TEMPERATURE CHANGES IN HUMAN SKELETAL MUSCLE DURING SINGLE LEG DYNAMIC KNEE EXTENSION EXERCISE

by

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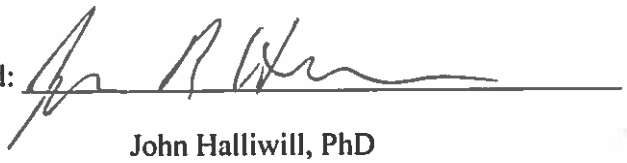
Approved: 

John Halliwill, PhD

The beneficial cardiovascular adaptations associated with endurance exercise are widely acknowledged, but the exact mechanisms mediating such adaptations are not well understood. Research has indicated that acute sustained postexercise vasodilation is induced by histamine receptor activation, and recent studies suggest that increased intramuscular histamine is responsible for this response. Currently, the specific exercise related factor or factors that trigger histamine release is unknown. Increased skeletal muscle temperature has been hypothesized as the key exercise condition that mediates histamine release; the current study is the first phase of an investigation that will ultimately address this hypothesis. This study examined the skeletal muscle temperature changes that occur during 60 minutes of single leg dynamic knee extension exercise at 60% of maximal work rate. The equilibration temperature specific to this exercise model was 39.15 ± 0.69 °C. The knowledge developed in this study will enable future studies to investigate the relationship between increased skeletal muscle temperature and histamine release.

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Introduction

Significance of Research

The leading cause of death in the world today is cardiovascular disease (CVD), one of several disease states that often manifests concurrently with obesity. In 2012 alone, roughly 17.5 million deaths were attributed to CVD, accounting for 31% of all global deaths (World Health Organization). Despite scientific knowledge indicating that a healthy diet and regular physical activity are tremendously helpful in preventing obesity and subsequent CVD, incidence of this devastating disease is increasing rapidly. Current treatments include lifestyle modifications and pharmaceutical interventions to diminish the damaging effects of the disease.

The beneficial effects of exercise on cardiovascular health are well documented and widely acknowledged by the general public. However, the exact physiological mechanisms underlying these advantageous cardiovascular adaptations are not completely understood. Our lab, led by Dr. John Halliwill, has been particularly interested in understanding the processes mediating the acute cardiovascular responses to exercise. While our research at times appears preoccupied with microscopic details, the big picture public health implications provide constant direction and motivation. Immediate goals of our work generally include understanding the effect of a specific factor on a specific mechanism mediating a specific response, but long-term goals strive to apply our detailed understanding of these mechanisms to improve health in aging or diseased populations.

Scientific Rationale

Two postexercise responses that have been investigated are hypotension and vasodilation. Hypotension describes a decrease in blood pressure. During normal conditions, blood pressure is maintained within a safe, normal range by the baroreflex, one of many homeostatic mechanisms controlled by the autonomic nervous system. After exercise, the operational range of the baroreflex is shifted to lower pressures, and blood pressure drops. This postexercise reduction in blood pressure is accompanied by vasodilation. Blood vessels, particularly arteries carrying blood from the heart to organs and tissues, have smooth muscle in their walls. Contraction or relaxation of this smooth muscle relative to baseline levels changes vessel diameter and corresponds to vasoconstriction or vasodilation. Postexercise, arteries leading to previously active skeletal muscle are vasodilated, and these wider vessels offer less resistance to blood flow. As a result, more blood is delivered to previously active skeletal muscle. Femoral artery blood flow is increased by ~50% following a single bout of moderate intensity dynamic exercise, such as cycling. The duration of this response – longer than 100 minutes – qualifies it as sustained postexercise vasodilation (Lockwood *et al.* 2005; McCord *et al.* 2006).

The mechanisms mediating these two postexercise responses have been studied extensively, but there are still gaps in the knowledge. Previous studies have indicated that histamine, a chemical most commonly associated with allergic reactions and the inflammatory response, plays a key role in postexercise vasodilation. As a signaling molecule, histamine binds to specific receptors, and these activated receptors can then evoke physiological responses. There are four histamine receptors in the human body:

H_1, H_2, H_3 and H_4. When histamine binds to any of these receptors, specific physiological effects are elicited according to the receptor type. The action of histamine receptors can be prevented by antagonists, biological molecules that bind to and block the receptor but do not provoke the normal physiological response. Anti-histamines, commonly taken to reduce allergy symptoms, are histamine receptor antagonists.

Previous research has shown that postexercise vasodilation is mediated by activation of H_1 and H_2 receptors, both of which are found on blood vessels. Blocking either receptor with fexofenadine (Allegra, a selective H_1 receptor antagonist) or ranitidine (Zantac, a selective H_2 receptor antagonist) significantly reduces postexercise vasodilation, and a combined H_1/H_2 receptor blockade using both drugs abolishes ~80% of the sustained postexercise vasodilation (McCord *et al.* 2006). These findings indicate that histamine receptor activation is a key mechanism in the pathway that leads to sustained postexercise vasodilation.

Although histamine receptor activation would seem to imply a corresponding increase in histamine concentration, this conclusion is not a given. Whereas antagonists bind to receptors and prevent their physiological action, a separate class of biological molecules known as agonists bind to and block receptors while eliciting their normal physiological responses. Agonists can be introduced via drug treatment, but they also exist under normal biological conditions. Therefore, histamine receptor activation may be achieved by either increased agonist concentration or increased histamine concentration. This uncertainty is confounded by the difficulty of measuring histamine concentration; when released or produced locally, histamine is metabolized rapidly or taken up by immune cells in the bloodstream (Halliwill, 2013). Several methods are

used to approximate histamine levels, and recent studies indicate that H_1 and H_2 receptor activation is indeed mediated by increased histamine concentration (Romero, 2015). Nonetheless, appreciating other potential explanations is essential to proper experimental design and analysis.

There are two potential sources of increased intramuscular histamine levels after endurance exercise: mast cell degranulation and de novo (new) formation. Mast cells are immune cells found in most tissues of the body, including skeletal muscle. Granules, or sacs, within mast cells contain several chemicals, including histamine, that initiate and mediate the inflammatory response (Hall, 2010). When mast cells degranulate, they release the contents of their granules. Mast cell degranulation, therefore, increases intracellular histamine concentration.

Alternatively, de novo formation could increase histamine levels. Histamine is synthesized from histidine with the help of an enzyme called histidine decarboxylase (HDC). As an enzyme, HDC facilitates the conversion of histidine to histamine. Studies have shown increased HDC mRNA expression and enzyme activity in mice following prolonged exercise; these responses would induce greater de novo formation of histamine (Graham *et al.* 1964; Ayada *et al.* 2000). Recent work has shown that inhibition of HDC significantly blunts the rise in histamine concentration after exercise. The same study also supports the notion that exercise induces mast cell degranulation (Romero *et al.* 2015). These findings suggest that both de novo formation via HDC and mast cell degranulation contribute to the rise in intramuscular histamine following exercise, but HDC appears to play a more significant role.

The specific exercise related factor that leads to histamine release, via either mast cell degranulation or de novo formation, is currently unknown. Exercise induces many physiological conditions, – mechanical stress, oxidative stress, increased temperature, etc. – several of which could potentially evoke histamine release. Previous work has hypothesized a link between mast cell degranulation and exercise related factors such as cytokines, reactive oxygen species, increased temperature and vibration. Additionally, oxidative stress and shear stress are hypothesized to increase HDC activity, facilitating de novo formation of histamine (Halliwill *et al.* 2013). Importantly, the factors listed here are merely hypothesized as potential explanations; there are many other possible causes of postexercise histamine release.

Cytokines, a potential stimulus for mast cell degranulation, are cell-signaling molecules involved in a variety of cellular responses. Reactive oxygen species are chemically reactive molecules that contain oxygen, and they are produced during exercise via aerobic metabolism. These molecules may cause mast cell degranulation. High concentrations of reactive oxygen species can damage cell structures; this process is known as oxidative stress and is hypothesized to increase HDC activity. Increased muscle temperature and vibration occur due to the mechanical and metabolic processes involved with endurance exercise, and both may lead to mast cell degranulation. Additionally, research using isolated cells has demonstrated a linear relationship between temperature and HDC activity (Savany and Cronenberger, 1982). Finally, shear stress, the force exerted on vessels as blood flows through, may increase HDC activity after exercise.

Purpose

Determining the specific exercise related factor that induces histamine release is the next key step in the understanding of sustained postexercise vasodilation. Recent research has indicated that oxidative stress is not the exercise related stimulus that triggers histamine release (Romero *et al.* 2015). The current investigation, part of which is included in this thesis, seeks to determine if increased skeletal muscle temperature is a stimulus for histamine release. The research presented in this thesis examines the temperature changes that occur in skeletal muscle during 60 minutes of single leg dynamic knee extension at 60% of maximal work rate. This investigation has developed the knowledge necessary to determine if increased skeletal muscle temperature induces histamine release. Ultimately, this research will add to our expanding knowledge of sustained postexercise vasodilation and position us one step closer to the central goal of exploiting these mechanisms to help aging and diseased populations.

Thermodynamic Principles

Anticipating and understanding muscles' temperature changes with exercise requires a comprehensive understanding of the principles of thermodynamics as they pertain to skeletal muscle. Thermodynamics is a branch of physics that studies the relationships between temperature, heat, work and energy. The foundational principles are encoded in the four laws of thermodynamics, two of which are particularly pertinent to this study.

The 0^{th} law of thermodynamics states that if two systems are each in thermal equilibrium with a third system, all three systems must be in thermal equilibrium (Giancoli, 2012). This basic principle underlies every day assumptions about

temperature, a measure of an object's average kinetic energy. Kinetic energy is energy associated with movement (Giancoli, 2012). Particles moving very rapidly have high kinetic energy and therefore a high temperature, while particles moving slowly have both low kinetic energy and temperature. When a collision occurs between two molecules, one of high kinetic energy and one of low kinetic energy, energy is transferred from a high to low energy state (Giancoli, 2012). This collision transfers heat. Heat is energy transferred between objects; once heat is acquired by an object it becomes internal energy. Internal energy is the sum of all the energy of all the molecules in an object, and it includes kinetic energy (Giancoli, 2012). Increasing the internal energy of an object increases its temperature, and the process of transferring heat to an object is properly termed heating. The tendency of higher energy molecules to transfer heat to lower energy molecules in a collision drives systems towards thermal equilibrium.

The 1st law of thermodynamics states that energy cannot be created nor destroyed (Giancoli, 2012). This law implies that kinetic energy can only be increased or decreased by way of energy transfers between systems. This law appears in various forms in many branches of physics, with the basic assumption that energy is conserved across all reactions and interactions.

Heat Exchange in the Natural World and Biological Systems

In the natural world, heat exchanges between systems occur via three possible processes: conduction, convection and radiation. These transfers only occur when systems are in different energy states; the energy differential is referred to as an energy

gradient, and heat travels down the gradient from a higher energy object to a lower energy object.

Conductive heat transfer occurs when atoms or molecules collide. Atoms or molecules of high kinetic energy vibrate rapidly, and they transfer heat to molecules of lower kinetic energy when collisions occur. This mode of heat transfer requires direct contact between two objects to permit molecular collisions to occur.

Convection describes heat transfer that is mediated by fluid movement. Fluids are substances with no fixed shape that are able to flow freely; both liquids and gases are categorized as fluids (Giancoli, 2012). With convective heat transfer, two systems not in direct physical contact may exchange kinetic energy via a fluid that bridges the space between them. In the human body, blood flow provides the ideal situation for convective heat exchange.

Radiation permits heat transfer by means of infrared waves. Unlike conduction and convection, no kinetic energy is transferred between adjacent particles; this allows radiation to pass through the vacuum of space. All objects emit and absorb infrared waves, but some surfaces have greater capacities for reflecting or absorbing those waves, and the actual amount of infrared radiation emitted by an object is proportional to its temperature (Giancoli, 2012). If two objects are composed of the same material, with the same capacity for emission and absorption of infrared waves, the warmer object will emit more radiation.

In biological systems, energy production and consumption also occur during chemical reactions. Chemical bonds contain varying amounts of energy; some are high energy bonds, others are low energy. Breaking a bond releases the energy associated

with it, and some of that energy is lost to the environment as heat. Energy generating reactions are termed exothermic, while reactions that form bonds and require energy input are endothermic (Giancoli, 2012). The ideal biological process would harness all of the energy produced in an exothermic reaction and apply it to an endothermic reaction. Alas, biological processes are not perfect, and some energy is always lost as heat.

Heat Production And Dissipation in Active Skeletal Muscle

In skeletal muscle, temperature is determined by the balance of local heat production and heat exchange via conduction and convection (Taylor, 2014). All metabolic processes, in muscles and all tissues of the body, require energy input; this energy is derived from exothermic chemical reactions, and some energy is lost as heat. Muscle contractions produce considerable amounts of heat that is dissipated by increased blood flow.

Heat Production During Exercise

Muscle contractions, the means by which movement is produced, require energy input and produce heat. Specifically, muscle contractions are powered by the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). ATP is often referred to as the biological energy currency; it stores a remarkable amount of energy and is almost always the preferred cellular energy source (Stock, 1999). Hydrolysis of ATP simply removes one of the three phosphate groups attached to adenosine; hence, triphosphate becomes diphosphate, but adenosine is unchanged. The reaction is catalyzed by the enzyme class known as the ATPases, and the balanced reaction yields

ADP and a free phosphate ion (P_i). High energy bonds are broken in this exothermic reaction, and energy is released. Some of the energy released from the hydrolysis of ATP is harnessed to power the muscle contraction, but up to 70% is lost as heat (Lim, 2008).

In order to sustain exercise, ATP must be produced at a rate equal to its consumption. During endurance exercise, ATP can be produced very effectively in the mitochondria, a specialized cell component, when oxygen is available. This ATP generating process is termed oxidative metabolism, and it involves many enzymatic steps that convert glucose to ATP. The complex pathway yields 32 ATP molecules per molecule of glucose input, but 66% of the potential energy stored in glucose is lost as heat during the process (Powers, 2007).

Challenges Posed to the System During Endurance Exercise

Endurance exercise presents many challenges that the body must manage adequately in order to maintain an environment conducive to sustained exercise. As ATP is hydrolyzed to liberate energy for contraction, it must be produced at an equal rate. ATP production via oxidative metabolism can only occur if adequate oxygen is available; strenuous exercises increases oxygen demand up to twenty times normal levels (Hall, 2010). Increased oxygen demand, therefore, is a significant challenge posed to the system during endurance exercise. Additionally, both ATP hydrolysis and oxidative metabolism produce considerable amounts of heat that increase local temperatures and pose a threat to the system. Increased oxygen demand and temperature are two of the many challenges associated with endurance exercise, and both can typically be controlled adequately with augmented blood flow.

Increased blood flow to active muscle improves convective heat transfer and dissipates heat to other regions of the body, the environment, or both. Ultimately, most of the heat produced during exercise must be dissipated to the environment in order to defend mean body temperature. The first step of this process is increased blood flow to the active skeletal muscle, which ushers the heat away from the site of production.

Mechanisms of Increased Blood Flow to Active Skeletal Muscle

Two situations must occur to permit increased blood flow to active skeletal muscle. First, cardiac output, the total volume of blood pumped by the heart per minute, must increase. Second, the augmented cardiac output must be redistributed towards the active skeletal muscle and away from inactive, non-essential tissues. Fulfillment of these two conditions yields increased blood flow to active skeletal muscle, a response known as exercise hyperemia.

Cardiac output increases linearly with work rate (Powers, 2007). Cardiac output (Q) is the product of stroke volume (SV), the volume of blood pumped by the heart per contraction, and heart rate (HR), $Q = HR \cdot SV$. Resting cardiac output is around 5 liters per minute; during exercise, cardiac output can increase to 13 liters per minute in an average individual and 30 to 40 liters per minute in a trained endurance athlete (Hall, 2010). This augmented cardiac output is mediated by increases in both stroke volume and heart rate. Both increase linearly with work rate, although stroke volume plateaus at roughly 40% of maximal work rate (Hall, 2010). Varied and complex mechanisms yield these responses, but increased sympathetic nervous system activity contributes significantly to both responses.

The sympathetic nervous system also plays a key role in redistributing the augmented cardiac output during exercise. Sympathetic control mediates vasoconstriction of the vessels leading to the skin, splanchnic organs, and all skeletal muscle; this results in diminished blood flow. Sympathetic control simultaneously vasodilates coronary vessels, providing increased blood flow to the contracting heart (Powers, 2007). Active skeletal muscles do not receive increased blood flow as a consequence of sympathetic control; instead, a mechanism known as functional sympatholysis blunts the sympathetic vasoconstriction while local substances induce vasodilation to match blood flow to metabolic demands.

Functional sympatholysis diminishes the effect of sympathetic vasoconstriction in vessels leading to active skeletal muscle. Sympathetic vasoconstriction is caused by the release of norepinephrine onto ∝-adrenergic receptors. This mechanism, just like the histamine receptor mechanism, yields a physiological response. In this case, the vascular smooth muscle contracts and vasoconstriction follows. Active skeletal muscle releases substances that impair the responsiveness of ∝-adrenergic receptors and, consequentially, blunt the sympathetic vasoconstriction (Saltin and Mortensen, 2012). Functional sympatholysis increases blood flow back towards resting levels, but it does not account for the increased flow seen in active skeletal muscle.

Two competing theories exist to explain the augmented blood flow to active skeletal muscle: the vasodilator theory, and the oxygen lack theory. The vasodilator theory contends that tissues with insufficient blood flow to match their metabolic demands release vasodilator substances, such as adenosine. These vasodilators increase the diameter of vessels leading to the specific tissue, and blood flow increases. The

oxygen lack theory asserts that blood vessels naturally dilate in hypoxic conditions, increasing blood flow and oxygen delivery.

Regardless of the specific stimulus, vasodilation of the vessels leading to the active skeletal muscle ultimately results in exercise hyperemia. Like fluid flow through any tube, blood flow is a function of the pressure difference (ΔP) between the two ends and the resistance to flow (R). Ohm's Law describes this relationship with the equation $F = \frac{\Delta P}{R}$. Resistance, in turn, is determined by the viscosity of the blood (η), the length of the vessel (L) and the radius of the vessel (r), given by the relationship $R \propto \frac{\eta \cdot L}{r^4}$. Resistance is inversely proportional to the fourth power of radius; this means that a slight change in vessel radius will significantly impact resistance. Integrating the equations for flow and resistance yields Poiseuilles's Law, $F \propto \frac{\Delta P \cdot r^4}{\eta \cdot L}$ (Hall, 2010). This relationship indicates that the single variable with the greatest influence over blood flow is vessel radius. Indeed, exercise hyperemia is mediated by increased radius in the vessels leading to active skeletal muscle.

Increased blood flow to active skeletal muscle supplies necessary oxygen and dissipates heat produced by contraction. Convective heat exchange between active muscle and circulating blood ushers heat away from the site of production, but further heat transfer to the environment is vital to defend mean body temperature.

Heat Dissipation to the Environment

Ultimately, the heat produced by muscular contractions travels to the skin to be dissipated to the environment, facilitated by the temperature gradient between deep

body tissues and the environment. This gradient is maintained due to constant heat production within the body's tissues and constant heat exchange with the environment.

The skin is highly effective at dissipating heat via radiation, convection, and evaporative cooling (sweating). In a normal room temperature environment, sweating accounts for 25% of heat loss and convective and radiative heat transfers contribute evenly to the remaining 75% (Arens and Zhang, 2006). When the body encounters thermal stress, sweating becomes a crucial mechanism to transfer heat from the body to the environment and regulate body temperature within a safe range. During physical exertion, roughly 80% of heat produced is dissipated to the environment by evaporative cooling (Gisolfi and Mora, 2000).

Resting Skeletal Muscle Temperature

Resting skeletal muscle temperature is not uniform. Instead, a temperature gradient exists from superficial to deep muscle, with superficial muscle typically at a lower temperature than deep (Kenny *et al.* 2003; Saltin *et al.* 1968). This gradient is consistent with the principles of thermodynamics that dictate heat exchange and dissipation towards superficial body tissues. Importantly, the temperature gradient in skeletal muscle is not universal; blood vessels create local temperature variations, as do metabolic processes. Nonetheless, a temperature gradient generally exists that drives heat dissipation towards superficial tissues and, ultimately, to the external environment.

At rest, average skeletal muscle temperature is lower than the body's core temperature (Webb, 1992; Snellen, 1969). According to the core-shell shell model of body thermodynamics, inactive skeletal muscle is part of the body's shell and contributes to heat dissipation (Webb, 1992).

Skeletal Muscle Temperature During Exercise

The balance of heat production and heat dissipation determines skeletal muscle temperature during exercise. Heat production is directly proportional to work rate; when work rate is fixed, heat production is constant as well (Taylor, 2014). Exercise hyperemia increases convective heat exchange and dissipates some, but not all, of the heat produced during contraction. During steady state exercise, an equilibration temperature is eventually reached that represents a harmonious matching of work rate and blood flow. Equilibration time and temperature are dependent on the relative workload (Taylor, 2014; Saltin *et al.* 1968).

The resting temperature gradient between body core and skeletal muscle reverses during intense exercise (Kenny *et al.* 2003; Webb, 1992; Snellen, 1969). Kenny *et al.* recorded intramuscular temperature and esophageal temperature, as a surrogate for core temperature, while subjects engaged in 15 minutes of bilateral knee extension at 60% of maximal oxygen consumption. The resting temperature gradient of – 1.15 °C between muscle and core reversed to +0.90 °C by the end of exercise. Other studies have suggested that the core may act as a heat sink during intense exercise to maintain skeletal muscle temperature within a functional range (Brooks *et al.* 1971).

Kenny *et al.* also found that the resting temperature gradient within skeletal muscle disappears rapidly at the onset of exercise. Prior to exercise, there was a distinct gradient between deep (36.14 °C), mid (35.86 °C) and superficial muscle (35.01 °C). Five minutes after the initiation of exercise, muscle temperature had nearly equalized at all three depths. Accordingly, the three depths exhibited different rates of temperature increase. Superficial muscle showed the greatest rate of temperature increase (0.61 ±

0.19 °C/min) and deep muscle showed the slowest rate of increase (0.22 ± 0.09 °C/min). The rate of temperature increase declined throughout exercise, but temperatures at all three intramuscular sites were still increasing at the end of the 15-minute exercise protocol. The three depths reached temperatures between 38.21 °C and 38.23 °C by the end of exercise. Presumably, intramuscular temperature would have continued to increase before eventually plateauing at the equilibrium temperature (Taylor, 2014).

Extensive work by Saltin *et al.* reported different intramuscular temperature responses to exercise (1968; 1972). The group's 1968 study found that the gradient between deep and superficial skeletal muscle is maintained during submaximal exercise. The 1972 study used a bicycle ergometer model to examine intramuscular temperatures during exhaustive exercise at three different relative work rates (90%, 100% and 115% of maximal oxygen uptake) and varying ambient temperatures (10 – 40 °C). Muscle temperatures of 39 – 40 °C were consistently observed at exhaustion, regardless of ambient temperature, work rate and exercise duration.

No existing study corresponds to the exercise model, intensity and duration used in previous investigations of postexercise responses. Further, previous studies have provided conflicting data regarding the nature and magnitude of skeletal muscle temperature changes during exercise.

Hypotheses

It was hypothesized that the resting skeletal muscle temperature gradient would equalize rapidly following the onset of exercise. Additionally, it was hypothesized that intramuscular temperatures at all three depths would reach an equilibration temperature of 40 °C. The long-term hypothesis driving the current investigation proposes that increased skeletal muscle temperature induces histamine release postexercise.

Methods

Experimental Outline

The studies included in this thesis were approved by the Institutional Review Board (IRB) at the University of Oregon (Protocol # 02172011.029). The ultimate question targeted with this protocol is does increased skeletal muscle temperature induce histamine release? This question can only be answered after performing two preliminary studies: experiment one to determine the muscle temperature response induced with the exercise model used in previous studies, and experiment two to develop the methodology to experimentally generate the appropriate temperature increase. Experiment three will combine the methodology established in experiment two with existing methods to determine if increased skeletal muscle temperature mediates histamine release. This thesis addresses experiment one; graduate students will continue the study with experiments two and three in the coming months. Experiment one aimed to understand the temperature changes that occur in skeletal muscle during 60 minutes of single leg dynamic knee extension (DKE) at 60% of maximal work rate.

Experiment One Methods

Experiment One Outline

Six subjects participated in experiment one, 5 males and 1 female. The experiment included three laboratory visits that lasted approximately five hours total: an initial prescreen, a peak test, and a study day. All study visits occurred in the Evonuk Environmental Physiology Core (EEP Core). Subjects were compensated at a rate of $15 per hour with funds from the National Institutes of Health, for a total of $75.

Prescreen Methods

The prescreen was performed by the lab coordinator and used to determine prospective subjects' eligibility for the study. Eligible subjects were healthy, non-smoking males and females, age 18-40, that were sedentary or recreationally active, but not engaging in more than five hours of exercise per week. Potential subjects were excluded if they were taking medications (oral contraceptives were acceptable), herbal remedies or dietary supplements, if they were pregnant or breastfeeding, or if they were using illegal or recreational drugs. Subjects were also excluded for known allergies to drugs or medications, especially lidocaine. Eligible subjects were informed of the experimental methods, risks and benefits and given the opportunity to ask any questions. Written informed consent was obtained at the end of this process, and subjects were reminded that they could withdraw from the study at any time. Enrolled subjects were assigned a 3-digit subject ID that was used to protect their privacy on all study documents.

Peak Test Methods

Prior to the peak test, subjects were instructed to refrain from exercise, alcohol, caffeine and medication (except birth control) for 24 hours. Subjects were also informed to arrive to the EEP Core fasted for at least two hours.

A pregnancy test was administered to female subjects upon their arrival to the lab, and a negative result was required to proceed with the visit. The height and weight of the subject was recorded, and a small venous blood sample of less than 10 ml was taken. A trained member of the lab completed the blood draws, and the samples were used in an ongoing large-scale genomic analysis.

Subjects were instrumented with a three lead electrocardiogram (ECG) that displayed heart rate and provided a representation of the electrical activity of the heart. Subjects then sat down on the kicking ergometer and any necessary seat and back adjustments were made.

Prior to initiation of the peak test, subjects were informed of the details of the test and given the opportunity to ask questions. Subjects were instructed to match their 30-second mean power output to the target power output while maintaining their kicking rate within the target range. All of these parameters were displayed on a computer display in front of the subject. Subjects were also instructed to continue with the peak test until they were physically unable to match the target work rate.

The peak test began with a 5-minute warm-up on the kicking ergometer at 5 watts of power; this time period also allowed subjects to familiarize themselves with the ergometer and the associated computer display. Following the 5-minute warm-up, the peak test ramp protocol was initiated. This protocol increased the subject's power output by 3 watts every minute, starting at 10 watts. Data collection during the peak test was done using a standardized data sheet. Work rate (power output, measured in watts) and heart rate were recorded every 30-seconds. Throughout the test, researchers offered words of encouragement and urged subjects to push themselves to their physical limits. The peak test was terminated when subjects felt they had reached their maximal exertion, or when subjects were no longer able to match the required power output.

Upon termination of the peak test, subjects continued kicking at a low work rate to recover. Once subjects were sufficiently recovered, their ECG electrodes were

removed. Subjects were offered a Gatorade and granola bar, thanked for their participation, and escorted out of the EEP Core.

The data collected during the peak test was used to determine the subject's work rate for the study day. 60% of the maximal work rate reached in the peak test was set as the subject's work rate for the study day. This methodology maintained the same relative exercise intensity for all subjects, regardless of their level of physical fitness.

Study Day Preparations

Prior to the study day lab visit, the thermocouples used to measure intramuscular, cutaneous and ambient temperatures were calibrated. The thermocouples were placed in a water bath of known temperature and the temperature readings were compared. Two calibrations were performed: one at roughly 21 °C and a second at approximately 41 °C. These two temperatures bracketed the anticipated intramuscular temperatures. Following the calibration, the thermocouples were sterilized and stored until their use.

On the study day, a Mayo tray with the necessary sterile items for the study was prepared before the subject's arrival. Once the tray was prepared, standard precautions were observed to maintain the sterile field.

Study Day Methods

Subjects were instructed to abstain from exercise, alcohol, caffeine and medication (except birth control) for 24 hours prior to the study day. Subjects were also asked to arrive to the EEP Core fasted for at least two hours.

Female subjects were administered a pregnancy test upon their arrival to the EEP Core, and participation in the study was incumbent on a negative result. All subjects were encouraged to use the restroom before beginning the study. Subjects were instrumented with a three lead ECG and blood pressure cuff and instructed to lie supine (face up) on a padded table. Ambient temperature, humidity and barometric pressure were recorded at the beginning of the study.

Longitudinal ultrasound was used to locate the pennation angle, or muscle fiber orientation, of the subject's vastus lateralis, the most lateral muscle of the quadriceps muscle group. Once the pennation angle was located with ultrasound, a sharpie marker was used to mark two dots corresponding to the fiber orientation.

Next, the sterile field was established. Two qualified researchers put on surgical masks and sterile gloves. A sterile drape was placed under the subject's experimental leg and chloraprep was applied to the leg in a circular fashion, radiating from the anticipated insertion site. Sterile scissors were used to cut a window in a sterile drape, and the drape was secured to the subject's skin with steri-strips. The window in the drape allowed access to the work area.

Next, the insertion area was anesthetized using a mixture of sodium bicarbonate and lidocaine. Using a 10 mL Luer Lock syringe, 2 ml of sodium bicarbonate was injected into 20 ml of lidocaine, and the vial was rotated to mix the solution. The addition of sodium bicarbonate neutralized the acidic lidocaine and decreased the pain associated with injection. 10 cc of the lidocaine sodium bicarbonate solution was drawn and approximately 1 cc was injected subcutaneously at the insertion site. Once the subject reported minimal cutaneous sensation at the insertion site, an additional

lidocaine injection of 1 cc was performed to anesthetize the fascia. Care was taken not to pass into the muscle body.

Once the subject's insertion site was adequately anesthetized, a sterile 18-gauge needle was used to pierce through the skin and muscle fascia and then removed. The needle was introduced parallel to the pennation angle marked on the skin surface. With the skin and fascia already pierced, an 18-gauge introducer needle was inserted through the skin, past the fascia and into the superficial layer of the vastus lateralis. The internal portion of the needle was removed, and the temperature probe containing all three intramuscular thermocouples was threaded through the needle in its place. The three thermocouples were located 0 mm, 15 mm and 30 mm from the tip of the probe. The remaining external layer of the needle was carefully removed, leaving only the probe placed in the vastus lateralis. The probe was secured to the subject's skin using sterile tegaderm; the tegaderm was placed directly over the insertion site to prevent the intramuscular thermocouples from moving during exercise.

Removing the introducer needle entirely was impossible because it was wrapped around the probe, which terminated in plastic adapters. For this reason, the needle was wrapped in microfoam tape to reduce the risks associated with an open needle. The terminal ends of the intramuscular thermocouples were attached to the WinDaq machine and intramuscular temperature recording began; a stopwatch was started concurrently.

Cutaneous thermocouples were placed on both the exercising and non-exercising legs. The air thermocouple was located directly behind the subject, and the

two cutaneous and air thermocouples were connected to the WinDaq machine and data recording began.

The thermocouples were allowed to stabilize for 1 hour before initiating the exercise portion of the study. During stabilization, temperature recordings from all six thermocouples were recorded at 15-minute intervals on a standardized data sheet, using the stopwatch started when the intramuscular thermocouples were connected. An ultrasound image of the temperature probe within the vastus lateralis was also obtained during the stabilization period. Upon stabilization of all six thermocouples, subjects were transitioned from the table to the ergometer. Researchers assisted subjects to ensure that the various wires transitioned to the ergometer safely and successfully.

Exercise was started 10 watts below the target work rate (60% of the work rate reached during the peak test). Work rate was increased by 1 watt every minute; using this ramp protocol, the target work rate was reached at 10 minutes. The target work rate was maintained for the remainder of the hour of exercise. Subjects were offered the option of watching Netflix or listening to music to pass the time. Researchers offered encouragement and regularly inquired about the subject's comfort and physical condition. Work rate, heart rate, systolic and diastolic blood pressure and temperature readings from all six thermocouples were recorded every five minutes during exercise using a standardized data sheet. A comment was also made in the WinDaq software at five-minute intervals. The insertion site of the thermocouples was monitored throughout exercise, and small amounts of blood and sweat were controlled with gauze.

After 60 minutes of exercise, subjects recovered passively while sitting on the ergometer. Once subjects recovered sufficiently, they transitioned back to the table. A

postexercise ultrasound image of the intramuscular temperature probe was obtained, and the probe was removed. The cutaneous thermocouples, ECG electrodes and blood pressure cuff were also removed. Subjects were offered a Gatorade and granola bar and compensated $75 for their time. Once any remaining questions or concerns were addressed, subjects were escorted out of the EEP Core and thanked for their participation in the study.

After each study, the thermocouples used were calibrated again to ensure accuracy. This calibration was performed in a similar manner to the first; one calibration was done in a cool water bath and one in a warm water bath.

Vastus Lateralis Depth Measurements

As supplementary knowledge for all three experiments in this protocol, ultrasound images of the vastus lateralis were taken to determine mean muscle depth. The subjects for this supplementary research were 9 consenting lab members (4 females and 5 males). Longitudinal ultrasound was used to obtain a quality image clearly showing the delineations between the subcutaneous fat, the superficial vastus lateralis, and the superficial aponeurosis.

Data Processing and Analysis

Thermocouple Calibration

A standard calibration with mean values from every thermocouple used in the study was applied to all thermocouples. Differences between individual thermocouples were slight, and did not warrant individualized calibrations. The standard calibration corrected each thermocouple by the same margin.

WinDaq Temperature Data

Mean temperature was extracted from the WinDaq file in five-minute intervals for each of the six thermocouple sites. Mean temperatures during the final five minutes of stabilization were used as resting measures. The comments placed at five-minute intervals during exercise expedited the procurement of five-minute mean temperature data during the 60 minutes of exercise. Five-minute mean data was chosen instead of instantaneous data to account for any transient temperature variations.

Ultrasound Image Analysis

The ultrasound images taken of probe placement and muscle depth were measured using the caliper tool integrated into the machine. Thermocouple location was approximated by tracing the length of the temperature probe and determining the location of the three thermocouples (0, 15 and 30 mm from the probe tip). Once the location of the thermocouples was determined, depth was measured by drawing a straight line to the top of the image (the skin surface) with the caliper tool.

Results

Subjects

Although six subjects participated in the study, one was unable to begin exercise. Results include data from the five subjects that completed the study.

Heart Rate

Mean heart rate at five minutes into exercise was 77.6 beats per minute (BPM). By the end of exercise, mean heart rate had reached a steady state of about 97 BPM. Considerable variation was seen between subjects, but every subject displayed the general pattern of rapid heart rate increase early in exercise followed by a relative plateau at steady state.

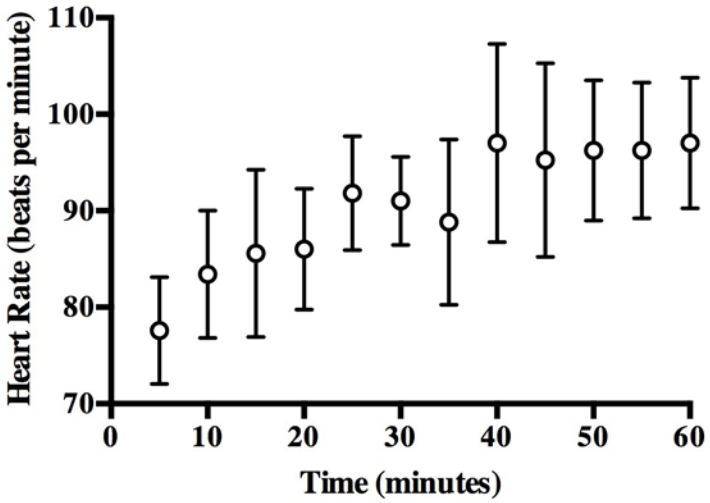

Figure 1: Heart rate mean and standard deviation during exercise

Intramuscular Thermocouple Depth

Ultrasound images of the temperature probe within the vastus lateralis indicate that the three intramuscular thermocouple sites did not correspond to three distinct

intramuscular depths. Ultrasound images of the probe placement were only obtained for three of the five subjects, and all three thermocouple sites were only visible on one ultrasound image; the other two images included only the two most distal sites. Depth measurements of these three images indicated that the three sites were more similar in depth than planned, and the slight depth differences observed did not correspond to the anticipated order from distal to proximal thermocouple sites. The deepest thermocouple measured was 1.33 cm, the mean depth was 0.88 cm and the median depth was 0.764 cm. All of the thermocouples visualized with ultrasound were clearly located within the superficial vastus lateralis.

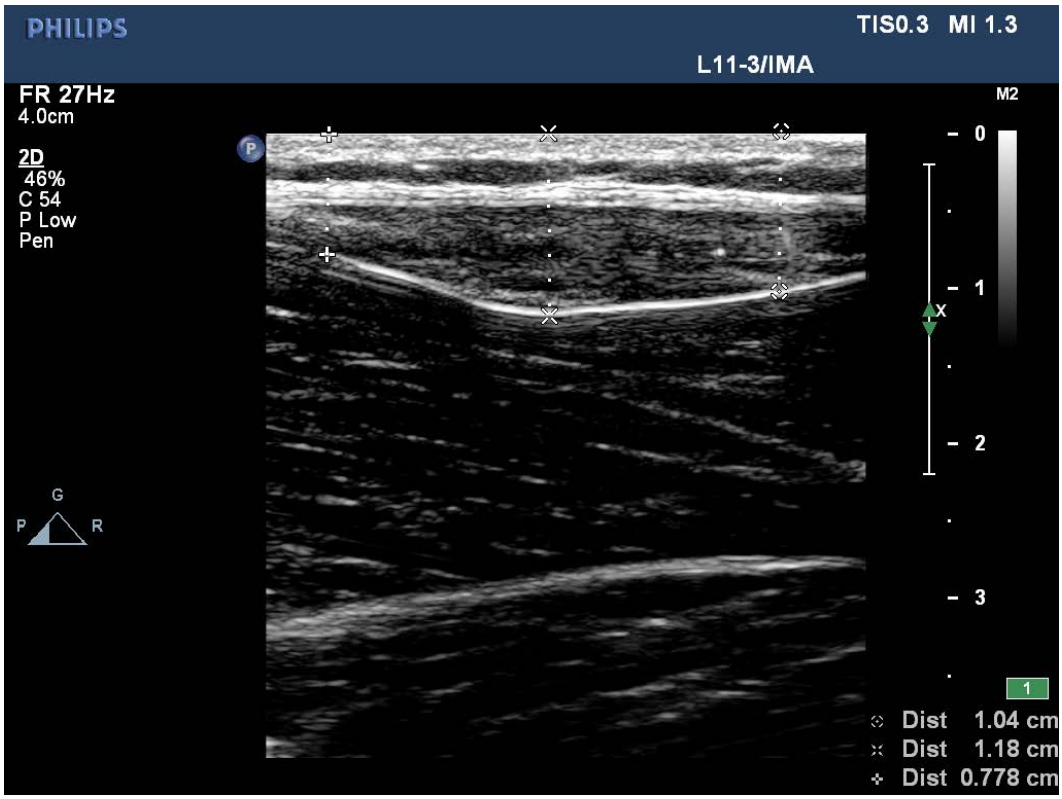

Figure 2: Representative ultrasound image of the temperature probe within the vastus lateralis

The probe appears bright white on ultrasound, and the tip is visible on the left side of the image. The three thermocouple sites are indicated with asterisks or crosses.

Vastus Lateralis Depth Measurements

Based on data from 9 subjects, mean subcutaneous fat depth was 0.68 ± 0.22 cm and mean aponeurosis depth was 2.69 ± 0.62 cm. The superficial vastus lateralis lies between the subcutaneous fat and the aponeurosis; its mean depth was 2.00 ± 0.57 cm. This data indicates that the intramuscular thermocouples visualized with ultrasound were positioned in the superficial part of the superficial vastus lateralis. Unfortunately, the three intramuscular temperature sites likely correspond more accurately to three

superficial locations, not three distinct muscle depths. For this reason, mean intramuscular temperatures reported here include all three sites.

Resting Skeletal Muscle Temperature

The mean intramuscular temperature at the end of the stabilization period was 34.59 ± 1.15 °C (range 33.71 °C to 35.43 °C).

Skeletal Muscle Temperature Changes During Exercise

Intramuscular temperature increased rapidly during the initial stages of exercise, and the rate of increase gradually declined until a steady state equilibration temperature was reached. Figures 3 and 4 demonstrate that the equilibration temperature was, on average, achieved after 30 minutes of exercise. The mean steady state intramuscular temperature during the last 30 minutes of exercise was 39.15 ± 0.69 °C (range 38.31 °C to 39.81 °C). The mean change in temperature from rest to equilibration during exercise was 4.59 ± 1.24 °C (range 3.28 °C to 6.44 °C).

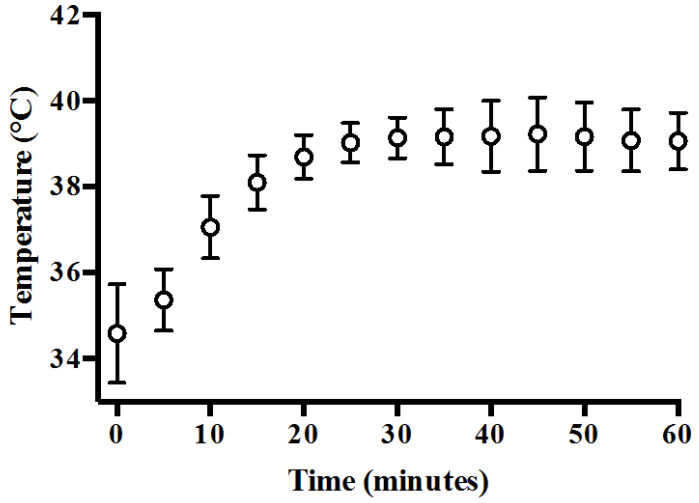

Figure 3: Mean intramuscular temperature during exercise

Mean intramuscular temperature increased rapidly during the initial stages of exercise and reached a steady state after 30 minutes. Mean intramuscular temperature at rest was 34.59 ± 1.15 °C, and the mean intramuscular temperature during the final 30 minutes of exercise was 39.15 ± 0.69 °C. Error bars represent standard deviation.

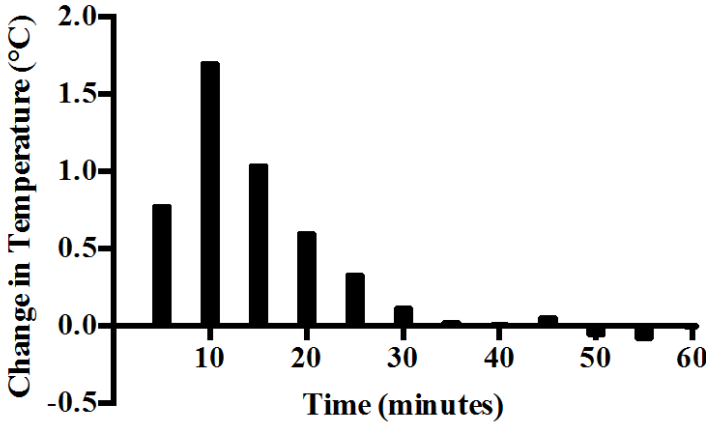

Figure 4: Change in mean intramuscular temperature at five-minute intervals during exercise

Mean intramuscular temperature changes over five-minute intervals were large during the first 10 minutes of exercise and then gradually decreased. From 30 minutes to 60 minutes, mean intramuscular temperature changes were minimal.

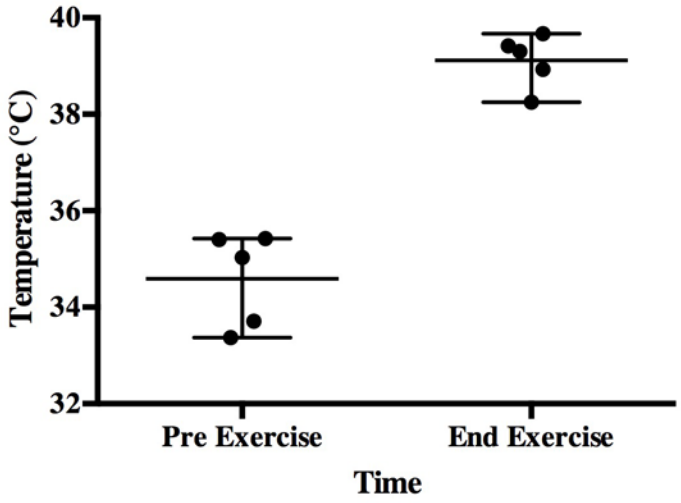

Figure 5: Mean intramuscular temperature pre exercise and end exercise with individual subject values

Mean intramuscular temperature is indicated by the long horizontal line, and the shorter horizontal lines indicate the range. Individual subject values are indicated by black dots. Mean intramuscular temperature increased from pre exercise (34.59 ± 1.15 °C) to end exercise (39.11 ± 0.55 °C).

Convergence of Intramuscular Temperatures

The temperature range of the three intramuscular sites decreased from rest to steady state exercise. During the last five minutes of stabilization, the mean range of intramuscular temperatures was 1.41 ± 0.90 °C. The mean range of intramuscular temperatures increased during the first 10 minutes of exercise to 1.51 ± 0.55 °C. However, intramuscular temperature range rapidly decreased after 20 minutes of exercise and remained consistent until the end of exercise. During this 40 minute interval, the mean intramuscular temperature range was 0.69 ± 0.49 °C.

Cutaneous and Ambient Temperatures

Mean ambient temperature fluctuated slightly during exercise, with a maximum temperature of 25.31 °C and a minimum temperature of 23.65 °C. Mean cutaneous temperatures were very similar between the exercise (32.69 ± 0.82 °C) and non-exercise leg (32.91 ± 0.97 °C) at rest. By the end of exercise, exercise cutaneous temperature increased to 34.31 ± 0.87 °C and non-exercise cutaneous temperature decreased to 30.72 ± 0.98 °C.

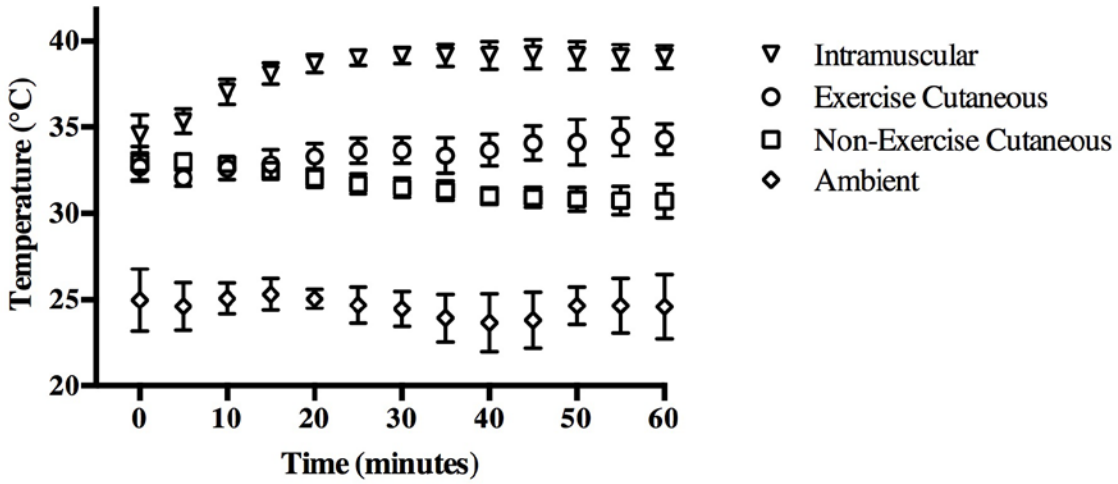

Figure 6: Intramuscular, cutaneous and ambient temperatures during exercise

Discussion

Cutaneous Temperatures

The cutaneous temperature response observed in this study is consistent with existing knowledge. A crucial component of increased blood flow to active skeletal muscle is an accompanying decrease in blood flow to other non-essential tissues, including the skin. Decreased cutaneous blood flow produces a corresponding fall in cutaneous temperatures. Torii *et al.* reported a mean skin temperature drop of 1 °C on the upper half of the body during during cycling exercise, regardless of ambient temperature (1992).

While cutaneous temperature decreased during exercise on the non-exercising leg, it increased on the exercising leg. This response can be attributed largely to vasodilation in response to heat production and conductive heat exchange between the muscle, subcutaneous fat and skin.

Probe Placement and Intramuscular Thermocouple Depth

Placement of the intramuscular temperature probe did not yield three distinct thermocouple depths. Care was taken to place the probe parallel to the muscle fibers to minimize muscle damage during exercise, and angling the probe deep into the muscle while orienting correctly relative to the pennation angle was challenging.

Kenny *et al.* used a different methodology to introduce a similar temperature probe into the vastus medialis to record intramuscular temperatures during exercise (2003). The group used ultrasound imaging to guide probe insertion to a depth 10 mm from the femur and deep femoral artery. In contrast to the current study, the probe was

introduced perpendicular to the surface with no regard for the pennation angle. This probe placement yielded excellent data on the depth dependent muscle temperature gradient. That said, in the current study parallel placement of the probe relative to the muscle fibers was prioritized to minimize muscle damage.

Ultrasound images confirming the location of the thermocouples within the vastus lateralis were obtained for three of the five subjects. For the remaining two subjects, ultrasounds images confirming the location of the probe were not possible due to the curvature of the probe and the dressings covering the insertion site. Given the mean muscle depths recorded in the supplementary investigation, the possibility of an intramuscular thermocouple inadvertently localizing within the subcutaneous fat must be considered. The mean subcutaneous fat depth was 0.68 cm, and the median thermocouple depth calculated from ultrasound was 0.764 cm. If a thermocouple were inadvertently placed within the subcutaneous fat, the temperature response would be blunted. Heat producing metabolic processes that power muscle contraction, namely ATP hydrolysis and ATP production, are restricted to the muscle tissue. Heat would transfer to neighboring subcutaneous fat via conduction, but the temperature response would be diminished.

Of the two subjects that ultrasound images were not obtained for, one displayed a temperature response that raises the suspicion of inadvertent probe placement within the subcutaneous fat. For that subject, the mean range of intramuscular temperatures during the final 40 minutes of exercise was 1.45 ± 0.29 °C and intramuscular temperatures at the end of exercise were 39.02 °C, 38.15 °C and 37.57 °C. In the other four subjects, every intramuscular temperature except two were above 39 °C at the

conclusion of exercise; the other two were above 38.5 °C. The lack of convergence and the abnormally low temperatures suggest that at least one thermocouple may not have been located within the vastus lateralis. However, without ultrasound imaging there is no evidence to support removing the subject's data from the results.

In fact, removing the subject of concern from the results influences the primary outcome data minimally. Mean intramuscular equilibration temperature increases from 39.15 ± 0.69 °C to 39.41 ± 0.46 °C, and mean change in temperature increases from 4.59 ± 1.24 °C to 4.92 ± 1.16 °C. Removing the subject's data also improves the convergence of temperatures during exercise; mean intramuscular temperature range during the final 40 minutes of exercise decreases from 0.69 ± 0.49 °C to 0.47 ± 0.24 °C.

While acknowledging this potential source of error, these calculations indicate that the inclusion or exclusion of the data in question has a minimal, though not insignificant, effect on the primary outcome measures. Given the lack of definitive evidence, removing the data would be an inappropriate action.

Convergence of Intramuscular Temperatures

Although a direct analysis of the depth dependent temperature gradient is not possible given the thermocouple placement, the convergence of intramuscular temperatures during exercise provides indirect evidence of depth dependent temperature dynamics. Previous work has indicated that the resting temperature gradient from deep to superficial muscle disappears rapidly with exercise, with thermal equilibrium between all muscle depths at steady state (Kenny *et al.* 2003). Although the current study did not produce data representative of a depth gradient, three different intramuscular sites were recorded in each subject, and temperatures at those three sites

converged from rest to exercise. This indicates that muscle temperature became more uniform during exercise, consistent with previous findings (Kenny *et al.* 2003).

Although intramuscular temperature was only recorded at relatively superficial depths, existing evidence supports the conclusion that deeper intramuscular sites reached a similar temperature. Muscle temperature across depths becomes more homogenous during exercise; this suggests that a temperature reading from any given location within the muscle is likely to also reflect muscle temperature at other locations (Kenny *et al.* 2003). Given this evidence, the superficial muscle temperatures recorded during exercise in this study are assumed to approximate deeper muscle temperatures as well.

Two Measures of Intramuscular Temperature Response

The objective of this study was to understand the changes in skeletal muscle temperature with the specific single leg exercise model. Muscle temperature response to exercise can be expressed in two ways: an absolute temperature (the steady state equilibration temperature) or a change in temperature from resting. Choosing which measure to apply to future studies is a key point of analysis for the current study.

Absolute equilibration temperature exhibited less variation between subjects than change in temperature. Change in temperature for individual subjects ranged from 3.28 °C to 6.44 °C (3.16 °C of variability), while absolute temperature fit into a narrower range of 38.31 °C to 39.81 °C (1.50 °C of variability). Although both measures come with inherent variability, the equilibration temperature was much more homogenous across subjects. Therefore, the muscle temperature response to exercise is best expressed by the mean equilibration temperature.

Intramuscular Equilibration Temperature

Mean intramuscular temperature reached an equilibration temperature of 39.15 ± 0.69 °C after 30 minutes of exercise. This temperature is significantly higher than the peak intramuscular temperature of ~38.22 °C reported in Kenny *et al.* (2003). That study required subjects to exercise for only 15 minutes, and temperatures appeared to still be increasing at the end of exercise. Saltin *et al.* reported muscle temperatures of 39 – 40 °C at exhaustion, regardless of ambient temperature, work rate and exercise duration (1972).

Muscle temperature can be viewed as an oversimplified marker of blood flow and concomitant nutrient delivery and waste removal. If blood flow is insufficient to deliver and remove the necessary substrates, exhaustion will follow and muscle temperature will increase, as convective heat exchange is impaired. The findings of Saltin *et al.* indicate that exhaustion is associated with a consistent muscle temperature ranging from 39 – 40 °C, regardless of other factors.

In contrast, the equilibration temperature reached in this study represents a balanced matching of heat production and heat removal. Heat production is directly proportional to work rate. This implies that heat production was constant from 10 minutes in to exercise (when the steady state work rate was reached) to the end of exercise. With heat production constant, only heat dissipation could influence intramuscular temperature. Increased blood flow to active skeletal muscle dissipates a considerable amount of the heat produced during contraction.

Previous work has indicated that equilibration time and temperature are dependent on relative workload (Taylor, 2014; Saltin *et al.* 1968); this study identified an equilibration time of 30 minutes and an equilibration temperature of 39.15 ± 0.69 °C for 60 minutes of single leg dynamic knee extension at 60% of maximal work rate.

Conclusions

This study endeavored to develop knowledge of the intramuscular temperature changes during 60 minutes of single leg dynamic knee extension at 60% of maximal work rate. In the context of ongoing research, the purpose of this study was to identify one number: the intramuscular equilibration temperature for the specific exercise stimulus. The experimental methods utilized to achieve that goal were not without their flaws, but this study succeeded in identifying the number vital for future research.

With a comprehensive understanding of the intramuscular temperature changes that occur during our particular exercise model, we are one step closer towards determining the specific exercise related factor that mediates histamine release and subsequent vasodilation. Phase two of the current study will develop the methodology to experimentally heat skeletal muscle to 39.15 °C, and phase three will determine if increased temperature leads to histamine release. Regardless of the eventual outcome, whether “increased muscle temperature” is circled in green or crossed out in red, this study will add significantly to our understanding of the postexercise hemodynamic response. Most importantly, adding to our knowledge of this specific mechanism positions us one step closer to the ultimate goal: exploiting our mechanistic understanding to improve health in aging and diseased populations.

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