ION CHANNEL GENE EXPRESSION AS OBJECTIVE BIOMARKERS OF TRAINING INDUCED FATIGUE

by

Timothy Allen VanHaitsma

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STATEMENT OF DISSERTATION APPROVAL

The following faculty members served as the supervisory committee chair and members for the dissertation of Timothy Allen VanHaitsma.

Dates at right indicate the members' approval of the dissertation.

Andrea T. White	, Chair	7/14/14 Date Approved
Alan R. Light	, Member	7/14/14 Date Approved
Russell S. Richardson	, Member	7/14/14 Date Approved
Markus Amann	, Member	7/14/14 Date Approved
Patricia Eisenmann	, Member	7/14/14 Date Approved

The dissertation has also been approved by James C. Hannon

Chair of the Department/School/College of Exercise and Sport Science

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The conditions and mechanisms that produce decrements in exercise performance have been an important area of research for decades. However, the sensation of fatigue that may persist for hours or days after exercise has received less attention. This long-lasting fatigue sensation is common in clinical populations. In chronic fatigue syndrome (CFS), moderate physical activity can produce increased fatigue sensations that last for several days. A recent study of CFS patients demonstrated significant increases in fatigue and pain sensations that were closely related to gene expression changes in metabolite-detecting, adrenergic, and immune markers following 25 minutes of moderate, whole body exercise. This exercise stimulus did not produce long-lasting fatigue or changes in gene expression in healthy age and gender-matched controls.

The graded exercise test (GXT) is commonly applied in CFS research. Although intense, the GXT rarely produces long-lasting fatigue in healthy individuals and its effect on CFS patients' postexertional fatigue is unclear. Thus, the goals of these studies were to examine gene expression and fatigue sensations in healthy, trained individuals during and after three different exercise stressors: a GXT, a 40k time trial in ambient conditions (AT), and the same time trial in adverse, hot conditions (HT). It was hypothesized that there would be larger changes in gene expression following both AT and HT, compared to GXT. The first study explored the differences in gene expression following GXT and AT. Following AT, there were larger decreases in metabolite-detecting mRNA, larger

increases in adrenergic, immunologic, and serotonin mRNA as compared to GXT. Further, these gene expression changes were different from postexercise responses of CFS patients. The goal of the second study was to add an additional stressor to the 40k time trial – heat. Because TRPV receptors are sensitive to heat, it was thought that the heat stress would cause larger increases in TRPV mRNA. However, because exercise power was reduced during HT, there were no differences in gene expression between the two trials except that IL6 mRNA decreased significantly more following AT compared to HT. Collectively, these results show that gene expression in healthy individuals is affected by the intensity and length of the exercise. The decreases in metabolite-detecting mRNA are thought to be an attempt to restore homeostasis in the fatigue and pain detecting receptors. The fact that there was no difference between AT and HT suggests that heat is an additional metabolite that activates the metabolite-detecting receptors, helping to regulate the intensity of exercise and the amount of fatigue during exercise. Adrenergic receptors increased, possibly causing a decrease in blood flow following exercise and increasing the resting levels of metabolites and subsequently increasing the resting fatigue signal. The change in the ratio of serotonin/dopamine may also contribute to the increased fatigue sensation following exercise.

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BACKGROUND

Muscle fatigue, from a physiological perspective, has been defined as any reduction in force-generating capacity of a muscle (24, 26). In the early 1900s, Mosso recognized three different possible causes of muscle fatigue that limited exercise performance: peripheral, central, and cognitive (51). Since Mosso described these three types of fatigue, researchers have attempted to determine the primary factors that limit exercise performance. In 1984, Bigland-Ritchie identified eight possible sites of muscle fatigue, including 1) excitatory input to higher motor centers, 2) excitatory drive to lower motor neurons, 3) motor neuron excitability, 4) neuromuscular transmission, 5) sarcolemma excitability, 6) excitation-contraction coupling, 7) contractile mechanisms, and 8) metabolic energy supply and metabolite accumulation (Figure 1.1) (12). These sites split nicely into two categories: central (1-3) and peripheral (4-8), but it seems that Mosso's idea of a separate category for cognitive, or mental, causes was ignored. For years, researchers have attempted to identify the main factor(s) responsible for muscle fatigue and hence the limitations to exercise performance. These arguments have generally been divided among those focused on a peripheral cause of fatigue versus a centrally mediated cause of fatigue (5, 22, 23, 39, 41, 49, 50, 54, 67).

Peripheral Causes of Muscle Fatigue

Peripheral mechanisms of fatigue are defined as factors that influence force production at the neuromuscular junction or within the muscle and may involve action potential failure, excitation-contraction coupling failure, or impairment in crossbridge cycling in the presence of unchanged or increased neural drive (62). Several factors within the muscle influence the force production capability. For years, it was thought

that the accumulation of metabolic byproducts limited energy production in the muscles and was the primary cause of fatigue. The accumulation of lactate was thought to be a major factor that limited muscle performance due to the belief that dissociated hydrogen ions reduced local pH to levels incompatible with muscle contraction (40, 42). However, this idea has largely been refuted, as lactate is a substrate that is quickly utilized by other tissues (16, 17). Further, at physiologic temperatures, the reduced pH during heavy exercise does not have a direct effect on contractility and fatigability (60, 63, 65, 66). Another metabolite, inorganic phosphate (P_i), may play a more substantial role in producing peripheral fatigue. There is evidence that P_i, which is released during intense exercise, may reduce crossbridge force production, inhibit sarcoplasmic Ca^{2+} reuptake, and reduce the amount of free Ca^{2+} available for release. All of these factors could be involved in increased muscle fatigue (65, 66). Recent advances in knowledge of Ca2+ handling in muscle supports this idea. For example, the ryanodine receptor located on the sarcoplasmic reticulum may play a major role in regulating Ca^{2+} release during exercise. Exercise causes remodeling of the ryanodine receptor by protein kinase A hyperphosphorylation, resulting in "leaky" calcium channels. In turn, this results in decreased Ca^{2+} reuptake and a subsequent decrease in exercise capacity (7, 8).

Coincident with the decrease in force production, there are subjective correlates that exercisers experience as peripheral fatigue. These include muscular sensations that have been described as pain, burning, heaviness, and the like. Interestingly, the misguided belief that these sensations are caused by the accumulation of lactic acid or "toxins" in the muscle still persists among lay practitioners.

Central Mechanisms of Muscle Fatigue

Central mechanisms of muscle fatigue are defined as mechanisms that cause a reduction in neural drive to the muscle, resulting in a decline in force production independent of changes in skeletal muscle contractility (62). The sites that may affect neural drive, using Bigland-Ritchie's nomenclature, include excitatory input to higher motor centers, excitatory drive to lower motor neurons, and motor neuron excitability (12). Because direct measurement of central processes is difficult at best, and in some cases impossible in humans, this area of research was largely ignored until recently. Even today, our understanding of the central nervous system's (CNS) role in fatigue is limited. To further complicate this aspect of fatigue, researchers have recognized that the role of central processes in producing fatigue varies greatly with the type of exercise performed, e.g. maximal vs. submaximal or sustained vs. intermittent (19, 38).

There are numerous ways in which central fatigue has been observed. Classically, central fatigue is demonstrated when maximal voluntary force can be augmented with a superimposed electrical stimulation of the muscle or nerve, thus showing a decrement in neural drive (6, 13). However, determining the cause of this decrement has proved to be more difficult. Central drive has both conscious and unconscious elements. Conscious elements include concepts such as motivation and effort that are problematic for physiologists to manipulate and measure. The unconscious elements include the sum of inhibitory and excitatory influences acting on the motor neurons, the motor cortex, and other structures "upstream" of the motor cortex, such as the prefrontal and cingulate cortices.

Similar to peripheral fatigue, it has been hypothesized that central fatigue may be influenced by "metabolites" produced from increased CNS demands during heavy or prolonged exercise. Thus, central fatigue may be due to the accumulation of metabolites such as serotonin and ammonia with concurrent decreases in dopamine and acetylcholine in the brain (58, 62). These changes may impair the ability of the brain to maintain or increase neural drive, resulting in decrements in muscle force (62).

Consistent with the idea that increased CNS demands may limit neural drive, it is possible that inadequate delivery of substrates to the brain produces central fatigue. There is some evidence that during strenuous exercise, cerebral blood flow [20, 21] is inadequate to key brain areas, resulting in decreased oxygenation and glucose availability (57). Either or both of these conditions have the potential to negatively impact neural drive and thus muscle force. Decreased glucose metabolism or availability within the brain could also impair conscious aspects of neural drive, decreasing central motor output (10, 11, 44). The neurotransmitter serotonin is also implicated in fatigue. An increase in systemic tryptophan leads to an increase in serotonin in the brain (53) activating the 5-HT receptor, which causes an increase in central fatigue (68).

Evidence also suggests that hyperthermia plays an important role in central fatigue. When brain, and more specifically, hypothalamic temperature increases, it is found that there is a decrease in both force output and central command (31, 55). However, this mechanism is probably only related to whole body exercise in which core temperature increases are common and is not a key factor underlying force decrements during isolated muscle activity.

As in peripheral fatigue, central fatigue is associated with several perceptual changes. As motor command increases in order to sustain a given force, the perception of effort also increases. Aspects of conscious awareness of fatigue are also present and will be discussed in more detail below.

Integrated Model of Muscle Fatigue

Originally, researchers primarily focused on fatigue mechanisms in the muscle. More recently, researchers began to focus on central mechanisms – or mechanisms involving corticospinal or higher brain centers. A number of researchers suggest that reduced central neural drive may be a result of inhibitory influences from muscle and/or other peripheral organs, thus integrating the central and peripheral models of fatigue. Decreases in central motor output during moderate exercise may be due to increases in afferent feedback from the Group III and IV afferent nerve fibers due to metabolic changes in the periphery (1, 27). Several recent studies have provided evidence to support this integrated model. When hypoxia is induced during exercise, causing an increase in metabolic by-products (15% O_2), there is a decrease in central motor drive as measured by iEMG, which suggests an increase in central fatigue as compared to normoxia (3). Further, there is a decrease in iEMG following a prefatiguing exercise, meaning that there is increased afferent feedback with more fatigue, perhaps limiting the degree of peripheral fatigue during an exercise bout (2). Another study attempted to examine whether feedback from the Group IV afferents was the primary contributor to central fatigue. This study used fentanyl, an opioid receptor antagonist, to selectively block the Group IV afferent response. It was found that the central motor drive was

increased throughout the subsequent exercise trial as a result of decreased afferent feedback to the central command center. Thus, central fatigue was substantially decreased while peripheral fatigue was greatly increased (4). These data suggest that afferent feedback plays a role in increasing central fatigue. The activation of these afferent nerve fibers by metabolites will be discussed in a subsequent paragraph.

An aspect of central fatigue that is important to evaluate is the cognitive perception of fatigue, or the perception of "feeling tired". Intense or sustained physical activity may alter the activity of brain neural networks, which in turn produces a conscious awareness of those alterations (61). The sensation of fatigue may arise from anatomical brain structures associated with conscious perception. These may include the motor and premotor complex, supplementary motor complex, basal ganglia, insula, anterior cingulate, and the cerebellum (61). The integration of the sensation of fatigue via the Group III and IV afferents may also occur in the brain stem or spinal cord (36, 59). Within the spinal cord, afferent neurons may activate inhibitory interneurons (14, 21, 30) or activate the spinal cord, signaling that the perfusion of the exercising muscle was inadequate to meet metabolic demand. Finally, afferent neurons may provide input to the brainstem, posterior hypothalamus, or midbrain, which in turn influence central command for autonomic and ventilatory output (36, 37). All of these factors may also contribute to the cognitive perception of fatigue.

Afferent feedback, from metabolite accumulation, substrate depletion, or cardiac and respiratory limitations, not only influences central motor drive but may also influence the cognitive perception of fatigue (18, 56, 61, 64). Motivational and psychological factors likely determine the degree to which the individual responds to sensations of

fatigue (61). When low motivation exists, the person doing the exercise may have an increased sensation of fatigue and a subsequent decrease in performance.

The integrated model of fatigue works through afferent nerve fibers in the periphery, sending signals to the central command center, which play a role in modulating the amount of fatigue. One of the questions that arise is what causes this increased afferent nerve activity with an increase in exercise intensity or duration. An in vitro study by Light and colleagues, using calcium imaging of mouse dorsal root ganglion neurons (DRGs), found that there were two classes of receptors on these cells. These receptors responded to low and high doses of metabolites that correspond with moderate and high intensity exercise and may signal for fatigue and pain, respectively (46). The metabolites to which these cells respond included a combination of increased lactate, extracellular ATP, and hydrogen ions (decreased pH).

There are several receptors on the afferent nerve endings that are sensitive to these metabolites, including ASIC3 (acid-sensing ion channel), TRPV (transient receptor potential vanilloid channels), and P2X (purinergic receptor). A combination of extracellular lactate and extracellular ATP increased the sensitivity of the ASIC3 receptor, making it more receptive to changes in metabolites within the physiological realm (52) while blocking the ASIC3 receptor using amiloride found that the Group IV afferent response to lactate and contraction was blocked, suggesting that ASIC3 receptor activation is essential for sending a fatigue signal to the central command center (34). The ASIC3 receptor may form heteromers with ASIC2 allowing activation at different pH levels, sending signals to the central command center that may be perceived as fatigue or pain (33). P2X receptors are also located on Group III and IV afferents that evoke the

exercise pressor reflex, which are the same afferent nerve fibers that are activated with central fatigue (35). The effects of P2X receptors are attenuated in an environment that simulates exercise – decreased pH and increased temperature (28, 29) – perhaps suggesting that the P2X receptors do not play as large of a role in signaling as the ASIC3 receptor or the TRP receptors. However, activation of the P2X receptors by extracellular ATP may help sensitize the ASIC3 receptor to lactate, allowing the metaboreceptors to sense a subtle decrease in extracellular pH (15). The TRPV1 and TRPV4 metaboreceptors may also play a role in the afferent nerve signal to the brain. These receptors are sensitive to an increase in acid, heat, and endocannaboids (9, 20). TRP receptors are sensitive to temperatures ranging from approximately 23-50 degrees Celsius (9), allowing a modulation of the afferent signal to the brain depending on the change in temperature of the body. Finally, the adrenergic β-receptors may enhance the metabolite signal on these afferents, especially in patients with fibromyalgia (48).

Fatigue: Clinical Perspective

From a clinical perspective, the symptom of "fatigue" is much broader than the physiological definition. Clinically, fatigue has been defined as "a subjective lack of physical and/or mental energy that is perceived by the individual or caregiver to interfere with usual and desired activities"(43). This definition relies, in part, on assessing the perceptions of individuals or their caregivers and determining the impact of these perceptions on physical and cognitive function.

In pathologic fatigue conditions, such as Chronic Fatigue Syndrome (CFS), evidence suggests that a dysregulation in afferent fatigue signaling may exist. CFS has

been defined by the Centers of Disease Control (CDC) as “persistent and relapsing fatigue not relieved by rest that severely compromises activities of daily life for 6 months or longer, for which other possible medical causes have been ruled out” (25). One of the main problems with CFS is that diagnosis relies on subjective perceptual responses. Objective biomarkers of fatigue would be extremely useful to diagnose CFS and to track responses to various therapeutic approaches. A study by Light et al. in 2009 examined metabolic (ASIC3, P2X4 and P2X5, and TRPV1), immunologic (IL-6, IL-10, TNF-α, TLR4, CD14), and adrenergic (α-2a, β-1, β-2, COMT) gene expression in leukocytes and found an upregulation in the mRNA for all of the genes following 25 minutes of moderate (70% of age-predicted maximum heart rate) exercise in CFS patients as compared to healthy, age-matched control participants (45, 47). It was also found that for subjective ratings of mental fatigue, physical fatigue, and pain (all on scales of 0-100 with 0 being no fatigue and 100 being the worst fatigue imaginable), patients with CFS reported a significantly higher rating of fatigue and pain throughout the 48 hours while also showing significantly higher increases in pain and fatigue from baseline following the exercise than healthy controls (45, 47). This paper suggested that an increase in the amount of P2X and ASIC3 mRNA following exercise may lead to an increase in the number of receptors and, subsequently, to an increased sensation of fatigue due to increased afferent nerve signal to the brain from even resting levels of metabolites (47). The study also suggested that the changes in mRNA in the leukocytes could increase the cytokines that sensitize muscle afferents, thus making them more sensitive to metabolites and increasing the sensation of fatigue following a bout of exercise (47).

The possible role of these biomarkers in objectively describing fatigue needs to be further elucidated. Therefore, this dissertation will explore two different aspects of perceptual fatigue and these biomarkers to better understand how they are affected by exercise in healthy individuals. The first paper will compare the responses of perceptual fatigue and biomarkers in trained cyclists following a high-intensity, short (graded exercise test) and long (40k time trial) exercise. The second paper will compare the responses of these biomarkers in trained cyclists following a 40k time trial at room temperature and a 40k time trial at 35 degrees Celsius.

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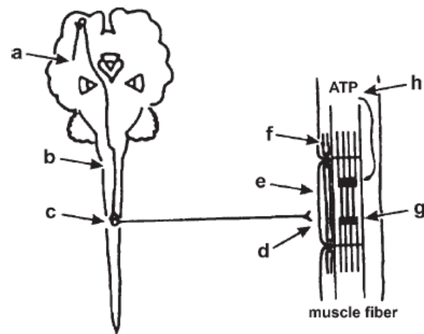

Figure 1.1: Potential sites of fatigue as described by Bigland-Ritchie (1984): *a*, excitatory input to the motor cortex; *b*, excitatory drive to lower motoneuron; *c*, motoneuron excitability; *d*, neuromuscular transmission; *e*, sarcolemma excitability; *f*, excitation-contraction coupling; *g*, contractile mechanism; *h*, metabolic energy supply (32). A-C are considered central while D-H are considered peripheral.

GENE EXPRESSION MARKERS OF FATIGUE AND PAIN SENSATIONS: A COMPARISON OF EXERCISE PARADIGMS

Abstract

Metabolite-detecting afferents and adrenergic receptors have been linked to pre- and postexercise fatigue within Chronic Fatigue Syndrome (CFS). It is unclear, however, whether these same pathways are altered during strenuous exercise in trained healthy subjects, and whether exercise duration or intensity more strongly influences gene expression. Previous studies have used both graded exercise tests (GXT) and moderate intensity time trials to examine mRNA changes during postexercise fatigue with CFS. Twenty moderately trained cyclists performed a GXT (mean duration 10.9 min) and a 40k time trial (TT, mean duration 74.3 min) on a bicycle ergometer. Following each trial, blood lactates were measured one and five minutes postexercise and blood draws were performed 0.5, 8, 24, and 48 hours postexercise. Leukocytes were separated for RNA extraction and qPCR analysis for gene expression (mRNA) of metabolite detecting, adrenergic, indolamine, and immune receptors. Physical fatigue and pain were greater following TT ($p<0.05$) but RPE and postexercise lactate were higher for GXT ($<.008$), indicating higher intensity. Mean mRNA changes were significantly greater following TT vs GXT, including: 1) decreases in gene expression for metabolite-detecting ASIC1, ASIC3, P2X4, and TRPV1; 2) increases in adrenergic receptors α2a, α2c, β1, but decreases in β2; 3) increases in indolamine receptor HTR1D and decreases in DRD4; and 4) increases in immune receptor IL-6 ($p<0.05$). These findings suggest that long duration, higher total workload exercise has more powerful effects on gene expression than does brief but higher intensity exercise.

Introduction

Traditionally, physiologists have viewed muscle fatigue as any exercise-induced decrement in maximal voluntary muscle force or power (8, 19). Clinically, the symptom of fatigue includes the sensation of tiredness and greater effort during muscular contraction that can extend for a period of time beyond exercise. In some clinical populations, fatigue is present at rest, manifested as decreased motivation, increased malaise, or even lack of mental focus (24, 49).

Two interrelated aspects of fatigue, central and peripheral, ultimately limit physical performance and may also influence postexercise recovery. Central fatigue, clinically defined, is "the failure to initiate and/or sustain attentional tasks and physical activities requiring self-motivation" (14, 15). It is not clear which specific CNS structures inhibit central motor drive (CMD); however, peripheral factors can influence central fatigue via afferent feedback. In vitro experiments have shown dorsal root ganglion cells (Group IV afferents) containing the receptors ASIC3 (Acid-sensing Inward Current), P2X (purinergic) types 4 and 5, and TRPV1 (transient receptor potential vanilloid) respond to physiologic combinations of lactate, protons, and extracellular ATP (26). These sensory receptors fall into two classes that encode for fatigue and/or pain based on metabolite concentrations (26). Thus, afferent feedback, in response to muscle metabolite accumulation, can inhibit CMD and also influence perceptual responses (12, 41, 49, 52).

Central and peripheral contributions to fatigue can be evaluated during maximal voluntary contraction by quantifying superimposed twitch characteristics and post-contraction twitch potentiation via supra-maximal nerve stimulation (1-4). In addition,

the sensation of fatigue and pain can be reliably assessed via a visual analog scale (VAS) during and after exercise (24, 25, 27, 54). The perception of tiredness or heaviness may persist for several hours or days following exercise, even when there is no change in muscular function or performance (45). In fact, Chronic Fatigue Syndrome (CFS) is, in part, defined by postexercise malaise (PEM) that interferes with normal physical and social functioning (18).

In combination with the effects of metabolite-detecting receptors on afferent fibers, fatigue perception is influenced by sympathetic nervous system (SNS) effects on central and peripheral sensory pathways (27). SNS effects on blood flow to working muscles can alter the local metabolite milieu, affecting peripheral afferent feedback (27). In CFS, researchers have shown alterations in leukocyte gene expression of adrenergic receptors, including α2a, α2c, β1, and β2 (22, 25, 27). The immune system may also directly affect muscle afferents, making them more sensitive to metabolites (27).

The neurotransmitters serotonin and dopamine have been implicated in fatigue (5, 6, 31, 40). Serotonin activates Group IV afferent fibers (51), increasing pain when injected intramuscularly (32). When serotonin was augmented in rats by administering a 5-HT agonist, exercise capacity decreased (5). Decreases in dopamine can increase fatigue (31). During exercise, dopamine metabolism is increased (10), decreasing levels of dopamine at exhaustion (5). Increased serotonin (35) and a concurrent decrease in dopamine may decrease central motor drive (42, 50, 55).

In efforts to identify objective markers of PEM, we examined mRNA expression of a group of molecular receptors involved in neuronal signaling of muscle fatigue and pain in CFS patients before and after exercise (25, 27, 54). Our exercise paradigm for

these studies was a 25 minute, moderate intensity (70% of age predicted maximal heart rate) combined arm and leg exercise, designed to resemble a typical daily activity. This paradigm resulted in significant increases in fatigue and pain sensations that persisted for up to 48 hours after exercise that were highly correlated to large changes in metabolite detecting, adrenergic, and immune receptor mRNAs. Small or no changes in gene expression were observed in healthy controls who also reported no increases in postexercise fatigue or pain (25, 27, 54). Other researchers have utilized a graded exercise test (GXT) to examine fatigue mechanisms in CFS (37, 43, 53), even though this type of exercise does not simulate activity that would likely be encountered by CFS patients.

The purpose of this investigation was to examine perceptual fatigue and pain as well as gene expression markers of fatigue and pain in healthy cyclists before, during, and after two exercise modalities; a GXT and a 40k TT. The GXT was selected for this protocol because it is commonly utilized as a model in CFS research. Although the GXT produces high metabolite levels in a short period of time, we predicted this exercise would not produce long-lasting fatigue and pain in healthy individuals. It was hypothesized that the 40k time trial would elicit greater and longer-lasting increases in fatigue and pain sensations that would be reflected in changes in gene expression, although we did not know whether these changes would be in the same direction as we have previously observed in CFS. We also utilized objective indices of central and peripheral fatigue before and after exercise to determine whether these were related to changes in gene expression.

Methods

Participants

The experimental procedures used in this investigation were reviewed and approved by the University of Utah Institutional Review Board. The procedures were explained verbally, and all participants provided written informed consent prior to testing. Twenty healthy, moderately trained male (n=10) and female (n=10) cyclists between the ages of 18-55 were recruited for this investigation (mean ± SD; age 36.1 ± 9.7 years, body weight 68.1 ± 9.2 kg, height 172.9 ± 6.3 cm, peak oxygen consumption ($VO2_{peak}$) 54.8 ± 5.9 $mL \cdot kg^{-1} \cdot min^{-1}$). Cyclists were excluded if any of the following applied: current, acute musculoskeletal injury; medications known to interfere with the sympathetic nervous system; unwillingness to comply with training interruptions mandated by the protocol; any uncontrolled chronic health condition.

Protocol Overview

Participants performed two tests, separated by at least one week: a graded exercise test to determine VO_{2peak} and a 40k time trial on a Velotron cycle ergometer (Racermate, Seattle WA) that was adjusted to match each participant's accustomed cycle position. All participants refrained from exercise for 24 hours prior to the exercise test. Venous blood samples were obtained from the arm at baseline and at 0.5, 8, 24, and 48 hours after each exercise test. To assess the severity of preexisting and exercise-related fatigue and pain symptoms at the time of each blood draw, participants provided numerical ratings of mental fatigue, physical fatigue, and overall body pain or soreness using a 0-100 scale, where 0 was defined as no pain or fatigue and 100 was defined as the

greatest amount of fatigue or pain the participant could ever imagine. Immediately after the baseline blood draw, participants began the exercise test as described below.

GXT Protocol

Participants performed an incremental cycling test (VO_{2peak}) beginning at 100W (50W for females) with resistance increasing 25W every minute until volitional exhaustion. Participants pedaled at their preferred pedaling rate and the test was terminated when the cadence dropped more than 10 rpm for more than 5 seconds despite strong verbal encouragement. Metabolic data were collected using open circuit calorimetry (Parvomedics, True Max 2400, Sandy, UT). Peak oxygen consumption ($VO2_{peak}$) was recorded as the highest VO_2 measured in a 15-second period.

40k Time Trial

On the second experimental day, participants performed a 40k time trial with instructions to cover the distance as fast as possible. Following a 10-15 minute self-selected warm-up, the participant began the test. The participants were able to fully control the gearing of the Velotron and were able to monitor speed, power, distance covered, and cadence. Heart rate, rating of perceived exertion (RPE), and work rate were recorded every 2.5 kilometers. Upon completion of the test, participants were encouraged to cool down for 5-10 minutes before cessation of exercise.

Neuromuscular Testing

A subset of participants (n=11; mean ± SD; age 41.5 ± 11.2 years, body weight 66.4 ± 8.2 kg, height 174.8 ± 11.2 cm, $VO2_{peak}$ 50.3 ± 7.7 $mL·kg^{-1}·min^{-1}$) completed a separate, follow-up study to examine central and peripheral contributions to muscle fatigue 5-10 minutes before and 2 minutes after each exercise test (GXT and TT). Supramaximal magnetic stimulation of the femoral nerve was superimposed on and applied 5 seconds after a series of maximal voluntary contractions (MVCs) of the quadriceps.

Participants sat in a semireclined position on a table, with the upper body supported by their elbows and a Pilates mini ball positioned under the lower back. Each participant's leg was marked with indelible ink and aligned with a mark on the table to assure proper positioning, with the knee joint angle set at 90° of flexion. A magnetic stimulator (Magstim 200^2; Wales, UK) connected to a 70mm double coil was used to stimulate the femoral nerve. After the coil position that elicited the maximal quadriceps response was located, it was marked to ensure consistent coil placement for postexercise assessments. The evoked quadriceps twitch force was obtained from a calibrated load cell (MLP-300; Transducer Techniques, Rio Nedo Temecula, CA) connected to a noncompliant strap, which was placed around the subject's right leg, just superior to the malleoli.

To verify that maximal magnetic stimuli were delivered, unpotentiated quadriceps single twitch forces (Q_{tw}) were obtained every 30 seconds at 70, 80, 85, 90, 95, and 100% of maximal stimulator output. A plateau in baseline Q_{tw} and M-wave amplitudes with increasing stimulus intensities was observed in every subject. The increase in Q_{tw}

from 90% to 95% of the stimulator output was 1.2 ± 3.2% ($P = .22$) and from 95% to 100% was -.08 ± 3.3% ($P = .91$). The stimulator was set at 100% for all subjects and trials.

We measured superimposed twitch force during and potentiated Q_{tw} ($Q_{tw,pot}$) force 5 seconds after a 5-second maximal isometric voluntary contraction of the quadriceps and repeated this procedure six times. Peak force, maximal rate of force development (MRFD), maximal rate of relaxation (MRR), contraction time (CT), and reaction time ($RT_{0.5}$) were analyzed for all $Q_{tw,pot}$. Voluntary activation of the quadriceps during the MVCs was assessed using a superimposed twitch technique (33). Briefly, voluntary muscle activation compares the additional force produced during a single twitch superimposed on the MVC with the force produced by the potentiated single twitch delivered 5 seconds after MVC (1-(superimposed twitch force/$Q_{tw.pot\ force}$)*100).

Blood Sample Processing

Blood samples obtained at baseline and 0.5, 8, 24, and 48 hours after each time trial were collected in EDTA tubes. Within 5 minutes of blood collection, the blood was centrifuged at 4100rpm (2.6rcf, Eppendorf Centrifuge 5702R) for 12 minutes. Immediately after, the white cell layer was carefully collected and stored in RLT + β-Me (Qiagen, Valencia, CA, USA) before being quickly frozen using a methanol-dry ice slurry and stored at -80°C. RNA was extracted using RNeasy kits (Qiagen) according to manufacturer's directions, and treated with RNase-free DNase-I (Qiagen). Immediately following extraction, RNA was converted to a cDNA library using the ABI High

Capacity cDNA Archive kit (Applied Biosystems, Inc., Foster City, CA, USA). The cDNA was stored at -20°C until analysis.

The cDNA libraries were analyzed using the ABI quantitative, real-time PCR system on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Inc.) using ABI Taqman Master Mix (Applied Biosystems, Inc.). Master mix/primer probe solutions were separately loaded onto 96-well preplates, with robot loading mixing these solutions when placed into 384 plates. Plates were centrifuged to remove air bubbles from the wells. Each sample was run in duplicate with the standards run in quadruplicate. No-template control samples were also run to examine for contamination. Each 384-well plate contained eight samples and all genes were analyzed on the same plate. Primer probes (all from Taqman Gene Expression Assays; Applied Biosystems, Inc.) were as follows;

Adrenergic A2A (α-2A) – HS00265081_s1; Adrenergic A2C (α-2C) - HS03044628_s1; Adrenergic B-1 - Hs02330048_s1; Adrenergic B-2 - Hs00240532_s1; ASIC1 - Hs00241630_m1; ASIC3 - Hs00245097_m1; CREB1 - Hs00231713_m1; DRD4 - Hs00609526_m1; HTR1D - Hs00704742_s1; IL10 - Hs00174086_m1; IL6 - Hs00174131_m1; P2X4 - Hs00175706_m1; TLR4 - Hs00152937_m1; TRPV1 - Hs00218912_m1; TRPV4 - Hs01099348_m1; and TF2B - Hs00155321_m1. TF2B (general transcription factor IIB) was used as the reference gene. All primer probes, with the exception of the adrenergic genes and HTR1D (these genes do not have introns), recognize sequences that cross splice sites and therefore make detection of genomic DNA unlikely. In all cases, we quenched the genomic DNA and ran no-template control wells to ensure that genomic DNA did not contaminate the final results. All of the primer

probes were designed and tested to be used together and have similar efficiencies to help eliminate inaccuracy. Evaluation of controls in this, and previous studies, indicated that TF2B had less intrinsic variation than other controls, had a count range that was similar to the genes of interest, and did not increase or decrease because of the exercise protocol (25, 27). Real-time results were analyzed using SDS 2.1 (Applied Biosystems, Inc.) and were inspected to determine artifacts (loading errors, robot errors, threshold errors, etc.). Count numbers were exported to an Excel spreadsheet and analyzed according to the ddCT method described in the ABI User Bulletin #2 (Applied Biosystems, Inc.). Baseline levels for each gene were computed relative to TF2B, and these values were used as the comparator for all measures taken after the baseline period.

Data Analyses

Ratings of physical fatigue, mental fatigue, and pain were analyzed using 2 (trial) x 5 (time) RM ANOVAs. Within-group time effects were examined with RM ANOVA with simple contrasts to determine which time points differed significantly from baseline. Between-trial differences in postexercise lactates, peak force, maximal rate of force development (MRFD), maximal rate of relaxation (MRR), contraction time (CT), reaction time (RT0.5), percent voluntary muscle activation (%VMA), potentiated twitch (Qtw.pot), and maximal voluntary contraction (MVC) were examined with RM ANOVA.

For each subject, postexercise values for gene expression measures were normalized to baseline values (1.00=baseline). Metabolite-detecting (ASIC1, ASIC3, P2X4, TRPV1, and TRPV4), immunologic (IL-6, IL-10, and TLR4), adrenergic (ADα2A, ADα2C, ADβ1, and ADβ2), and indolamine (HTR1D and DRD4) mRNAs

were examined using 2 x 5 RM ANOVAs with planned contrasts for evaluate time and trial effects. All data were presented as means and standard deviations, with significance set at $\alpha<0.05$.

Results

Exercise Performance and Subjective Responses

By design, GXT was significantly shorter than TT (10.9 vs 74.3 min, $p<0.001$), but was more intense, as evidenced by significantly higher postexercise lactates ($p<0.001$), peak RPEs ($p<0.05$), and peak heart rates ($p<0.01$; see Table 2.1). Significant time ($F_{(4,48)} > 4.8$, $p<0.003$), exercise (GXT vs TT, $F_{(1,12)} > 8.3$, $p<0.014$), and exercise by time ($F_{(4,48)} > 6.9$, $p<0.002$) effects were found for ratings of postexercise physical fatigue and pain. Following TT, significant increases in physical fatigue were seen at 0.5, 8, and 24 hours postexercise ($p<0.005$), and increases in pain were evident at 0.5 and hours postexercise ($p<0.008$, Figure 2.1). For mental fatigue after TT, a borderline significant time effect was noted ($p=0.092$), with trends for increases seen at 8 and 24 hours postexercise. After GXT, there were no significant increases in mental fatigue, physical fatigue, or pain following exercise (Figure 2.1).

Neuromuscular Responses

Peak force during the 5-second contraction was significantly lower than baseline values after both GXT and TT ($p < 0.05$), though there was not a significant difference between trials ($p = 0.85$). Following both trials, $Q_{tw.pot}$ was significantly attenuated from pre-exercise values ($p < 0.001$) and to a greater extent following TT as compared to GXT

($p < 0.05$, Table 2.2). Additionally, MRFD and MRR decreased significantly from baseline after both trials, though there were no significant differences between trials. Contraction time (CT) decreased significantly after GXT but not after TT. Percent muscle activation was significantly lower than baseline following both GXT (85.5%; $p < 0.001$) and TT (91.4%; $p < 0.01$), but the degree of change was not different between the trials ($p = 0.18$).

Gene Expression Measures

Metabolite-detecting receptors. Significant exercise (GXT vs TT, $F_{(1,17)} > 6.1$, $p<0.02$), time ($F_{(4,68)} > 14.8$, $p<0.001$), and exercise by time ($F_{(4,68)} > 4.4$, $p<0.003$) effects were noted for ASIC3 and P2X4 (Figure 2.2). Across both treatments, ASIC3 and P2X4 decreased significantly at 0.5 hours postexercise, with significantly larger decreases seen after TT ($p<0.001$). In addition, ASIC3 and P2X4 mRNA levels remained significantly lower than baseline values at 8 hours post-TT ($p<0.012$). For TRPV1, significant time ($F_{(4,68)} = 19.1$, $p<0.001$) and exercise by time ($F_{(4,68)} = 5.5$, $p=0.003$) effects were observed, with significant decreases occurring at 0.5 and 8 hours after both GXT and TT; however, at 24 and 48 hours postexercise, TRPV1 levels returned to baseline after TT but remained significantly decreased at these time points after GXT. Postexercise ASIC1 and TRPV4 mRNA levels decreased significantly ($F_{(4,68)} >4.2$, $p<0.012$) at 0.5 hours and at both 0.5 and 8 hours postexercise for ASIC1 and TRPV4, respectively. Exercise and exercise by time effects were not significant for ASIC1 or TRPV4.

Adrenergic and indolamine receptors. In contrast to the metabolite-detecting receptors, the adrenergic receptors, except for β2, increased their expression following exercise. RM ANOVAs yielded significant time ($F_{(4,68)} > 4.3$, $p<0.004$) and exercise by time ($F_{(4,68)} > 3.3$, $p<0.016$) effects for α2C, β1, and β2. Across both exercise trials, α2C mRNA increased significantly at 0.5 and 8 hours postexercise ($p=0.042$); however, examination of the exercise by time interaction indicated that only the increases following TT were statistically significant ($p<0.006$, Figure 2.3). Similarly, β1 mRNA increased significantly at 8 hours postexercise following both GXT and TT ($p<0.004$), but significant increases in β1 mRNA were also seen at 0.5 hours postexercise after TT ($p=0.011$). In addition to significant time and exercise by time effects, a significant exercise effect was also evident for β2 ($F_{(1,17)} = 7.8$, $p=0.013$). β2 mRNA decreased significantly at 0.5 hours postexercise after TT, returning to baseline by 8 hours postexercise. After GXT, β2 mRNA was not significantly different from baseline at any postexercise time point. A significant time effect ($F_{(4,68)} = 4.7$, $p=0.004$) was noted for α2A, with significantly higher levels observed at 0.5 and 8 hours postexercise across both exercise trials.

Significant effects of exercise ($F_{(1,17)} = 5.3$, $p=0.034$), time ($F_{(4,68)} = 4.3$, $p=0.004$), and exercise by time ($F_{(4,68)} = 4.1$, $p=0.005$) were also found for the serotonin receptor HTR1D. At 0.5 and 8 hours after TT, HTR1D expression increased significantly above baseline ($p<0.003$) and then decreased toward baseline at 24 and 48 hours postexercise (Figure 2.4). In contrast, HTR1D expression increased from 0.5 to 24 hours postexercise after GXT, with significant increases in expression occurring at 8 hours postexercise ($p=0.001$). There was not a significant exercise effect for DRD4, although

time ($F_{(4,68)}$ = 28.4, $p<0.001$) and exercise by time ($F_{(4,68)}$ = 4.8, $p=0.006$) effects were significant. After TT, DRD4 mRNA was significantly lower than baseline levels at 0.5 and 8 hours postexercise ($p<0.042$), while after GXT, DRD4 expression tended toward an increase at 0.5 hours postexercise ($p=0.075$) and decreased at 8 hours postexercise ($p<0.001$).

Immune markers. A significant exercise effect ($F_{(1,17)}$ = 5.6, $p=0.031$) was observed for IL-6 mRNA, which increased at all postexercise time points after GXT but not after TT (Figure 2.5). For TLR4, significant exercise by time ($F_{(4,68)}$ = 9.1, $p<0.001$) and time ($F_{(4,68)}$ = 10.9, $p<0.001$) effects were evident. TLR4 increased earlier after TT, with significant increases occurring at 0.5 and 8 hours postexercise ($p<0.001$) and returning to baseline levels at 24 and 48 hours postexercise. After GXT, TLR4 mRNA increased significantly at 8 hours postexercise ($p<0.001$) and decreased more gradually toward baseline at 24 and 48 hours postexercise. A significant time effect was observed for IL-10 ($F_{(4,48)}$ = 21.4, $p<0.001$), with postexercise increases seen at 0.5, 8, and 24 hours across both exercise treatments ($p<0.025$).

Discussion

Main Findings

The primary purpose of this study was to examine the perceptual fatigue and pain responses as well as the gene expression markers associated with fatigue following two different exercise trials; a graded exercise test and a 40k time trial. By design, GXT was shorter, but more intense than TT as evidenced by higher postexercise lactate levels, peak heart rates, and peak RPE ratings. However, the perceptual ratings of both physical

fatigue and pain were significantly increased for 24 hours following TT while unchanged following GXT. This suggests that the longer duration, less intense TT causes long-term fatigue while GXT is not sufficient to produce lasting fatigue. The higher lactate values following GXT suggest that the muscle was more acidic and had higher extracellular ATP concentrations as compared to TT (7, 21), though the exposure to heightened levels of these metabolites was more than 6 times longer during TT. The increased levels of metabolites at the end of GXT should cause a greater activation of the Group III/IV afferent nerve fibers and may contribute to the cessation of exercise seen at the end of the GXT(26); however, the longer exposure of the nerves to metabolites during TT may provide the impetus for the more pronounced sensation of fatigue and the larger changes seen in postexercise mRNA gene expression.

Mental fatigue, physical fatigue, and pain in healthy, trained individuals did not increase to the same absolute levels, nor did it last as long as our previous work in CFS patients, though our exercise trials were more intense (25, 27). However, the relative increases in physical fatigue and pain from baseline to peak values following TT in healthy individuals roughly matched the relative increases from baseline in CFS patients following a 25-minute exercise trial (25, 27). In CFS, perceptual responses tended to peak 8-24 hours after exercise and remain elevated for at least 48 hours (25, 27), while in healthy individuals, perceptual responses peaked at 0.5-8 hours before returning to baseline levels. These increases in perceptual responses mirrored the largest gene expression changes in both healthy and CFS individuals as described below.

Using more objective assessments of the contributions to muscular force, the level of peripheral fatigue as assessed by magnetic stimulation was significantly higher

following TT. The level of peripheral fatigue following TT were similar to those found during a 5k time trial (3), suggesting that the participants in this trial reached a similar individual critical threshold of peripheral fatigue, even though the trial was approximately 10 times longer. Despite lower lactate levels, TT demonstrated a larger change in $Q_{tw.pot}$ and subsequent peripheral fatigue than GXT. While lactate contributes to afferent signaling(26), in a brief exercise test such as a graded exercise test, a long-lasting sensation of fatigue (distinct from muscle fatigue as defined by a decrease in muscle performance) is unlikely to be explained solely by the metabolic contribution to central command via afferent signaling (1, 13). This suggests that there may be additional central mechanisms for the cessation of exercise, perhaps supporting the idea of a central governor (38, 39).

Gene Expression Changes Following GXT and TT Trials

We expected that a 40k time trial would produce greater sensory fatigue perception following exercise, eliciting greater changes in gene expression, though we were unsure that the direction of change would mirror that of the changes that were previously found to occur with CFS.

Metabolite-detecting Receptors

The current study demonstrated marked decreases in mRNA for 8 hours postexercise before returning to baseline for both trials, though there were larger decreases in ASIC3, P2X4, and TRPV1 following TT. In contrast to the transient decreases in mRNA for the metabolite-detecting receptors found in the current study, our

previous work demonstrated marked increases in CFS patients (25, 27, 54). The decreases in metabolite-detecting receptors in healthy individuals may be indicative of a healthy adaptive down-regulation following receptor activation in an attempt to reduce receptor sensitization and the sensation of fatigue following exercise (54). Though peak metabolite levels may be lower during TT than GXT, the activation of the receptors may be prolonged due to the extended exercise duration, leading to increased perceptual fatigue and subsequently larger decreases in gene expression. These larger decreases in mRNA following TT may lead to a greater exercise adaptation through an attempt to 'reset' the fatigue signal to pre-exercise levels. Finally, our data suggest that the magnitude of change in the mRNA for metabolite-detecting receptors may be work related, with larger magnitudes, or durations, of work leading to larger decrements in gene expression.

The decreases in metabolite-detecting mRNA following exercise are consistent with a recent viewpoint paper by Smith (48) that suggested the "no pain, no gain" colloquial phrase may in fact rely on the ability to push through fatigue in order to maximize the benefits of exercise training. In healthy individuals, a single bout of exercise reduces the amount of mRNA for fatigue and pain sensing metabolite detectors, with a more strenuous and painful exercise causing larger changes. With consistent training, there may be a further down-regulation in the number and perhaps sensitivity of these metabolite detecting receptors, reducing the perception of fatigue and pain felt during subsequent similar exercise bouts, leading to performance increases independent of cardiovascular adaptations.

Adrenergic Receptors

Following exercise, expression of the adrenergic receptors, with the exception of β2 following TT, increased following exercise. The general increases during this study were similar to the changes that were found with CFS (25, 27, 54), though our increases were attenuated and returned to baseline levels by 24 hours after exercise. However, the CFS group had increases in adrenergic β2 (25, 27, 54), whereas we found an initial decrease or no change following exercise in healthy individuals. Further, it appears that the intensity and duration of the GXT was not great enough to cause significant changes in gene expression.

One of the primary roles of β-adrenergic receptors is the control of blood flow, both by enhancing contractility and heart rate via the β1 receptor and by causing dilation of arteries supplying the working muscle by activation of the β2-receptor. β2 receptors are also important in exercise training, as activation enhances anaerobic metabolism and muscle oxidation (47). Within the arteries, alpha-2 receptor activation is known to cause vasoconstriction and reduction in blood flow (16). During a healthy exercise response, it may be expected that the adrenergic receptors would change to increase blood flow during both exercise and rest to reduce metabolite accumulation and afferent signal. However, following the time trial, the increase in α-adrenergic mRNA and decrease in β2 receptors may reflect a decrease in blood flow for up to 0.5-8 hours following exercise, leading to increased metabolite levels during both rest and subsequent exercise. The adrenergic receptors may also aid in the sympathetic nervous system response on the immune system (17) and enhance the fatigue signal mediated by the muscle sensory afferents (28).

Immune Receptors

We determined that TLR4 mRNA was upregulated after both GXT and TT. TLR4 was upregulated immediately after TT, but not until 8 hours after GXT. We also determined that IL-6 mRNA was upregulated after GXT as compared to no change after TT. TLR4 is an indicator of inflammation; whereas IL-6 is an anti-inflammatory myokine (44) that may increase cortisol release following exercise (20), may increase the sensitivity of the vasculature to the sympathetic signal (23), or cause vasodilation of the arterioles (34). When compared to a study by Nieman et al., in which cyclists completed a 2-hour cycling bout at 60-65% of $Watts_{max}$ in which no change in IL-6 mRNA was found, we found increased gene expression following GXT but not TT (36). The primary difference between the two trials was the GXT duration was less than 11 minutes and the intensity was much greater than TT. The moderate decrease in IL-6 gene expression beginning at 8 hours and lasting until 24 hours, as well as the increases in TLR4, following TT suggests that the cyclists may have been undergoing a temporary depression in immune function or sympathetic tone, allowing an increased blood flow following exercise (23).

Indolamine Receptors

Following TT, there were larger increases in the serotonin receptor and larger decreases in the dopamine receptor than after GXT. Serotonin and dopamine have been implicated in fatigue, though HTR1D and DRD4 gene expression have not been examined in CFS. Previous studies have described how serotonin and dopamine may cause central fatigue (5, 29-31, 35), but have ignored the possible effects in the periphery

and the sensory afferent nerves. We found that the serotonin receptor had 8-30 fold increases following exercise and remained elevated for at least 8 hours postexercise, with larger increases following TT. During exercise, elevated quantities of serotonin lead to decreased motivation (11) and serotonin quantities tend to peak at the point of fatigue. Activation of the 5-HT receptor increases afferent signal in rats (51) and causes a short, but modest, increase in pain when directly injected into human muscle (32). Unlike serotonin, the dopamine receptor had decreases of almost 50% at 8 hours after exercise. Activation of the dopamine receptor has been shown to inhibit depolarization of afferent nerves (9, 46). Thus, a decrease in the number of dopamine receptors could lead to increased activation of the afferent nerve and, even at rest, an increase in fatigue sensation. The combination of an increase in serotonin receptor and the concurrent decrease in dopamine receptor mRNA may lead to increased sensations of fatigue and reduced motivation following exercise (31).

Methodological Qualifications

A limitation of this study is that gene expression was assessed in leukocytes and may not represent changes in brain, muscle, or other tissues. In addition, it is unclear how changes in gene expression would affect protein synthesis or quantities with any of the genes under study. Future studies should determine the relationships between changes in mRNA and the effect on protein synthesis and quantity.

Conclusions

The longer, less intense TT produced longer-lasting perceived fatigue and pain, more peripheral fatigue, and greater mRNA gene expression changes than the shorter, but more intense GXT. Also, the normal exercise response is different in healthy individuals than individuals with CFS, even when the increase in perceived physical fatigue during exercise is matched between groups. CFS patients have large increases in metabolite-detecting genes following exercise while a healthy response consists of a decrease in these same genes. CFS patients also demonstrate an increase in adrenergic β2 mRNA while a healthy response consists of a smaller increase in α2a, α2c, and β1 and no change or a decrease in β2 mRNA. These findings aid in describing the normal gene expression response to exercise as compared to the abnormal response seen in patients with CFS. These gene alterations suggest that increases in adrenergic and serotonin gene expression and the concurrent decrease in dopamine gene expression may lead to an increased resting afferent signal, leading to increased fatigue and decreased motivation. The decrease in metabolite-detecting receptor gene expression may be an attempt to return the fatigue signal to homeostasis. The combination of changes in all of the receptors following exercise may possibly contribute to the prolonged sensation of fatigue experienced by individuals following intense exercise.

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Table 2.1. Subject and exercise characteristics.

Age	36.1 ± 9.7		
Weight (kg)	68.1 ± 9.2		
Training History (miles/week)	77.6 ± 37.6		
Riding experience (years)	8.2 ± 9.3		
$VO2_{peak}$ (mL/kg/min)	54.8 ± 5.9		
	GXT	TT	p-value
Exercise Time (min)	10.9 ± 1.2	74.3 ± 6.0 *	***<0.001***
Total Work (kJ)	2.18 ± 0.3	13.93 ± 2.9 *	***<0.001***
1-min Post-ex Lactate (mmol/L)	12.4 ± 0.8	8.9 ± 0.9 *	***<0.001***
5-min Post-ex Lactate (mmol/L)	10.0 ± 1.2	6.7 ± 0.8 *	***0.008***
Peak HR (bpm)	183.9 ± 9.7	176.2 ± 13.5 *	***0.003***
Peak RPE (6-20)	18.6 ± 1.0	17.7 ± 1.7 *	**<0.05**
Average HR (bpm)	-	164 ± 15.0	N/A
Average RPE (6-20)	-	15.5 ± 1.46	N/A

Values are mean ± SD; n = 20. HR = heart rate, RPE = ratings of perceived exertion. * Denotes significant difference from GXT ($p < 0.05$)

Table 2.2. Effects of GXT and TT on quadriceps muscle function

	Percentage change from pre- to 2 min postexercise		
	GXT	40k time trial	p-value
$Q_{tw.pot}$ (N)	-26.5 ± 12.4	-32.7 ± 11.1	**.04**
MRFD ($N \cdot s^{-1}$)	-28.9 ± 15.1	-34.6 ± 16.9	.29
MRR ($N \cdot s^{-1}$)	-31.9 ± 19.2	-35.6 ± 15.9	.09
CT (s)	-3.8 ± 5.5	2.9 ± 8.3 †	.07
$RT_{0.5}$ (s)	3.1 ± 19.1 †	-3.0 ± 13.0 †	.44
MVC peak force (N)	-10.1 ± 7.8	-9.5 ± 8.6	.85
Percentage voluntary muscle activation	-14.5 ± 11.0	-8.6 ± 8.5	.18

Peripheral fatigue was assessed via supramaximal magnetic stimulation of the femoral nerve before and 2 min after exercise. Changes in fatigue variables are expressed as a percentage change from pre-exercise baseline. Values are expressed as means ± S.D. Abbreviations: $Q_{tw.pot}$, potentiated single twitch; MRFD, maximal rate of force development; MRR, maximal rate of relaxation; CT, contraction time, $RT_{0.5}$, one-half relaxation time; and MVC, maximal voluntary contraction. Percentage muscle activation is based on superimposed twitch technique. Most of the variables changed significantly when comparing baseline and 2 min after exercise ($p < 0.05$). † Not significantly different from pre-exercise baseline. Pre-exercise, resting mean values before GXT for potentiated single twitch, MRFD, MRR, CT, and $RT_{0.5}$ were 165.3 ± 32.0 N, 3006.5 ± 511.4 N s^{-1}, 2221.5 ± 958.1 N s^{-1}, .175 ± .0172 s, and .045 ± .006 s, respectively. Pre-exercise, resting mean values before TT were 164.1 ± 39.7 N, 2948.9 ± 849.3 N s^{-1}, 2272.5 ± 1132.4 N s^{-1}, .174 ± .0164 s, and .044 ± .009, respectively.

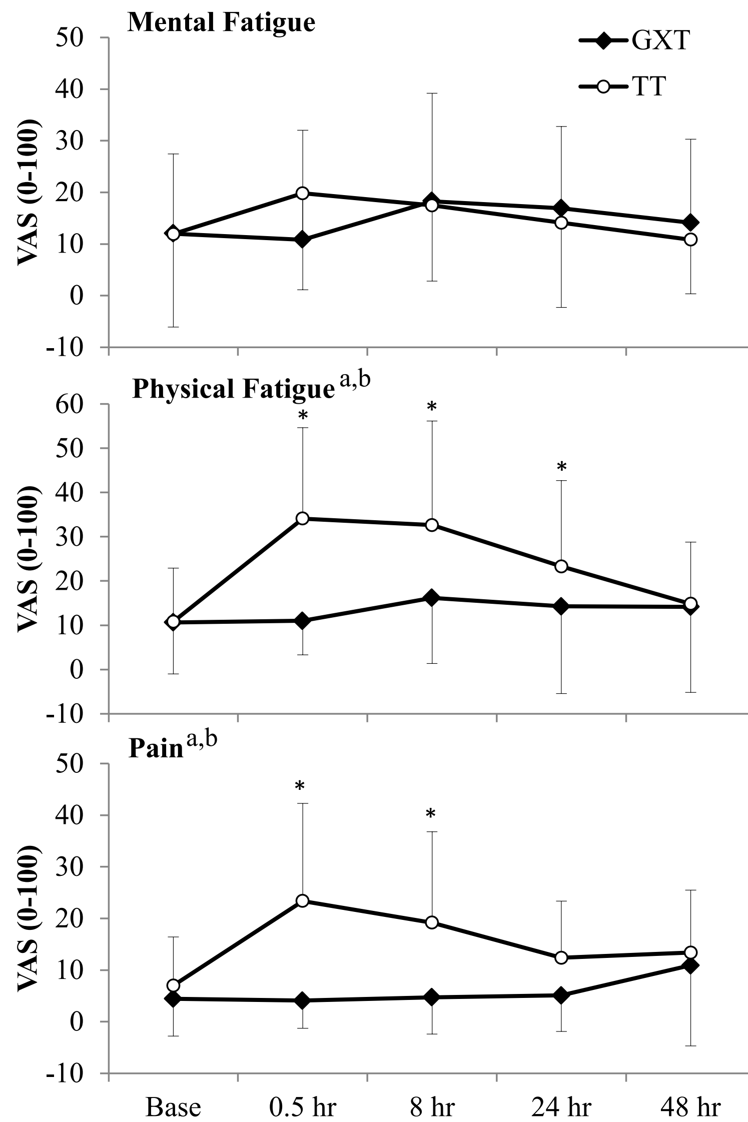

Figure 2.1. Ratings of mental fatigue, physical fatigue, and pain at the times indicated. Ratings were based on a 0 to 100 scale. Baseline indicates before exercise; 0.5 = one half hour after exercise; 8 = 8 hours after exercise; 24 = 24 hours after exercise; 48 = 48 hours after exercise.

[a] refers to a significant overall exercise effect ($p<0.05$)

[b] refers to a significant overall exercise by time interaction ($p<0.05$)

* refers to a significant within trial difference from baseline ($p<0.05$)

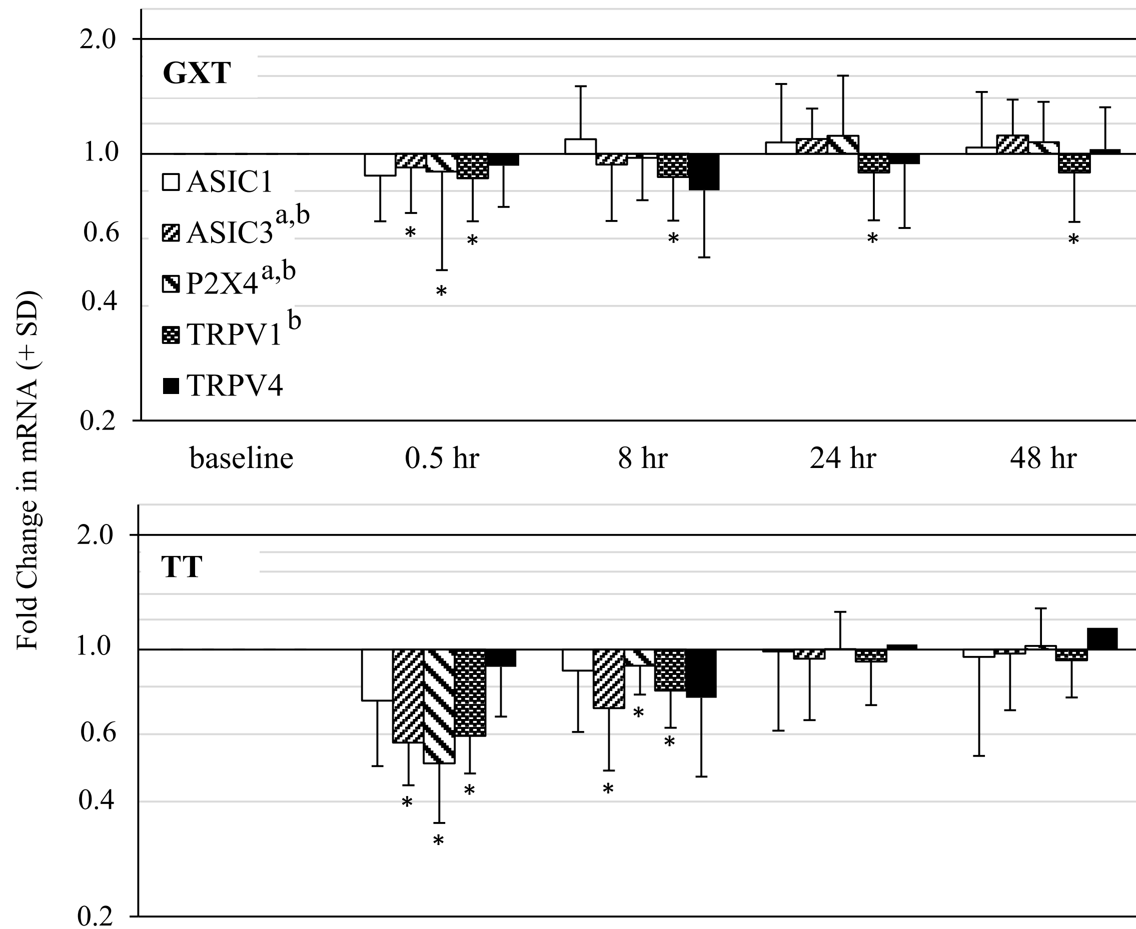

Figure 2.2. Amount of mRNA for ASIC1, ASIC3, P2X4, TRPV1, and TRPV4 expressed as fold changes relative to baseline levels at each of the times indicated before (baseline) and after the indicated exercise (GXT = graded exercise test; TT = 40k time trial).

[a] refers to a significant overall exercise effect ($p<0.05$)

[b] refers to a significant overall exercise by time interaction ($p<0.05$)

* refers to a significant within trial difference from baseline ($p<0.05$)

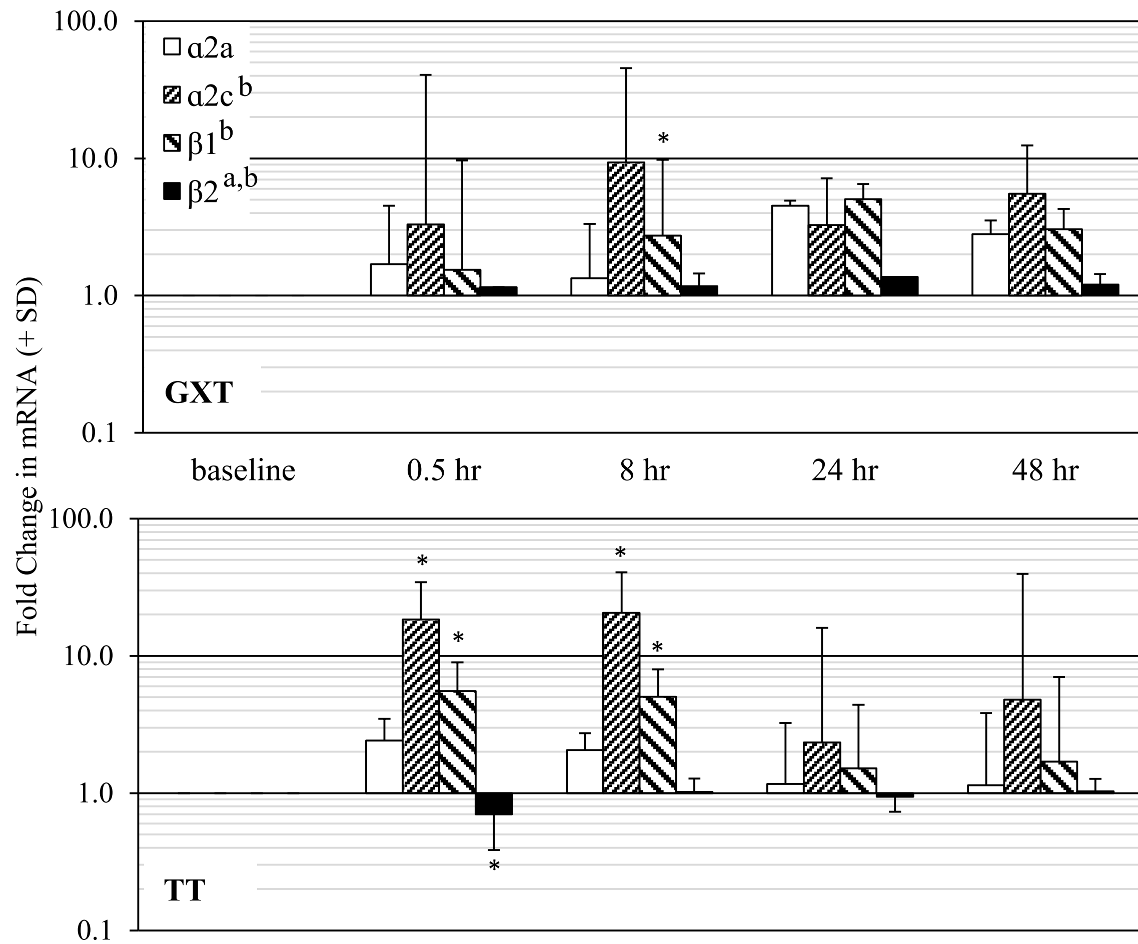

Figure 2.3. Amount of mRNA α2a, α2c, β1, and β2 adrenergic receptors expressed as fold changes relative to baseline levels at each of the times indicated before (baseline) and after the indicated exercise (GXT = graded exercise test; TT = 40k time trial).

[a] refers to a significant overall exercise effect ($p<0.05$)

[b] refers to a significant overall exercise by time interaction ($p<0.05$)

* refers to a significant within trial difference from baseline ($p<0.05$)

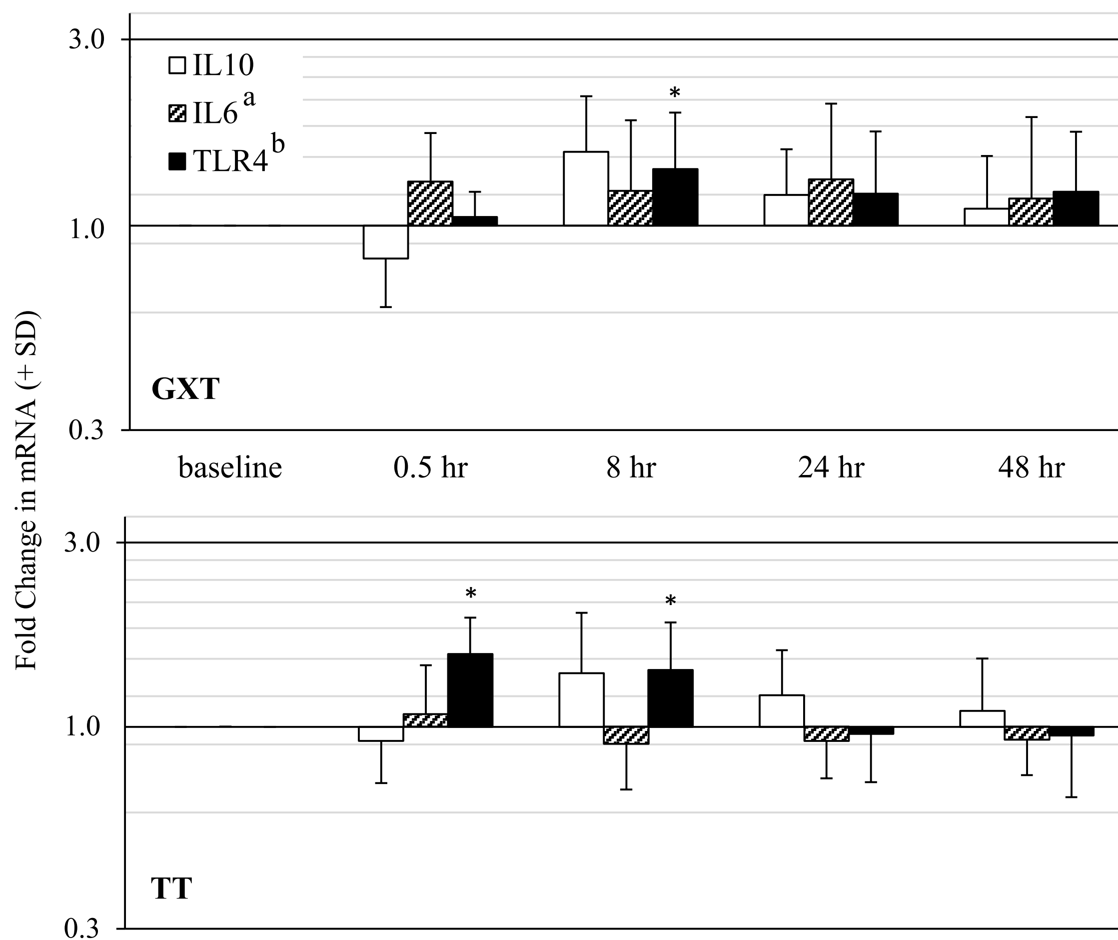

Figure 2.4. Amount of mRNA for HTR1D and DRD4 indolamine receptors expressed as fold changes relative to baseline levels at each of the times indicated before (baseline) and after the indicated exercise (GXT = graded exercise test; TT = 40k time trial).

[a] refers to a significant overall exercise effect ($p<0.05$)

[b] refers to a significant overall exercise by time interaction ($p<0.05$)

* refers to a significant within trial difference from baseline ($p<0.05$)

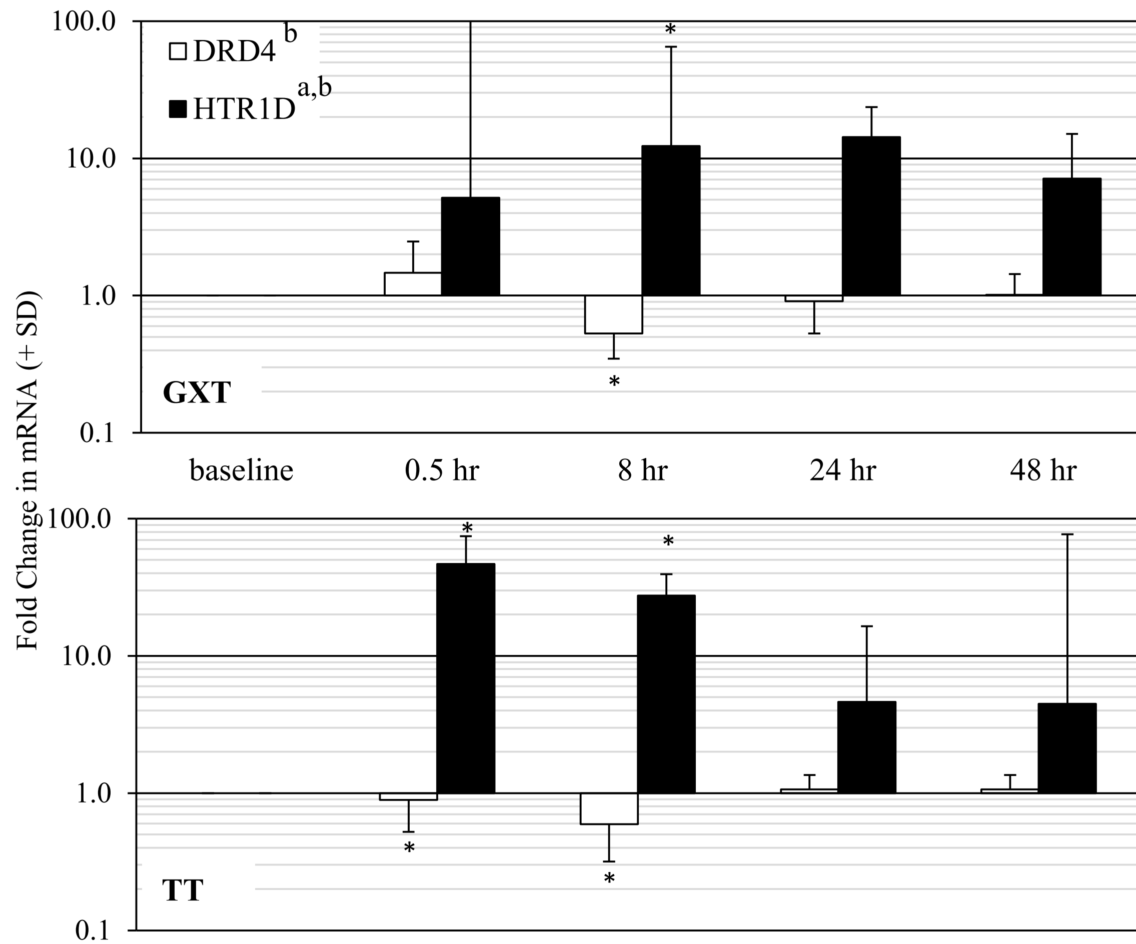

Figure 2.5. Amount of mRNA for IL-6, IL-10, and TLR4 immune function genes expressed as fold changes relative to baseline levels at each of the times indicated before (baseline) and after the indicated exercise (GXT = graded exercise test; TT = 40k time trial).

[a] refers to a significant overall exercise effect (p<0.05)

[b] refers to a significant overall exercise by time interaction (p<0.05)

* refers to a significant within trial difference from baseline (p<0.05)

EFFECT OF HEAT STRESS ON FATIGUE SENSATION

AND GENE EXPRESSION IN TRAINED CYCLISTS

FOLLOWING A 40K TIME TRIAL

Abstract

Heat stress has a negative impact on endurance exercise performance, resulting in decreased work output and increased effort and fatigue perception. We examined, in healthy trained cyclists (n= 20), the effect heat stress (35°C; HT) compared to ambient conditions (21°C; AT) on 40k time trial performance, immediate and longer term postexercise perceptions of fatigue and pain, and transcriptional alterations in the mRNA of exercise and immune function-related genes. Blood lactates were measured 1 and 5 minutes postexercise and blood draws were performed at baseline and at 0.5, 8, 24, and 48 hours postexercise. Leukocytes were separated for RNA extraction and qPCR analysis was performed for metabolite detecting, adrenergic, indolamine, and immune receptors. Core temperature was significantly higher during HT ($p<0.05$) while power and lactates were significantly higher during AT ($p<0.05$). Physical fatigue and pain sensations were increased for 8 hours following both AT and HT ($p<0.05$). Both trials resulted in significant postexercise decreases in metabolite detecting receptors ASIC1, ASIC3, P2X4, TRPV1, and TRPV4; increases in adrenergic receptors α2a, α2c, and β1; decreases in adrenergic β2, the immune receptor TLR4, and dopamine (DRD4); and increases in serotonin (HTR1D) and IL-10 ($p<0.05$). Postexercise IL-6 differed between AT and HT, with significantly greater increases observed following HT ($p<0.05$). Although lactates were higher following AT, postexercise changes in mRNA were similar between the trials. Thus, heat stress may 'act' like a metabolite, increasing inhibitory afferent signaling during exercise, causing decreased motor command that results in decreased power output.

Introduction

Heat stress has a negative impact on endurance exercise performance, resulting in decreased work output and increased effort and fatigue perception (12, 21, 23, 24, 31, 54). Cardiovascular strain likely contributes to performance decrements during heat stress (13), but hyperthermic fatigue is multifactorial (12, 16, 21, 31). Increases in core temperature and heat storage rates during exercise alter central nervous system (CNS) function, resulting in reduced voluntary muscle activity and increased fatigue perception (19, 21, 35, 36, 57).

Heat-related alterations in CNS function may be mediated by several mechanisms. Inhibitory feedback from thermoreceptors in the hypothalamus may inhibit central motor drive (39, 40). For example, when the head is cooled during exercise, decreasing the temperature of the brain and hypothalamus, time to fatigue is increased, suggesting that directly reducing inputs from brain heat sensors prevents decreases in CNS drive (4).

Several neurotransmitters, particularly the indolamines dopamine (DA) and serotonin (5-HT), may play a role in the development of fatigue during prolonged exercise (39). Serotonergic activity influences arousal levels that may improve exercise performance. It has also been shown that endurance performance is impaired in rats that had received a 5-HT agonist (7). In addition, tissue levels of DA and 5-HT have been shown to increase during exercise, but DA levels decrease substantially at exhaustion (6). This suggests that the ratio of 5-HT/DA may be important in the development of central fatigue (6, 16, 50, 58, 60). Further evidence supports the idea that these neurotransmitters play an important role specifically during endurance exercise in the heat. Administration of bupropion, a DA/noradrenaline uptake inhibitor, resulted in improved time trial

performance in the heat (50).

Another factor that may increase central fatigue during exercise is stimulation of the group III/IV afferents by a combination of metabolites, including protons, ATP, and lactate, which activate five molecular receptors, including ASIC3 (Acid-sensing Inward Current), P2X (purinergic) type 4 and 5, and TRPV (transient receptor potential vanilloid) type 1 and 4, signaling both fatigue and pain (26, 29, 47). In addition to being sensitive to metabolites, TRPV receptors are highly responsive to heat, with a threshold as low as 25°C and increased responsiveness to temperatures as high as 50°C (10, 14). It is possible that TRPV1 molecular receptors influence responses of metaboreceptive neurons innervating skeletal muscle as muscle temperature increases. This is consistent with observed increases in sympathetic response with muscle heating (48). The overall effect would be that heat-stress is perceived as a metabolite, and along with changing concentrations of protons, lactate, and ATP, amplifies the peripheral fatigue signal during exercise in the heat, potentially adding to the brain thermoreceptor signal to increase central fatigue.

The purpose of this investigation was to examine the effects of a 40K time trial in two different environments, a typical ambient environment (21 °C, ambient trial (AT)) and a warm (35°C, heat trial (HT)) environment, on immediate and longer term postexercise responses of fatigue and pain sensations (using visual analog scales- VAS) and transcriptional alterations in the mRNA of exercise and immune function-related genes in healthy, fit individuals.

We hypothesized that performance of a 40K time trial under heat-stressed conditions would produce higher levels of fatigue and pain sensations compared to

exercise during ambient conditions. Further, we expected that fatigue and pain perception would remain elevated longer after HT compared to AT. In addition, we hypothesized that mRNA for genes implicated in transducing the additional fatigue and pain signaling produced by heat stress (ASIC3, P2X4, TRPV1, and TRPV4) would be increased in blood leukocytes to a greater extent following HT than AT. Along with these sensory receptors, we hypothesized that proinflammatory cytokines (putative mediators of the sickness response) and their receptor mRNAs for IL6 and TLR4 would be elevated following time trials in the heat because the "sickness response" can be induced by working in the heat. Finally, we hypothesized that adrenergic receptors (α2a, α2c, β1, and β2) and the serotonin receptor (HTR1D) that may mediate sympathetic responses to increased muscle and core temperatures, and/or may also play a role in signaling leukocytes to mount a protective response to heat stress, would also increase. We expected these increases would be seen immediately following exhausting exercise and would be larger and longer lasting following exercise in the heat.

Methods

Participants

All experimental procedures in this investigation were reviewed and approved by the University of Utah Institutional Review Board. The protocols and procedures were explained, and all participants provided written informed consent prior to testing. Twenty healthy, moderately trained male (n=10) and female (n=10) cyclists between the ages of 18-55 were recruited for this investigation (mean ± SD; age 36.1 ± 9.7 years, body weight 68.1 ± 9.2 kg, height 172.9 ± 6.3 cm, peak oxygen consumption (VO_{2peak})

54.8 ± 5.9 mL/kg/min). Cyclists were excluded if any of the following applied: current acute musculoskeletal injury; medications known to interfere with the sympathetic nervous system; unwillingness to comply with training interruptions mandated by the protocol; any uncontrolled chronic health condition.

Protocol Overview

Participants performed three exercise tests, separated by at least 1 week: a graded exercise test to determine VO_{2peak} and two 40K time trials, one in a typical ambient environment (21 °C, ambient trial (AT)) and one in a warm environment (35°C, heat trial (HT)) on a Velotron cycle ergometer (Racermate, Seattle WA). All participants refrained from exercise for 24 hours prior to the exercise test. The ergometer was adjusted to match each participant's accustomed cycle position, and participants used their own cycle shoes and pedals. Venous blood samples were obtained from the arm at baseline and at 0.5, 8, 24, and 48 hours after the two 40K time trials. To assess the severity of preexisting and exercise-related fatigue and pain symptoms at the time of each blood draw, participants provided numerical ratings of physical fatigue and overall body pain or soreness using a 0-100 scale, where 100 was defined as the greatest amount of fatigue or pain the participant could ever imagine experiencing. Immediately after the baseline blood draw, participants began the exercise test as described below.

Experimental Protocol

On the first experimental day, participants performed an incremental cycling test (VO_{2peak}) beginning at 100W (50W for females) with resistance increasing 25W every

minute until volitional exhaustion. Participants pedaled at their preferred pedaling rate, and the test was terminated when the cadence dropped more than 10 rpm for more than 5 seconds despite strong verbal encouragement to increase the cadence. Metabolic data were collected using open circuit calorimetry (Parvomedics, True Max 2400, Sandy, UT). Peak oxygen consumption (VO_{2peak}) was recorded as the highest VO_2 recorded in a 15-second period.

On the next two experimental days, in random order, participants performed a 40K time trial at typical ambient temperature (AT; 21 °C, 20-30% humidity) or in a warm environmental chamber (HT; 35 °C, 25-35% humidity) in order to simulate an intense training ride. Following a 10-15-minute self-selected warm-up, the trial began, with participants able to fully control the gearing of the Velotron. Participants received feedback of speed, power, distance covered, and cadence and were strongly encouraged to cover the distance as fast as possible. Core temperature (T_{core}) was measured via a rectal thermistor (YSI Precisions 4400 Series; Yellow Springs Instruments, Yellow Springs, OH, USA) inserted to a depth of 15 cm from the external sphincter. Heart rate, T_{core}, time, and power were recorded every 2.5 km. Thermal sensation (20), thermal comfort, rating of perceived exertion (RPE), and VAS ratings of physical fatigue and pain were recorded every 5 km. Upon completion of the test, participants were encouraged to cool down for 5-10 minutes before cessation of exercise.

Blood samples obtained at baseline and 0.5, 8, 24, and 48 hours after each time trial were collected in EDTA tubes. Samples were quickly centrifuged (within 5 minutes of collection) at 4100rpm (2.6rcf, Eppendorf Centrifuge 5702R) for 12 minutes. Immediately after, the white cell layer was carefully collected in RLT + βME (Qiagen,

Valencia, CA, USA) and quickly frozen using a methanol-dry ice slurry and stored at -80°C. RNA was extracted using RNeasy kits (Qiagen), according to manufacturer's directions, and treated with RNase-free DNase-I (Qiagen) to eliminate possible genomic contamination. Immediately following extraction, RNA was converted to a cDNA library using the ABI High Capacity cDNA Archive kit (Applied Biosystems, Inc., Foster City, CA, USA). The cDNA was stored at -20 °C until analysis.

The cDNA libraries were analyzed using the ABI quantitative, real-time PCR system on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Inc.) using ABI Taqman Master Mix (Applied Biosystems, Inc.). Master mix/primer probe solutions were separately loaded onto 96-well preplates, with robot loading mixing these solutions when placed into 384 plates. Plates were centrifuged to remove air bubbles from the wells. Each sample was run in duplicate with the standards run in quadruplicate. No-template control samples were also run to eliminate potential contamination. Each 384-well plate contained eight samples and all genes were analyzed on the same plate. Primer probes (all from Taqman Gene Expression Assays; Applied Biosystems, Inc.) were as follows;

Adrenergic A2A (α-2A) – HS00265081_s1; Adrenergic A2C (α-2C) - HS03044628_s1; Adrenergic B-1 - Hs02330048_s1; Adrenergic B-2 - Hs00240532_s1; ASIC1 - Hs00241630_m1; ASIC3 - Hs00245097_m1; DRD4 - Hs00609526_m1; HTR1D - Hs00704742_s1; IL10 - Hs00174086_m1; IL6 - Hs00174131_m1; P2X4 - Hs00175706_m1; TLR4 - Hs00152937_m1; TRPV1 - Hs00218912_m1; TRPV4 - Hs01099348_m1; and TF2B - Hs00155321_m1. TF2B was used as the reference gene. All primer probes, with the exception of the adrenergic genes, and HTR1D, (these genes

do not have introns), recognize sequences that cross splice sites and therefore make detection of genomic DNA unlikely. All of the primer probes were designed and tested to be used together and have similar efficiencies to allow cross comparisons of quantities. Evaluation of controls in this and previous studies indicated that TF2B had less intrinsic variation than other controls, had a count range that was similar to the genes of interest, and did not change in response to the exercise protocol (28, 30). Real-time qPCR results were analyzed using SDS 2.1 (Applied Biosystems, Inc.) and were inspected to determine artifacts (loading errors, robot errors, threshold errors, etc.). Count numbers were exported to an Excel spreadsheet and analyzed according to the ddCT method described in the ABI User Bulletin #2 (Applied Biosystems, Inc.). Baseline levels for each gene were computed relative to TF2B, and these values were used as the comparator for all measures taken after the baseline period.

Data Analysis

To evaluate treatment (AT vs HT) and time effects, as well as time by treatment interactions during exercise, repeated measures (RM) ANOVAs were performed for physiological (T_{core}, power, and heart rate) and perceptual (RPE and ratings of physical fatigue and pain) responses. If time effects were significant, planned contrasts were used to determine which points differed from baseline. Significant treatment effects and time by treatment interactions were followed up with *post hoc* paired t-tests. Treatment differences for exercise duration, thermal sensation, thermal comfort, and lactates were determined by paired t-tests.

For each subject, postexercise values for gene expression measures were normalized to baseline values (1.00=baseline). Metabolite-detecting (ASIC1, ASIC3, P2X4, TRPV1, and TRPV4), immunologic (IL-6, IL-10, and TLR4), adrenergic (ADα2A, ADα2C, ADβ1, and ADβ2), and indolamine (HTR1D and DRD4) mRNAs were examined using 2 (treatment) x 5 (time) RM ANOVAs with planned contrasts to evaluate time and treatment effects and time by treatment interactions. Postexercise ratings of physical fatigue and pain were analyzed using RM ANOVAs. All data were presented as means and standard deviations, with significance set at $\alpha<0.05$.

Results

Time trial physiological and perceptual responses are shown in Table 3.1. Peak T_{core} was significantly higher during exercise in the heat (HT; 39.2 vs 38.3 °C), and significantly higher ratings for thermal comfort (more discomfort) and thermal sensation (hotter) were reported during HT ($p<0.001$). Compared to AT, HT resulted in significantly longer exercise duration (79 vs 75 min) and higher RPE (18.4 vs 17.4) respectively, while average power was significantly lower (157 vs 187 W) ($p<0.01$). Postexercise lactate measurements were significantly higher following the AT trial compared to HT (8.8 vs 6.4 and 6.6 vs 4.2 mmol L^{-1}, at 1 and 5 minutes postexercise ($p<0.01$, Table 3.1).

Significant overall time effects across AT and HT trials were seen for T_{core}, power, heart rate, and RPE ($F_{(2.4, 42)} > 9.7$, $p<0.001$, Figure 3.1). For T_{core}, a significant treatment (AT vs HT) by time interaction was noted ($F_{(2.3, 44)} = 7.8$, $p=0.001$), with between-trial differences observed from 25 to 40 km (Figure 3.1, panel A). Significant

treatment ($F_{(1,19)} = 33$, $p<0.001$) and treatment by time interactions ($F_{(3,58)} = 2.9$, $p=0.038$) were evident for power output, with significant between-trial differences observed from 10 to 35 km of exercise (Figure 3.1, panel B). Similarly, a significant treatment effect ($F_{(1,18)} = 20.1$, $p<0.001$) was evident for RPE, with significant between-trial differences seen from 20 to 40 km (Figure 3.1, panel D). Heart rate responses during AT and HT trials did not differ (Figure 3.1, panel C).

Perceptions of physical fatigue and pain increased significantly across both AT and HT, from 5 km to the end of the trials ($F_{(2.3, 41)} > 42$, $p<0.001$, Figure 3.2, panels A and B, respectively). Additionally, significant time effects were observed for postexercise physical fatigue and pain ratings ($F_{(4,64)} > 7$, $p<0.001$). Physical fatigue was significantly higher at 0.5, 8 and 24 hours postexercise, and pain remained significantly elevated at 0.5 and 8 hours postexercise ($p<.005$, Figure 3.2, panels C and D). There were no treatment differences (AT vs HT) for perceptions of physical fatigue or pain during or after the trials.

Gene Expression Changes

Metabolite-detecting receptors. There were no significant overall treatment or treatment by time interactions for any metabolite-detecting receptor mRNA (Figure 3.3). *S*ignificant overall time effects across AT and HT trials were noted for ASIC3, P2X4, TRPV1, and TRPV4 ($F_{(4,64)} > 7.5$, $p < 0.001$), but not for ASIC1 ($F_{(4,64)} = 3.1$, $p = 0.054$). Planned contrasts comparing postexercise time points to baseline indicated that ASIC3 and P2X4 mRNA levels were significantly decreased at 0.5 and 8 hours postexercise ($p < 0.001$). TRPV1 mRNA levels were significantly reduced at all postexercise time points (p

< 0.001), while TRPV4 was significantly lower than baseline only at 8 hours postexercise ($p < 0.001$).

Adrenergic and indolamine receptors. For all adrenergic receptor mRNAs, overall treatment and treatment by time interactions were not statistically significant (Figure 3.4). However, overall time effects were significant for all adrenergic receptors across both AT and HT trials ($F_{(4,64)} > 6.4$, $p < 0.001$, Figure 3.4). Contrasts indicated that adrenergic α2A, α2C, and β1 mRNA expression was significantly higher at 0.5 hours following the time trials ($p < 0.006$), with α2C and β1 remaining elevated at 8 hours postexercise ($p < 0.001$). Finally, β2 mRNA significantly decreased at 0.5 and 24 hours following exercise ($p < 0.007$).

For serotonin (HTR1D) and dopamine (DRD4) receptor mRNA, a significant overall time effect was observed ($F_{(4,64)} > 9.3$, $p<0.001$, Figure 3.5), but treatment and treatment by time effects were not significant. Following exercise, HTR1D mRNA expression was significantly higher at 0.5- and 8-hour time points ($p<0.001$). DRD4 mRNA was significantly decreased at 0.5, 8, and 24 hours postexercise ($p < 0.05$). The treatment main effect for DRD4 approached statistical significance ($F_{(1,16)} = 4.4$, $p = .054$), and it appears that DRD4 decreases tended to be more pronounced following HT.

Immune receptors and cytokines. Changes in immune gene mRNA after both trials are presented in Figure 3.6. For TLR4, significant time ($F_{(4,64)} = 14.8$, $p < 0.001$) and treatment by time ($F_{(4,64)} = 4.0$, $p = 0.025$) effects were observed. Contrasts indicated that TLR4 expression was significantly decreased at 0.5 and 8 hours following AT and HT; however, TLR4 levels remained decreased at 24 and 48 hours after HT but had returned to baseline at these time points after AT ($p < 0.001$). A significant overall time

effect was observed for IL-10 ($F_{(4,64)} = 10.7$, $p < 0.001$), with contrasts indicating significant increases at 8 and 24 hours postexercise across both groups ($p < 0.01$). For IL-6, the only significant main effect observed was for treatment ($F_{(1,16)} = 6.1$, $p = 0.026$), indicating higher IL-6 levels for HT across all postexercise time points.

Discussion

As has been previously documented, time trial performance during heat stress (HT) was significantly slower than during ambient conditions (AT) (42, 46, 49, 54, 56). Power output during HT was significantly lower than AT beginning at 10k into the trial, well before differences in core temperature were evident (beginning at 25k, Figure 3.1) (46). This suggests that the drive to reduce exercise intensity was not immediately caused by core temperature increases (brain thermoreceptor activation) but may have been mediated, at least in part, by thermoreceptors in the skin and/or muscle or by changes in cardiovascular function (42, 46). It is also possible that the reduced power observed during HT may have been due to a change in pacing strategy in anticipation of challenges associated with heat stress, as has been demonstrated before (49). Interestingly, between-trial differences in power were absent during the final sprint, indicating the ability to override sensory fatigue signals when the knowledge that the end of the trial is near (38).

Although we hypothesized that time trial performance during heat stress would produce greater fatigue sensations, subjective ratings of physical fatigue and pain increased similarly during and after both trials; however, rating of perceived exertion (RPE) increased more over the course of HT (see Figure 3.2). Previous research has shown that the faster rate of change in RPE observed during heat stress was closely

related to pacing strategy (15). Our data are consistent with this observation. Thus, the cyclists presumably adjust their pace to achieve the same sensory fatigue level in both conditions, sacrificing their power (and therefore their performance). Remarkably the cyclists recognize increasing effort in the HT condition, suggesting that motor command to activate the working muscles is more difficult to generate in this condition, independent of the amount of sensory fatigue the cyclists perceive (33). It is possible that RPE is influenced by thermal sensation and discomfort, which were both significantly higher during HT.

While there has been much research focused on causes of fatigue during exercise, there has been relatively little attention to postexercise fatigue, which can represent normal recovery processes or, in some clinical populations, may indicate pathology. In this study, both trials resulted in similar postexercise increases in physical fatigue and pain ratings, lasting 8 hours after exercise. These perceptual responses suggest that overall work produced during an exercise trial does not completely explain postexercise responses, as HT power output was significantly lower.

Gene Expression Following AT and HT Trials

We expected that the addition of heat stress would produce greater sensory fatigue perception during and after exercise; however, HT and AT produced similar fatigue perceptions. The lack of difference in fatigue ratings matches the general lack of between-trial differences in mRNA expression.

Metabolite-Detecting Receptors

During exercise, Group III/IV afferents have been implicated in contributing to the decline in motor command and subsequent decrease in power (1-3). Researchers in our group have determined that ASIC, P2X, and TRPV receptors, present on afferent fibers innervating skeletal muscle, may be responsible for detecting combinations of metabolites (H+, lactate, and ATP). Signaling from these afferent neurons to the brain is then interpreted as fatigue and/or pain (29). In combination with adrenergic, immunologic, and other receptors, changes in metabolite-detecting receptors may contribute to an increased fatigue sensation at rest for an extended period following vigorous exercise (30).

In the present study, both trials resulted in decreases in metabolite-detecting receptor mRNA at 0.5 and 8 hours postexercise, that, except for TRPV1, returned to baseline levels by 48 hours postexercise. These results are in strong contrast to postexercise responses observed in patients with pathologic fatigue, who show sustained increases in these markers after only 25 minutes of moderate exercise (70% of predicted max heart rate) (28, 30). In those studies, controls demonstrated very small (if any) decreases in the mRNA of these receptors following the 24 minutes of moderate exercise.

The postexercise decreases in metabolite-detecting receptor mRNAs observed in the present study may represent signaling to decrease metabolite-detecting receptor sensitivity in an attempt to reduce the afferent signal following exercise. In contrast, patients with chronic fatigue syndrome (CFS) experience severe postexercise fatigue perception in conjunction with amplified metabolite-detecting receptor mRNA expression

during the same period; thus, the receptors may have increased fatigue and pain sensitivity for up to 48 hours after exercise in these patients (59).

We hypothesized that after HT, there would be an increase in TRPV, P2X, and ASIC receptor mRNA, because TRPV receptors respond to heat and also influence the sensitivity of P2X and ASICs (6, 11). Interestingly, higher postexercise lactate levels following AT did not result in any greater change in metabolite-detecting mRNAs compared to HT even though lactate is one of the essential metabolites that help signal muscle fatigue and pain (47). If heat is one of the metabolites that contributes to sensory muscle fatigue, it appears that in both trials cyclists chose work rates that produced very similar metabolite loads, leading to similar sensory fatigue levels in both the AT and HT trials. This concept is also supported by the similar heart rate in both trials. These cyclists chose to match the sensory perception of fatigue, rather than attempt to match the performance speed. They apparently did this even in the face of slightly increased perceived exertion. Thus, it appears, at least for this sample of cyclists, that maintaining a certain level of sensory fatigue is more important than maintaining either perceived exertion or performance speed.

The results of this study would likely have been different if we had matched the work rates between the two trials, rather than matching the participants' effort. With this paradigm, we may have seen an increased response of genes to the exercise in the heat as the overall metabolite load would have been increased as compared to the exercise in ambient conditions.

Adrenergic Receptor

The adrenergic receptors alpha-2a, alpha-2c, and β2 are important in the regulation of blood flow. β2 receptors are also important in the exercise training response, as their activation enhances muscle oxidative and anaerobic metabolism (51). These receptors can co-localize with ASIC and P2X and thereby directly influence the sensitivity of these metabolite-detecting receptors (30). The observed significant increases in alpha-2C, alpha-2A, and β1 receptor expression following both time trials may have increased the sensitivity of metabolite-detecting receptors, thus contributing to fatigue sensations persisting after exercise. It is also possible that the changes in expression of alpha and beta adrenergics were a cause of or a response to decreased blood flow at rest (18). In this case, if the quantity of adrenergic α2-receptors is increased in muscle blood vessels, just as in the leukocytes, there may be an increased resting vasoconstriction. The decreased muscle blood flow may be enhanced by a decrease in β2 receptor expression. In skeletal muscle, previous research has demonstrated that the β2 receptor is the primary β-adrenoceptor controlling blood flow (22). Thus, the increased vasoconstriction from the increase in alpha receptors, coupled with the decreased vasodilation from the β2 receptors, may lead to an increased resting metabolite load. The combination of increased metabolites and an increased sensitivity of the afferent nerve may lead to an increased sensation of fatigue postexercise.

Indolamine Receptors

Serotonin and dopamine also play a role in fatigue, particularly in conjunction with heat stress (49). Activation of the dopaminergic system is necessary to perform

exercise. Further, pharmacologic manipulation of dopamine levels via the dopamine reuptake inhibitor bupropion resulted in improved endurance performance (25, 58). Conversely, elevated levels of serotonin are associated with decreased motivation (11). During sustained aerobic exercise, serotonin levels peak at the point of fatigue. In the present study, we observed significant decreases in transcription of the dopamine receptor DRD4 at 0.5, 8, and 24 hours following exercise. Significant postexercise increases in transcription for the serotonin receptor HTRD1 were observed following both time trial conditions. This may be in response to the increased blood serotonin levels that are seen during exercise (7, 41). Increased serotonin in the periphery may increase the afferent signal during and following exercise. Previously, it has been shown that serotonin activates the 5-HT receptor on afferent nerves leading to an increased firing rate (53). There may also be an increase in 5-HT receptors in the brain following exercise, increasing the sensitivity of the brain to endogenous serotonin (8).

Immune Function in Fatigue

For these genes, the only postexercise difference between the AT and HT treatments was increased expression of IL-6 in the HT group. Unlike previous research that utilized a less intense exercise protocol (32), we found an increase in IL-6 mRNA postexercise These differences may be due to utilization of more intense exercise as well as the thermal challenge. The increase in IL-6 mRNA may contribute to increases in circulating IL-6, much of which is released directly from the muscle (52). IL-6 may play a role in lipolysis, fat oxidation, and insulin-mediated glucose disposal (44) as well as an anti-inflammatory role, decreasing the amount of inflammatory TNF-α released into

circulation (45). The increase in IL-6 mRNA following exercise in the heat, but not ambient conditions, suggests that there may be increased inflammation following exercise in the heat. In addition, IL-6 has been associated with pain in a number of chronic conditions, and may play a direct role in increasing fatigue and pain sensations (17).

Heat shock proteins (HSPs), in particularly HSP70, are increased following vigorous activity (34). One way in which HSPs work is by binding to TLR4, causing an increase in NF-κB activation (5). NF-κB is a pro-inflammatory transcription factor that may play a role in chronic inflammatory diseases (9). This study found an approximately 1.5-fold increase in TLR4 mRNA. If this increase in TLR4 mRNA leads to an increase in TLR4 protein, an increased postexercise inflammatory response would result. Interestingly, following HT the TLR4 response remained elevated above baseline at 24 and 48 hours postexercise, while values normalized after AT during this period. This suggests that heat stress may produce a longer lasting postexercise inflammatory stimulus.

Following strenuous exercise, plasma anti-inflammatory cytokine IL10 levels have been shown to increase by as much as 27-fold (43), perhaps to counteract the concurrent large increases in inflammatory cytokine levels. However, this study found an initial 10% decrease in IL10 receptor mRNA following exercise at ambient temperatures followed by a 1.5-fold increase 8 hours after both AT and HT. Previously, it has been shown that IL10-deficient mice had increased levels of fatigue as compared to wild-type mice (27). In the same way, this initial decrease in IL10 receptor mRNA may contribute to the increased postexercise fatigue. By 8 hours after exercise, IL10 receptor mRNA

levels increased, perhaps restoring the sensitivity of the body to the anti-inflammatory cytokine, reducing inflammation and fatigue levels. The sum total of an increase in TLR4 mRNA and an initial decrease in IL-10 should be an increased inflammatory response following exercise, perhaps leading to an increased fatigue response. By 8 hours after exercise, the increase in IL-10 mRNA could mediate decreased inflammation and fatigue sensations.

Methodological Qualifications

A limitation of this study is that gene expression was assessed in leukocytes and may not represent changes in brain, muscle or other tissues. In addition, it is unclear how changes in gene expression would affect protein synthesis associated with any of the genes under study. Future studies should determine the relationship between changes in mRNA and the effect on protein synthesis.

Conclusions

The two time trials under AT and HT conditions produced similar and significant increases in fatigue, effort, and pain perceptions during and following exercise, despite the fact that significantly less work was produced during HT. Although the AT resulted in significantly higher postexercise lactate levels, changes in metabolite-detecting mRNAs were the same for both trials. This suggests that heat may inhibit exercise performance much like other metabolites, possibly due to its effects on TRP expression, increasing the overall afferent signal via group III and IV afferent during exercise (37), causing decreased motor command and central motor drive (55) leading to the observed

decreased power output. The increase in serotonin and the adrenergic receptors along with the decrease in metabolite-detecting receptors and dopamine may play a pivotal role in the fatigue that occurs in many individuals following exercise, possibly persisting for several days.

Understanding how exercise affects gene expression may lead to a better understanding of why athletes, healthy individuals, and even diseased populations perceive fatigue differently before, during, and after exercise, and how fatigue perception may be altered by training, diet, and other day to day changes.

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Table 3.1. Mean physiological and perceptual responses to time trials during ambient (AT) and heat-stressed (HT) time trials.

	AT	HT	p-value
Physiological Responses			
Time (min)	**75.2 ± 6.58**	**79.0 ± 7.20**	**0.002**
Power (W)	**187 ± 40.3**	**157 ± 32.3**	**<0.001**
Heart Rate (bpm)	164 ± 15.7	165 ± 15.7	0.697
Lactate – 1min post-ex ($mmol \cdot L^{-1}$)	**8.8 ± 3.2**	**6.4 ± 1.7**	**0.002**
Lactate – 5min post-ex ($mmol \cdot L^{-1}$)	**6.6 ± 2.7**	**4.2 ± 1.5**	**0.001**
Perceptual Responses			
RPE (6-20)	**15.5 ± 1.46**	**16.4 ± 1.29**	**0.004**
Physical Fatigue (0-100) *	45 ± 21.7	51 ± 22.4	0.311
Pain and Soreness (0-100)*	34 ± 22.9	36 ± 24.4	0.730
Thermal Comfort	**2.3 ± 0.68**	**3.6 ± 0.42**	**<0.001**
Thermal Sensation	**5.0 ± 0.93**	**7.0 ± 0.41**	**<0.001**

Values are mean ± SD. AT = Ambient Time Trial (21 °C); HT = Heat Time Trial (35 °C)

* increase from baseline to end of time trial

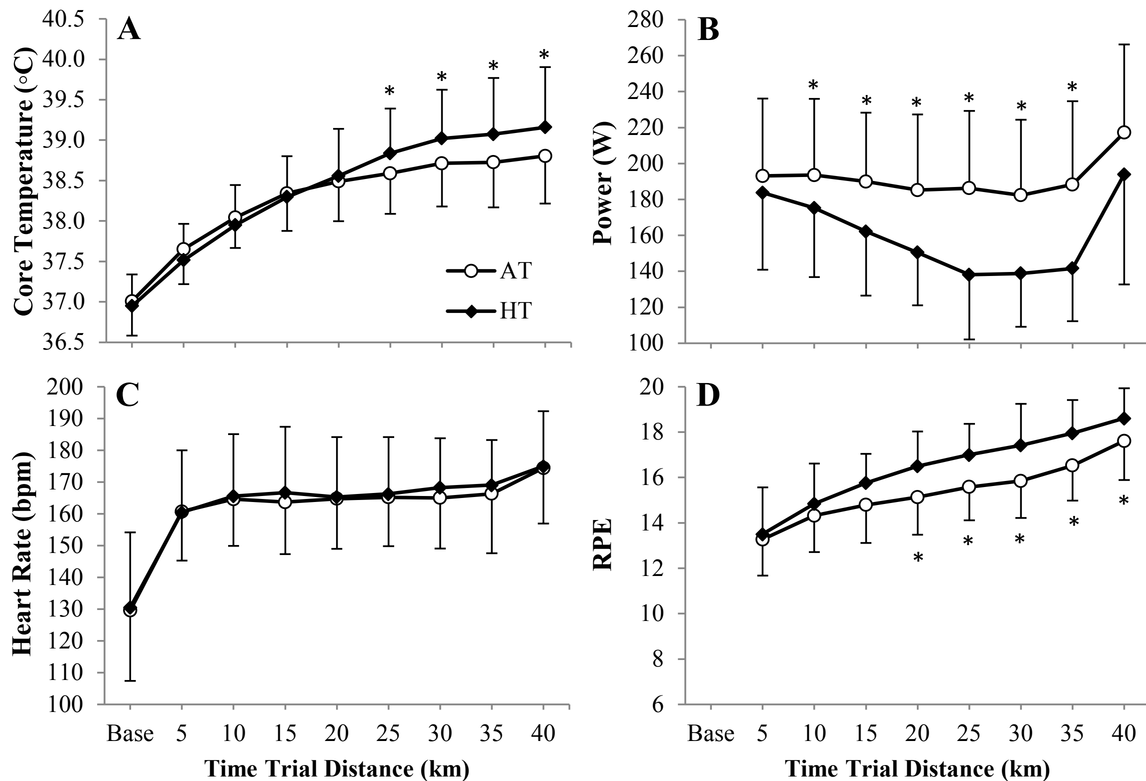

Figure 3.1. Groups means (± SD) for core temperature (panel A), power (panel B), heart rate (panel C), and RPE (panel D) at 5 km intervals during ambient (AT) and heat-stressed (HT) time trials. Significant overall time effects were noted for each of these variables, $p<0.001$ with significant increases observed from 5 km to the end of the trials. For core temperature, a significant treatment by time interaction was noted, $p<0.001$, with significantly greater increases observed during HT after 25 km. Both treatment and treatment by time effects were significant for power ($p<0.005$), with treatment differences evident between 10 and 35 km. There were no treatment differences for heart rate.

* indicates significant differences between trials assessed by post hoc paired t-tests ($p<0.05$).

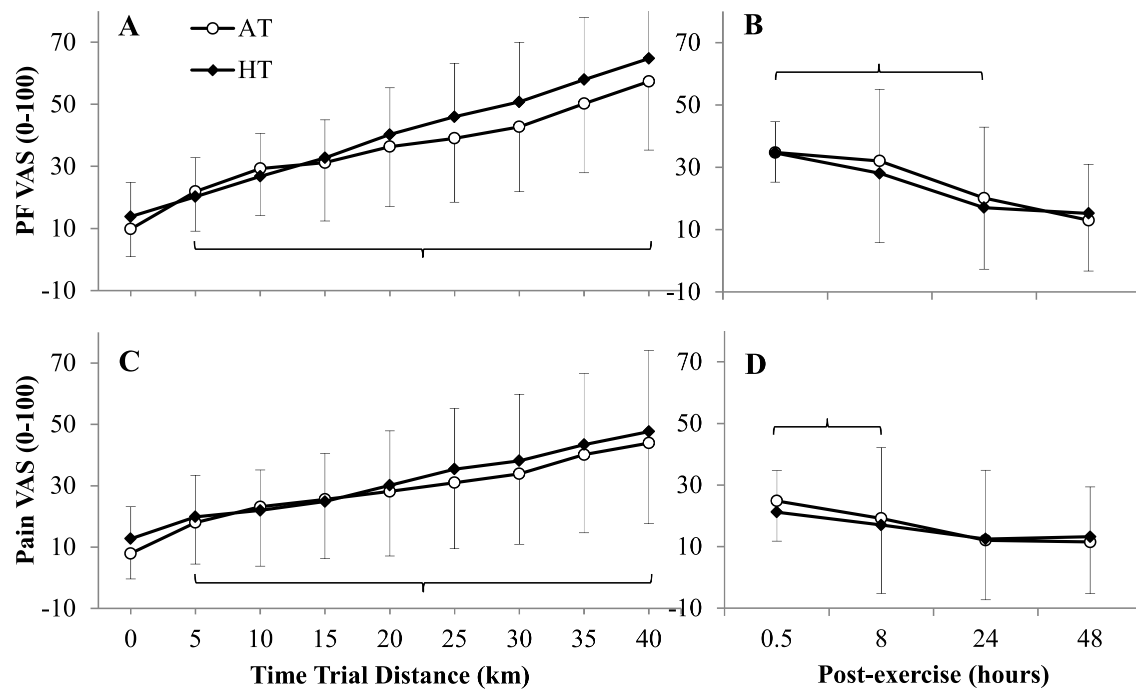

Figure 3.2. Ratings of physical fatigue (PF) during time trial exercise and recovery (panels A and B) during ambient (AT) and heat (HT) trials. Ratings for pain are depicted in panels C and D. Significant time effects were noted for both variables during exercise and postexercise periods, p<0.001. Treatment (AT vs HT) effects were nonsignificant. Brackets enclose time points that are significantly different from baseline across both treatments, p<0.05.

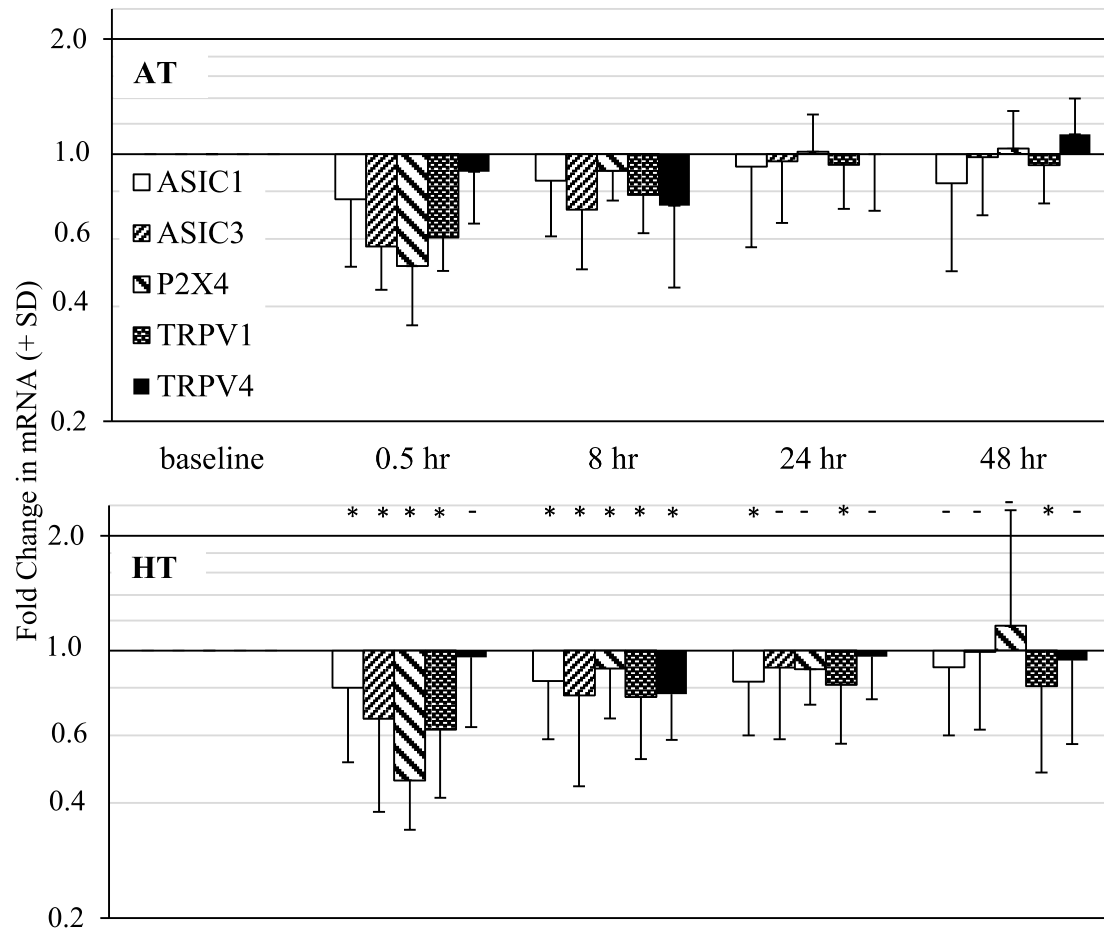

Figure 3.3. Metabolite-detecting receptor gene expression for ambient (AT) and heat (HT) trials expressed as fold changes relative to baseline (1.0). Values are mean ± SD

* indicates significant difference from baseline across both trials, p<0.05

- indicates nonsignificant time effects

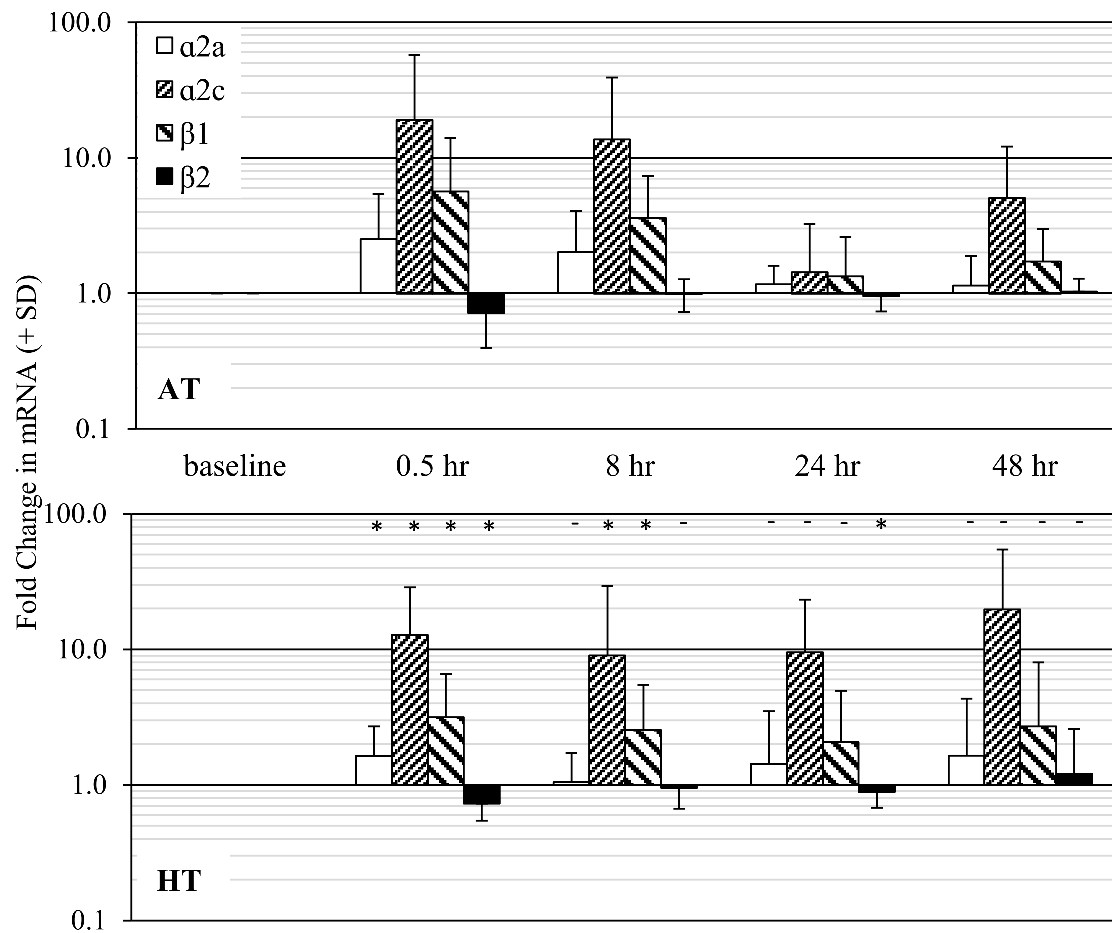

Figure 3.4. Adrenergic receptor gene expression for ambient (AT) and heat (HT) trials expressed as fold changes relative to baseline (1.0). Values are mean ± SD

* indicates significant difference from baseline across both trials, $p<0.05$;

- indicates nonsignificant time effects

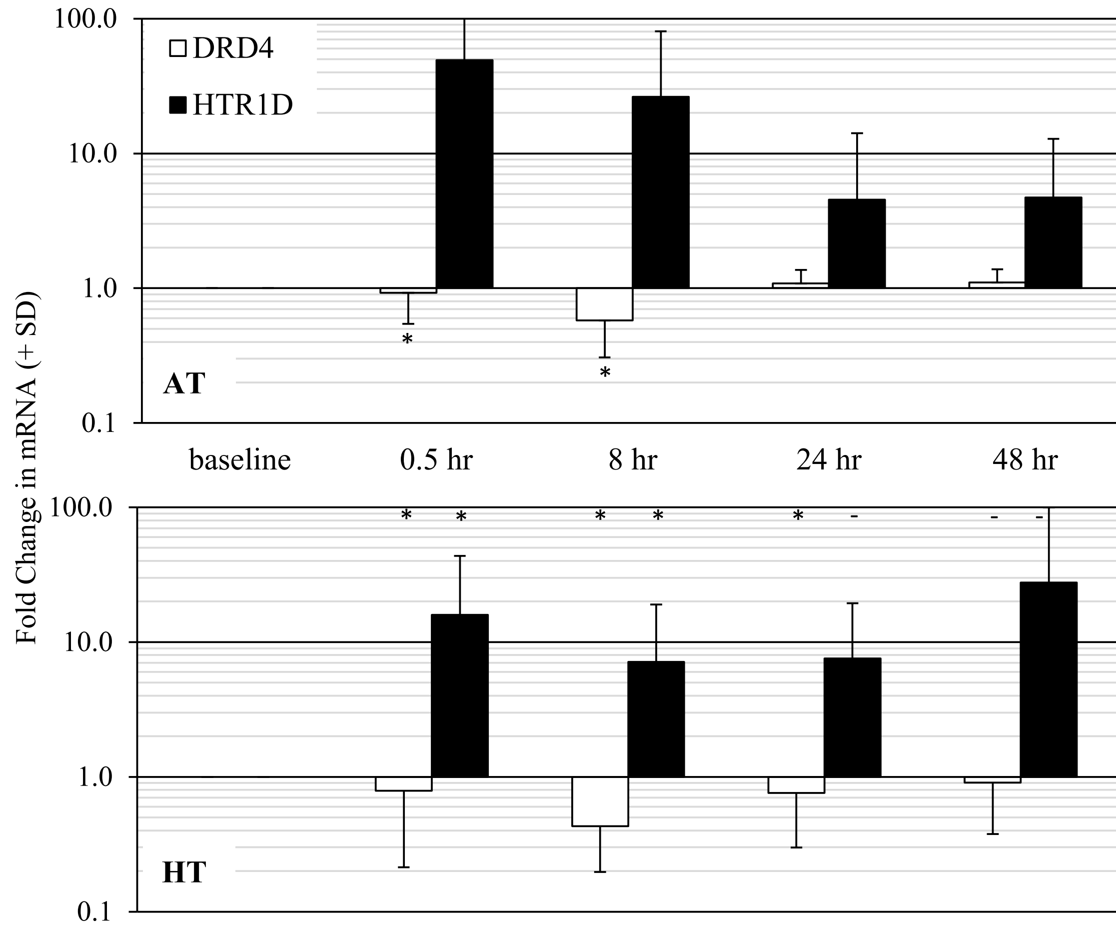

Figure 3.5. Indolamine receptor gene expression for ambient (AT) and heat (HT) trials expressed as fold changes relative to baseline (1.0). Values are mean ± SD

* indicates significant difference from baseline across both trials, p<0.05

- indicates nonsignificant time effects

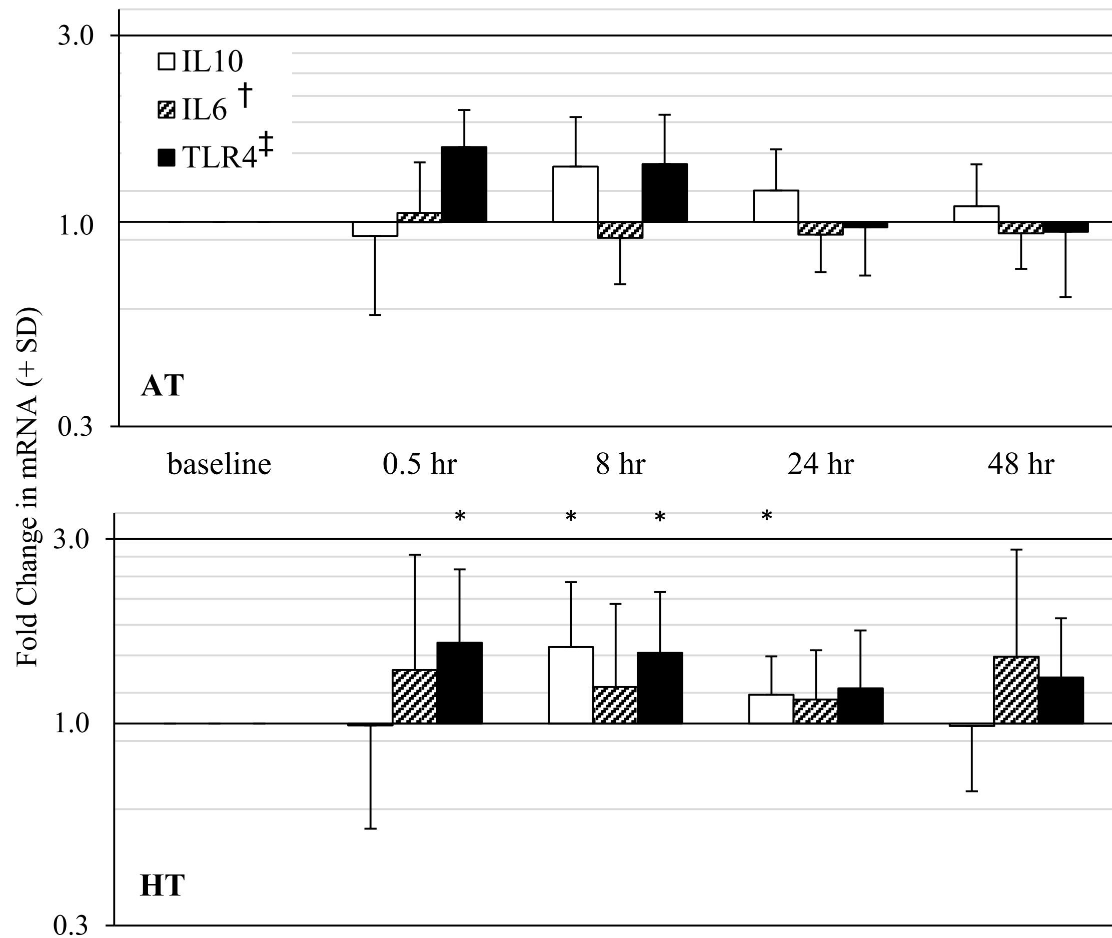

Figure 3.6. Immune function gene expression for ambient (AT) and heat (HT) trials expressed as fold changes relative to baseline (1.0). Values are mean ± SD

* indicates significant difference from baseline across both trials, $p<0.05$

† indicates significant treatment effect across all time points, $p<0.05$

‡ indicates significant time by treatment interaction, $p<0.05$

CONCLUSION

Fatigue is a complex and difficult phenomenon to characterize. Much of the current research has focused on the decrease in force-generating capacity of the muscle and whether fatigue is central or peripheral in nature. However, since the early 1900s when Mosso described fatigue, including the sensation, or perception, of fatigue as an important component (11), this component of fatigue has been ignored by researchers. Therefore, the overarching purpose of these studies was to examine the perceived sensation of fatigue for several days following exercise and the gene expression responses to the same exercise. It was initially hypothesized that strenuous exercise would cause an increase in metabolite-detecting mRNA, similar to the increases seen in chronic fatigue syndrome (CFS) (6, 10).

Because these particular studies had their start in the CFS literature (6, 10, 12, 13, 20, 21), we first wanted to examine how a graded exercise test and a time trial differed in both perceived fatigue and gene expression. We also wanted to explore how the additional stressor of increased temperature would affect perceived fatigue and gene expression. We found that a normal gene expression response to exercise in a healthy population consisted of a decrease in metabolite detecting genes (ASIC1/3, P2X4, and TRPV1/4), dopamine, IL-10, and adrenergic β2 as well as an increase in adrenergic genes (adrenergic α2a/c and adrenergic β1), serotonin, IL-6, and TLR4. The magnitudes of these changes were affected by a combination of the intensity and duration of the exercise. The changes in gene expression that occurred were larger following the time trial in ambient conditions than the graded exercise test, though the changes following the ambient time trial were similar to the time trial in the heat. Even though exercise intensity was decreased during exercise in the heat, as evidenced by decreases in lactate

levels during exercise as well as postexercise, suggesting a decreased metabolite load, the metabolite load and subsequent afferent signal may actually be the same between these trials if heat is considered an additional metabolite, in addition to H+ ions, lactate, and ATP. This suggests that the metabolite load, and subsequent afferent signal, is important in regulating the perceived effort level and subsequent power output during the time trial (19).

When normal gene expression following exercise that causes a prolonged sensation of fatigue in healthy individuals is compared to a disease that exhibits abnormal pain or fatigue for several days following mild exercise, such as chronic fatigue syndrome or fibromyalgia, we found that the diseased response was vastly different than the healthy response (7, 10). This suggests that there is an abnormal response to exercise by individuals who have these diseases, and it is possible to differentiate and diagnose these diseases using gene expression. Further, it may be possible to use gene expression to determine the underlying causes of these diseases, perhaps allowing future treatment opportunities that address these different gene expression responses.

The metabolite-detecting genes exhibited general decreases following each of the trials, irrespective of the length, intensity, or temperature of the trial. The smallest decreases in gene expression followed the GXT while the longest lasting decrease followed the time trial in the heat (HT). Because of this, it follows that the changes in gene expression may be related to work. The smallest gene expression changes occurred following the exercise trial in which the least work occurred. The decrease following each of these trials may represent a normal, adaptive response to exercise (22) and an attempt to 'reset' the fatigue signal to return the afferent signal to baseline levels of

fatigue and pain.

While much of the pain and fatigue response is mediated through the metabolite-detecting ASIC, P2X, and TRPV receptors (8), heat may also enhance the fatigue signal from any given level of metabolites by activating the TRPV receptors (1, 3). The length of exercise may also play a role in the size of the response, as the duration of activation enhances the afferent signal during exercise. Even though the metabolite levels were decreased during the 40k time trial in ambient conditions as compared to the graded exercise test, the changes in gene expression were much larger following the 40k time trial. This may suggest that long durations of exercise may sensitize these metabolite detecting receptors, increasing the afferent signal, and thus increasing the sensation of fatigue during mild, prolonged exercise.

The majority of the adrenergic receptors exhibited pronounced increases in mRNA gene expression following exercise, with the one exception being adrenergic β2, which decreased following both time trials, but not GXT. Adrenergic receptors may influence fatigue in several different ways. Adrenergic receptors, particularly β-adrenergic receptors, may directly influence the sensitivity of the sensory afferents, increasing the signal sent by the metabolite detecting neurons (9). Not only may postexercise sensitivity of the afferents be affected, but blood flow may be decreased by activation of the α-adrenergics within the blood vessels (5), leading to a decrease in arterial radius, increasing the concentration of metabolites and subsequent afferent signal.

Serotonin and dopamine have long been suspected of playing a role in central fatigue during exercise. The results from these studies also suggest that they may contribute to playing a role during long-term, postexercise fatigue. The large increase in

mRNA for serotonin following exercise may increase the fatigue levels felt during the days following exercise. When the serotonin receptor was activated in rats, the afferent signal was increased (18). Dopamine has been shown to inhibit the depolarization of the afferent nerve when activated; thus, a decrease in dopamine mRNA may decrease the inhibitory affect, leading to an increased perception of fatigue (2, 16). Thus, the concurrent increase in serotonin and decrease in dopamine mRNA may combine to accentuate the afferent signal, contributing to the increased perception of fatigue following exercise.

The immune system may also play a role in the fatigue sensation following exercise, as there appears to be an inflammatory response with an increase in TLR4 and a decrease in IL-10. IL-6, in particular, has been associated with pain in chronic conditions, and may also directly influence fatigue sensations (4). IL-6 may also play a role in fat oxidation, lipolysis, and glucose disposal, particularly after exercise in the heat, suggesting a prolonged energy need, and perhaps indirectly reflecting an enhanced fatigue sensation (14).

One of the main concerns with any gene expression study is trying to determine the cause for the change in gene expression and what subsequently happens to the underlying protein levels. Gene expression is not necessarily directly linked to protein levels as gene expression may increase to restore a decrease in protein levels, such as when proteins are utilized and lost. Gene expression may also increase in order to increase protein expression, perhaps to attempt to restore homeostasis within the body, such as a gene involved with blood flow trying to increase blood flow and subsequently decrease the metabolic milieu following exercise.

Future studies with gene expression and fatigue are needed to examine the effects of abnormal fatigue within otherwise healthy, trained individuals. This may be able to be accomplished by examining the effects of "overtraining" or perhaps the less severe, initial stages of overtraining – overreaching. Examining the beginning effects of abnormal fatigue such as overreaching may allow a better understanding of abnormal fatigue in diseased individuals, perhaps allowing an understanding of the causes of this fatigue. Recent research has more clearly linked the metabolites produced from exercise with the sensations of fatigue and soreness associated with exercise (15). A response to this article has also questioned whether the "no pain, no gain" adage of exercise applies to these receptors in the adaptations that follow (17). In order to better understand the adaptation to exercise in these metabolite detecting receptors, it is necessary to do a training study to determine the changes in baseline metabolite-detecting receptors as well as to determine whether the exercise-induced gene expression differs after training. It is also necessary to further examine intensity, duration, and temperature effects. This can be done by holding intensity or duration constant, and examining gene expression effects while exercising at 3 different intensities or durations. Temperature should also be further examined by holding intensity and duration constant while only changing the temperature in which exercise is performed. Finally, it is necessary to examine how changes in gene expression ultimately affect subsequent protein expression.

In conclusion, the sensation of fatigue following exercise, as well as the central and peripheral fatigue observed during exercise, is likely caused in part by a combination of changes in each of the systems observed. It is more than likely not just one system that causes the increase in fatigue observed following exercise. Also, the changes

observed following exercise seem to be enhanced when exercise is performed at a high intensity for a longer duration, such as that seen during a time trial, though heat does not seem to further enhance the gene expression, possibly due to a decreased intensity during the time trial in the heat.

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