9-9-2010

Sex Differences in Heat Shock Protein 72 Expression and Inflammatory Response to Acute Exercise in the Heat.

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BY

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B.S., Health and Exercise Science, University of Oklahoma, 2004
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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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ABSTRACT

This study evaluated possible sex differences in intracellular heat shock protein 72 (Hsp72), intracellular cytokines, and extracellular Hsp72 (eHsp72) before and after exercise in the heat**.** Nine non-heat acclimated women (W) (age 23 ± 3, BF 21 ± 2%, VO_{2max} 58 ± 5 ml/kgFFM/min) and nine non-heat acclimated men (M) (age 25 ± 5, BF 12 ± 5%, VO_{2max} 60 ± 7 ml/kgFFM/min) completed 2 treadmill bouts at 60% VO_{2max} for 60 min in a 42°C, 20% RH environment. The W had normal menstrual cycles and were tested in counterbalanced order during follicular (fol) and luteal (lut) phases. M and W's duplicate trials were separated by 12 ± 2 days. Blood samples were drawn pre, 0, 1, and 4 hrs post-exercise. Mononucleated cells were analyzed for Hsp72, IL-1ra, IL-6, and TNF-α using flow cytometry. eHsp72 was analyzed using ELISA. In trial 1, Hsp72 content increased in M by 37% 4 hrs post exercise ($p<0.05$), but did not change significantly in W at any time after exercise. When Hsp72 expression was normalized to

baseline, M expressed greater Hsp72 than W ($p<0.05$) after exercise. Baseline Hsp72 increased by 26% in M from trial 1 to trial 2 ($p<0.05$), but this effect did not occur in W. eHsp72 did not change after exercise, but there was a main effect for M having higher levels than W ($p<0.05$). While cytokines did not change during exercise, W consistently expressed less IL-1ra than M ($p<0.05$). IL-6 and TNF-α were higher in the fol than lut phase at 4 hrs post exercise ($p<0.05$). Our findings suggest that unacclimated M and W differ in their expression of Hsp72 and eHsp72 after exercise in the heat. M up-regulate Hsp72 after a single bout of exercise in the heat, which persisted for 12 days, suggesting an acquired cellular thermotolerance. The inhibition of Hsp72 expression in W after exercise could be due to a known effect of estrogen to stabilize the cell membrane or to its action as an anti-oxidant.

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Chapter I

Introduction

The heat shock protein family (HSP) is a family of highly conserved proteins located in all cells. HSPs demonstrate cytoprotective properties and are categorized according to their molecular weight. In a non-stressed cell, HSP are bound to heat shock factor (HSF) in the cytosol of the cell. However, upon stressful stimuli, HSPs are released from HSF allowing HSP to bind to the damaged protein and permitting HSF to be translocated to the nucleus to begin transcription of heat shock 72 protein (Hsp72) (Ellis, 1996). Hsp72 is the most inducible form of HSP (Kregel 2002). The induction of Hsp72 can arise from a variety of stressors that include oxidative stress (Adrie 2000), energy depletion (Sciandra 1983), ischemia reperfusion injuries (Marber 1995), or hyperthermia (Mizzen 1988), among others. Stressors can result in the release of Hsp72 into the circulation typically from the splanchnic tissue (Febbraio 2002), but can also be released by other healthy (Lancaster 2005) and necrotic cells (Basu 2000). Extracellular Hsp72 (eHsp72) acts as a danger signal activating the immune system (Asea 2002), stimulating inflammation (Dybdahl 2002), and it is sometimes used as an indirect marker of heat stress (Ruell 2006).

Hsp72 has been implicated in cellular protection against increases in core temperature with exercise in the heat. This increased protection is known as acquired thermotolerance and is defined as a single exposure to heat stress that leads to future protection. This process involves an increased basal expression of intracellular Hsp72 (Landry 1982, Li 1982). When comparing heat tolerant to heat intolerant subjects, those who were heat

intolerant were found to have lower post exercise intracellular Hsp72 levels in lymphocytes than heat tolerant subjects. Heat intolerant subjects also demonstrated decreased heat shock factor-1 (HSF-1) in lymphocytes, the primary transcription factor for Hsp72, throughout exercise and during recovery (Moran 2006).

There is increased gut permeability with exercise in the heat (Rowell 1973). Hsp72 may be responsible for a greater heat tolerance due to its role in reducing heat-induced gut permeability and in decreasing pro-inflammatory cytokines released from peripheral blood mononucleated cells (PBMC) (Snyder 1992). Specifically, due to the increased demand of blood flow to the skin and active skeletal muscles during exercise in the heat, blood is shunted away from the gut and blood flow to the splanchnic region receives less than 20% of its normal blood flow (Rowell 1973). This decreased blood flow can cause ischemia and increased permeability of the gut (Rowell 1973). Increased gut permeability can allow lipopolysacharide (LPS) to enter the systemic circulation. When LPS, a gram-negative bacteria found in the intestinal lumen enters the systemic circulation, it forms a complex with lipopolysacharide binding protein (LBP) that binds to toll-like receptors (TLR) on immune competent cells (Schumann 1992). The binding of this complex to its receptor activates NF- kb and the synthesis of pro-inflammatory cytokines in circulating leukocytes (Liu 2006). Endotoxin release from the gut and its subsequent inflammatory cascade can lead to systemic inflammation and eventually multiple organ dysfunction syndrome (Chen 2005). This is one of the main causes of heat stroke (Leon 2007, Lim 2006). Hsp72 has been shown to decrease gut permeability by maintaining intestinal tight junctions (Dokladney 2006), suggesting a potential role of Hsp72 in decreasing intestinal

permeability. This decrease in intestinal permeability may limit LPS release into the circulation. LPS tolerance, the diminished inflammatory response to repeated LPS exposure**,** has been linked to heat tolerance (Selkirk 2008). Trained subjects demonstrated greater intracellular Hsp72 in monocytes during heat stress than untrained subjects, and this corresponded with greater LPS tolerance and decreased monocyte apoptosis at exhaustion (Selkirk 2009). Furthermore, NF-kb activation occurred at higher core temperatures in trained subjects compared to untrained, suggesting an important role of Hsp72 in heat acclimation and cellular heat tolerance. (Selkirk 2008). Taken together, the increased expression of Hsp72 may serve a two-fold purpose: 1) Increased Hsp72 in the gut cells may decrease permeability by maintaining tight junctions and limit LPS movement through the gut. This could increase tolerance to exercise in the heat. 2) Hsp72 may decrease pro-inflammatory cytokines released from PBMCs by inhibiting NF-kB activation and the subsequent release of pro-inflammatory cytokines.

Serum Hsp72 (eHsp72) acts as a danger signal activating the immune system (Asea 2002) and promotes the release of pro-inflammatory cytokines (Dybdahl 2002). eHsp72 can bind to TLR on antigen presenting cells stimulating the release of cytokines (Asea 2008) and are also involved in the maturation of dendritic cells (Wang 2005). It is thought that the release of Hsp72 comes from the splanchnic tissue (Febbraio 2002) and not the exercising muscle. However, the release of Hsp72 can come from healthy PBMC (Hunter-Lavin 2004) and B cells (Clayton 2005), and under situations of extreme stress, Hsp72 can also be released from necrotic cells (Basu 2000). eHsp72 can serve as a

marker of heat illness as higher levels of eHsp72 were detected in runners with severe heat illness (Ruell 2006).

Scope of the Study

Given that increased Hsp72 expression may confer protection against heat-induced perturbations, it may be significant that animal studies have shown a difference in the expression of Hsp72 between sexes. Under resting conditions, basal levels of Hsp70 in cardiac and renal tissue are higher in female compared to male rats (Voss 2003, Fekete 2006). However, in response to an assortment of stressors in a variety of tissues, male animals are able to express higher Hsp72 levels than females. Specifically, cardiac tissue of male rats exhibited a two-fold higher increase in Hsp70 after one hour of running compared to their female counterparts (Paroo 2002a). Whole body hyperthermia (43°C, 20 min) resulted in male rats demonstrating a greater expression of cardiac Hsp72 after heating than females (Shinohara 2004). In both studies, ovariectomized female rats displayed similar post stress Hsp70 levels as males. Ovariectomized rats treated with estrogen showed decreased Hsp70 similar to intact females. Furthermore, both studies revealed that males, who induced a larger expression of cardiac Hsp70 than intact females, had better protection against an ischemic insult to the heart given after the stress. It should be noted that the ischemic insult in the above experiments were performed on euthanized rats 24 hrs after exercise. Also, the gastrocnemius muscle of male rats doubled the Hsp70 protein content and increased Hsp70 mRNA almost 9 fold after 60 min of running, while female rats did not increase either Hsp70 content or mRNA. Furthermore, placebo treated ovariectomized animals revealed higher post

exercise Hsp70 content and mRNA levels than estrogen treated animals (Paroo 2002b). In response to acute tail shock, the pituitary gland, mesenteric lymph nodes, and liver of male rats had higher tissue content of Hsp72 levels post stress than females, while the adrenal glands, spleen, and heart did not express a sex specific response. Moreover, there was no fluctuation in Hsp72 expression at rest or in response to stress in any of the tissues surveyed across the estrous cycle (Nickerson 2006). Little is known about sex differences in Hsp72 expression in leukocytes after exposure to heat.

Based upon the above results in animals, the presence or absence of estrogen may influence both the basal and the stress response of Hsp72. Ovariectomized rats treated with estrogen had greater basal Hsp70 expression in the soleus and less Hsp70 expression after 90 min of downhill running compared to rats treated with progesterone or sham (Bombardier 2009). Such results suggest that estrogen may be the primary sex-specific hormone responsible for the increased Hsp70 expression at rest and the blunted Hsp70 response after exercise. An increased basal expression of Hsp70 may reduce the muscle's stress-response to downhill running and thus limit the need for additional Hsp70 production after the stress (McArdle 2004).

The anti-oxidant actions of estrogen could be a factor involved in the blunted Hsp70 response to stress. In skeletal muscle, estrogen has been shown to be a powerful antioxidant (Tiidus 1999) and a vital cell membrane stabilizer (Whiting 2000). Estrogen can be directly incorporated into the cell membrane, and this can serve to fortify the cell (Kendall 2002). Stabilizing the cell membrane has been shown to maintain calcium

homeostasis, which can decreases calpain activity (Belcastro 1998). Calpian activation leads to neutrophil infiltration and oxidative stress (Stupka 2001). Thus, estrogen may protect the cells during exercise against oxidative damage (Tiidus 2001), potentially resulting in less tissue damage and a blunted Hsp70 inflammatory response.

Taken together, the results from animal studies suggest two things regarding Hsp and estrogen: 1) Estrogen may be responsible for the increased basal expression of Hsp72 in certain tissues (cardiac, renal, skeletal muscle) and 2) because of the protective anti-inflammatory effect of estrogen, the expression of Hsp72 may be reduced after exposure to a severe stress, such as unaccustomed or high-intensity exercise. In addition, estrogen could be protecting the cell from perturbations that would otherwise result in increased expression of Hsp72. As a result, females may have a smaller induction of Hsp72 in response to a stress than males.

Cellular Actions of Estrogens

The increased basal expression of Hsp70 seen in females may be due to estrogen's interaction with HSF-1, the key transcription factor for HSP. Estrogen can initiate the Hsp72 cascade through HSF-1. *Ex-vivo* treatment with 17-b estradiol activated HSF-1 and led to increased expression of Hsp72 in male and female cardiac myocytes from rats (Hamilton 2004, Knowlton 2001). It has been shown that Hsp90 forms a complex with HSF-1 and the intracellular estrogen receptor. Treatment with estrogen disassociates HSF-1 from Hsp90 allowing it to activate the transcription of Hsp72 (Knowlton 2001).

The exact mechanism behind the relationship between estrogen and the blunted Hsp70 response to stress is currently not known. However, it has been suggested that estrogen mediates its effect on Hsp70 through a nongenomic hormonal pathway. Treating animals with tamoxifen, a known estrogen receptor agonist, showed the same blunted post exercise Hsp70 expression as ovariectomized animals treated with 17β and 17α estradiol (Paroo 2002b). Since tamoxifen, 17β, and 17α estradiol all acted to suppress the post exercise expression of Hsp70, researchers suggest that these estrogen related compounds stabilized the cell membrane and attenuated oxidative stress (Wiseman 1993). As described above, stabilizing the sarcolemma protects the cell against exercise-induced damages and could result in a blunted Hsp72 expression.

Purpose

Currently, there are no human studies that have tested for possible sex differences in the stress response of Hsp72. Therefore, we aim to compare the expression of intracellular Hsp72 and eHsp72 at rest and in response to exercise in the heat in men and women. Furthermore, we will examine if females in follicular and luteal phases of the menstrual cycle differ in their amount of Hsp72 expressed at rest and in response to exercise in the heat. Since Hsp72 mediates inflammatory cytokine production, we will also compare the responses of intracellular inflammatory markers (TNF-α, IL-1ra, and IL-6) between men and women, and between women in follicular (low estrogen) versus luteal (high estrogen) phases of their menstrual cycle.

Hypothesis

In this study we will test the following hypotheses:

1. Women will have higher basal levels of intracellular Hsp72 than men.
   Results from animal studies have shown that estrogen is associated with increased basal Hsp72. For our studies we will obtain blood samples from men and women matched for age and fitness. We will assess Hsp72 levels in PBMC.

2. In response to a severe exercise stress (60 minutes of treadmill exercise at 60% VO_{2peak} in the heat), men will express greater amounts of Hsp72.
   In animal studies, estrogen has been shown to protect cells from stressors that are known to increase Hsp72 production. Also, estrogen causes higher baseline levels of Hsp72, and thus possibly limits the need for increased production during a stressor. Therefore, we predict that increases in Hsp72 in PBMC after a severe exercise stress will be smaller in women than men.

3. Intracellular Hsp72 levels will be higher at baseline in women during the luteal phase of their menstrual cycles, but in response to severe exercise the expression of Hsp72 will be blunted in luteal compared to the follicular phase.

Since estrogen is higher in the luteal phase, women will express higher baseline Hsp72 compared to the follicular phase, when estrogens are minimal. After exercise, women in the luteal phase will have a smaller increase in intracellular HSP72 due to the protective effects of the estrogens.

4. Pro-inflammatory cytokines (TNF-α) will be reduced in conditions where intracellular Hsp72 is elevated.

   Since men have a greater Hsp72 response to exercise, they will express less TNF-α and IL-6 than women after exercise. Also, since women in the follicular phase have a greater Hsp72 response to exercise they also will have a greater inflammatory response compared to during the luteal phase.

5. There will be no difference between groups in eHsp72. eHsp72 is released with exercise, but this process does not appear to be mediated by estrogen.

   eHsp72 has been shown to be released from the liver with exercise. There is no animal or cell data that has examined how eHsp72 differs betweens sexes. Further, more eHsp72 is released with heat illness. There is no research to suggest that women are more inclined to experience heat illness symptoms than men, therefore we expect no difference in eHsp72 between sexes.

To confirm the above hypotheses, healthy men and regularly menstruating women will perform two bouts of treadmill running in a hot environment (42°C, 30% RH). All subjects will be un-acclimated to the heat and data collection will occur in the fall and

winter months. The men and women will be matched for age, fitness level, and will exercise at a similar relative exercise intensity (60% VO_{2peak}) for 60 minutes. Women will exercise during their follicular and luteal phases, and the menstrual phase order will be counterbalanced. Men will exercise on two occasions separated by two weeks. Venous blood samples will be obtained at rest before exercise, immediately post exercise, 1, and 4 hrs after exercise. Hsp72 and inflammatory cytokines will be determined from PBMC cells by flow cytometry and extracellular Hsp72 will be determined by ELISA kits.

Limitations

The vast majority of studies that have sought to examine differences in Hsp72 expression between sexes have quantified Hsp72 in tissues (animals) or with muscle biopsies (humans). In this study we will quantify Hsp72 expression in PBMCs and in the plasma. Therefore, an assumption of our study is that the Hsp72 expressed in circulating lymphocytes gives us an accurate representation of changes that are occurring in other tissues of the body.

Our subjects will be tested during the time of year when we assume they are not heat acclimated. Once acclimated, cellular levels of Hsp72 may be up-regulated and the differences in Hsp72 between men and women, or between follicular and luteal phase, could be lost.

We are testing college age, healthy and relatively fit men and women. Less fit individuals may have a different Hsp72 response: such as lower basal levels of Hsp72 and a greater stress response (Gjovaag 2006).

We are examining the stress response to exercise in the heat. Although there is evidence of carry-over for the protective function of Hsp72 to other forms of stress, our sex difference may only apply to this specific form of stress.

Significance

If females have a blunted increase in Hsp72 during exercise and heat stress, they may be more susceptible to cellular injury. Specifically, a decreased synthesis and release of Hsp72 might increase susceptibility to heat or impair their ability to become heat acclimated. Alternatively, sex differences in the Hsp72 response to stress may highlight a novel method of acquired thermotolerance if Hsp72 is not needed to protect cells from heat-induced perturbations.

Definitions

Luteal phase testing: 19-22 days after the onset of bleeding for a normal 28 day cycle (Timmons 2005) with values >500 pmol/L and progesterone values > 4 nmol/L.

Follicular phase testing: 3-8 days after the onset of bleeding for a normal 28 day cycle (Timmons 2005). Estrogen values between 100-500 pmol/L and progesterone values between 1-4 nmol/L.

Core temperature: internal temperature measured rectally with the thermistor placed 8-10 cm past the anal sphincter. Rectal temperature was used because subjects were given water during exercise. If core temperature was measured in the esophagus, then consuming water would alter the temperature being recorded.

Peripheral blood Mononucleated cell (PBMC): Blood cells with a round shaped nucleus. These include lymphocytes, monocytes, macrophages, and granulocytes.

Pro-inflammatory cytokines: Tumor necrosis factor- a (TNF-a) was measured in PBMC before and after exercise.

Anti-inflammatory cytokines: interleukin -1 receptor antagonist (IL-1ra) and interleukin-6 (IL-6) were measured in PBMC before and after exercise.

Hydration: Urine assessments of hydration will occur prior to each exercise test. Subjects will be considered hydrated enough to begin the test if their urine osmolality is below 800 mOsm/kg.

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This paper is prepared for submission according to the guidelines of the International Journal of Sports Medicine.

Chapter II: A Review of Sex Differences in Immune Function After Aerobic Exercise

by

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A Review: Sex Differences in Immune Function After Aerobic Exercise

Abstract

When menstrual phase and oral contraceptives are controlled for, males and females display marked differences in immune response to an exercise stress. In highly controlled research studies, sex differences in immune cell changes, cytokine alterations, along with morbidity and mortality after inoculation are apparent. Exercise has been hypothesized to serve as a model of various clinical stresses by inducing similar hormonal and immunological alterations. Thus, a greater understanding of sex differences in post exercise non-specific immune function may provide insight into more effective clinical approaches and treatments. This paper reviews the recent evidence supporting sex differences in post exercise immune response and highlights the need for greater control when comparing the post exercise immune response between sexes.

Introduction: exercise as a model to assess immune function

Exercise modulates the non-specific (innate) (52) and specific (acquired or adaptive) (12) arms of the immune system with an intensity dependent response. Moderate bouts of exercise have been shown to enhance immunity (51). However, intense exercise depresses the immune system (52, 8). More specifically, during moderate and intense bouts of exercise there are transient increases in circulating pro- and anti-inflammatory cytokine levels (55), concentration of lymphocytes and lymphocyte

sub-sets (46), and macrophage activity (22). Recently, researchers (75, 76, 9, 53) have suggested there are sex differences in the immune response to moderate and intense exercise.

Exercise has been hypothesized to serve as a model for certain clinical stresses. In a review article, Dr. BK Pederson wrote:

> "Physical exercise can be regarded as a prototype of physical stress. Many clinical physical stressors (e.g. surgery, trauma, burn, sepsis) induce a pattern of hormonal and immunological responses that have similarities to that of exercise (60)."

Clinical physical injury, similar to exercise injury, displays marked sex differences (4). For example, females have higher levels of mortality than males in response to burns of similar size (31). Females have a lower incidence of multiple organ dysfunction syndrome (MODS) and sepsis in response to shock compared to males (17). It is thought that the disparity in sex outcomes results from interactions of sex hormones with various aspects of the immune system. Since exercise induces similar immune response, it may provide a useful model to study sex differences in immune response to clinical stressors. However, to understand this relationship, studies that control for menstrual phase, oral contraceptive (OC) use, and fitness levels between men and women are needed. The focus of this review will be to discuss what is currently known about sex differences in non-specific immune responses to exercise.

This review will consist of both animal and human studies that have examined the post exercise immune response.

Sex Difference in Immune Function in Non-Exercising Conditions

Several aspects of immunity have marked sex differences in non-exercising conditions. T cells, macrophages, and monocytes possess estrogen receptors (4) with two different subtypes, ERα and ERβ (61). ERα is mainly found in the uterus and mammary glands, while ERβ prevails in the central nervous, cardiovascular, and immune systems (32). Through these receptors, estrogen led to greater survival against herpes simplex virus 1 (HSV-1) in inoculated rats (9). In addition, in vitro stimulation of lymphocytes with phytohemagglutinin, a toxin used to elicit cytokine production from immune competent cells, found that females produce more Th2 (IL-4, IL-10) cytokines than males (29). Th2 cytokines are responsible for secretion of antibodies and this may play a role in the higher incidence of autoimmune diseases in women (85). Furthermore, females have a higher percentage of T lymphocytes within the total lymphocyte pool (5), and have more active circulating polymorphonuclear leukocytes (neutrophils) and macrophages (64, 65). Overall, physiologic levels of estrogen stimulate humoral and cell-mediated immune responses, but large increases in estrogen (either from pregnancy or supraphysiologic doses) can suppress cell-mediated immunity (54). Taken together, results imply that females of reproductive age have a more active immune system than age matched males. This could account for females having a lower incidence of, and mortality rates from, certain types of infection (bacteria septlcemai, pneumonia/influenza, bacterial meningitis) (28) and

lower rates of atherosclerosis (79). Similarly, this could also explain the increased incidence of autoimmune diseases.

Sex Difference in Immune Response to Exercise: Inoculation Studies

Inoculating animals with viruses has previously been used as a model to study upper respiratory infections in animals by inducing illness (33). Inoculation purposefully infects the animal by transferring the causative agent into the animal. In this manner, whole body responses can be measured after inducing a specific illness. With this methodology, female mice experienced lower mortality after intranasal inoculation with herpes simplex virus 1 (HSV-1) at rest and after exercise than males. HSV-1 was delivered after the third bout of running to exhaustion or after 3 non-exercising control sessions. Though exercise resulted in greater morbidity (illness symptoms) than control, both sexes experienced the same degree of morbidity. Despite males and females having a similar rate of infection by HSV-1 after inoculation, fewer females died (9). Similarly, female mice that exercised at a moderate intensity had a greater macrophage resistance to HSV-1 than their male counterparts (8). However, both males and females experienced suppressed macrophage function after exhaustive exercise, and experienced this suppression to a similar degree. Thus, it is plausible that the decreased mortality after HSV-1 inoculation seen in female mice may be due to increased macrophage function. Since more females survived HSV-1 inoculation than males, the presence of estrogen could be an important determinant of this response. However, ovariectomized mice supplemented with estrogen experienced higher mortality than intact female mice after HSV-1 inoculation (7). Despite the

better protection of intact mice, there was only a trend (p=0.1) toward intact females having greater macrophage resistance than the estrogen treated ovariectomized group. Therefore, the authors suggested that antiviral macrophage resistance is not responsible for the lower mortality (7). Since estrogen supplementation did not restore the protective effects of intact mice, other female hormones could be responsible for this added fortification of female mice. Taken together, animal research with HSV-1 inoculation demonstrates that male and female mice are equally susceptible to an infection at rest or after exhaustive exercise. However, more females survive. The greater macrophage activity may be responsible for this effect, but future studies should incorporate other immune parameters. The mechanism behind greater female survival with HSV-1 may be related to other ovarian hormones besides estrogen.

Sex Difference in the Cytokine Response to Exercise

The local response to a tissue injury involves the release of cytokines. Cytokines are released from the site of inflammation. The local response of cytokine release is supplemented by the release of cytokines from the liver, termed the acute phase response. The acute phase cytokines are TNF-α, IL-1β, and IL-6. These pro-inflammatory cytokines cause the movement of lymphocytes, neutrophils, and monocytes to the injured site. These leukocytes ultimately infiltrate the damaged muscle and serve to repair the tissue (2). Initially, exercise leads to increased release of pro-inflammatory cytokines (TNF-α, IL-1β,) and this is counteracted quickly by

the release of cytokine inhibitors (IL-1ra, TNF receptors) and anti-inflammatory cytokines (IL-10), which limit the inflammatory response of exercise (60). With chronic exercise and training, there is a decrease in cytokine production during an acute bout of exercise (69). Decreased cytokine release may contribute to immunosuppression and lead to a greater risk of bacteria and infection that is often evident in endurance-trained athletes (51). However, this decrease in inflammation could be a key link between exercise and health through a possible reduction in the risk of chronic disease.

Generally, cytokines are released after prolonged exercise or exercise that causes muscle damage (10, 60). The intensity and duration of exercise, along with fitness level, determines the cytokine profile (30). Interestingly, exercise does not cause an alteration in pro-inflammatory gene expression in peripheral blood mononucleated cells (PBMC) (81), suggesting that this is not a primary site for cytokine release. Recently, researchers demonstrated IL-6 is released from the exercising muscle (38, 67). IL-6 can increase 100 fold after exercise making it the most responsive cytokine to exercise and perhaps underscoring its biological significance. IL-6 has been shown to regulate metabolic factors such as glucose uptake and fatty acid oxidation (59). Recently, IL-6 released from the exercising muscle has been shown to have anti-inflammatory properties through its up-regulation of anti-inflammatory cytokines IL-1ra (56) and IL-10 (55), in addition to inhibiting TNF-α release (66). For a detailed review of IL-6 and exercise, see *Febbraio, 2005* (21).

Sex differences in the regulation of cytokines have been previously demonstrated in non-exercising conditions. After lymphocytes were stimulated with

phytohemaglutinin, a toxin used to elicit cytokine production from immune competent cells, a greater Th1 profile, characterized by increased release of IFN-γ and IL-2, was shown in lymphocytes drawn from men compared to women. Women possessed a greater Th2 cytokine release (IL-4, IL-10) than men, but there were no differences across the menstrual cycle (29). Th2 cytokines are responsible for humoral mediated immunity and lead to increased secretion of antibodies. Similarly, IL-1 release from mononucleated cells is lower in males and is menstrual phase dependent in females(44). More specifically, the balance of the IL-1 family (IL-1-α, IL-β - agonist, IL-1ra – antagonist) is menstrual phase dependent. The ratio of agonist (IL-1-α, IL-β) to antagonist (IL-1ra) was equal during the follicular stage, but the agonist was ~45% higher in the luteal phase. Thus, the activity of IL-1α/β was greater in the luteal phase. IL-1β may influence reproductive functions like endometrial development and preparing the birth canal for parturition. IL-1β has also been shown to block leutenizing hormone and ovulation in rats (28). After trauma-hemorrhage injury, ovariectomized mice had decreased cytokine expression (IL-2, IL-3, and IFN-γ) from macrophages compared to ovariectomized mice treated with 17-β estradiol. The estradiol treated group maintained cytokine release after injury and this suggests that estrogen is capable of preventing immunosuppression that had been previously demonstrated with male mice and enhancing survival (41).

Currently, there are a handful of studies that have compared the cytokine response to exercise between sexes. There was no difference reported in serum IL-10, IL-1ra, IL-6, and IL-8 between men and women immediately and 1.5 hours after completing a marathon (50). The in-vitro production of IL-1, IFN-γ, and IL-4 from cultured whole

blood showed no differences between genders in response to continuous incremental cycling at 55%, 70%, and 85% VO_{2peak} (49). Similarly, 90 minutes of cycling at 65% VO_{2max} resulted in no difference in serum IL-6 levels between men and women (75). There was however, a trend ($p=0.06$) of increased IL-6 in women who took OC and those who were not taking OC and exercising in the follicular phase (75). The change in IL-6 values could be due to altered carbohydrate (CHO) oxidation rates. It was shown that whole body CHO oxidation during 50 min of cycling at 70-90% of lactate threshold is higher in the follicular phase (89). This higher rate of CHO oxidation could have lead to a greater depletion of CHO. In response to low CHO availability, IL-6 production will increase (38). In contrast, Edwards found that 60 minutes after a maximal cycling test, female IL-6 values were greater than men (18), although there were no differences between genders at baseline, immediately, or 30 minutes post exercise. At 60 minutes post exercise, the male IL-6 values decreased towards baseline while the female values continued to rise. The exercise-induced IL-6 response is directly linked to the duration and intensity of exercise, along with the number of muscle fibers recruited (increased release) and the fitness level of subjects (decreased response) (57). Thus, methodological differences could account for the current disparity in the literature regarding IL-6.

At the transcriptional level, Northoff et al found a gender and menstrual phase difference in mRNA inflammatory gene expression in response to a 60 min run at 93% of the individual's anaerobic threshold (53). Women in the luteal phase demonstrated a greater condition of pro-inflammation than women in the follicular

phase or men immediately after exercise. This pro-inflammatory state was characterized by an increase in inflammatory genes (interferon-γ, IL-12 receptor β1, and prostaglandin D2 receptor) and a decrease in anti-inflammatory genes (IL-6, IL1R2, IL1-ra) in PBMC. The authors state that the increase pro-inflammatory condition in the luteal phase could be a "mechanism designed to end a very early pregnancy in case of major external stress input. After all, human females get a new chance to conceive in the next month and nature may prefer to destabilize a pregnancy under influence of stress rather than carry it on under high risk." Furthermore, women in the luteal phase regulated over 200 genes (129 genes up-regulated, 143 genes down-regulated), while women in the follicular phase regulated 80 genes (48/32) and men regulated only 63 genes (34/29). Interestingly, post exercise IL-6 mRNA was down- regulated in the luteal phase, while up-regulated in the follicular phase after exercise. Future studies that control for menstrual cycle are needed to assess the expression of the specific proteins before any conclusions can be drawn.

Thus, in limited research on aerobic exercise, it appears the overall cytokine response to exercise is not markedly different between sexes. However, few studies controlled for either menstrual phase or oral contraception. Some work has demonstrated a greater up-regulation of inflammation (129 genes up-regulated, 143 genes down-regulated) in the luteal phase at the transcriptional level after exercise (53). Potential sex differences in IL-6 may exist after maximal exercise (18) and further research is

needed to confirm the IL-6 response at longer time points after exercise while controlling for menstrual phase and oral contraceptive use.

Sex Differences in Leukocyte Response to Exercise

Moderate aerobic exercise results in a transient increase in both innate (monocytes, macrophages, neutrophils, NK cells) and specific (B and T lymphocytes) cells of the immune system. The effector cells of the innate immune system are monocytes, macrophages, neutrophils, and a subset of lymphocytes called natural killer (NK) cells. These cells represent the first line of defense against infections by neutralizing microbes or pathogens that have entered the circulation through phagocytosis (monocytes, macrophages, neutrophils) or by directly lysing the pathogen (NK cells). T cells recognize specific antigens presented to them to create memory cells, and B cells secrete antibodies to kill extracelluar pathogens. B cells are fundamental for eradicating bacterial infections. The number of total leukocytes, lymphocytes, granulocytes (neutrophils), and monocytes increase in a biphasic response (46). The immediate increase of leukocytes is characterized by increases in lymphocytes, monocytes, macrophages, and neutrophils, and is then followed by a delayed response of additional neutrophils 2 hours post exercise (46, 87).

Both the duration and intensity of exercise combine to determine the specific increase in leukocytes with exercise. Exercising for up to 30 minutes leads to increased lymphocytes (CD4+T cells, CD8+T cells, CD19+ B cells, CD16+ NK cells, CD56+ NK cells), which return to baseline values within 10-30 minutes after cessation of

exercise (46). Longer duration exercise requires longer time periods for leukocytes to return to baseline. Specifically, CD8+ lymphocytes increase more with exercise than CD4+ cells (60). CD8+ lymphocytes can directly kill foreign or infected cells, whereas CD4+ are helper cells that mainly produce cytokines to magnify the immune response. Also, memory lymphocytes are recruited into the circulation more so than naïve lymphocytes (27). Memory cells are more likely than naïve cells to relocate to non-lymphoid tissues or possible locations of infection, like the vasculature of the skin, lung, liver, and gut.

The increases in epinephrine release and cardiac output associated with exercise are thought to contribute to the exercise-induced leukocytosis through de-margination from vascular pools and immune organs (26, 24, 80). The delayed increase in neutrophils may be mediated by an increase in Granulocyte colony-stimulating factor (G-CSF) more so than epinephrine or cardiac output (87). Epinephrine release in response to submaximal exercise has been shown to be sex dependent, with males demonstrating a greater release compared to mid-follicular females (11, 15, 34). However, an overall greater expression of β_2-adrenergic receptors on lymphocyte have been found in women compared to men (84, 43). The majority of previous research suggests there are no post exercise sex differences in leukocytes (49, 1), lymphocytes (49, 1), natural killer cells (6, 48) monocytes (1) or neutrophils (1). However, the above studies did not control for menstrual cycle phase, oral contraceptives, or matching male and female subjects for activity or fitness level.

In one of the few studies to examine immune cell changes that controlled for menstrual phase, oral contraception, and fitness, Timmons *et al* showed that women taking OC had a greater post exercise increase in lymphocytes and neutrophils compared to men and non-OC users after 90 min of cycling at 65% of VO_{2max} (75). Women taking OC experienced cycle specific (follicular and luteal phases that corresponded to triphasic OC) exercise induced changes in total leukocytes, neutrophils, monocytes, and lymphocytes, whereas non OC users had no fluctuations across the menstrual cycle. The increase in immune cells after exercise were greater in OC users on days taking the pill, and these increases were always greater than the post-exercise changes seen in men. There were no differences in total leukocytes, neutrophils, and monocytes between men and regularly menstruating women not taking OC. However, non-OC users had a greater post exercise increase in lymphocytes than men. Taken together, this study demonstrated immune cell changes between men and women that are specific to OC use. There was a greater increase in immune cells after exercise in the high progesterone phase of women taking OC than men and non OC using women. Also, non OC using women had more lymphocytes circulating post exercise than men.

Since there were no changes in lymphocyte number across the menstrual cycle in non-OC users, sex hormones probably do not account for sex differences. While the authors corrected for exercise-induced changes in plasma volume, there was no mention of correcting for contraceptive induced changes in plasma volume. Previous research has found an increase in plasma volume in women taking OC (83). A

difference in plasma volume between woman taking OC and those who did not could influence the results not only of the previous study, but also much of the preceding literature.

Thus, with moderate to intense aerobic exercise, the circulating leukocyte populations change dramatically. However, the majority of research suggests that there is no difference between sexes in the leukocyte response to aerobic exercise. Currently, Timmons *et al* is the only study to control for OC use, and the only study to show a difference between men, OC, and non OC users. Future research is warranted.

Sex Differences in Natural Killer Cell Response to Exercise

Natural Killer (NK) cells are a subset of lymphocytes produced in the bone marrow and are part of the innate immune system. NK cells kill virally infected cells or tumor cells through direct cytolytic mechanisms, without activation. NK cells account for 10-15% of circulating blood mononuclear cells. During exercise, NK cells are transiently increased by 186- 344% of initial resting value, following both maximal and sub-maximal bouts (63). NK cells are the most responsive leukocyte to exercise due to their catecholamine sensitivity (25). The magnitude of increase in NK cells is more responsive to the intensity than duration of exercise. Generally, NK cell number and activity will decline only in intense exercise lasting at least 1 hour (58). At rest, men have a higher NK cell activity despite no difference in NK cell numbers than regularly menstruating women or women using OC. Women using OC had the lowest NK cell activity (88). Furthermore, IL-1 release from monocytes, an activator

of NK cell activity, has been shown to be both sex and menstrual phase dependent (44).

Previous research supports the notion that there are no sex differences in NK cell number or activity in response to incremental or continuous exercise (6, 48). However, neither study controlled for menstrual phase or OC use. In contrast, adolescent girls not taking OC and tested in the mid-follicular phase had a greater increase in NK cell count than adolescent boys during (77) and after (78) cycling exercise for 60 min at 70% VO_2. Also, NK cell subset expression was significantly different between sexes (77). NK cells can be divided into 2 unique groups: $CD56^{dim}$, representing 90% of the circulating NK cells, and $CD56^{bright}$ cells that are more responsible for inflammation (13). The ratio of $CD56^{dim}$: $CD56^{bright}$ have been shown to play a role in reproduction as the concentration of NK cells in the uterine mucosa changes across the menstrual cycle and with pregnancy (40). For an in depth review of NK cell subset changes with exercise see *Timmons, 2008* (74). NK cell activity was not assessed in either study. Since results from Yovel 2001 (88) suggest there is both a sex and OC effect on NK cell activity at rest, future controlled studies are needed to quantify NK cell activity during and after exercise in an adult population.

Sex Differences in Neutrophil Response to Exercise

Neutrophils are a large subset of granulocytes, comprising ~90% of all granulocytes. Granulocytes are characterized by the granules in their cytoplasm and consist also of basophils and eosinophils. Neutrophils are members of the innate immune system.

They are part of the acute inflammatory response and are the first cells recruited from the blood to the site of injury or infection (5). Neutrophils attack microbes that have entered the circulation by phagocytosing the microbe or releasing oxidative bursts to destroy the pathogen. Neutrophils also produce cytokines to recruit more neutrophils and other immune cells to the site of injury and enhance both specific and innate immunity. Granulocytes are higher in the luteal phase compared to the follicular phase (19) and have been shown to increase during pregnancy (82). There is evidence that with pregnancy there is a decrease in cell-mediated immunity (36). As a compensation mechanism, the pregnant women increase activity of the innate system, most notably granulocytes.

Acute exercise causes a mild inflammatory response to repair damaged tissue, which is characterized first by neutrophil infiltration, followed by macrophage infiltration several hours later (23). While the current data on sex differences in neutrophil infiltration after exercise are equivocal (45, 70, 71), generally females rats have a blunted post exercise inflammatory response that leads to less neutrophils infiltrating skeletal muscles and less muscle soreness (70, 72). From animal studies, it seems that estrogen is limiting neutrophil infiltration by acting as a cell membrane stabilizer and antioxidant. However, data from human studies are less compelling. For a review of sex differences in neutrophil infiltration *see Point – Counterpoint, Tiidus & Hubal 2009* (35, 73).

Higher numbers of circulating neutrophils were observed both at rest and after 90 min

of cycle ergometry in women taking OC compared to men and non-users. Furthermore, the greatest increase in neutrophils after exercise in OC users was seen in the luteal phase when estradiol levels were lowest (75). Since estrogen has been shown to inhibit the inflammatory response to exercise (70, 72), it makes sense that neutrophils would be highest when estrogen was lowest. Previously, data from males suggested that increased IL-6 levels during exercise lead to increases in cortisol, which ultimately are responsible for exercise neutrophilia (68). However, data from sex comparison studies suggest there is no correlation between IL-6 levels and cortisol during exercise (75, 18). OC users had higher cortisol and neutrophil levels compared to men and non-users, but equivalent resting and post exercise levels of IL-6 (75). This could potentially highlight differences in regulation of anti-inflammatory mediators between men and women and future research should be conducted to understand this response.

Potential Mechanisms of Action for Sex Differences in Immune Response to Exercise

Given the post-exercise sex differences in immune function, estrogen may be responsible for this disparity. However, results from a few well-controlled studies suggest other physiologic variables account for the sex discrepancies. The sex differences in IL-6 during maximal exercise could potentially be mediated by a difference in the amount of adipose tissue (42). Mohamed-Ali showed that adipose tissue released IL-6 (47). Furthermore, increases in catecholamines during exercise are related to IL-6 release from adipose tissue (39). Thus, the greater IL-6 response in women could be due to their greater fat content (18).

The disparity in post-exercise leukocyte and neutrophil responses between women who took OC, non-OC users, and men could be related to differences in growth hormone and cortisol levels. Both growth hormone (3) and cortisol levels (75) are higher in women taking OC. Furthermore, both growth hormone (37) and cortisol (14) have been shown to increase circulating neutrophil levels. However, in Timmons *et al* (75), cortisol levels did not differ between menstrual cycle phases, only between groups. Thus, cortisol alone could not be responsible for the increased post exercise immune cell response of the OC users. Exercise induced leukocytosis seen in both men and women appear to be associated with the increased circulating catecholamines (60). Thus, as noted by Timmons *et al*, the greater increase in lymphocytes in women during exercise may be due to their greater density of lymphocyte β_2-adrenergic receptors. (84, 43). Furthermore, the number of β_2-adrenergic receptors on lymphocytes decreases over 10 wks of aerobic training (62). Thus differences in training also may be responsible for some of the sex differences reported in studies that did not control for fitness.

Intact female mice had lower mortality rates to post-exercise HSV-1 inoculation compared to males or ovariectomized females (8, 7). Yet, when estrogen was replaced after ovariectomy, ovariectomized females were still more susceptible than the intact group. Therefore, the authors concluded that physiologic doses of estrogen (1μg/day) are not responsible for the enhanced immunity seen in intact female animals. Further research is warranted to confirm this finding and to identify the

cause for the greater immune response of the female animals. Similarly, 8 days of supplementing men with estradiol had no effect on resting or post exercise cortisol, IL-6, or neutrophil counts after 90 min of cycle ergometry at 60% of aerobic capacity (76). This study reinforces the suggestion that estrogen alone is not responsible for immune sex differences, and could potentially point to a difference in the expression of estrogen receptors (ER) on cells throughout the body. Both males and females have ERα and ERβ in skeletal muscle, with ERα mRNA 180 fold greater than ERβ (86). Females exhibit greater ERα expression in the lungs than men (20), while ERβ mRNA is higher on adipocytes in women (16). Taken together, these data suggests a sex difference not only in ER quantities, but also a site-specific preferential expression of ER isotypes.

Future Research Considerations and Conclusions

When menstrual phase and oral contraceptives are controlled, males and females display marked differences in immune response to exercise (Table 1). Sex differences in immune cell changes, cytokine alterations, along with morbidity and mortality are apparent after submaximal and maximal aerobic exercise stressors. The primary mechanism for many of the sex differences does not appear to involve the presence of estrogen. Thus, future research should clarify which specific ovarian-related changes are responsible for these immune response differences and their specific actions. By using exercise to model the stress responses to certain clinical traumas, this avenue of research may provide valuable insight into new approaches and sex-specific treatments.

Table 1. Gender differences in immune function in studies that controlled for menstrual phase and OC use.

Author	**N size**	**Exercise**	**Immune changes**
Timmons, 2005	12 women (6 OC users, 6 NOC users), 12 men	90 min cycling, 65 % VO_{2max}	38% > lymphocyte increase post exercise in NOC women compared to men.
Northoff, 2008	9 women, 12 men	60 min treadmill run, 93% AT	>Pro-inflammatory gene expression in LP compared to men or FP.
Brown, 2004	89 female mice, 86 male mice.	3 consecutive days of treadmill running after HSV-1 inoculation until volitional fatigue.	>morbidity for males (28%) compared to females (16%).
Brown, 2006	36 female mice, 36 male mice	3 days of moderate (90 min) or exhaustive (volitional fatigue) treadmill running after HSV-1 inoculation.	>macrophage antiviral resistance in moderately exercised females compared to males.
Gonzalez, 1998	9 women	80 min walking, 32% VO_{2max} in cold (-5°C) environment.	41% decrease in IL1-β after exercise in LP compared to FP. No change in IL-6 or TNFα.
Timmons, 2006a	25 girls, 33 boys	60 min cycling, 70% VO_{2max}.	>Leukocyte count at 30 & 60 min post exercise in T5 boys compared to T4/5 girls. >NK cell response immediately post exercise in T4/5 girls compared to T3/4 boys.

Table 1. (Cont.)

Timmons, 2006b	11 girls, 11 boys.	60 min cycling, 70% VO_{2max}.	> Lymphocyte count in girls at 30 min (29%) and 60 min (23%) of exercise. $CD56^{dim}$ cells (105%) and $CD56^{dim}$ expressed as proportions (67%) greater in girls. $CD56^{bright}$ cell counts 82% greater in girls but not $CD56^{bright}$ proportions.
Ferrandez, 1999	60 female, 60 male mice	Swimming until exhaustion	>chemotaxis index in females compared to age matched male mice

OC – oral contraceptive user. NOC non oral contraceptive user. LP – Luteal Phase. FP- Follicular Phase. T5 – Tanner stage 5. T4/5 – Tanner stage 4 and 5.

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This chapter is prepared according to the guidelines for the Journal of Applied Physiology.

Chapter III: Sex Differences in Heat Shock Protein 72 Expression and Inflammatory Response to Acute Exercise in the Heat.

by

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Abstract

This study evaluated possible sex differences in intracellular heat shock protein 72 (Hsp72), intracellular cytokines, and extracellular Hsp72 (eHsp72) before and after exercise in the heat**.** Nine non-heat acclimated women (W) (age 23 ± 3, BF 21 ± 2%, VO_{2max} 58 ± 5 ml/kgFFM/min) and nine non-heat acclimated men (M) (age 25 ± 5, BF 12 ± 5%, VO_{2max} 60 ± 7 ml/kgFFM/min) completed 2 treadmill bouts at 60% VO_{2max} for 60 min in a 42°C, 20% RH environment. The W had normal menstrual cycles and were tested in counterbalanced order during follicular (fol) and luteal (lut) phases. M and W's duplicate trials were separated by 12 ± 2 days. Blood samples were drawn pre, 0, 1, and 4 hrs post-exercise. Mononucleated cells were analyzed for Hsp72, IL-1ra, IL-6, and TNF-α using flow cytometry. eHsp72 was analyzed using ELISA. In trial 1, Hsp72 content increased in M by 37% 4 hrs post exercise ($p<0.05$), but did not change significantly in W at any time after exercise. When Hsp72 expression was normalized to baseline, M expressed greater Hsp72 than W ($p<0.05$) after exercise. Baseline Hsp72 increased by 26% in M from trial 1 to trial 2 ($p<0.05$), but this effect did not occur in W. eHsp72 did not change after exercise, but there was a main effect for M having higher levels than W ($p<0.05$). While cytokines did not change during exercise, W consistently expressed less IL-1ra than M ($p<0.05$). IL-6 was higher in the fol than lut phase at 4 hrs post exercise ($p<0.05$). Our findings suggest that unacclimated M and W differ in their expression of Hsp72 and eHsp72 after exercise in the heat. M up-regulate Hsp 72 after a single bout of exercise in the heat, which persisted for 12 days, suggesting an acquired cellular thermotolerance. The inhibition of Hsp72 expression in W after exercise could be

due to a known effect of estrogen to stabilize the cell membrane or to its action as an anti-oxidant.

Keywords: Heat shock protein, sex differences, thermoregulation, cytokines, immune function

Introduction

The cellular stress response is characterized by increased expression of intracellular heat shock protein 72 (Hsp72). The actions of Hsp72 serve to fortify the cell against perturbations by maintaining proper protein folding (13), inhibiting intracellular pro-inflammatory cytokine synthesis (5, 34, 35, 45), and limiting cellular apoptosis (34). The expression of Hsp72 increases during (34) and after (9) exercise in the heat, and Hsp72 expression has been linked to heat tolerance (24). After heat exposure, baseline Hsp72 increases and cells are protected against normally lethal increases in core temperature (19, 20).

Given that Hsp72 expression is up-regulated in response to stress, it is interesting that animal studies have shown a difference in the expression of Hsp72 between sexes. Under resting conditions, basal levels of Hsp70 in cardiac and renal tissue are higher in female compared to male rats (10, 41). However, in response to running (28, 29) and hyperthermia (36), male animals were able to express higher Hsp72 than females in the gastrocnemius and cardiac tissue. In the above studies, ovariectomized females treated with sham displayed similar post stress Hsp70 levels as males, while ovariectomized females treated with estrogen showed decreased Hsp70 similar to intact females. An increased basal expression of Hsp70 may reduce the response to stress and thus limit the need for additional Hsp70 production after the stress (22). Little is known about sex differences in Hsp72 expression in PBMC after exposure to heat.

Currently, there are no human studies that have tested for possible sex differences in the cellular stress response to exercise in the heat. Therefore, our purpose was to compare the expression of Hsp72, circulating Hsp72 (eHsp72), and intracellular cytokines at rest and after exercise in the heat in men (M) and women (W). Furthermore, we examined if W in follicular (fol) and luteal (lut) phases of the menstrual cycle differ in their amount of Hsp72, eHsp72 and intracellular cytokines expressed at rest and in response to exercise in the heat.

Materials and Methods

Subjects

Eighteen (9 men and 9 women) active, but untrained subjects completed two treadmill sessions in a hot, dry environment (42.3±1°C, 22.5 ±12% RH). M were matched with W for fitness and age (Table 1). The W were followed for three months and demonstrated regular menstrual cycles. They were not taking hormonal contraceptives. W were tested in the fol (day 7±2) and lut (days 20±1) phases of their menstrual cycle. The same length of time elapsed between the 2 exercise trials (M: 12 ±4 days, W: 12 ±2 days). The University of New Mexico's Institutional Review Board approved this protocol and the subjects provided informed, written consent prior to participation.

Preliminary Testing

Body composition and cardiorespiratory fitness were assessed for all subjects. Three site skinfold (Lange, Beta Technology, Santa Cruz, CA) measurements (M: chest, abdomen, thigh; W: triceps, suprailiac, thigh) were used to determine percent body fat. Each site was measured in triplicate and the mean value was used to calculate percent body fat. A continuous graded treadmill test in a temperate room (22-24°C, 30% RH) was used to

determine VO_{2peak}. VO_{2peak} was assessed through open circuit spirometry (ParvoMedics, Sandy, UT) and defined as the highest 30 second value when 2 of the following criteria were met: 1) a plateau in VO_2 (change in VO_2 <150 ml min^{-1}) with increased workload, 2) a maximal respiratory exchange ratio greater than 1.1, and 3) heart rate greater than 95% of the age predicted maximum (220-age).

Experimental Design

Each subject performed 2 exercise trials (treadmill running at 60% VO_{2peak}) in the heat for 60 min. Female subjects were counterbalanced so that 4 subjects performed the first exercise bout in the lut phase while 5 subjects performed the first exercise bout in the fol phase. Fol testing was done between days 5-9 and lut testing occurred between days 19-21. The onset of bleeding was considered day 1. Plasma estrogen and progesterone values were obtained to corroborate the appropriate menstrual cycle phase (Genway Bioscience, San Diego, CA).

Experimental Protocol

All subjects were un-acclimated to the heat and data collection took place during the fall and winter months (October – February). Subjects were instructed to avoid exercise and alcohol for 24 hrs and to avoid caffeine for 12 hrs prior to each exercise test. Subjects were given a list of high carbohydrate foods to consume for dinner the night before the test and for breakfast on the morning of the test. Subjects were asked to consume the same foods for the second trial.

On the day of the trial, nude body weight was recorded to the nearest 0.1kg (Seca Scale, Birmingham, UK) and urine osmolality was used to assess hydration levels (Advanced Osmometer, Model 303, Advanced Instruments Inc, Norwood, MA).

Core temperature (T_{core}) was measured by inserting a thermistor (YSI precision 4400 Series, Yellow Springs Inc, Yellow Springs, OH) 10 cm past the anal sphincter. An intravenous catheter was inserted in an antecubital vein and kept patent by infusing 3 ml of isotonic saline every 15 min during and after the exercise bout. Samples were drawn pre, immediately post, 1 hour post, and 4 hours post exercise. All blood was stored in EDTA treated tubes (Vacutainer, Franklin Lakes, NJ) for future analysis.

The exercise intensity was set to elicit 60% of VO_{2peak}. VO_2 was measured every 15 min during exercise. The workload was adjusted during the first 15 min of trial 1 to ensure 60% of VO_{2peak}. After the first 15 min, the workload did not change. When subjects repeated the exercise trial, the intensity was identical to the first exercise trial. During the first exercise trial, subjects were allowed to drink water *ad libitum*. The subjects ingested the same volume of water during their second trial. 1 male and 1 female subject was unable to complete 60 min of exercise due to heat related issues. When the trial was repeated, these subjects exercised for the same duration as in trial 1.

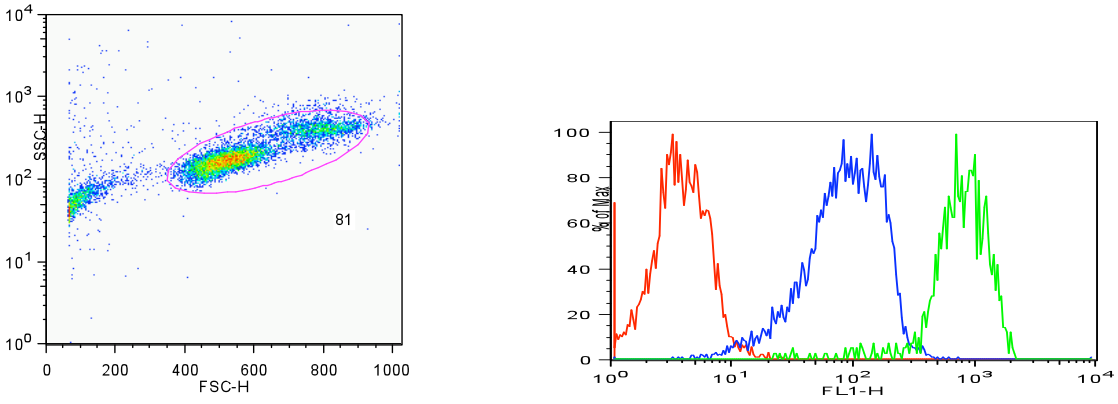
Blood Analysis

Hsp72

Three ml of blood was drawn at each time point. Half of the blood was aliquotted into EDTA treated tubes and used to measure hematocrit, Hsp72, estrogen, and progesterone. Hsp72 was quantified using flow cytometry. Briefly, cells were separated from 1 ml of blood using density gradient centrifugation (15 min, 2100 RPM, 0 ACC) with 1.077 g/ml

Histopaque (Sigma-Aldrich, St. Louis, MO). Peripheral blood mononucleated cells were washed with PBS and then treated with Reagent A (Fix and Perm kit, Invitrogen, Carlsbad, CA) and incubated at room temperature for 15 min. Cells were then washed and treated with Reagent B (Fix and Perm kit, Invitrogen, Carlsbad, CA) combined with a monoclonal Hsp72 FITC antibody (Assay Designs, Ann Arbor, MI) at 100 µg/ml. Cells were incubated for 20 minutes in the dark at room temperature. Cells were washed a final time, and then diluted in 300 µl sheath fluid and analyzed using a FACSCAN cytometer (BD Scientific, San Jose, CA). Ten thousand events were collected. Data was analyzed using Cellquest software (BD Scientific, San Jose, CA). The amount of protein produced per cell population was quantified as mean fluorescent intensity (MFI). To determine MFI, cells were gated and corrected for auto-fluorescence based upon unstained control. The gating strategy is shown in figure 1 To serve as a positive control, 1 ml of post exercise blood was placed in a 42°C water bath for 2 hours.

Figure 1. Gating strategy: SSC vs FSC for PBMC. Expression of Hsp72 in unstained cells, pre exercise, and after 42ºC incubation.



Cytokines

For cytokine analyses, 1.5 ml of blood was treated with 4.5µg Brefeldin A (ebiosciences, San Diego, CA) then placed in EDTA treated tubes. The same protocol was used to

quantify cytokine expression except that Reagent B (Fix and Perm kit, Invitrogen, Carlsbad, CA) was combined with an APC conjugated monoclonal antibody for TNF-α (concentration: 0.2 mg/ml), a FITC conjugated anti-human IL-1ra antibody (0.5 mg/ml), and a PE conjugated anti-human IL-6 antibody (0.2 mg/ml). To determine MFI, cells were gated and corrected for auto-fluorescence based upon unstained control. To serve as a positive control, 1 ml of post exercise blood was treated with 1μg of LPS.

eHsp72

Plasma was analyzed for extracelluar Hsp72. Plasma was obtained after density gradient centrifugation using 1.077 g/ml Histopaque (Sigma-Aldrich, St. Louis, MO). Plasma was immediately stored in -80°C until future analysis. An Hsp72 ELISA kit (Assay Designs, Ann Arbor, MI) was prepared according to manufacturer's instruction to quantify eHsp72. Minimum detection of eHsp72 was 0.20 ng/mL with an inter-assay precision of 12.8% and an intra-assay precision of 3.9%. Hematocrit was analyzed within 15 min of the blood draw and used to correct for plasma volume changes with exercise as outlined in Van Beaumont, 1972 (40). eHsp72 results are corrected for plasma volume changes from the exercise bout.

Estrogen and Progesterone

Plasma was analyzed for estrogen and progesterone using EIA kits (Genway Bioscience, San Diego, CA) according to manufacturer's instruction. Minimum detectable concentration of estradiol was 5 ± 2 pg/mL and 0.08 ± 0.03 ng/mL for progesterone. Inter-assay variability was 6% for estradiol and 8.8% for progesterone. Intra-assay variability was 4.6% for estradiol and 9.7% for progesterone.

Statistical Analysis

To determine if Hsp72, eHsp72, or cytokines changed with exercise for a given condition, a 1 factor (time) ANOVA was used to analyze each condition independently. A total of 6 independent 1-way ANOVA's were run. The conditions were: trials 1 and 2 for M (n = 9), trials 1 and 2 for W (n = 9), fol (n = 6), and lut (n = 6).

To analyze if there was an order effect, we compared trial 1 vs. trial 2 for the M (n=9) and trial 1 vs. trial 2 for the W (n=9) using a 2 factor (time x trial) repeated measures ANOVA.

To test for differences due to the menstrual cycle, a 2 factor (time x phase) repeated measures ANOVA was used. Here we compared fol vs. lut data, n=6.

To test for sex differences in Hsp72, eHsp72, and cytokines, a 2 factor (time x condition) ANOVA was used. In this analysis, we compared each condition (M trial 1, M trial 2, W trial 1, W trial 2, fol, and lut) to all other conditions.

Baseline Hsp72 MFI on trial 1 was compared to baseline Hsp72 on trial 2 using a paired t-test. This was done to assess acquired cellular thermotolerance.

Statistical analysis was performed by using SPSS version 16. Statistical significance was set at $\alpha = 0.05$. If the sphericity assumption was violated, the Huynh–Feldt correction was applied to the degrees of freedom of the *F* ratio. When appropriate, Tukey's *post hoc* tests were performed. All data is expressed mean ± SD.

Results

Subject characteristics. M and W differed significantly for height ($p = 0.01$), weight ($p = 0.01$), % body fat ($p = 0.01$), and aerobic capacity expressed as ml/kg/min ($p = 0.03$). However, when aerobic capacity was expressed relative to fat free mass, there was no difference between groups (Table 1). For M, trial 2 was repeated 12 ± 4 days after trial 1. For W, trial 2 was repeated 12 ± 2 days after trial 1.

Table 1. Descriptive Data (n=9 women, 9 men) (Mean ± SD).

	Age (yr)	Height (cm)	Weight (kg)	VO_{2peak} ($ml \bullet kg^{-1} \bullet min^{-1}$)	VO_{2peak} ($ml. \bullet kg^{-1}$ FFM $\bullet$ min^{-1})	% Body Fat
Men	26±5	182± 7*	81.0±15*	52.6±8*	60.0±7.7	12.0±5.5*
Women	24±3	170±3	63.1±12	44.9±5	58.8±5.2	21.4±2.5

* difference ($p < 0.05$) between groups.

Exercise Response. M and W did not differ in starting or ending T_{core}, HR, pre or post urine osmolality, or relative exercise intensity. However, M had significantly greater sweat rates than W ($p = .01$) (Table 2). 1 female subject experienced heat illness issues at 45 min on trial 1, and 1 male subject was forced to stop exercising for similar reasons at 30 min on trial 1. The second bout for both subjects was identical in duration and intensity to the first bout. All other subjects completed 60 min of exercise.

Table 2. Thermal and cardiovascular responses to exercise (Mean ± SD).

	Pre Tc (°C)	End Tc (°C)	Pre Urine Osm (mOsm/ kg)	Post Urine Osm (mOsm/ kg)	End HR	End $\%VO_{2pk}$ (ml/kg/ min)	Sweat Rate (ml/min)
Women Combined (n=9)	37.25±.2	38.93±.5	459±333	407±255	161±12	59±4	15.6±7$^{\Omega}$
Fol (n=6)	37.19±.1	39.06±.5	394±346	351±245	161±11	59±5	16.2±5$^{\Omega}$
Lut (n=6)	37.34±.2	38.97±.4	516±287	464±235	162±10	60±3	15.0±8$^{\Omega}$
Men							
Trial 1 (n=9)	37.04±.2	39.36±.3	532±298	520±266	162±17	57±4	29.1±11
Trial 2 (n=9)	36.99±.3	39.26±.1	442±317	547±307	158±16	58±5	26.4±13

$^{\Omega}p< 0.05$ from Men's Trial 1 & 2

Hormones during the Menstrual Phase. Three subjects were removed from only the menstrual cycle analyses after examining their estrogen and progesterone values. In these subjects, estrogen and/or progesterone values were not higher in the lut compared to fol phase. Therefore, all menstrual phase analysis are shown with n = 6. Of the six subjects analyzed for menstrual phase differences, four completed their first exercise trial in the fol phase, and two subjects exercised in the lut phase first. Estrogen and progesterone levels were significantly higher in the lut compared to fol phase ($p = .01$) (Tables 2 and 3). There was no difference in starting or end of exercise T_{core}, HR, or VO_2 between phases.

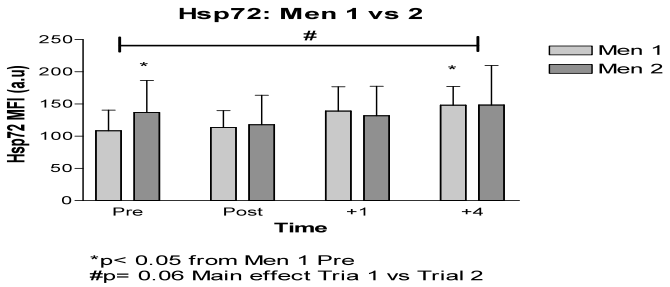
Table 3. Sex hormones and menstrual cycle phase (n = 6) (Mean ± SD).

	Estrogen (pg/ml)	Progesterone (ng/ml)	Ending HR	% VO_{2max}	Pre Tc (°C)	End Tc (°C)
Follicular	86±27*	.85±.64$^{\Omega}$	160±16	57±5	37.19±.1	39.06±.5
Luteal	159±56	5.5±2.9	165±15	59±3	37.34±.2	38.97±.4

*p< 0.05 from Lut; $^{\Omega}$p< 0.05 from Lut

Effect of exercise on Hsp72 and cytokines. During exercise, intracellular protein content of Hsp72 expressed as MFI increased in M trial 1 (p = 0.03) (Figure 2). Post hoc tests revealed a significant difference between baseline and 4 hrs post exercise (p = 0.05). Hsp72 did not increase in trial 2. M trial 1 and 2 showed a strong tendency towards statistical significance (p = 0.06) (Figure 2). Pre Hsp72 MFI for the M in trial 2 was 26% higher than pre trial 1 (p = 0.01) (Table 4).

Figure 2. Hsp72 Response to Exercise: Men's trial 1 and 2.



For W, MFI of Hsp72 did not change in trial 1 or trial 2 (Figure 3). There was no difference between trial 1 and 2. Pre Hsp72 MFI for W trial 2 was not higher than pre trial 2 (Table 4).

Figure 3. Hsp72 Response to Exercise: Women's trial 1 and 2.

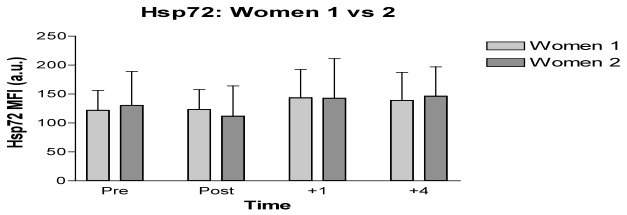


Table 4. Baseline Hsp72 content (MFI): Trial 1 vs Trial 2. Mean ± SD.

	Basal Hsp72 Content	% Increase from Trial 1
Men's trial 1	108 ± 31	
Men's trial 2	136 ± 49*	26%
Women's trial 1	121 ± 34	
Women's trial 2	130 ± 58	7%

*p< 0.05 from Men's Trial 1.

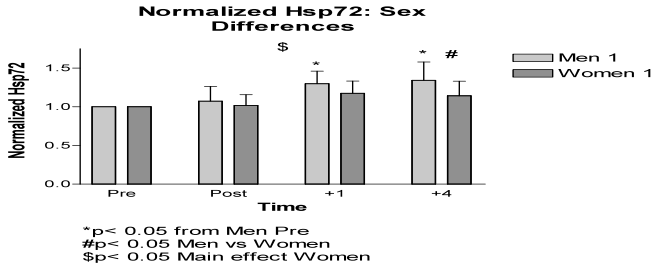
Hsp72 data was normalized to baseline to describe the percent increase observed with exercise. Normalized Hsp72 increased in M trial 1 (p = 0.00). Post hoc tests revealed significant increases from baseline after 1 (p = 0.00) and 4 hrs of exercise (p = 0.00). During M trial 2, normalized Hps72 did not increase significantly from baseline. M trial 1 differed from M trial 2 (p = 0.00).

Normalized Hsp72 increased from baseline in W trial 1 (p = 0.02), however, none of the post hoc tests were significant. In W trial 2, normalized Hsp72 did not increase from baseline. W trial 1 differed from W trial 2 (p = 0.00).

Cytokines and eHsp72 did not change with exercise.

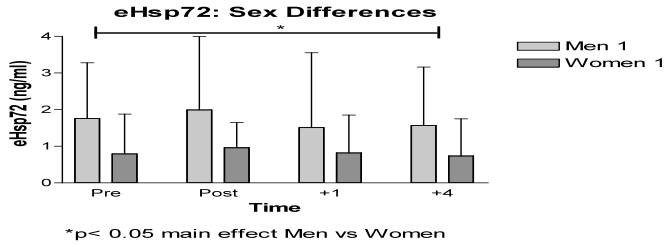
Effect of sex on Hsp72 and cytokines. There was no difference between M and W trials, including menstrual phase, on Hsp72 expressed as MFI. For normalized Hsp72, there was a main effect for condition (p = 0.00). Post hoc test showed M trial 1 was significantly different than W trial 1 (p = 0.00), fol (p = 0.00), and lut (p = 0.00) (Figure 4). M trial 2 was not different than W trial 2, fol, or lut.

Figure 4. Normalized change in Hsp72: Men vs Women – Trial 1.



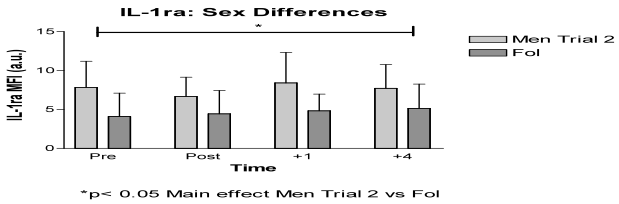
eHsp72 expressed a main effect for condition (p = 0.05). Post hoc test showed M trial 1 was higher than W trial 1 (p = 0.05) (Figure 5).

Figure 5. eHsp72 Sex Differences. Corrected for plasma volume change with exercise.



There was a main effect for condition for IL-1ra ($p = 0.03$). Post hoc test showed that M trial 2 ($p = 0.03$) was significantly higher than fol. There was no difference between M and W trial 1 and M and W trial 2 (Figure 6).

Figure 6. IL-1ra: Sex Differences.



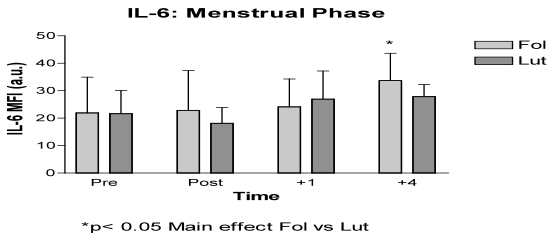
There was no main effect for condition for TNF- α or IL-6.

Effect of menstrual phase on Hsp72 and cytokines. Hsp72 MFI did not change during exercise in the fol or lut phase and the response was similar in both fol and lut phases. Normalized Hsp72 was not different when exercise was done in the fol compared to lut phase. Normalized Hsp72 did not change from baseline during exercise in fol or lut phase. eHsp72 expression did not change with exercise in the lut or fol phase, and there was no differences in eHsp72 expression between menstrual cycle phases.

IL-1ra did not change during exercise in the fol or lut phase and the response was similar in both fol and lut phases.

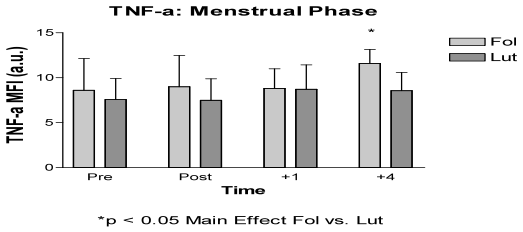
W expressed higher IL-6 in the fol compared to lut phase ($p = 0.03$) (Figure 7). Paired t test revealed a significant increase in fol at 4 hrs post exercise ($p = 0.04$). Exercise in the fol phase and lut phase did not change IL-6.

Figure 7. IL-6 Menstrual Phase.



W expressed higher TNF- α in the fol compared to lut phase (p = 0.01) (Figure 8). Paired t test revealed a significant increase in fol at 4 hrs post exercise (p = 0.03). Exercise in the fol phase and lut phase did not change TNF- α.

Figure 8. TNF-α Menstrual Phase.



Discussion

Our major finding is that W did not increase Hsp72 in response to exercise in the heat, nor was baseline Hsp72 increased after trial 1(Figure 3). M expressed a greater cellular stress response to exercise in the heat than W. This is evident by the fact that M expressed greater Hsp72 than W. Furthermore, baseline Hsp72 content was up-regulated by 26% from trial 1 to trial 2 in M (Table 4). This increase in baseline Hsp72 led to a significant fall in post exercise Hsp72 when the trial was repeated after ~12 days (Figure 2). Thus, for a given level of stress, women had a lower cellular stress response than men. These sex differences may highlight the pleiotropic effects of estrogen in mediating the stress response to exercise in the heat.

Hsp72

Hsp72 expression plays a significant role in maintaining cellular homeostasis during and after stress (13, 42). While there have been numerous animal studies that have shown sex differences in Hsp72 expression with hyperthermia, to our knowledge ours is the first study to examine this effect in humans. Results from animal studies imply that estrogen is responsible for increased baseline Hsp72 expression (3, 10, 41) and this up-regulation exerts protective effects that limit Hsp72 production during stress (22, 28, 29, 36). In our study in humans, baseline Hsp72 did not differ between sexes (Table 4). M expressed increased Hsp72 4 hrs post exercise (Figure 2) and this is consistent with previous findings (4, 9, 11, 26, 33) that have shown increased Hsp72 content within 4 hrs after a stressor. In contrast, W did not increase Hsp72 (Figure 3). Further, M had higher normalized Hsp72 compared to W in trial 1 (Figure 4). Thus, while it is understood that M increase Hsp72 in response to stress, we report for the first time that W do not. The lack of increase in Hsp72 could potentially demonstrate increased protection from exercise in the heat exerted by estrogen, thereby inhibiting the need for additional Hsp72.

Acquired cellular thermotolerance occurs when a single exposure to a severe, but sub-lethal heat stress leads to protection against future, more severe heat exposure. This process involves the increased expression of basal Hsp72 (19, 20) and leads to decreased Hsp72 induction in response to a second exposure to heat (30). In this fashion, Hsp72 can act as a marker for thermal history (33). Our data showed a 26% increase in baseline Hsp72 content in M, but not in W (Table 4). Thus, it appears that Hsp72 is regulated differently after an acute bout of exercise in the heat in un- heat acclimated M and W. To

our knowledge, it has not been previously appreciated that baseline Hsp72 could be up-regulated for 12 days after a single acute bout of exercise in the heat.

This paper also is the first to examine possible effects of the menstrual cycle on Hsp72 expression. If estrogen is responsible for the decreased Hsp72 induction during exercise, then the variation of estrogen across the menstrual cycle could alter baseline or stress induced Hsp72 expression. The mechanism behind the relationship between estrogen and the blunted intracellular Hsp70 response to stress is currently not known. It was suggested that estrogen mediates this effect through a nongenomic hormonal pathway. Treating animals with tamoxifen, a known estrogen receptor agonist, caused the same blunted post exercise Hsp70 expression as in ovariectomized animals treated with 17β and 17α estradiol (28). Since tamoxifen, 17β, and 17α estradiol all suppress the post exercise expression of Hsp70, researchers suggest that these estrogen related compounds stabilize cell membranes and attenuate oxidative stress (44). Such an effect could protect thermal sensitive cells against exercise-induced damage, and thereby result in a blunted Hsp72 expression.

However, we found no difference in baseline or post-exercise Hsp72 content when W exercised in the fol compared to the lut phase. Thus, while these unacclimated W did not increase Hsp72 in response to exercise in the heat, and this inhibition has been theorized to be mediated by estrogen (3, 28, 29, 36), the physiologic variations in estrogen and progesterone in these women during the menstrual cycle may not be sufficient to alter Hsp72 expression.

Cytokines

Exercise results in a rise in extracellular pro- and anti-inflammatory cytokines. It is known, however, that PBMC are not the main origin of circulating cytokines (18, 27, 31, 37, 38). Despite this, recent studies have demonstrated robust changes in cytokine gene expression in PBMC after exercise (6, 16, 15, 30). Furthermore, inflammatory gene expression is menstrual phase dependent (25). Therefore, we sought to determine if M and W differed in the intracellular inflammatory response to stress. Hsp72 has been shown to abrogate pro-inflammatory cytokine synthesis (TNF-α, IL-1), but has little effect on IL-6 (8). While we found no change in cytokine expression with exercise in the heat, cytokines were affected by menstrual phase and sex.

Our results demonstrate a menstrual phase effect on IL-6 as W expressed higher IL-6 after exercise in the follicular phase (Figure 7). This result is consistent with previous work in which W who exercised in the fol phase up-regulated IL-6 mRNA in PBMC, but with exercise in the lut phase, IL-6 mRNA was down-regulated (25). The change in IL-6 values between menstrual phases may be due to altered carbohydrate (CHO) oxidation rates. Zderic *et al* found that whole body CHO oxidation during cycling at 70-90% of lactic threshold for 50 min is higher in the fol phase (46). This higher rate of CHO oxidation could have lead to a greater use of CHO. In response to low CHO availability, IL-6 production will increase (18).

IL-1ra is a cytokine that exerts anti-inflammatory effects through binding to the IL-1 receptor thus inhibiting the biological activities of IL-1α and IL-1β. Circulating IL-6 can

trigger the release of IL-1ra (39). Unlike IL-6, which is released from the skeletal muscle with exercise (27), IL-1ra is released from PBMC's (27). It appears that the increase in post exercise IL-1ra mRNA and protein plays a vital, paradoxical role to limit the effects of inflammation seen with exercise. However, we found that intracellular IL-1ra in PBMC did not change with exercise. One reason for the lack of IL-1ra production in our study could be due to hyperthermia. Previous work has shown that stress hormones limit the intracellular cytokine production from monocytes (31). With exercise in the heat, stress hormones are elevated (12) and could thus limit IL-1ra production. Our results are consistent with Selkirk *et al* who found that classic monocyte's ($CD14^{++}CD16^{-}$) intracellular IL-1ra MFI was unchanged in response to walking in the heat until exhaustion (34).

Contrary to our results, Northoff *et al* reported an increased IL-1ra mRNA synthesis after exercise in the fol compared to lut phase (25). Further, PBMC have been shown to release less (21) or the same amount (43) of IL-1ra during the lut compared to the fol phase. Our results suggest IL-1ra does not change across the menstrual cycle, but that M have higher IL-1ra than W in the fol, but not the lut phase (Figure 6).

Similar to IL-6, it appears that circulating immune cells are not responsible for the increased serum TNF-α which accompanies strenuous exercise (37). Our data support this theory, as there was no increase in intracellular TNF-α after exercise in PBMCs. Hsp72 has been shown to inhibit TNF-α through the NF-κB pathway (5, 45). Despite the smaller increase in Hsp72, M and W expressed similar amounts TNF-α. However, W had higher TNF-α in the fol compared to lut phase (Figure 8). Previously, it was found that

progesterone plays a role in inhibiting TNF-α mRNA and protein in macrophages (23). This decrease in TNF-α occurred through progesterone's ability to inhibit the Nf-Kb pathway. Progesterone was significantly higher in the lut phase (Table 3) and potentially limited TNF-α production. In addition, this highlights a different mechanism of TNF-α regulation that could account for similar TNF-α values between M and W, despite differences in Hsp72 expression.

eHsp

Another novel finding in this study was that the eHsp72 response was different between M and W in trial 1 (Figure 5). eHsp72 values in W were almost half of M's values. Unlike intracellular Hsp72 that has been shown to down-regulate inflammatory cytokines, eHsp72 stimulates pro-inflammatory cytokine release from monocytes (2). eHsp72 has been linked to LPS tolerance (1) and may serves as a marker for heat illness (32).

The release of eHsp72 is through an α_1 adrenergic pathway (17). Epinephrine release in response to submaximal exercise is greater in males compared to mid-fol females (7, 14). Epinephrine was not measured in the current study, yet this could be a plausible explanation for the greater eHSP in M than F. Future studies should address the different eHsp72 response between M and W.

Conclusions

We found that un-acclimated M and W differ in the amount of Hsp72 expressed in PBMC in response to exercise in the heat. M increased Hsp72 after 1 bout of exercise in the heat, while W did not. For M, one bout of exercise in the heat increased basal Hsp72 content, suggesting acquired cellular thermal tolerance. W did not demonstrate this

response. During trial 1, eHsp72 was higher in M. Despite these sex related differences in Hsp72 expression, M and W do no differ in their ability to tolerate or acclimate to heat. Thus, estrogen could provide cellular protection and thus decrease the need for Hsp72. Though cytokines did not change with exercise, IL-6 and TNF-α were menstrual phase dependent, while IL-1ra regulation could be dependent on sex.

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Chapter IV

Summary, Conclusions, and Recommendations

Summary: Hypotheses

1. *Women will have higher basal levels of intracellular Hsp72 than men.*

   Our results showed that baseline Hsp72 in PBMC did not differ between sexes. Future research should examine Hsp72 content in tissues to determine baseline sex differences.

2. *In response to a severe exercise stress (60 minutes of treadmill exercise at 60% VO_{2peak} in the heat), men will express greater amounts of Hsp72.*

   Our data supports this hypothesis as men increased Hsp72 expression (MFI) 4 hrs post exercise, while women did not increase Hsp72. Furthermore, normalized Hsp72 was significantly higher in men than women.

3. *Intracellular Hsp72 levels will be higher at baseline in women during the luteal phase of their menstrual cycles, but in response to severe exercise the expression of Hsp72 will be blunted in luteal compared to the follicular phase.*

   Neither baseline nor post exercise Hsp72 levels were altered by menstrual phase. Our results are supported by animal research (Nickerson 2006) that showed no change in Hsp72 content after tail shock between phases of the menstrual cycle. Thus, while it appears that estrogen is responsible for the inhibition of Hsp72 after a stress, physiologic changes across the menstrual cycle do not alter baseline or post stress Hsp72 expression.

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4. *Pro-inflammatory cytokines (TNF-α) will be reduced in conditions where intracellular Hsp72 is elevated.*

   Intracellular cytokines did not change in response to the exercise stress. Catecholamine release is greater with exercise in the heat, and increase catecholamines have been shown to inhibit pro-inflammatory in PBMC (Rhind 2004). However, IL-1ra was higher in men compared to follicular phase women. In addition, both TNF-α and IL-6 were higher in the follicular compared to luteal phase.

5. *There will be no difference between groups in eHsp72. eHsp72 is released with exercise, but this process does not appear to be mediated by estrogen.*

   eHsp72 was significantly higher in males compared to females in trial 1. The release of eHsp72 is through an α_1 adrenergic pathway (Johnson 2005), and epinephrine release in response to submaximal exercise is greater in men (Davis 2000). Thus, the greater epinephrine release in men could potentially explain the increased eHsp72 release in men compared to women.

Limitations

In this study Hsp72 was only assessed in PBMC's and not in tissues as in most of the animal studies. However, previous work in humans showed no difference between M and W after 6 weeks aerobic conditioning in Hsp70 expression in the vastus lateralis (Morton 2009). Unfortunately, baseline Hsp70 expression was not reported in this study.

One potential reason for a blunted increase in Hsp72 in W may have been due to their lower T_{core} response during exercise. Although not statistically significant, W were ~ 0.3°C cooler at the end of 60 min of exercise than M. This is most likely due to issues with the rectal probe not reading appropriately or not being inserted to the proper depth. Despite this difference, the M and W had similar cardiovascular and metabolic exercise responses (Table 2). Previous work has shown that other factors such as oxidative stress (Adrie 2000), energy depletion (Sciandra 1983), disruptions in calcium homeostasis (Kiang 1994), and ischemia reperfusion injuries (Marber 1995) increase Hsp72. Finally, all menstrual phase analyses were conducted with an *n* of 6. Therefore, it is plausible that with a larger samples size and thus more power, changes in Hsp72 or cytokine expression may emerge.

Conclusions and Applications

Our data demonstrates that in response to exercise in the heat, men express more Hsp72 than women. Also, men had higher eHsp72 values than women. Despite these sex related differences in Hsp72 expression, men and women do no differ in their ability to tolerate or acclimate to heat. Thus, estrogen could provide cellular protection and thus decrease the need for Hsp72, highlighting a novel role of estrogen in conferring cellular thermotolerance. Though cytokines did not change with exercise, IL-6 and TNF-α were menstrual phase dependent, while IL-1ra regulation could be dependent on sex.

Recommendations

Future research should examine sex differences in Hsp72 after repeated bouts of exercise or during a heat acclimation process. Our data suggests that women are protected from stressful stimuli without the need for additional Hsp72. It is our contention that estrogen is mediating this protection. Therefore, studying the Hsp72 responses to exercise after pharmaceutically inhibiting estrogen and progesterone release could reveal important insights into cellular thermotolerance. Finally, it would be interesting to compare post-menopausal women to regularly menstruating women to determine the effect of age and estrogen on Hsp72 expression.

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Detailed Flow Cytometry Protocol

1. Blood that will be tested for cytokines must be treated with Brefeldin A (golgi transport inhibitor). Add 3 µl of BFA/1 ml of blood. Thus, add 3 µl directly to EDTA treated tubes for each ml of blood. From the beginning, you have to alliquot blood for Hsp and blood for cytokines.

2. I separate the blood using Histopaque (1.077 Sigma-Aldrich), however there is a red cell lysis that you can add directly to whole blood that is quicker and does not require histopaque.

3. If using histopaque, spin 2100 RPM, 15 min, 0 ACC.

4. Remove plasma and store for extracellular proteins of interest.

5. Remove buffy coat and place in a new 15 ml conical tube. Wash in 8 ml of cold PBS (1300 RPM, 7 min, 6 ACC). Set aside 1 sample to use as the blank (unstained) control.

6. Remover supernatant.

7. Add 75 μl reagent A (from fix and perm kit - Invitrogen) to pellet to fix cells. Re-suspend pellet and incubate 15 min at room temp. Don't add reagent A to the blank sample. Simply remove the supernatant and place in fridge until flow analysis.

8. Wash in 3 ml wash buffer (PBS, 10% FBS, and 1% NaN_3). Spin 1200 RPM, 5 min, 6 ACC.

9. Remove supernatant.

10. Alliquot 75 μl reagent B (from fix & perm kit - Invitrogen) along with appropriate amount of Hsp72 Ab (3 μl/ 10^6 cells – Hsp72 FITC – Assay Designs). Re-suspend pellet and incubate in the dark 20 min.

Do the same for cytokines. 75 ul of reagent B mixed with appropriate volumes of IL-1ra (1 μl/10^6 cells - eBioscience), IL-6 (2 μl/10^6 cells – eBioscience), and TNF-α (1 μl/10^6 cells – eBioscience) antibody. All antibodies are added to each sample. Re-suspend and incubate in the dark for 20 min.

For isotype controls, each pellet can only be treated with one Ab. Thus, you need 4 different samples (one for Hsp72 isotype control, 1 for TNF isotype control, 1 for IL-6 isotype control, and one for IL-1ra isotype control). The isotype controls are added with the same concentration as the corresponding Hsp72/cytokine antibody. Re-suspend and incubate in the dark for 20 min.

11. Wash 3 ml wash buffer.

12. Remover supernatant.

13. Re-suspend in 500 ml Sheath fluid.

14. Measure on Flow Cytometer.

15. To quantify MFI, gate on blank or isotype control. Use “median” statistic to quantify intracellular protein content. Subtract blank MFI (for auto-flouresence) from median MFI value.

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The University of New Mexico Health Sciences Center
Consent to Participate in Research

Gender difference in heat shock protein expression in response to acute exercise in the heat

Introduction

You are being asked to participate in a research study that is being done by Dr. Schneider, PhD, who is the Principal Investigator and her associates. from the Department of Health, Exercise, and Sports Science. This research is studying the effect of gender on heat shock protein expression in response to exercise in the heat.

Heat shock proteins (HSP) are a family of proteins that serve to protect the cell. HSP can be found in all cells of the body. The activities of HSP are vital for cells to resist environmental stressors like heat. Because of the protective nature of HSP, recent research has implicated HSP in preventing damage to the heart.

You are being asked to participate in this study because HSP can increase protection from stressors like exercise and heat, but animal studies suggest women produce less HSP in response to stress than men. Therefore, if we can understand the reason behind this response, it could potentially lead to better cell protection. 24 people (8 men and 16 women) will take part in this study at the University of New Mexico.

This form will explain the research study, and will also explain the possible risks as well as the possible benefits to you. We encourage you to talk with your family and friends before you decide to take part in this research study. If you have any questions, please ask one of the study investigators.

What will happen if I decide to participate?

If you agree to participate, the following things will happen:

Your cardiovascular fitness will be assessed through a preliminary exercise trial (VO_2 max test) where you will run on a treadmill while breathing into a mouthpiece. Specifically, you will run until you become too tired to continue. During the test, we will collect your expired air which allows us to calculate the amount of oxygen your body is consuming. To collect your expired air, you will breathe through a sterile, rubber mouthpiece and wear a nose-clip. This may cause you to experience some discomfort (dry mouth) and you may feel confined. A heart rate monitor will be used to measure your heart rate. This test will be done in a comfortable environment. You should have at least an average value of aerobic fitness for your age and gender to qualify for this study. We will calculate your fitness based upon the amount of oxygen you breath in during the test.

Your body composition will be assessed through hydrostatic weighing and skin fold measurement. During hydrostatic weighing, we will weigh you on a normal scale, and then also weigh you underwater.

We will take 3 skin fold measurements on your body: One on your upper arm, one by your belly button, and one on your thigh. During the skin fold measurement, you will feel a slight pinch for 1-2 seconds.

These measurements allow us to determine your body density, which we can use to find your % body fat. Your percent body fat is the amount of fat that makes up your total body weight.

Female subjects will exercise in both phases of the menstrual cycle. In the follicular phase (before ovulation), estrogen is low. In the luteal phase (after ovulation), estrogen and progesterone are high. Thus, by exercising in both phases of the cycle then we can see what effect estrogen has on HSP. To determine the phase of the menstrual cycle, females subjects will keep a record of the number of days of each menstrual cycle. The start of bleeding is considered day one of the menstrual cycle. The exercise test for the follicular phase will occur between days 2-4 of the menstrual cycle. The exercise test in the Luteal phase will occur between days 22-25 of the menstrual cycle. To help determine when ovulation occurs, female subjects will record their oral temperature each day before getting out of bed. Males and females will be tested twice over the course of 4 weeks.

Females will not be involved in this study if they are using any form of hormonal contraceptives, or have used them within the past three months. Also, females will not be allowed to participate in this study if they are pregnant. Prior to each exercise trial, female subjects will be given a pregnancy test. This involves subjects urinating on a pregnancy detection kit. If the subject is pregnant, they will not be exercised and will not participate in this study.

If you meet the criteria to be included in this study based upon the above assessments, then the study will require you walk or jog in a hot (100° F, 37.78°C) environment until you reach a temperature of 39.0 C (102.2° F). This should take 45-60 minutes. During the exercise test we will measure:

-Core body temperature. This requires that you insert a small, sterile, rubber catheter 4 inches into your rectum.

-Heart rate. We will use a telemetric heart watch monitor which consists of a transmitter belt worn around the chest and a receiver watch worn on your wrist.

-Urine samples. One tablespoon of urine will be required prior to the exercise challenge to check your hydration status. These samples will be coded, analyzed, and properly disposed on the same day.

Skin Temperature. We will place a flat thermometer on your upper right arm, chest, right thigh, and right calf to measure how hot your skin gets. They will be placed on top of your skin and be held there with plastic.

-Blood samples. Twenty milliliters (approximately 1.35 tablespoons) of blood will be drawn with a needle from an arm vein before, immediately after exercise, and 1, 4 and 24 hours after exercise. Each visit 7 tablespoons, or 100 ml of blood will be drawn. The total experiment will require about ¾ of a cup, or 200 ml of blood. This will require a catheter to be placed in your arm so we only have to insert the needle once per exercise session. Every 15 minutes during exercise, we will prick your finger to get one drop of blood. This will measure lactate and tell us how intensely you are exercising. This will occur 3 times for each exercise trial.

How long will I be in this study?

Participation in this study will take a total of 10 hours over a period of two 24 hour periods.

What are the risks or side effects of being in this study?

Every reasonable precaution will be taken to minimize risks during this study. As in any testing situation, there are risks involved. Some of these risks include muscle soreness or injury, heart attack (1 in every 10,000 exercise tests) or other heart events during the exercise trials. The risk for healthy young people during exercise testing is much lower. There may be some discomfort and feeling of confinement from the use of the mouthpiece and nose clip.

- During the cardiovascular fitness test you may experience a strong sense of fatigue, fainting, breathlessness, psychological stress (i.e., panic). There may be some discomfort and feeling of confinement from the use of the mouthpiece and nose clip. During the exercise trials in the hot room, you may feel hot, sweaty, and tired.

- Blood samples will be drawn from an arm vein (1 baseline blood sample, and a total of 4 samples after the exercise). Blood sampling may cause minor pain and discomfort from the needlestick and there is a slight risk of bruising or infection at the site of sampling. Sterile equipment and standard procedures done by experienced staff will be used to minimize these risks.

- Insertion of the rectal probe may lead to additional discomfort or feelings of awkwardness during the exercise trail. You, the subject, will insert the probe.

- There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study.

There may be unforeseeable risks to subject or unforeseeable risks to embyro if subject is pregnant or becomes pregnant before the end of the exercise testing.

For more information about risks and side effects, ask your study doctor.

What are the benefits to being in this study?

There are no direct benefits to you for participation in this study.

Your participation in this study will help us to determine if a gender difference in heat shock protein is present in humans. Potential results from this study could lead to methods for increasing protection for women against cardiovascular disease and certain types of tumors.

What other choices do I have if I do not want to be in this study?

The only alternative is not participating in this study.

How will my information be kept confidential?

We will take measures to protect your privacy and the security of all your personal information, but we cannot guarantee confidentiality of all study data. You will be given a coded number to protect your identity. We will keep a link between your code and your name. A link will be kept for the duration of the study and will be stored in a separate file away from the data. This code is stored in a locked file cabinet in the investigator's office. The link will be destroyed at the end of data analysis.

Information contained in your study records is used by study staff. The University of New Mexico Health Sciences Center Human Research Review Committee (HRRC) that oversees human subject research will be permitted to access your records. There may be times when we are required by law to share your information. However, your name will not be used in any published reports about this study. A copy of this consent form will be kept in your medical record.

What are the costs of taking part in this study?

There is no cost to you for taking part in this study.

What will happen if I am injured or become sick because I took part in this study?

No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study. If you are injured or become sick as a result of this study, UNMHSC will provide you with emergency treatment, at your cost. It is important for you to tell your study doctor immediately if you have been injured or become sick because of taking part in this study. If you have any questions about these issues, or believe that you have been treated carelessly in the study, please contact the Human Research Review Committee (HRRC) at the University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131, (505) 272-1129 for more information.

Will I be paid for taking part in this study?

No. There is no form of compensation for participating in this study.

How will I know if you learn something new that may change my mind about participating?

You will be informed of any significant new findings that become available during the course of the study, such as changes in the risks or benefits resulting from participating in the research or new alternatives to participation that might change your mind about participating.

Can I stop being in the study once I begin?

Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study without affecting your future health care or other services to which you are entitled.

If you choose to withdraw from the study, please contact Trevor Gillum at (580) 380-1486 or tgillum@unm.edu. Subjects will be removed from the study they fail to comply with the standard, high carbohydrate diet, start taking vitamin supplements, or in the case of women, do not maintain a normal menstrual cycle.

Whom can I call with questions or complaints about this study?

If you have any questions, concerns or complaints at any time about the research study, Dr. Schneider, PhD, or her associates will be glad to answer them at (505) 277-3795, Monday through Friday 8:00-5:00. If you need to contact someone after business hours or on weekends, please call (580) 380 1486 and ask for Trevor Gillum. If you would like to speak with someone other than the research team, you may call the UNMHSC HRRC at (505) 272-1129.

Whom can I call with questions about my rights as a research subject?

If you have questions regarding your rights as a research subject, you may call the UNMHSC HRRC at (505) 272-1129. The HRRC is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving human subjects. For more information, you may also access the HRRC website at http://hsc.unm.edu/som/research/hrrc/.

CONSENT

You are making a decision whether to participate in this study. Your signature below indicates that you read the information provided (or the information was read to you). By signing this consent form, you are not waiving any of your legal rights as a research subject.

I have had an opportunity to ask questions and all questions have been answered to my satisfaction. By signing this consent form, I agree to participate in this study. A copy of this consent form will be provided to you.

_____ _____

_____

Name of Adult Subject (print) Signature of Adult Subject

Date

.

_____

INVESTIGATOR SIGNATURE

I have explained the research to the subject or his/her legal representative and answered all of his/her questions. I believe that he/she understands the information described in this consent form and freely consents to participate.

_____

Name of Investigator/ Research Team Member (type or print)

_____

_____

(Signature of Investigator/ Research Team Member) Date

HEALTH HISTORY AND PHYSICAL ACTIVITY QUESTIONNAIRE

Name ______________________________ D.O.B ___/___/___
Date___/___/___

Age____ Height ______ Weight _______ Gender____
Ethnicity_______

Sitting blood pressure _______________

♦♦♦

MEDICAL HISTORY

Physical
injuries:____
_____

Limitations____
____

Have you ever had any of the following problems? Please check all that apply.

Heart attack/Myocardial Infarction____ Heart surgery ____ Valve
problems ______
Chest pain or pressure ____ Swollen ankles ____ Dizziness ____
Arrhythmias/Palpitations ____ Heart murmur ____ Shortness
of breath ______ Congestive heart failure ____
Blurred Vision ____ Abnormal anxiety____ Palpitations ____
Tingling/numbness in extremities ____ Leg cramps ____
Gastrointestinal Ulcers _____

Have you ever had any of the following? Please check all that apply.

Hepatitis/HIV _____ Depression _____ Cancer (specify type) ____
Rheumatic fever _____ High blood pressure_____ Thyroid problems ____
Kidney/liver disease _____ Obesity _____ Total cholesterol
>200 mg/dl _____
Diabetes (specify type) _____ Asthma _____ HDL cholesterol <35
mg/dl _____
Emphysema _____ Stroke _____ LDL cholesterol >135
mg/dl _____
Trigylcerides>150
mg/dl _____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.

____
____

Is your mother living? Y N   Age at death______ Cause____________________
Is your father living? Y N   Age at death______ Cause____________________

Do you currently have any condition not listed that may influence test results? Y N

Details____
____

____
____

Indicate level of your overall health. Excellent ____ Good ____ Fair ____ Poor____

Are you taking any medications, vitamins or dietary supplements now? Y N

If yes, what are
they?____
_

Do you have allergies to any medications? If yes, what are they?

____

Are you allergic to latex? Y N

Have you been seen by a health care provider in the past year? Y N

If yes, elaborate

____

Have you had a prior exercise test? Y N If yes, when?______________ What were the results?

____
____

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes,
elaborate.____

____
____

♦♦

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type _________________

How long?____________ Quantity____/day Years since quitting_______________

How often do you drink the following?

Caffeinated coffee, tea, or soda _______oz/day Hard liquor _______oz/wk Wine _______oz/week

Beer _______oz/wk

Indicate your current level of emotional stress. High____ Moderate ____ Low____

♦♦♦

PHYSICAL ACTIVITY/EXERCISE

Physical Activity

Minutes/Day (*Weekdays*) Minutes/Day (*Weekends*)

_______/_______ average _______/______ average

Do you train in any activity (eg. jogging, cycling, swimming, weight-lifting)? Y N

How well trained are you? ____

Vigorous Exercise (>30 Minute sessions)

__________Minutes/hours a week

How many days/wk do you exercise or do vigorous work outside? ________ average days/wk

♦♦

WOMEN ONLY

Please check the response that most closely describes your menstrual status:

_____ Post-menopausal (surgical or absence of normal menstrual periods for 12 months)

_____ Eumenorrheic – Normal menstrual periods (~every 28 days)

_____ Amenorrheic – Absence of normal menstrual periods for at least 3 months

_____ Oligomenorrheic – Irregular menstrual periods with occasional missed cycles.

Measured Height (cm) __________________ Weight (kg)________________
BMI (kg/m^2) _____________

Blood Pressure (mmHg) _______/_______

♦♦

Positive cardiovascular risk factors include:

1. Family history: Myocardial infarction, coronary revascularization or sudden death before 55 years in father or first degree male relative or before 65 in mother or first degree female relative.

2. Current cigarette smoker or quit within the previous 6 months.

3. Hypertension: systolic blood pressure greater or equal to 140 mmHg or diastolic pressure greater or equal to 90 mmHg, or on antihypertensive medication.

6. BMI>30 kg/m2

7. Sedentary lifestyle: persons not participating in a regular exercise program or not meeting the minimal physical activity recommendations for the US Surgeon General's Report.

If subjects have two or more cardiovascular risk factors as outlined above, then will be excluded from the study. Furthermore, subjects need to fall within the following criteria to be included:

-Healthy, between the ages of 18 and 34 years*

-VO2max > 30 for women and > 35ml/kg FFM/min for men, as determined in our lab using a graded, cycle protocol.

-Moderately active lifestyle (exercise vigorously at least 30 min, 3 times a week)

- Not pregnant or not trying to become pregnant