter performance lasting six to eight minutes in the severe domain. While histamine receptor blockade appears not to influence relatively short-duration, high-intensity exercise, its negative impact on longer-duration, variable-intensity exercise (noted in a prior study) warrants future investigation. Finally, this study demonstrated that the zero-slope protocol can be leveraged for interventional studies and is a valuable tool for exploring physiological adjustments to an intervention.

CHAPTER VII
SYNTHESIS OF IDEAS

Histamine is a primordial signaling molecule, highly conserved across multiple genera and species. Its existence appears to predate multicellular organisms, dating back to unicellular eukaryotes, for example, *Tetrahymena pyriformis,* which expresses a gene similar to mammalian histidine decarboxylase (HDC) and both histamine H_1 and H_2 receptors [212,213]. In unicellular eukaryotes, histamine is crucial for survival and proliferation, playing a role in important processes like phagocytosis, glucose metabolism, cell growth, and chemotaxis [213]. The origin of mast cells, which are capable of storing, synthesizing, and releasing histamine, dates back over 500 million years to some of the earliest multicellular organisms [214]. When stimulated, mast cells release their contents, one of the most prominent being histamine, which then acts through its receptors on the surrounding environment, playing an important role in both innate and adaptive immunity [215]. While their original function centered on host defenses, as organisms evolved, mast cells differentiated into a more complex entity with roles in immunomodulation, tissue-remodeling and repair, and angiogenesis [216]. To date, mast cells have been found in all vertebrate species [216] , indicating that their physiological importance extends beyond solely host defenses. The release and subsequent action of their mast cell-derived mediators is a fundamental component of the physiological response to stress. Specifically, regarding exercise-induced stress, histamine appears important in response, recovery, and adaptation.

The first investigations into histamine's physiological role came from Dale and Laidlaw in 1910, when they identified β-iminazolylethylamine (the chemical name for histamine) as capable of reducing blood pressure, increasing glandular secretion, and constricting smooth muscle in animals [217]. In 1935, Anrep and Barsoum presented evidence of histamine during exercise, demonstrating that in dogs, venous concentrations increased in response to muscular contractions in an intensity- and time-dependent manner [218]. Subsequent investigations in humans found that plasma histamine concentrations increase during exercise, although the sources of that histamine were unknown [219,220]. However, until the late 1990s and early 2000s, much research surrounded its role in allergic reactions, gastric acid secretion, and inflammation. It was around this time that two seemingly unrelated lines of research, hyperalgesia of muscle

overuse syndrome [221,222] and post-exercise hypotension [5,6,184] , reinvigorated interest in histamine's influence during and following exercise.

Overlooked in the traditional exercise physiology zeitgeist, the release of histamine in exercising muscle and its subsequent actions may be integrally important to transducing exercise adaptation. During exercise, histamine concentrations increase in skeletal muscle [26,1221417223], leading some to facetiously ask, "Are we allergic to exercise?"[79] These questions, while amusing, appear to vastly undersell the role of histamine in exercise responses. Mast cell-mediated histamine release appears to be a potent vasodilatory mechanism driving post-exertional vasodilation , hypotension, and, in extreme cases, syncope [4–6,28,184,224]. Further, histamine itself appears integral in regulating transcription and cellular crosstalk following exercise [7,122] and in facilitating adaptations to exercise [8,225]. Clearly, painting histamine as solely involved in host defenses or immunologic interactions ignores its important role as an exerkine and greatly undersells its physiological importance.

Sources of histamine in skeletal muscle

Histamine is synthesized by the decarboxylation of the amino acid L-histidine by the enzyme HDC. This enzyme is widely expressed across numerous cells in the body, including gastric mucosa, neurons, parietal cells, mast cells, and basophils [27,226]. Of these, only basophils and mast cells are known to produce and store large amounts of histamine, which is done so in granules [227]. During exercise, histamine concentrations increase in skeletal muscle, which appears to arise from both *de novo* synthesis of histamine from histidine by HDC and mast cell degranulation, a process in which mast cells secrete the contents of their granules into the external environment [2,122]. Transcriptomic work initially suggested that HDC mRNA was expressed in isolated human vascular smooth muscle cells and endothelial cells [228], and in bulk human skeletal muscle samples [7], and thus multiple cell types within skeletal muscle may contribute to *de novo* histamine synthesis. However, a recent investigation integrating isolated single-cell transcriptome profiling compared to bulk skeletal muscle RNA-sequencing indicates that HDC is only expressed in mast cells, pockets of which are located within skeletal muscle [122]. It appears that these mast cells are solely responsible for the increases in skeletal muscle histamine during exercise.

Once released, histamine is primarily metabolized by histamine-N-methyltransferase (HNMT) and diamine oxidase, which account for 97% of histamine breakdown [154,227,229,230]. Of these, only HNMT is expressed within skeletal muscle, meaning it is likely responsible for histamine breakdown in skeletal muscle. Because of its local release and rapid enzymatic degradation, histamine has a relatively short half-life *in vivo,* and circulating histamine concentrations are generally low, even in response to exercise [5,6,184,231,232] , making the quantification of the histaminergic response to exercise difficult. Measuring circulating concentrations of 1-methylhistamine and 1-methylimidazole acetic acid, breakdown metabolites of histamine, are promising indicators of exercise-induced histamine release [232] but their validation and characterization in exercise responses requires further investigation.

Upstream mechanisms of histamine release during exercise

Histamine is released within exercising skeletal muscle [22], but the upstream factors that drive its formation and release remain unknown. One potential stimulus may lie in exercise-induced increases in muscle temperature. Mechanistically, the rise in temperature may activate transient receptor protein vanilloid (TRPV) channels 1-4, which are expressed on the surface of mast cells, and the stimulation of which contributes to calcium influx necessary for degranulation or selective release of histamine [153]. In this way, heating may contribute to mast cell degranulation, although increases in temperature alone do not induce mast cell degranulation *in vitro* [10]. Multiple stimuli, such as osmotic swelling, decreases in pH, and proinflammatory cytokines, can act on TRPV channels, exerting a synergistic effect to increase calcium influx and induce degranulation [153]. All of these have been shown to be altered during and following exercise, and it may be that *in vivo*, these other changes in the local environment can induce appreciable degranulation. Ex vivo, the activity of HDC harvested from rodents is elevated at higher temperatures [34], suggesting that in vivo HDC activity is also elevated at higher temperatures, thereby increasing *de novo* histamine synthesis and contributing to exercise-induced histamine release. In humans, passive heating of skeletal muscle to temperatures reached during exercise with pulsed short-wave diathermy increased histamine concentrations in skeletal muscle in one [10] but not another investigation (AIM 1).

Other potential mediators of exercise-induced histamine release include oxidative stress, cytokine release, or the release of soluble factors that may trigger mast cell degranulation. Of

these, reactive oxygen species do not cause appreciable mast cell degranulation and histamine release in humans [29]. Preliminary data from our lab found that mast cells exposed to dialysate from exercising skeletal muscle degranulate to a greater extent than controls, suggesting that a soluble factor released during exercise may induce degranulation [66]. The identity of the soluble factor remains elusive; however, identification of receptor targets may provide insight into the factor(s) that elicit histamine release.

Mast cells express numerous surface receptors that, when bound to their ligand, initiate second-messenger signaling, activating the mast cell and resulting in the release of the mast cell contents into the external environment [36,92]. This can occur via degranulation, when mast cells release the contents of their granules via exocytosis, or selective release when only certain cytokines, chemokines, or biologic amines are discharged [36]. The most commonly characterized mast cell receptors are FcεRI receptors, which are stimulated by IgE-bound foreign antigens and cause degranulation [65,88]. These are largely responsible for degranulation in response to foreign pathogens, such as during allergic reactions. However, exercise-induced mast cell degranulation in skeletal muscle is unlikely to be mediated by IgE-bound antigens. Mast cells express numerous other receptors with affinities for numerous cytokines, chemokines, neurokines, and peptides. Of these, special consideration has been given to the mas-related g-protein coupled receptors (MRGPR), specifically MRGPRX2 [94,126,127]. The MRGPRX2 receptor is activated by binding to numerous endogenous peptides[36,94,126] , making it a popular target for drug development to treat disorders related to mast cell activation [100–103,128]. This promiscuity, combined with the high affinity it has for neuropeptides [129], implicates MRGRPRX2 as important in exercise-induced degranulation. For example, substance P, a key mediator of MRGPRX2-induced degranulation [129] , increases during exercise [124,180] , representing a possible trigger of exercise-induced mast cell degranulation. More work is necessary to identify soluble factors that act as intermediaries, thereby identifying the trigger for the proverbial smoking gun of exercise-induced mast cell degranulation.

Histamine in the acute exercise response

Once released, histamine exerts its actions by binding to one of its G-protein-coupled receptor types, H_1, H_2, H_3, and H_4. Of these, H_1 and H_2 receptors are commonly found within skeletal muscle and are expressed on myeloid cells, smooth muscle cells, and the vascular

endothelium [26,27,122,226]. H_1 receptors are G_q-coupled receptors and their downstream effects are mediated by the increased activity of phospholipase C [233]. H_2 receptors are G_s-coupled receptors with downstream effects mediated by increases in intracellular cAMP [234]. Through binding to these receptors, histamine relaxes smooth muscle, dilating the arterioles [26,27] , and also increases capillary permeability [39,235] within skeletal muscle. This response alters the local environment and has implications for systemic responses to exercise, most notably sustained post-exercise vasodilation. Histamine binding mediates sustained post-exercise vasodilation following both large- and small-muscle-mass aerobic exercise [4–6,184] , and in extreme cases, mast cell degranulation and histamine release have been implicated as underlying post-marathon syncope [231] . Findings are mixed regarding histamine's influence following resistance exercise, as some have shown that blockade has no impact on post-exercise femoral blood flow [83], although unpublished data from our laboratory indicate that H_1 and H_2 receptor blockade reduces femoral blood flow and vascular conductance immediately post-exercise [84]. Explanations for these differing findings are not readily apparent but may be due to the type and intensity of resistance exercise (four unilateral sets each of leg press, leg curl, and leg extension at 12-repetition maximum [83] versus six sets of ten repetitions at 70% 1-reptition maximum barbell squat [84]).

Further insight into the role of histamine comes from its effect on the post-exercise recovery process. Following an exercise bout, previously active tissue begins processes to remove metabolites and replenish previously depleted energy stores. Blood flow is important because it enables, among other things, glycogen resynthesis and metabolite washout. H_1 and H_2 receptor blockade reduces post-exercise blood flow to [28] and within [145] previously active muscle. In doing so, blockade also reduces glucose and oxygen delivery [28], insulin sensitivity [82], interstitial glucose concentrations [145], and glycogen resynthesis [122] following exercise. As such, histaminergic signaling appears important in exercise recovery by facilitating nutrient delivery and metabolite washout. Histamine receptor blockade does not impact glucose uptake by skeletal muscle [28] or activation of insulin signaling mediated GLUT4 translocation [122]. Taken together, these findings indicate that the influence of histamine in post-exercise recovery is mediated largely by its role in regulating blood flow within tissue. However, the role of histamine in exercise recovery may go beyond regulating delivery and removal.

Following dynamic knee extension exercise, biopsy samples show differential expression of ~3000 protein-coding genes within bulk muscle tissue, roughly 25% of which are altered with

histamine receptor blockade [7]. This altered response includes genes pertaining to improvements in cellular maintenance, inflammation, and metabolic and vascular function. However, much of this response appears mediated by cross-talk stemming from tissue-resident inflammatory cells within skeletal muscle. Compared to the transcriptomic response in bulk tissue, the transcriptomic response of individual myofibers is relatively subdued [122], indicating a complex intracellular communication network, key to exercise responses in skeletal muscle. It appears that histamine, secreted from small mast cell populations residing within the skeletal muscle, acts in a paracrine fashion, binding to H_1 and H_2 receptors and initiating a substantial portion of the transcriptional exercise response [122]. Even partial dysregulation of this transcriptional response alters subsequent protein expression [83] , and, when sustained, these cellular responses have implications for exercise training.

Histamine as a signaling molecule in exercise adaptation

When balanced with sufficient recovery, regular exercise training elicits functional adaptations that improve both health and performance. Many of these adaptations are mediated by exerkines, which are signaling molecules released in response to exercise and exert their effects through autocrine, paracrine, and endocrine pathways [21]. Histamine's role in the exercise responses implicates it as an exerkine. Blocking H_1 and H_2 receptors prior to training attenuates many of the health and performance benefits of aerobic exercise training, including improvements in insulin signaling, vascular function, muscle capillarization, and oxidative enzyme capacity [8,225]. It is important to note that these studies employed complete blockade of the histamine H_1 and H_2 receptors using 540 mg fexofenadine (H_1 receptor antagonist, common dosage 180 mg) and 40 mg famotidine (H_2 receptor antagonist, common dosage 20 mg). While the high dosages provide substantial blockade necessary to untangle the importance of histaminergic signaling, they do lack the ecological validity of investigations employing more conventional dosing schemes.

To date, only one study has employed a conventional dosing scheme, using H_1 receptor blockade at the recommended dose (i.e., 180 mg fexofenadine) to investigate the influence of histamine on adaptations to resistance training. H_1 antagonism altered the differential protein expression induced by a 10-week resistance training program, although it did not impact changes in strength or muscle size [83]. It is important to note that this investigation was conducted in

untrained individuals, who typically have robust responses to resistance training interventions and experience initial gains in strength due to neural adaptations during strength training [85,86]. Going forward, it will be important for more studies to confirm these findings surrounding histamine's influence on resistance exercise adaptation and to investigate trained individuals or longer durations of training. Further, it is important to evaluate singular blockade (i.e., H_1 only or H_2 only) and lower, more ecologically valid doses of aerobic training, which help provide insight to athletes and habitual exercisers looking to manage allergy symptoms in training and competition.

Influence of histamine on exercise performance

Exercise elicits a coordinated physiological response necessary to meet the multifaceted demands and sustain a given workload. The inability to meet these demands necessitates a reduction in external workload or results in task failure. Initial studies in rodents indicated that histamine is crucial to endurance capacity and protects against exercise-induced fatigue [136,138]. Following histamine H_1 and H_2 receptor antagonism, exercising skeletal muscle fatigued earlier and had lower levels of intramuscular glycogen and nitric oxide [138] , providing insight into the mechanistic influence of histamine in exercising skeletal muscle. Histamine upregulates nitric oxide synthase and nitric oxide production in endothelial cells [236,237], which may explain why blockade reduces nitric oxide in skeletal muscle. Nitric oxide is a potent vasodilator and facilitates blood flow to and within skeletal muscle during exercise, enhancing oxygen and nutrient delivery and improving metabolite washout. The greater depletion of muscle glycogen with blockade may indicate either an increased bioenergetic cost of a given workload or a greater reliance on intramuscular substrates, both of which may be affected by worsened or dysregulated blood flow. These initial findings in rodents indicated histamine is important in facilitating exercise responses and set the stage for future inquiry into its role during exercise in humans.

To date, few studies have examined the influence of histamine physiological responses during exercise or exercise performance in humans. Because measuring histamine in circulation is difficult, all studies to date have employed histamine receptor blockade to evaluate its effects. Early investigations indicated that H_1 antagonist administration does not affect muscular strength and endurance measured by knee extension and flexion torque [203] and does not affect physiological responses to moderate-intensity steady-state or high-intensity interval running [204].

H_1 and H_2 receptor blockade did not impact oxygen uptake, heart rate, or rating of perceived exertion during 45 minutes of downhill running [135]. Investigations into histamine's role during cycling found that in trained individuals, combined H_1 and H_2 receptor blockade slows 10-kilometer cycling time trial performance [9], but does not impact time-to-exhaustion in the severe domain [114]. There were also no differences in oxygen uptake, heart rate, or rating of perceived exertion between blockade and control conditions in either study [9,114]. It is interesting to note that the durations of these performance tasks were different (~1000 seconds for the 10-km and ~475 seconds for the time to exhaustion). Based on the evidence from rodents [136,138], one might expect histamine to have a greater influence on exercise performance in longer-duration exercise, where glycogen depletion, nutrient delivery, and metabolite washout may have more of an influence. During dynamic knee extension, H_1 and H_2 receptor blockade increased femoral blood flow but had no effect on vascular conductance and decreased intramuscular pH [37]. In resistance exercise, neither H_1 and H_2 receptor blockade nor H_1 receptor blockade alone impairs maximal leg strength in untrained individuals [83]. This finding is not surprising, given that maximal leg strength is likely limited by force generation and not fatigue

The understanding of histamine's influences on exercise responses and performance is still in its infancy. Taken together, these findings paint a complex, but incomplete, picture of the role of histamine during exercise. In fact, the increased femoral blood flow but decreased intramuscular pH following blockade during dynamic knee extension [37] creates a sort of 'histamine paradox' by which, during exercise, blocking histamine's receptors in skeletal muscle increases bulk blood flow in conduit vessels but may impair muscle microvascular perfusion. The idea that blocking histamine's actions dysregulates the microvasculature is supported by findings that blockade reduces blood flow and nutrient delivery following exercise [28,145] and may implicate this 'histamine paradox' in the mixed findings regarding histamine's effects on exercise performance. However, exercise elicits complex physiological responses that are highly dynamic and vastly different from the post-exercise period. Without further investigation, it is difficult to draw conclusions about the influence of histamine on physiology during exercise or on exercise performance.

Concluding remarks

The stress of exercise triggers a well-coordinated, multifaceted physiological response necessary to meet the demands of the activity. With sufficient recovery, exercise proves hermetic, eliciting adaptations that enhance physiological function, improve health, and exercise performance. A key component of this process is the responses within tissue, which underpin the systemic improvements following exercise training. Histamine is a key mediator of this local response and is important in the acute cellular [7,122], intramuscular [58,122], and systemic [5,79,224] exercise responses. Blocking histamine's actions likely blunts adaptation and alters the recovery process, attenuating the beneficial adaptations from aerobic [8,225] and resistance [83] exercise training. As such, histamine is an exerkine important in facilitating both acute exercise responses and the health benefits obtained from exercise training. The full scope of impact surrounding histamine receptor blockade requires further investigation, as do dosing schemes (e.g., H_1 vs H_2 only, lower doses, etc.), and the effects of antihistamine use versus training with allergies. Given the importance of histaminergic signaling in functional exercise adaptation and the higher rates of injuries seen in athletes with allergies [45,238,239], one might speculate that alterations in histamine signaling may be implicated in other issues arising from a stress-recovery imbalance, such as the development of overtraining syndrome. However, the scope and magnitude of its impact, as well as its role in recovery, require further investigation.

While histamine is important in acute post-exercise responses and may transduce many of the adaptations to exercise training, the causes of its release and its impact on physiological function and exercise performance warrant future exploration. Histamine may not be integral to maximum force generation, but only one study has evaluated its influence in resistance training. The mixed findings regarding histamine's influence on aerobic exercise performance highlight the need for future investigations, particularly those employing diverse exercise modalities (e.g., running, swimming) and varying exercise durations. These findings can serve as a basis to help provide insight to athletes, coaches, and clinicians on the use of atropine during training and competition. Further, they more fully characterize the role of histamine as an exerkine, improving understanding of the mechanisms underlying exercise adaptation and providing foundational insight into the fundamental physiological processes governing stress, recovery, and adaptation.

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