**Original grant title:** Exercise, Histamine Receptors, and Vascular Health in Aging

**Possible changes to title:**

1) Histamine as a Molecular Transducer of Exercise Adaptations

2) Exercise, Histamine, and Vascular Health

3) Exercise, Histamine Receptors, and Vascular Health

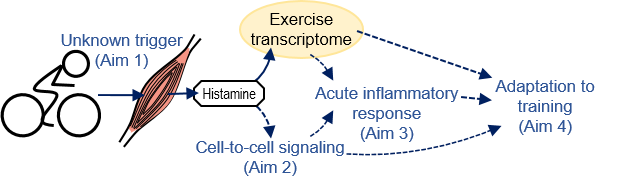
# PROJECT SUMMARY/ABSTRACT – LIMIT IS 30 LINES

Over 46 million Americans are over the age of 65, a population that is expected to double by the year 2060. With aging, there is a generalized vascular dysfunction. The long-term goal of this proposal is to understand the link between exercise, histamine, and alterations in endothelial and vascular function so that we can exploit this link to generate novel interventions to improve or maintain health in the aging population. In this context, exercise stimulates the release of histamine from mast cells within skeletal muscle, turning on a pathway that appears to trigger a broad range of cellular adaptations in response to exercise. It is unknown what exercise-related signal causes mast cell degranulation. However, subsequent activation of histamine receptors may play a role in improving endothelial function and stimulating vascular remodeling, as more than 25% of exercise-responsive genes are affected by histamine blockade. How these histamine-mediated responses impact on endothelial cell function and vascular remodeling is unknown. Further, the histamine footprint on the exercise transcriptome, which crosses many cellular functions, has an extensive domain associated with cytokine pathways, but it is unknown how histamine interacts with the inflammatory response to exercise. Lastly, it is unknown whether activation of histamine receptors is a necessary step on the path to the positive vascular adaptations to endurance exercise training. These unknowns will be addressed in the following specific aims: 1) Identify the exercise-related signal that causes release of histamine in skeletal muscle, 2) Determine the histaminergic component of endothelial and vascular smooth muscle cell responses to exercise, 3) Determine the impact of histamine on short-term inflammatory responses to exercise, and 4) Determine the histamine component of longer-term adaptations to exercise training. These aims are supported by the PI’s prior work and will be addressed using state-of-the-art techniques in humans. Information from these studies will prove valuable on four fronts. First, these studies will identify a mechanism (the signal for mast cell degranulation) that can be exploited by future interventions, to mimic or enhance the beneficial effects of exercise. Second, these studies will identify a key pathway that contributes to the cardiovascular health benefits of lifetime physical activity by modulating critical signals for endothelial and vascular health. Third, these studies will advance understanding of histamine’s role in exercise inflammatory responses, which may suggest how and when to intervene in the inflammatory process, preserving beneficial and avoiding deleterious effects of inflammation in the context of exercise. Last, these studies may prove that histamine, generally associated with pathophysiological responses (e.g., asthma, allergies, anaphylaxis, and tumor growth), is an important molecular transducer of exercise responses, both in the short and long term. In summary, these studies will contribute to the understanding of exercise as a therapeutic modality in treatment and prevention of cardiovascular disease and set the stage for new vascular health interventions targeted at older individuals.

# PROJECT NARRATIVE

Exercise has beneficial effects on cardiovascular health that go beyond what can be explained by the improvement of traditional risk factors. The studies in this application are designed to understand the mechanisms that underlie some of these beneficial effects of exercise. These studies may provide information that is pertinent to the use of exercise in the prevention and treatment cardiovascular diseases, including coronary heart disease, high blood pressure, and peripheral artery disease, and it sets the stage for new vascular health interventions targeted at older individuals.

# SPECIFIC AIMS

Americans, as a population, are getting older and this is associated with a rise in generalized vascular dysfunction. The long-term goal of this proposal is to understand the link between exercise, histamine, and alterations in endothelial and vascular function so that we can exploit this link to generate novel interventions to improve or maintain vascular health in the aging population. In this context, we have shown that exercise stimulates the release of histamine from mast cells within skeletal muscle1,2, turning on a pathway that potentially triggers a broad range of cellular adaptations in response to exercise3,4. We speculate that this histaminergic pathway may play a role in improving endothelial function and stimulating vascular remodeling that could be exploited to improve cardiovascular health and benefit older individuals. To fill essential gaps in knowledge and move toward this goal, we propose the following Specific Aims which are illustrated in **Fig. 1**.

**Fig. 1: Aims**

**Aim 1: Identify the exercise-related signal that causes histamine release in skeletal muscle.** It is unknown what exercise-related signal causes histamine release from exercising muscle1. Our prior work under this grant found no role for acute oxidative stress (i.e., increased reactive oxygen species) in the response. We hypothesize that **1a)** the rise in muscle temperature with exercise and/or **1b)** a soluble factor released in muscle triggers mast cell degranulation. We expect these studies will identify an exercise-specific trigger for the localized release of histamine in exercising skeletal muscle, which will become a tool for driving this pathway (gain-of-function experiments) and will lead to health interventions which augment or mimic exercise responses.

**Aim 2: Determine the histaminergic component of endothelial and vascular smooth muscle responses to exercise.** In other contexts, histamine exerts an influence on endothelial and vascular muscle cells, but it is unknown how histamine-dependent signaling influences such cellular responses to exercise. We hypothesize that both **2a)** histamine receptor activation and **2b)** histamine-induced myokines stimulate the expression of pro-angiogenic responses *in vitro*. We expect these studies will identify a key pathway that contributes to the cardiovascular health benefits of lifetime physical activity by modulating cell-to-cell signaling between exercising muscle and endothelial and vascular smooth muscle cells. These studies will also advance understanding of histamine’s role in exercise training (Aim 4).

**Aim 3: Determine the impact of histamine on short-term inflammatory responses to exercise.** Exercise generates pro- and anti- inflammatory signals that appear necessary for the full expression of the health benefits of exercise. Histamine drives a large number of exercise-responsive genes, including an extensive domain associated with cytokine pathways3, but it is unknown how histamine affects the acute inflammatory response to exercise. We hypothesize that histamine-blockade **3a)** reduces pro-inflammatory and pro-resolving cytokine levels, **3b)** alters the pattern of peripheral mononuclear blood cell (PBMC) phenotypes and pro-angiogenic cells, and **3c)** reduces cellular infiltration into exercised muscle. We expect these studies will identify histamine as a key regulator of exercise inflammatory responses, and may suggest tools to intervene in the inflammatory process, preserving beneficial and avoiding deleterious effects of inflammation in the context of exercise.

**Aim 4: Determine the histamine component of longer-term adaptations to exercise training.** The mechanisms that drive the positive adaptations and health benefits of exercise training are inadequately understood5. Histamine exerts a broad impact on the exercise transcriptome3, but it is unknown whether activation of histamine-receptors is necessary to fully express the training-induced adaptations. We hypothesize that histamine-blockade will reduce training-induced gains in **4a)** capillarization of skeletal muscle, **4b)** endothelial function, **4c)** cardiovascular fitness, and **4d)** markers of cardiovascular health. We expect these loss-of-function studies will show that histamine, generally associated with pathophysiological responses (e.g., asthma, allergies, anaphylaxis, and tumor growth), is an important molecular transducer of exercise adaptations.

*Overall, this work advances mechanistic understanding of exercise as a therapeutic modality in treatment and prevention of vascular disease, setting the stage for novel interventions targeted at older individuals.*

# RESEARCH STRATEGY – 12 page limit, including progress report

This is the competitive renewal of a highly productive 4-y R01. Aims in this proposal are strongly informed by publications generated by the original grant, summarized in the Progress Report and throughout the proposal.

# A. SIGNIFICANCE

Over 46 million Americans are over the age of 65, a population that is expected to double by the year 20606. Advancing age is associated with considerable impairments of endothelial and vascular function which can cause functional limitations to activities of daily living and predispose individuals to cardiovascular disease including coronary heart disease, hypertension, and peripheral artery disease. Studies in older men and women indicate that endothelial function of conduit arteries and the microcirculation are frequently diminished, but may be preserved in older highly fit individuals7–10. Another hallmark of vascular aging is the reduced blood flow and perfusion of skeletal muscle, which has functional consequences. Skeletal muscle blood flow is reduced ~ 20-30% in older individuals11,12, and there is compelling evidence of a decreased capillary supply to skeletal muscle. Microcirculatory capacity decreases 4-6% per decade13,14, but can respond positively to exercise interventions in the elderly15,16. Thus, regular endurance exercise can reduce or prevent cardiovascular disease in the aged by stimulating vascular and endothelial adaptations; however, the signals and mechanisms which transduce these effects are poorly understood. NIH’s Director has highlighted this as*a critical gap in knowledge*by establishing the NIH Common Fund program on “Molecular Transducers of Physical Activity in Humans” to identify novel mechanisms for exercise-induced health benefits.

*The contribution of the proposed research is expected to provide information that directly addresses multiple gaps in knowledge regarding the link between exercise, histamine, and alterations in endothelial and vascular function in humans.* In this context, we have shown that exercise stimulates the release of histamine from mast cells within skeletal muscle1,2, turning on a pathway that potentially triggers a broad range of cellular adaptations in response to exercise3,4. We speculate that this histaminergic pathway may play a critical role in improving endothelial function and stimulating vascular remodeling in response to exercise (i.e., histamine may be an important molecular transducer of physical activity). Expected outcomes that will move the field forward include: 1) identification of a mechanism that can be exploited by future interventions, to mimic or enhance the beneficial effects of exercise, 2) identify a key pathway that, by modulating critical signals for endothelial and vascular health, contributes to the cardiovascular health benefits of lifetime physical activity, 3) advance understanding of histamine’s role in exercise inflammatory responses, which may suggest how and when to intervene in the inflammatory process, preserving beneficial and avoiding deleterious effects of inflammation in the context of exercise, and 4) prove that histamine, generally associated with pathophysiological responses (e.g., asthma, allergies, anaphylaxis, and tumor growth), is an important molecular transducer of exercise responses. The Scientific Rationale for these outcomes is further developed under Approach.

*These contributions are significant because we can exploit this link to generate novel interventions to improve or maintain vascular health in the aging population.* They are also significant because they will contribute to the understanding of exercise as a therapeutic modality in treatment and prevention of cardiovascular disease.

# B. INNOVATION

Our work on the role of histamine in exercise is tightly aligned with the goals of NIH’s “Molecular Transducers of Physical Activity in Humans” program, but precedes the call for R01 applications on this topic. *The proposed research is innovative, in our opinion, by its targeting histamine as a novel molecular transducer of physical activity-induced health benefits*. The dogma is that histamine is released only during muscle damaging exercise or with injury. In contrast, we are showing a role for histamine in the day-to-day response to routine physical activity in the absence of muscle damage. In humans, we have shown that aerobic exercise results in activation of histamine H1 and H2 receptors within the previously exercised muscle, triggering vasodilation and a broad range of responses to exercise2–4. We have also shown that histamine affects the availability of glucose to skeletal muscle, glucose uptake by skeletal muscle, and insulin sensitivity following exercise17–19. Further, of the more than 3000 protein-coding genes which are exercise-responsive, we have shown that histamine modifies > 25%, including ones involved in such physiological domains as inflammation, vascular function, metabolism, and cellular maintenance3,4, as shown in **Fig. 2**. *For these reasons, we believe histamine is an unrecognized molecular transducer of physical activity responses.* To the best of our knowledge, we are the only researchers exploring the role of histamine receptors in the context of exercise and vascular function.

In addition, the proposed studies are part of a novel and rapidly growing literature on the *post-exercise mileu* as target for intervention20,21, as indicated by recent Featured Topics22,23. We believe the mechanisms responsible for the active resolution of exercise, evident in recovery (e.g., histamine-mediated vasodilation), are linked to chronic adaptations that occur with exercise training. Further, we are suggesting a novel link from vascular responses to inflammatory signaling, which may relate to the anti-inflammatory properties of routine physical activity24–26. Lastly, we use rigorous state-of-the-art methods, combined in innovative ways, to answer important questions *in vivo* and *in vitro* in humans and human cell cultures.

**Fig. 2: Combined H1/H2 blockade reveals broad histamine footprint on exercise transcriptome.** A single bout of exercise alters the expression of thousands of protein-coding genes (represented by yellow circle). Much of this response depends on the activation of H1 or H2 receptors by histamine, as combined histamine H1/H2-receptor blockade markedly reduces the transcriptome response to exercise (represented by green circle). Histamine modulates many cellular functions: vascular function, metabolism, cellular maintenance, and inflammation. Reproduced from4, based on data from3.

# C. APPROACH

The general approach for this research is to address our aims in a series of protocols that will be conducted in cohorts of healthy normotensive men and women. We will study sedentary individuals, excluding individuals who participate in aerobic exercise ≥ 30 min per session and ≥ 3 sessions per week, based on their exercise habits over the prior 12 months.

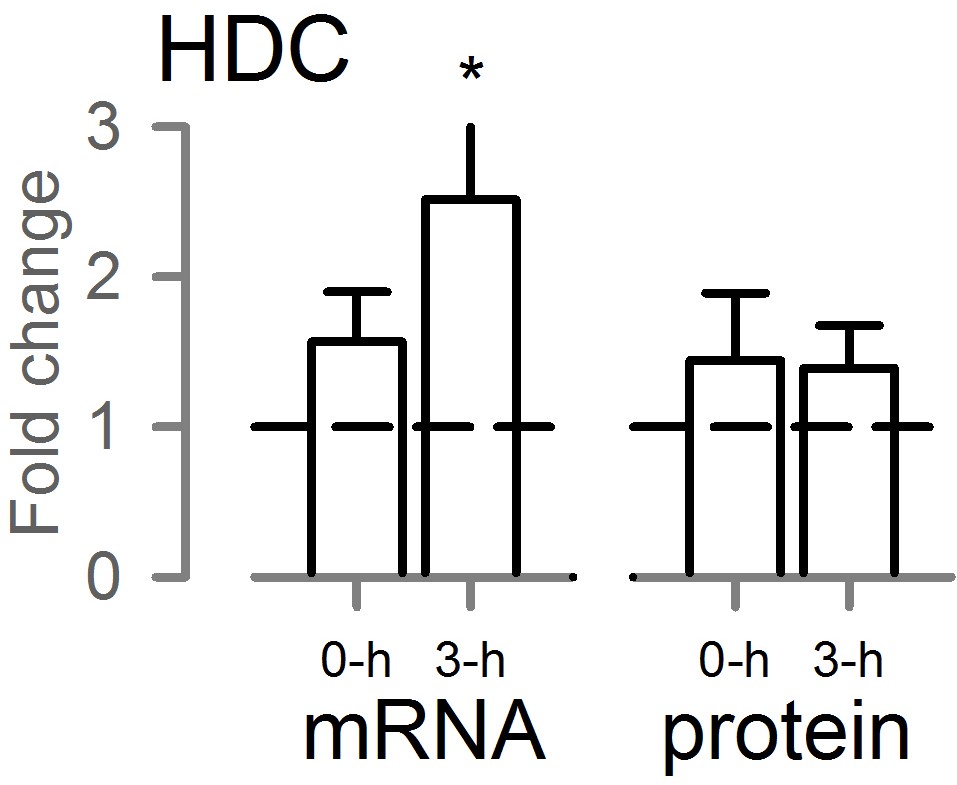
**Abbreviations:** α-FMH, α-fluoromethylhistidine; CCL2, chemokine (C-C motif) ligand 2; HDC, histidine decarboxylase; IL1β, interleukin 1β; IL6, interleukin 6; IL8, interleukin 8; IL10, interleukin 10; MMP9, matrix metallopeptidase 9; MCP1, monocyte chemoattractant protein 1; MVEC, cardiac microvascular endothelial cell; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOS3/eNOS, endothelial nitric oxide synthase; PBMC, peripheral mononuclear blood cell; THSB1, thrombospondin 1; TNFα, tissue necrosis factor α;

## C:\Users\halliwil\AppData\Local\Microsoft\Windows\INetCacheContent.Word\Source of Histamine.jpgAim 1: Identify the exercise-related signal that causes histamine release in skeletal muscle.

**Relevant Literature & Scientific Rationale:** Under the original grant, we demonstrated that histamine is released from skeletal muscle during and after exercise1. Specifically, we documented that interstitial histamine is elevated in response to 1 h of unilateral dynamic knee-extension exercise (**Fig. 3A**). Blockade of histidine decarboxylase (the enzyme that produces histamine) reduces histamine release within skeletal muscle, and tryptase (a biomarker of mast cell degranulation) is also increased in response to exercise. Collectively, these observations show that both *de novo* formation of histamine and mast cell degranulation are triggered by exercise. We also found that histidine decarboxylase (HDC) mRNA expression is elevated in human skeletal muscle following exercise3 (**Fig. 4**), an observation that is consistent with previous work in rodent models after prolonged exercise27–29, and suggests that signaling mechanisms related to *de novo* formation are upregulated by acute exercise. Taken together, our findings support the idea of parallel histaminergic pathways that are triggered by exercise, as first postulated by Endo and colleagues27–30 (Their elegant work over the last decade has suggested that the induction of histidine decarboxylase replenishes the pool of mast cell histamine lost with degranulation). *At present, we do not know the upstream signal that results in release of histamine within exercising skeletal muscle*. Several exercise-related factors such as oxidative stress31, cytokine release,32 and elevated temperatures33 have been shown in other contexts to induce mast cell degranulation in an antigen-independent manner. Additionally, oxidative stress34 and the transcription factor hypoxia-inducible factor-1α35, both of which are induced by exercise, are associated with upregulated transcription of histidine decarboxylase. Histidine decarboxylase also appears to function optimally at elevated temperatures and reduced pH, two conditions that are well-associated with exercise36. Lastly, a study by DeForrest *et al*. suggests that shear stress may increase *de novo* histamine synthesis within blood vessels through a shear stress dependent mechanism37. To test one of these potential exercise-related mechanisms in the original grant, we infused the potent antioxidant N-acetylcysteine prior to and during exercise and found no effect on this pathway21, suggesting that exercise-induced oxidative stress is not a necessary signal for histidine decarboxylase and/or mast cells activation. *To date, the trigger remains elusive*. To attack this **gap in knowledge**, we will test two hypotheses: one based on the rise in temperature in exercising muscle, and one based on the presence of a soluble signal driving histamine responses.

**Fig. 4: Histidine decarboxylase (HDC) expression and abundance within skeletal muscle.** Samples were obtained before and at 3-h post-exercise and are expressed at the fold change relative to pre-exercise3. Values are means ± SE; For expression, n=8, \*P<0.05 for exercise effect; For abundance, n=3 to 6, and statistics have not been calculated due to the preliminary nature of results (unpublished data).

**Fig. 3: Effect of exercise on intramuscular histamine and tryptase release.** Samples were obtained by intramuscular microdialysis during 1 h of dynamic knee extension1. **A)** Histamine increases with exercise, and this is partially blocked by inhibition of histidine decarboxylase with α-FMH. Values are means ± SEM. \*P<0.05 vs. Pre-exercise; †P<0.05 vs. Control. **B)** Tryptase, biomarker of mast cell degranulation, increases with exercise. Values are means ± SEM. \*P<0.05 vs. Pre-exercise.



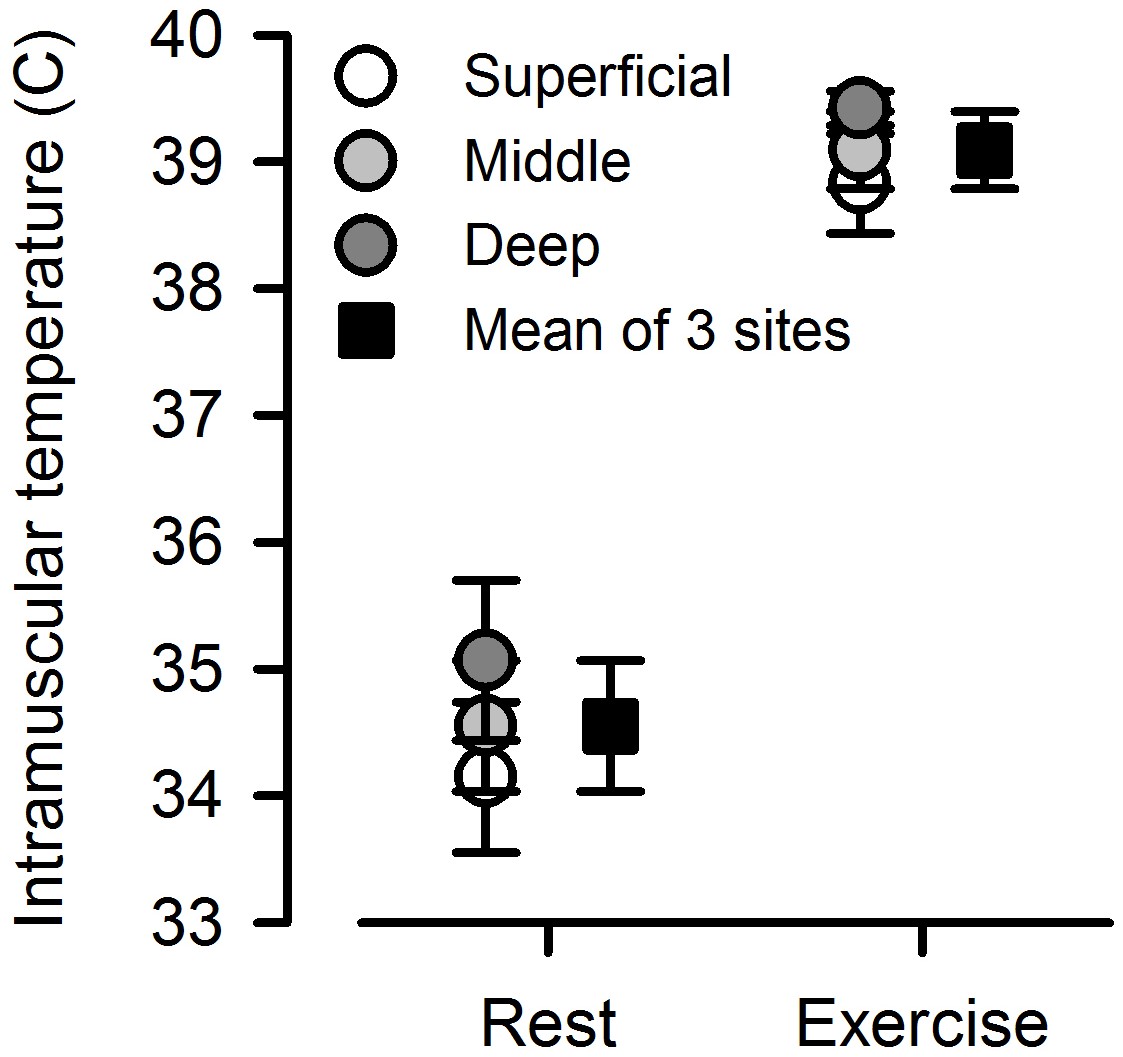
**Experience & Preliminary Studies**: We are experienced with intramuscular microdialysis1,18, intramuscular temperature (**Fig. 5 & 6**), using heating interventions38,39. **Fig. 5** shows the rise in intramuscular temperature in response to 1 h of unilateral dynamic knee-extension exercise (i.e., a dose of exercise which consistently generates histamine release). As muscle temperature rises, temperatures of superficial, middle, and deep muscle converge, resulting in a homogenous steady-state muscle temperature of 39.2°C (for this intensity of exercise). This sets the target temperature to be obtained in Aim 1a. We have recently heated the *vastus lateralis* to this target temperature using short-wave diathermy (a therapeutic heating modality) while sampling from the muscle via microdialysis (**Fig. 6**). This supports Aim 1a.

**Fig. 7** shows the response of a mast cell bioassay to dialysate obtained via intramuscular microdialysis of exercising subjects40,41. In brief, β-hexosaminidase released into culture medium by mast cells in response to dialysate is quantified to determine the % of degranulation *in vitro* (see Details of Methods section). The figure shows the presence of a soluble factor released by skeletal muscle which increased mast cell degranulation by ~60% in the assay. This supports Aim 1b.

**Research Design & Rigor:** After screening, which includes medical history, subjects will undergo intramuscular microdialysis to collect dialysate, but under one of two different protocols. For Aim 1a, we will use short-wave diathermy to heat the muscle around the microdialysis probe to 39.2°C for 1 h, replicating the thermal effects of exercise, to test the hypothesis that this rise in temperature elicits histamine release from skeletal muscle. A microdialysis probe in the contralateral unheated leg will serve as a control. Dialysate histamine concentrations from before, every 20-min during heating, and post-heating will be quantified by ELISA as in our prior research1. For Aim 1b, subjects will perform 1 h of unilateral dynamic knee-extension exercise1,42, to test the hypothesis that exercise generates release of a soluble factor from skeletal muscle which elicits mast cell degranulation. A microdialysis probe in the contralateral resting leg will serve as a control. Dialysate from before, every 20-min during exercise, and post-exercise will be tested in a mast cell degranulation bioassay for its potential to activate mast cells (**Fig. 7**) (see Details of Methods section). Primary outcome variables for Aim 1 are as follows:

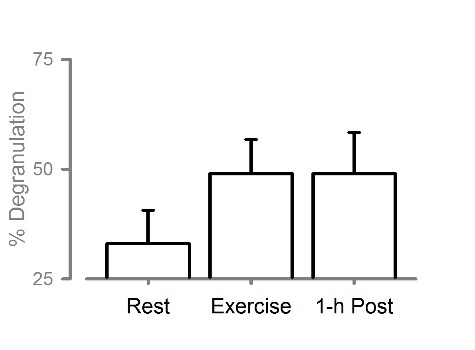
**COMING SOON…… FIGURE 6 HERE**

**Fig. 6: Histamine release in response to muscle heating.** Work in progress… (unpublished data).



**Fig. 5: Intramuscular temperature during exercise.** Multi-site thermocouples registered temperature at three depths within the *vastus lateralis* during exercise. n=6, statistics have not been calculated due to the preliminary nature of results (unpublished data).

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| **Aim 1a.** Histamine concentration in dialysate in response to muscle heating | **Aim 1b.** Mast cell degranulation in bioassay in response to dialysate from exercised muscle |

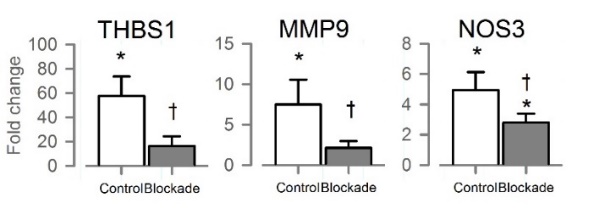
**Analysis & Expected Outcomes:** For each sub-aim, our primary outcome variables are measured within subjects across time and condition (control vs. heat; or control vs. exercise). We will use a two-way repeated-measures model … We expect that either muscle heating will result in histamine release, or a soluble factor released by exercising muscle will result in mast cell degranulation. It is also possible that both outcomes will occur, as there may exist multiple triggers for histamine release in response to exercise. In summary, we expect these studies will identify an exercise-specific trigger for the localized release of histamine in exercising skeletal muscle.

**Fig. 7: Mast cell degranulation in response to dialysate from exercising muscle.** In vitro bioassay for mast cell degranulation in response to soluble factors in dialysate obtained from *vastus lateralis* during exercise. n=5, statistics have not been calculated due to the preliminary nature of results (unpublished data).

**Potential Problems & Alternative Strategies:** We have done considerable work setting up the methods for this protocol, and our team has extensive experience with microdialysis in humans. There are no hurdles to overcome in initiating this protocol, and should challenges arise in their conduct, we have extensive experience upon which to draw in troubleshooting those problems. For 1a, we considered use of ultrasound for heating, but decided diathermy is superior as it does not involve the mechanical affects associated with therapeutic ultrasound. For 1b, other strategies which we have considered include “omic” style searches for factors known to degranulate mast cells, but the advantage of the current protocol is that it feeds directly into future studies that can “pharmacodisect” the response in cell culture.

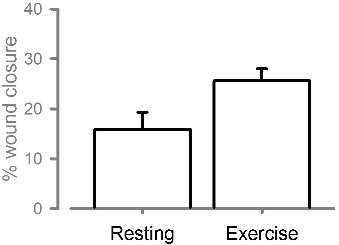
**Future Directions:** Identification of a physiological tool (e.g., modest tissue heating) which can drive this pathway sets the stage for future “gain-of-function experiments” such as adding heating to exercise and defining the dose-response relationship. If we find that a soluble factor, and not temperature, is the trigger, we would next pharmacodisect the response quickly via the mast cell bioassay.

## Aim 2: Determine the histaminergic component of endothelial and vascular smooth muscle cell responses to exercise.

**Relevant Literature & Scientific Rationale:** In other settings, histamine has been shown to up-regulate endothelial nitric oxide synthase (NOS3/eNOS)43, implying that histamine receptors may underlie improved endothelial function observed following acute and chronic exercise. Likewise, histamine increases endothelial permeability and plays a critical role in angiogenesis in the setting of wound healing, tumor growth, and pregnancy44 by up-regulating vascular endothelial growth factor (VEGF)45,46 and other proangiogenic signals such as monocyte chemotactic protein 1 (MCP1)47, matrix metalloproteinase 2 (MMP2)48, and basic fibroblast growth factor (bFGF)49. It is worth highlighting that the complex balance of pro- and anti-angiogenic factors plays a critical role in exercise-induced angiogenesis, and histamine may act synergistically with vascular VEGF to facilitate this physiological angiogenesis50. However, the interaction of H1/H2 receptor activation and the angiogenic response to exercise has not been explored. Endothelial and vascular smooth muscle cells possess H1/H2 receptors that when activated, result in vasorelaxation and increased vascular permeability51,52. Our prior research3 indicates the exercise-induced expression of mRNA in skeletal muscle for many angiogenic signal molecules are blocked or markedly blunted by histamine blockade (see **Fig. 8 & 9**). But this work highlights some of the challenges in the literature, in that *in vivo* studies represent an integrated physiological response, but may mask cell-type specificity of responses, whereas *in vitro* studies may illuminate cell-type specific responses, they may lack the synergism of complex stimuli like exercise. For example, histamine in exercising muscle may work through myokines to promote angiogenesis, a feature which would not be recognized by applying histamine *in vitro*. *Thus, an important question is whether histamine blockade will reduce or block components of endothelial and vascular smooth muscle cell responses to exercise, both by direct histamine-receptor involvement and by indirect pathways such as release of myokines.* To overcome these limitations and fill the gap in knowledge regarding the role histamine in exercise responses, we will employ a hybrid “*in vivo* to *in vitro”* model in which we can pharmacodisect integrated responses to exercise with cell-type specific measurements.

**Fig. 9: Angiogenic gene expression within skeletal muscle.** Expression of mRNA for THSB1, MMP9, and NOS3. Open bars denote the control condition; Gray bars denote the H1/H2 blockade condition. Values are means ± SE; For expression, n=8, \*P<0.05 for exercise effect, and † P<0.05 blockade vs control3.

**Fig. 8: Heat map showing effect of exercise and histamine blockade on angiogenesis pathways.** Differentially expressed protein-coding genes for H1/H2 blockade vs control at 3 h post-exercise were mapped to Panther pathway P00005 “Angiogenesis”138. Shown are the log2 fold changes from pre-exercise under control and H1/H2 blockade, and the difference between control and blockade for the top 14 responsive genes. Numerous genes were upregulated by exercise under control conditions (green), but not during blockade. Thus, the difference between conditions is a relative downregulation (red). This provides compelling preliminary evidence for an exercise-angiogenesis link that is dependent on histamine. Based on data from3.

**Experience & Preliminary Studies**: We are experienced with intramuscular microdialysis1,18, endothelial cell angiogenic assays53–56, and are working with our consultant, Dr. Cynthia Meininger, to optimize endothelial migration and proliferation assays for testing angiogenic properties of dialysate, obtained from exercising humans, in cardiac microvascular endothelial cells (MVECs) and human vascular smooth muscle cells (VSMCs) to support Aim 2 (**Fig. 10**).

**Research Design & Rigor:** After screening, which includes medical history, subjects will undergo intramuscular microdialysis to collect dialysate, using two probes (control vs. blockade). Subjects will perform 1 h of unilateral dynamic knee-extension exercise1,42, to test the hypotheses histamine receptor activation and histamine-induced myokines stimulate the expression of pro-angiogenic responses *in vitro*. Dialysate from before versus during exercise (resting vs. exercise), and with and without combined histamine H1/H2 blockade will be applied to MVECs and VSMCs in vitro (see Details of Methods section). In addition, combined histamine H1/H2 blockade *in vitro* will be used to differentiate between direct effects of histamine, mediated through H1/H2 receptors on MVECs and VSMCs, versus indirect effects, mediated by factors (such as myokines) released *in vivo* in response to activation of H1/H2 receptors. Primary outcome variables for Aim 2 are as follows:

**Fig. 10: Scratch test for endothelial cell migration.** Wound closure at 6 h post-scratch, showing 40% increase in migration with exposure to dialysate from exercising vs resting muscle. Values are means ± SE; n=3. Statistics have not been calculated due to the preliminary nature of results (unpublished data).

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| MVEC and VSMC proliferation and migration |
| Expression and abundance of…. |

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| --- | --- | --- |
| **Key Comparisons** | | |
| No blockade | *In vitro* blockade | *In vivo* blockade |
| Direct + | Direct - | Direct - |
| Indirect + | Indirect + | Indirect - |
| ^------- Aim **2a** -------^ | |  |
|  | ^------- Aim **2b** -------^ | |

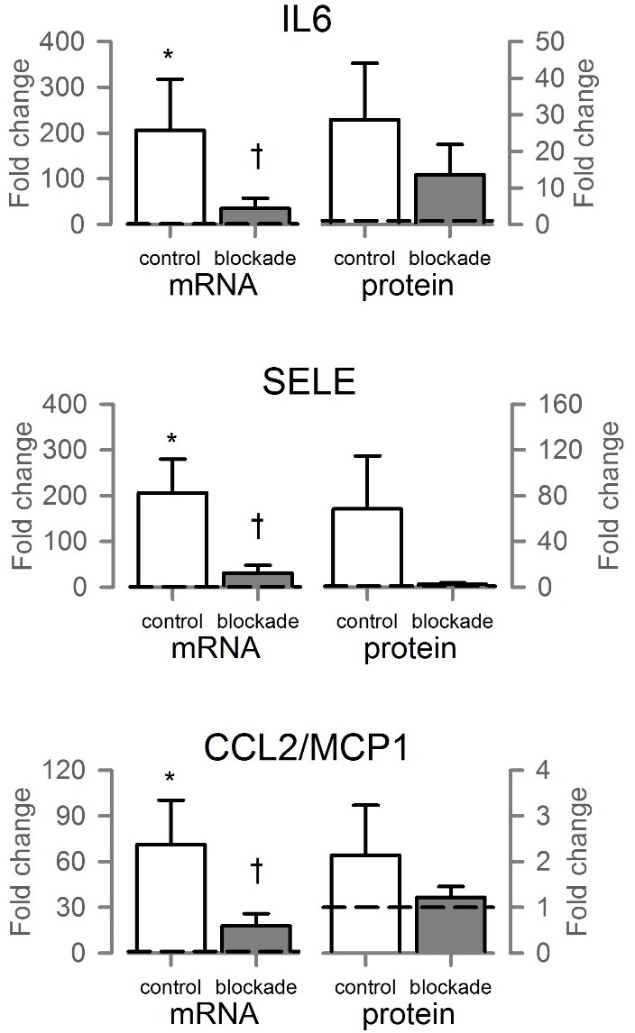
**Analysis & Expected Outcomes:** Our primary outcome variables are measured within subjects across exercise condition and blockade condition (resting vs. exercise; no blockade vs. *in vivo* blockade vs. *in vitro* blockade). We will use a two-way repeated-measures mode… As shown to the right, the key comparison to address Aim 2a (Direct effects of histamine receptor activation) is “no blockade” vs “*in vitro* blockade”, as both conditions will exhibit any indirect effects of histamine that were generated *in vivo*, but only “no blockade” will exhibit direct effects of histamine*.* The key comparison to address Aim 2b (Indirect effects of histamine receptor activation) is *“in vivo* blockade”vs *“in vitro* blockade”, as *in vitro* but not *in vivo* blockade will exhibit any indirect effects of histamine that were generated *in vivo.* We expect that there will be demonstrable direct and indirect effects of histamine that are pro-angiogenic in both MVEC and VSMC.

**Potential Problems & Alternative Strategies:**

**Future Directions:** We expect these studies will identify a key pathway that contributes to the cardiovascular health benefits of lifetime physical activity by modulating cell-to-cell signaling between exercising muscle and endothelial and vascular smooth muscle cells. These studies will also advance understanding of histamine’s role in exercise training (Aim 4). If we demonstrate substantial indirect effects of histamine, we will be in a position to further explore the pathways involved (i.e., identify what myokine, etc, is responsible) .

## Aim 3: Determine the impact of histamine on short-term inflammatory responses to exercise.

**Relevant Literature & Scientific Rationale:** Aerobic exercise generates pro-inflammatory and pro-resolving) signals, including the release of cytokines, chemokines57, and lipid mediators 58, and mobilizes immune cells59 and circulating angiogenic cells60 that are relevant to improving or maintaining cardiovascular wellness. There is considerable evidence that exercise plays a role in preserving endothelial and vascular health by inhibiting chronic low-grade vascular inflammation61–64, perhaps through the resolution of inflammation, which is now recognized as an active (not passive) process65–67. Thus, understanding the exercise-dependent mechanisms of inflammatory regulation is highly relevant to cardiovascular disease as it impacts atherosclerotic processes, arterial stiffening, and hypertension68,69. Further, dysregulated inflammatory responses to exercise (e.g., unresolved inflammation, exercise-induced urticaria, anaphylaxis, and asthma) are considerable disincentives to routine physical activity in affected individuals70.

Exercise-associated pro-inflammatory cytokines/chemokines including fractalkine, interleukins IL1β and IL8, monocyte chemoattractant protein 1 (MCP1), and tissue necrosis factor α (TNFα), which are involved in chemotaxis, adhesion, and transmigration of PBMCs, and generally affect the endothelium as part of the process of cellular infiltration71–85. Interestingly, IL1β has the potential to induce expression of histidine decarboxylase86, the enzyme which produces histamine in skeletal muscle (which could be a positive feedback loop); IL8 is also pro-angiogenic74,75. Exercise also increases levels of anti-inflammatory/pro-resolving interleukins IL6 and IL10. IL6, considered by many to be the first recognized myokine, has anti-inflammatory effects in skeletal muscle after exercise, while having pro-inflammatory effects in other tissues57,87–89. IL10 inhibits the actions of NF-κB90,91 (serving as a counter to TNFα which activates NF-κB83–85), and promotes resolution of inflammation. Lipid mediators of inflammation that respond to exercise include classical pro-inflammatory prostaglandins and leukotrienes, and recently identified anti-inflammatory/pro-resolving lipoxins, resolvins, protectins, and maresins58. Our prior research3 indicates the exercise-induced expression of mRNA in skeletal muscle for many of these signal molecules are blocked or markedly blunted by histamine blockade (see **Fig. 11**). *Thus, an important question is whether histamine blockade will reduce or block the response of these signals to exercise.* These same signals are believed to mobilize sub-populations of PBMCs and alter the cell-surface expression profile of others, resulting in a sustained elevation in circulating neutrophils and the subpopulation of monocytes expressing pro-inflammatory markers (Mon1 “classical monocytes”) which lasts approximately 24 h59,92. Further, Mon1 express the receptor for monocyte chemoattractant protein 1 (CCL2/MCP1)93, suggesting that they will be responsive to the impact of histamine blockade on CCL2/MCP1 within skeletal muscle. *Thus, an important question is whether histamine blockade will modulate the circulating populations of neutrophils and monocytes*. During recovery from exercise, neutrophils, followed by macrophages, infiltrate skeletal muscle tissue. The processes by which circulating PBMCs extravasate to skeletal muscle tissue (margination, adhesion, transmigration) involve many of the factors discussed above, along with changes in capillary permeability. Thus, by directing the response of cytokines, chemokines, and regulating capillary permeability, histamine has the potential to substantially alter the infiltration of immune cells into skeletal muscle tissue following exercise. It is unclear which signal initiates this process94, so *an important question is whether histamine, with its natural history of chemotaxis, contributes to the exercise-induced infiltration of neutrophils and monocyte/macrophages into skeletal muscle.*

**Fig. 11: Heat map showing the effect of exercise and histamine blockade on inflammatory pathways.** Differentially expressed protein-coding genes for H1/H2 blockade vs control at 3 h post-exercise were mapped to Panther pathway P00031 “Inflammation mediated by chemokine and cytokine signaling pathway”138. Shown are the log2 fold changes from pre-exercise under control and H1/H2 blockade, and the difference between control and blockade for the top 30 responsive genes. Numerous genes in this pathway are upregulated by exercise under control conditions (green), but not during blockade. Thus, the difference between conditions is a relative downregulation (red). This provides compelling preliminary evidence for an exercise-inflammation link that is dependent on histamine. Based on data from3.

In addition to effects on the exercised muscle groups, exercise promotes vascular health beyond the active muscle vascular beds61–63. One potential mechanism for these distant effects is circulating angiogenic cells expressing endothelial specific markers60,95–97. Of note, CD14+ PBMCs expressing E-Selectin (CD62E) are a novel exercise-induced population which are highly pro-angiogenic (“angiogenic monocytes”). Lansford et al.98 demonstrated that CD62E+ PBMCs are elevated 57% after 50 minutes of moderate intensity cycling. E-Selectin is an inducible adhesion molecule involved in recruitment of cells to promote angiogenesis99. In vitro, these CD62E+ cells stimulate greater pro-angiogenic responses from cultured endothelial cells than are generated by CD34+/VEGFR2+ “endothelial progenitor cells”, the archetype for circulating angiogenic cells98,100. It is unknown which monocyte subpopulations markers are expressed by these angiogenic monocyte, or how they overlap with the three main monocyte subpopulations. Thus, we hope to further characterize this PBMC subpopulation. Moreover, the exercise factor that stimulates their activation is unknown. As our prior studies3 demonstrated an increased expression of E-Selectin mRNA which was blocked by anti-histamines, *an important question is whether histamine released from exercising skeletal muscle could be the exercise factor which promotes the CD62+ “angiogenic monocyte” subpopulation.*

**Fig. 12: Inflammatory signaling expression and abundance within skeletal muscle.** Expression of mRNA and protein abundance for IL6, SELE (E-selectin), and CCL2 (MCP1). Samples were obtained before and at 3-h post-exercise and are expressed at the fold change relative to pre-exercise. This demonstrates our ability to use muscle biopsies to quantify expression and abundance and provides compelling preliminary evidence for an exercise-inflammation link that is dependent on histamine. Open bars denote the control condition; Gray bars denote the H1/H2 blockade condition. Values are means ± SE; For expression, n=8, \*P<0.05 for exercise effect, and † P<0.05 blockade vs control3; For abundance, n=3 to 6, and statistics have not been calculated due to the preliminary nature of results (unpublished data).

In summary, we know that histamine drives a large number of exercise-responsive genes, including an extensive domain associated with cytokine, chemokine, and lipid mediator pathways3, but it is unknown how histamine affects the acute inflammatory response to exercise. The **gap in knowledge** is whether histamine is a necessary signal for expression of the many components of exercise inflammatory responses, including cytokines, lipid mediators, circulating immune and angiogenic cells, and cellular infiltration into muscle.

**Experience & Preliminary Studies**: We are experienced performing muscle biopsies3,101,102, assessing inflammation103–105, and using flow cytometry103,104, performing vascular studies including venous congestion plethysmography38,106–113, and using histamine blockade in exercise protocols3,21,105,114–117. Our prior research3 indicates the exercise-induced expression of mRNA for IL1β, IL6, IL8, MCP1, Fractalkine, and Eoxatin-1 in skeletal muscle are all blocked or markedly blunted by histamine blockade at 3-h post-exercise (**Fig. 11**). In contrast, elevations in TNFα expression were not affected by blockade, and IL10 was not yet elevated at 3-h post-exercise. Likewise, exercise-induced expression of mRNA for enzymes linked to lipid mediators (e.g., PLCB2, ALOX5AP, **Fig. 11**). We have preliminary data showing that histamine blockade translates into altered protein abundance as early as 3 h post-exercise (**Fig. 12**). This supports our belief that histamine serves as a master regulator for many of the cytokine and chemokine signals released by skeletal muscle tissue in response to exercise. We have the methods for imaging cellular infiltrates into skeletal muscle tissue, such as neutrophils and macrophages. **Fig. 13** shows a recent image documenting the presence of M1 (CD86+) and M2 (CD163+) macrophages within skeletal muscle. In short, we have experience conducting all aspects of this study.

**Research Design & Rigor:** After screening, which includes medical history, subjects will undergo a preliminary visit to determine VO2 peak and workload (see Details of Methods section). Subsequently, subjects will undergo a randomized crossover experiment which will include identical exercise sessions and follow-up measurements under placebo control versus combined histamine H1/H2 blockade conditions. Exercise will consist of a 60-min bout of seated upright cycling at 60% VO2 peak. Prior to exercise, subjects will ingest either placebo pills (**control**) or both the selective H1-receptor antagonist fexofenadine hydrochloride (540 mg Allegra, Aventis Pharmaceuticals Inc.) and the selective H2-receptor antagonist ranitidine hydrochloride (300 mg Zantac, GlaxoSmithKline) to produce combined H1- and H2-receptor antagonism (**blockade**). The order of control versus blockade for will be randomized and double-blinded. The peak test and the subsequent protocols will be performed in the morning after an overnight fast, with subjects having abstained from caffeine for 12 h and from alcohol and exercise for 24 h prior to the study. We will obtain venous blood samples before and at 3, 6, 12, 24, and 48 h after performing a 60-min bout of seated upright cycling at 60% VO2 peak under control and blockade conditions. Similarly, we will obtain muscle biopsy samples from the *vastus lateralis* muscle before and at 3 and 24 h after exercise. These blood and biopsy samples will be used to measure the following key outcome variables (for details on measurements, see Details of Methods section).

**Fig. 13: Labeling of M1 and M2 Macrophages within skeletal muscle.** M1 are labeled as CD86+ (red), M2 as CD163+ (green), and myocytes are outlined by laminin (blue). This demonstrates our ability to use muscle biopsies to quantify cellular infiltration of immune cells. (unpublished data).

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| **Aim 3a.** Cytokines: Plasma levels of IL1β, IL6, IL8, IL10, TNFα, MCP1, Fractalkine, Eotaxin-1 |
| **Aim 3b.** PBMC phenotypes: Cell counts for neutrophils CD14+/CD86-, Mon1 “classical monocytes” CD14+/CD16-/CD192+, Mon2 “intermediate monocytes” CD14+/CD16+/CD192+, Mon3 “non-classical monocytes” CD14+/CD16+/CD192-, “Angiogenic monocytes” CD14+/CD86-/CD62E+ |
| **Aim 3c.** Cellular infiltration: Cell counts for neutrophils CD66b+, M1 macrophage CD86+, M2 alternatively activated macrophages CD163+ |

In addition to our key outcome variables, we will measure the corroborating variables listed below. We will measure mRNA expression and protein abundance for select targets in skeletal muscle. We will measure skeletal muscle blood flow and endothelial function (flow-mediated vasodilation), which may be altered by histamine-dependent changes in endothelial nitric oxide synthase (NOS3/eNOS) expression. We will collect plasma samples for a targeted lipidomic panel to generate hypothesis regarding lipid mediators.

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| mRNA expression (qPCR) in muscle: IL1B, IL6, IL8, IL10, TNF, CCL2, CCL8, CX3CL1, CCL11, SELE, IL1RL1, CXCR1, CXCR2, CCR1, NOS3 |
| Protein abundance (Western), muscle homogenate: IL6, IL8, L10, TNFα, MCP1, E-Selectin, eNOS |
| Hemodynamics: Blood flow, vascular conductance, endothelial function, capillary permeability as described in Details of Methods section. |
| Lipid mediators: Plasma levels, lipid mediator panel (a targeted “lipidomics” approach) |

**Analysis & Expected Outcomes:** Our primary outcome variables are measured within subjects across time and condition (control vs. blockade). We will use a two-way repeated-measures model…. We expect that histamine-blockade will reduce circulating pro- inflammatory and pro-resolving cytokine levels. Further, we suspect that a subset of cytokines will show blunted exercise responses with histamine blockade, and that this subset will consist largely of myokines (i.e., those arising from the exercised muscle, such as IL6) and chemokines (e.g., MCP1). We expect blockade will alter the pattern of peripheral mononuclear blood cell (PBMC) phenotypes and pro-angiogenic cells, and that the CD62E+ “angiogenic monocyte” may be particularly sensitive to blockade. Lastly, we expect blockade willreduce cellular infiltration into exercised muscle of both neutrophils and macrophages. In summary, we expect these studies will identify histamine as a key regulator of exercise inflammatory responses, and may suggest tools to intervene in the inflammatory process, preserving beneficial and avoiding deleterious effects of inflammation in the context of exercise.

**Potential Problems & Alternative Strategies:** We have already documented our ability to block histamine-receptor dependent responses to exercise, including vascular and transcriptome effects. Thus, we have done considerable work setting up our primary protocol. Our team has extensive experience performing the proposed measurements. There are no hurdles to overcome in initiating this protocol, and should challenges arise in their conduct, we have extensive experience upon which to draw in troubleshooting those problems.

**Future Directions:** Knowledge obtained from this aim may set the stage for future studies on how and when to intervene in the inflammatory process, preserving beneficial and avoiding deleterious effects of inflammation in the context of exercise. For example, anti-histamines may provide a way to moderate pro-inflammatory signals without impact on pro-resolving signals, typing the balance toward recovery. Thus, one potential future direction is to generate ideas on how to reduce the deleterious sequelae associated with inflammation.

## Aim 4: Determine the histamine component of longer-term adaptations to exercise training.

**Aim 4: Determine the histamine component of longer-term adaptations to exercise training.** The mechanisms that drive the positive adaptations and health benefits of exercise training are inadequately understood5. Histamine exerts a broad impact on the exercise transcriptome3, but it is unknown whether activation of histamine-receptors is necessary to fully express the training-induced adaptations. We hypothesize that histamine-blockade will reduce training-induced gains in **4a)** capillarization of skeletal muscle, **4b)** endothelial function, **4c)** cardiovascular fitness, and **4d)** markers of cardiovascular health. We expect these loss-of-function studies will show that histamine, generally associated with pathophysiological responses (e.g., asthma, allergies, anaphylaxis, and tumor growth), is an important molecular transducer of exercise adaptations.

**Relevant Literature & Scientific Rationale:**

**Experience & Preliminary Studies**:

**Research Design & Rigor:**

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| **Aim 4a.** Capillary density; Peak vasodilatory capacity |
| **Aim 4b.** Flow-mediated dilation |
| **Aim 4c.** VO2 peak, lactate threshold, metabolic enzyme activity |
| **Aim 4d.** Arterial pressure, femoral β-stiffness |

**Analysis & Expected Outcomes:** Mixed 2x3 and 2x2 design [Measurements before training, half-way through (3 weeks), and post training (6 weeks), except biopsy based measures will only be pre and post training].

Repeated measures across time within group (Placebo vs Blockade).

Heirachy on outcomes.

Issues: clinical trial designation; subject compliance; subject dropout; missing data

**Potential Problems & Alternative Strategies:**

**Future Directions:**

## Details of Methods

**Sex as a Biological Variable.** Since responses may differ between sexes and throughout the menstrual cycle/oral contraceptive use118, we will study equal numbers of men and women with enough subjects of each sex to perform rigorous statistical comparisons across sex (treating Sex as a Biological Variable). To minimize the potential confound of differences across the menstrual cycle and with oral contraceptive use, all women subjects will be studied during the early follicular phase of the menstrual cycle or placebo phase of oral contraceptive use119,120, unless otherwise noted.

**Short-wave diathermy.**

**Intramuscular microdialysis.**

**Intramuscular temperature.**

**Mast cell degranulation bioassay.**

**femoral b-stiffness**

**Biopsy: Enzyme activity, capillary density**

**Endothelial cell proliferation/migration assays**

**VO2 peak testing.** VO2 will be measured using a mixing chamber (Parvomedics) and mass spectrometer (Perkin-Elmer MGA 1100) with subjects on an electromagnetically braked cycle ergometer (Lode Excalibur). Heart rate will be measured by 3-lead ECG (Cardiocap/5 Critical Care Monitor) and arterial pressure with an automated auscultametric device (Tango+, SunTech Medical).

**Histamine-receptor blockade.** Combined H1- and H2-receptor antagonism will be produced by oral administration of both the selective H1-receptor antagonist fexofenadine hydrochloride, administered as a single oral dose of 540 mg (Allegra, Aventis Pharmaceuticals), and the selective H2-receptor antagonist ranitidine hydrochloride, administered as a single oral dose of 300 mg (Zantac, GlaxoSmithKline). This combination of fexofenadine and ranitidine reduces sustained post-exercise vasodilation by ~ 90% following unilateral dynamic knee extension exercise115. This dosage of oral fexofenadine has been shown to selectively block H1 receptors (time to peak concentration 1.15 h and half-life 12 h), while the dose of oral ranitidine has been shown to selectively block H2 receptors (time to peak plasma concentration 2.2 h and 2.6 h half-life)121,122. Responses are 90% inhibited within 1 h and remain inhibited 6 h after administration121,123. Fexofenadine and ranitidine do not appear to cross into the central nervous system or possess sedative actions123. Furthermore, these drugs do not have any direct cardiovascular effects in the absence of histamine receptor stimulation (i.e., when given under normal resting conditions, these drugs do not elicit any changes in heart rate, blood pressure, or smooth muscle tone)114–117. Subjects will ingest the histamine receptor antagonists with water 1 h prior to exercise. We have considered the use of other H1- and H2-receptor antagonists, including those that can be administered intravenously (e.g., promethazine); however, most alternative antagonists are “first generation” antagonists that have significant cardiovascular, central neural, or anti-muscarinic effects. These doses and their effectiveness have been established in our prior work3,21,105,114–117.

**Multiplex immunoassays.** Quantification of circulating pro- and anti-inflammatory cytokines will be performed by multiplex bead-based immunoassays on a Gallios flow cytometer. Plasma samples will be diluted 1:4 prior to assay using a commercial manufacturer-validated cytokine panel (Firefly multiplex human immunoassay, Abcam). The panel will include IL1β, IL6, IL8, IL10, TNFα, MCP1, Fractalkine, and Eotaxin-1, key responders to exercise124–126.

**Lipid mediator panel.** (a targeted “lipidomics” approach)

**Flow cytometry.** Quantification of circulating CD14+ PBMCs and their subpopulations will be performed with a 10 color/3 laser Gallios flow cytometer and Kaluza Analysis Software (BD Biosciences). In brief, blood samples obtained via standard venipuncture will be prepared using a standard no-wash whole blood lysing and fixing kit (ImmunoPrep reagent system, BD Coulter) and stained with fluorochrome-labelled antibodies to identify the target cell populations. We will use lineage negative stains to remove T, B, NK, and dead cells (CD3, CD19, CD56, DAPI) and CD45 to identify leukocytes. Our target lines and probes are: 1) general neutrophil population (CD14+/CD86-/CD16+), 2) Mon1 “classical monocytes” (CD14+/CD86+/CD16-), 3) Mon2 “intermediate monocytes” (CD14+/CD86+/CD16+/CD192+), 4) Mon3 “non-classical monocytes” (CD14+/CD86+/CD16+/CD192-)92,127, and 5) “Angiogenic monocytes” (CD14+/CD86-/CD62E+) as recently identified98–100. We will also label for endothelial progenitor cells (CD34+/CD309+)95–98,128,129. We have experience with flow cytometry103,104.

**Muscle biopsies** will be obtained using the investigators standard techniques3,101,102 before and at 3 and 24 h post-exercise. The biopsies will be performed using a 5-mm Bergström biopsy needle, under sterile procedure and local anesthesia (1% lidocaine). Samples will be immediately blotted and frozen in isopentane cooled by liquid nitrogen (within seconds) and stored at –80°C until analysis.

**mRNA analysis.** Total RNA will be isolated by standard methods of homogenization, chloroform/isopropanol extraction, and re-suspension in nuclease-free water. RNA will be reverse transcribed into cDNA according to the manufacturers' directions (iScript, BioRad). Primer pairs customized using Beacon Designer 5.0 software (Premier Biosoft, Palo Alto, CA) will be designed to avoid homology (BLAST analysis) and secondary structures will be considered optimal if they produce: 1) primer efficiencies between 90 and 100% and 2) a single DNA product of predicted size as identified with a melt analysis and DNA agarose gel. Determination of relative mRNA expression will then be performed by RT-PCR using the iQ5 Multicolor Real-Time PCR cycler (BioRad). The geometric means of GAPDH and β2M, which do not change with exercise, will be used to normalize the genes of interest. We have extensive experience with these methods3,101–104.

**Protein analysis.** Portions of muscle biopsies and cell cultures will be homogenized and used to determine protein abundance in targets of interest by standard immunoblotting procedures. Briefly, homogenates will be loaded into duplicate using pre-cast any kD gels (TGX Bio-Rad) and primary and secondary antibodies will be applied on membranes after transfer. Images will be obtained with a ChemiDoc XRS imaging system (Bio-Rad). Quantification will be completed using Quantity One 1-D Software (Bio-Rad) to quantify the densitometry analysis of protein abundance and all results will be made relative to loading control (GAPDH). We have extensive experience with these methods101–104.

**Histological analysis**. Identification of selected infiltrates into skeletal muscle will be performed using portions of skeletal muscle biopsies. Sections of skeletal muscle biopsies will be cut at 8µm using a Leica Cryostat (CM1850UV) set to -20ºC. Sections will be blocked in PBS and primary/secondary antibodies will be added. Sections will be imaged on a Leica Epiflourescence microscope (Leica DM4000B). The number of positive cells for each target of interest and the total number of muscle fibers from each area of examination will be counted and then expressed as number of positively stained cells per 100 skeletal muscle fibers. We have extensive experience with these methods101,102.

**Blood flow.** Femoral artery blood flow will be measured with a Doppler ultrasound equipped with a high-resolution 10 Mhz linear array vascular probe as routinely done by the investigators19,21,114–117,130. We will simultaneously document arterial pressure, so as to calculate femoral vascular conductance.

**Flow-mediated dilation (FMD).** FMD will be measured in the brachial and superficial femoral arteries. The brachial artery and coronary arteries show remarkable similarity, and thus, analysis of the brachial artery by high-resolution ultrasound is an accepted proxy of the function of the coronary circulation131. We will measure brachial and superficial femoral artery diameter and blood velocity at baseline and for 3 min following a 5-min arterial occlusion, achieved using an occlusion cuff placed just distal to the elbow and proximal to the knee. Following release of the cuff, blood flow and shear stress are increased, and dilation of the artery occurs, largely due to the release of nitric oxide132,133. FMD will be calculated as the percent change in diameter from baseline to peak dilation, indicative of conduit vessel endothelial function. FMD is a well-established predictor of future cardiovascular events134,135 and has been performed extensively by the investigators38,106–110.

**Capillary permeability by venous congestion plethysmography.** Changes in limb volume will be measured with mercury-in-silastic strain gauges which are electronically calibrated in situ. A venous collectingcuff will be placed around the thigh proximal to the knee, and thestrain gauge will be placed around the calf at the point of maximumgirth. Our collecting cuffs use low-volume, circumferential airbladders. Pressure in the venous collectingcuff will be measured with a pressure transducer connected to thecuff via an airway. The transducers will be calibrated before theexperimental protocol, and the calibrations verified aftertheprotocol. Using the protocol of Gamble et al., the collectingcuff will be inflated to 10, 20, 30, and 40 mmHg for 4 min at each pressure (continuous protocol)136,137. Congestion cuff pressure greater than existing venous pressure causes a rapid volume change, attributable to vascular distension. When the pressure exceeds the transmicrovascular equilibrium pressure (Pvi) and the lymphatic drainage capacity, a gradual, sustained increase in limb circumference is observed. This is attributable to net fluid filtration (Jv) from the vascular bed into the interstitium. At pressure greater than Pvi, additional cuff pressure results in increases in Jv. The slope of the linear relationship between cuff pressure and Jv represents the capillary filtration capacity. The investigators have extensive experience with venous congestion plethysmography measurements111–113.

# PROGRESS REPORT: June 2013-April 2017

TEXT

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