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ARE THERMOTOLERANCE AND HEAT ACCLIMATION RELATED THROUGH THE HEAT SHOCK RESPONSE?

BY

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ABSTRACT OF DISSERTATION

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Are Thermotolerance and Heat Acclimation Related Through the Heat Shock Response?

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ABSTRACT

Thermotolerance (cellular adaptations that allow survival after an acute, severe heat exposure) and heat acclimation (systemic adaptations that improve heat dissipation following chronic heat exposure) have traditionally been considered separate phenomena. However, recent studies in animals suggest these adaptations may be related through the heat shock response. **METHODS:** We evaluated the effects of a standard laboratory heat shock response-inhibitor (QUERCETIN) on established markers of thermotolerance [gastrointestinal barrier permeability, plasma TNF-a, Il-6, and Il-10 concentrations; leukocyte HSP70 content(HSP70)] and heat acclimation [reduced body temperatures, heart rate, and physiologic strain in response to exercise/heat stress] in male subjects (n=8) completing a 7-day heat acclimation protocol. Thermotolerance markers were assessed in blood drawn before (pre), after (post), 2hr after (2-post) and 4hr after (4-post) exercise on day 1 and day 7 of heat acclimation. Heat acclimation markers were assessed by a standard heat tolerance test, performed at baseline, and on day 6 of heat acclimation. Subjects completed an identical protocol under placebo supplementation (PLACEBO), in

counter-balanced order, under double-blind conditions, with sufficient washout. **RESULTS:** QUERCETIN increased gastrointestinal barrier permeability and TNF-a on the 1st day of exercise/heat stress, no differences in these variables were reported in PLACEBO. 7 days of exercise/heat stress in PLACEBO decreased subjects' exercise Il-6 and Il-10 and increased HSP70; it also reduced exercise body temperatures, heart rate, and physiologic strain. Striking differences were noted in these same subjects under QUERCETIN. Here gastrointestinal barrier permeability remained elevated, Il-6 and Il-10 were not reduced, HSP70 was not increased, and exercise body temperatures were not reduced. While exercise heart rate and physiologic strain were reduced, this occurred much later in exercise. **CONCLUSIONS:** Consistent with the concept of whole-organism adaptation, repeated exercise/heat stress reduces circulating cytokine concentrations and increases cytoprotective HSP70, contributing to reductions in systemic markers of heat strain. Exercising under the influence of a heat shock response inhibitor may prevent these responses, contributing to loss of benefits normally incurred by a standard heat acclimation protocol in humans.

TABLE OF CONTENTS

SYMBOLS / ABBREVIATIONS

≥: greater than or equal to

>: greater than

<: less than

±: plus or minus

~: approximately

^{o}C: degrees Celsius

μg/ml: migrogram per milliliter

um: micromolar

μL: microliter

μmol: micromole

ANOVA: analysis of variance

ATP: adenosine triphosphate

bpm: beats per minute

BM: body mass

BW: bodyweight

cm: centimeter

Da: dalton

dH_2O: distilled water

eHSP72: extracellular heat shock protein 72

EIA: colorimetric enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

g: gram

g/d: gram/day

g/kg: grams per kilogram

GPX: glutathione peroxidase

H_2O: water

HA: heat acclimation

HR: heart rate

HR max: maximal heart rate

HRP conjugate: horseradish peroxidase conjugate

HSE: heat shock element

HSD: honestly significant difference

HSF-1: heat shock factor 1

HSP: heat shock protein

HT: heat tolerance test

Ig: Immunoglobin

iHSP70: intracellular heat shock protein 72

Il-1β: interleukin 1 beta

Il-6: interleukin 6

Il-10: interleukin 10

IP: intestinal permeability challenge

kcal: kilocalorie

kDa: kilodalton

kg: kilogram

LAL: limulus amebocyte assay

LPS: lipopolysaccharide

M: molar

mg: milligram

mg/d: milligram per day

ml: milliliter

mM: millimolar

mOhm: milliohm

mOsmol/kg: milliosmolar per kilogram

n: number of subjects

Na: sodium

NaCl: sodium chloride

NAD: nicotinamide adenine dinucleotide

nm: nanomolar

nmol/min/ml

NP40: Nonidet P-40

PBS: phosphate buffered saline

pg: picogram

pg/ml: picogram per ml

PV: plasma volume

Q: quercetin

r^2: coefficient of determination

RH: relative humidity

RM ANOVA: repeated measures analysis of variance

SD: standard deviation

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: standard error

Tb: mean body temperature

TBW: total body water

TNFα: tumor necrosis factor alpha

Tre: rectal temperature

Tris: tris(hydroxymethyl)aminomethane

Tsk: skin temperature

VO_2 max: maximum oxygen uptake

CHAPTER 1

Introduction

The pathophysiology of exertional heatstroke is not well understood. Current research suggests that increased intestinal cell permeability may predispose humans to this potentially fatal illness (Bouchama et al. 2002; Lim et al. 2006). Performance of physical work in hot ambient conditions increases blood flow to active skeletal muscle and skin (Rowell, 1974). The additional blood flow is obtained by shunting blood from the splanchnic tissues. While not a problem at moderate exercise intensities, the combination of severe exercise and heat stress have been shown to reduce splanchnic perfusion by >70%, potentially causing ischemia in intestinal barrier cells (Rowell et al. 1965). Reduced splachnic blood flow reduces heat loss by convection to the circulating blood and leaves splanchnic tissues with inadequate oxygen supply (Lambert, 2008). The resultant rise in splanchnic tissue temperature and oxygen radical generation damages lipid membranes and fragments DNA (Finaud, 2006), reducing the structural integrity of the intestinal barrier (Ding et al. 2004; Kriegel et al. 1988; Lambert et al. 2002).

The gastrointestinal tract is the major source of endogenous endotoxin, and epithelial cell damage allows endotoxin entry into the portal circulation (Baker et al. 1988). Fixed tissue macrophages in the liver (Kupfer cells) respond to circulating endotoxin by secreting pro-inflammatory cytokines (TNF-a, Il-6) that target endotoxin removal (Jaeschke, 1997). Cytokine release by Kupfer cells activates circulating leukocytes and has a profound impact on surrounding tissues (Nathan, 1987). These cytokines increase concentrations of adhesion molecules on the surface of leukocytes and endothelial cells, facilitating reduced leukocyte rolling rates, firm adhesion, and

subsequent leukocyte migration into the interstitum (Sumagin & Sarelius, 2006). These processes are important in the pathogenesis of microvascular injury. Neutrophils are the first responders to bacterial infection. Following activation by circulating inflammatory cytokines (Il-1b, Il-6, TNF-a) (Robertson et al. 1996), neutrophils produce microbicidal agents that allow them to phagocotize LPS. Increased release of these agents is problematic, causing damage to epithelial and endothelial barriers in small intestine and liver tissues, and perpetuating the inflammatory response (Bannerman et al. 2003).

Barrier damage facilitates increased LPS extravasation from the gut, over-whelms the protective mechanisms of the portal circulation and immunoregulatory mediators in the liver, and results in a release of LPS into the systemic circulation. Plasma LPS concentrations as low as 4 ng/kg have been shown to induce a pyretic and hypotensive response in otherwise healthy humans (Suffredini et al. 1989). Greater LPS titers have been implicated in sustained inflammatory responses (Bouchama et al.1991), disseminated intravascular coagulation (al-Mashhadani et al. 1994), adult respiratory distress syndrome (Streiter et al. 1990), and diffuse liver injury (Deaciuc et al. 1999). All four morbidities are associated with severe heatstroke (Moseley & Gisolfi, 1993), which culminates in multiple organ failure and death (Lumlertgul et al. 1992).

Conversely, exposure to lesser thermal and oxidative stressors produces a state of cell thermotolerance. Much of this effect can be attributed to activation of the heat shock response, more specifically to increased heat shock protein (HSP) synthesis. Briefly, HSP function as molecular chaperones, ensuring newly synthesized proteins achieve their proper configuration and cellular locale (Moseley, 1997). In unstressed and/or quiescent cells, HSP localize to the cytosol. Here they bind the heat shock factor 1 (HSF-1),

transcript, retaining HSF-1 in its native inactive conformation. Stress exposure results in translational arrest, causing aggregation of protein fragments within the cell (Hildebrandt et al. 2002). Protein accumulation competes HSP from HSF-1,binding HSP and leaving HSF-1 free to trimerize and enter the nucleus. Binding of HSF-1 to promoter elements on DNA stimulates new HSP synthesis (Wu, 1995).

HSP family members range from 27 to 110 kDa, and expression varies by cellular locale and tissue type (Moseley, 1997). The 72-kDa protein (HSP72) has been examined extensively, and has been shown to be highly inducible by both thermal and oxidative stressors (Sonna et al. 2002; Horowitz et al. 2004). Exposing rats (Flanagan et al. 1995; Kokura et al. 2007), rabbits (Manzerra et al. 1997) and pigs (Sepponen et al. 2006) to these stressors increases HSP72 expression in small bowel, colon, and liver tissues. HSP72 upregulation in these tissues reduces inflammation, morbidity, and mortality from LPS challenge (Dong et al. 2005; Hung et al. 2005; Kluger et al. 1997). It also improves survival in ischemic (Juel et al. 2008; Stojadinovic et al. 1995) and thermal (Weshler et al. 1984) heat stroke simulations. In contrast, animals that have been pharmacologically or genetically manipulated to suppress HSP72 induction receive little or no benefit from prior stress exposure (Dokladny et al. 2001; Huang et al. 2001). Their response is similar to or worse than control animals upon subsequent challenge.

Tissue culture studies suggest a potential mechanism for the improved stress tolerance seen in these animals. Here human epithelial cells are cultured to produce monolayers, which are an accepted in-vitro simulation of intact epithelium. It was first shown that exposing cultured Madin-Darby Canine Kidney Cell (MDCK) monolayers to preconditioning heat stress (42ºC for 90 minutes) increases the temperature required for

barrier disruption, as measured by an increase in mannitol permeability (Moseley, 1994). This benefit was later tied to paracellular barrier improvements, including showing greater integrity of tight junctions between adjacent cells and reductions in paracellular transport of large molecules in the period following preconditioning stress exposure (Dokladny, 2006a; 2006b). This benefit was blocked by suppressing the heat shock response with quercetin during the preconditioning heat exposure. More recently occludin, a tetra-span protein vital to paracellular barrier function in the gastrointestinal epithelium was also shown to be up-regulated by a physiologically relevant heat stress (39ºC) (Dokladny, 2008). Taken together, these studies indicate that a physiologically relevant hyperthermia (between 39 and 42ºC) increases HSP72 and occludin expression in simulated intestinal epithelium, the combination of which cause paracellular barrier improvements.

Commonalities between these cell culture experiments and exercise/heat stress in human subjects suggest a similar mechanism. First, intestinal barrier disruption is often noted in humans following acute bouts of exercise in desert heat (Camus et al. 1997; Jeukendrup et al. 2000; Ng et al. 2008; Pals et al. 1997). Second, repeating this exercise/heat stress in animal or human subjects over multiple days causes significant increases in HSP_{72} expression (Amorim et al. 2008; Horowitz et al. 2004; McClung et al. 2008; Yamada et al. 2007). Third, as compared to a control population, heat intolerant subjects exhibit lower HSP72 titers both inside and outside of the cell in response to thermal stimuli (Moran et al. 2006; Wang et al. 2001; Wu et al. 2001). Finally, following severe exercise/heat stress trained subjects report lower circulating LPS concentrations than their untrained counterparts (Selkirk et al. 2008). Collectively, these studies suggest

that increased tolerance afforded to human subjects by repeat exercise/heat stress may result from intestinal cell barrier improvements and reductions in LPS extravasation into circulation. Like the cell culture and animal models discussed previously, HSP72 overexpression is vital to this response.

Application to US military personnel:

Carrying heavy rucksacks and hot environmental exposure cause military soldiers significant oxidative stress (Bachkosky et al. 2007). This stress has been implicated in skeletal muscle microtrauma, sustained inflammation, and non-specific immunosupression (Brown et al. 2007; Malm, 2006; Murphy et al. 2008; Nieman et al. 1990; Powers et al. 2008). Quercetin (3,3'4',5,7-pentahydroxyflavone) is a dietary flavanol that affords powerful antioxidant (Boots et al. 2008), anti-inflammatory (Nair et al. 2006), and antiviral (Davis et al. 2008; Nieman et al. 2007) effects in-vitro and in-vivo. In early 2009 military oversight committees adapted quercetin into energy bars as a nutraceutical countermeasure to aid soldiers during high stress military deployments (Natick, 2009).

Although used here to improve soldier health, it would surprise many that quercetin was originally examined for its cytotoxic effects. A 1990 article in "Cell Structure and Function" provides a good summation. It states:

"A number of biological effects of quercetin have been reported; the inhibition of cultured cell growth, inhibitory effects on glycolysis, macromolecule synthesis, activity of protein kinases, and ATPases… Quercetin is reported to be a hyperthermic sensitizer in HeLa cells." (Hosokowa et al. 1990).

This statement is troubling, particularly the mention of hyperthermic sensitization. This sensitivity is the result of suppressed protein kinase signaling, which also contributes to reduced HSP synthesis in response to physiologic stressors (End et al. 1987). Cell and tissue culture experiments indicate quercetin reduces HSF-1 phosphorylation, trimerization, and nuclear entry, preventing HSP_{72} expression and restitution of paracellular barrier integrity following physiologic stress (Dokladny et al. 2006a; 2006b; 2008). The results of these tissue culture experiments have also been extended to animal models. Pharmacological increases in liver HSP72 expression have been reported to protect rats against LPS-induced liver damage, pro-inflammatory cytokine production (TNF-a and Il-6), and LPS-induced mortality (Nakada et al. 2005). However, combining oral quercetin supplementation with pharmacological HSP72 stimulators prevented the increase in liver HSP72 and left rats no more protected then controls (Nakada et al. 2005). Pharmacological HSP72 overexpression has also been adapted to an intestinal wounding model. Here HSP72 over-expressing rats were better able to restitute the gastric barrier following wounding, resulting in reduced infiltration of inflammatory cells. In contrast, when oral quercetin supplementation was combined with the stimulus for HSP72 overexpression, HSP72 was not increased. This prevented rats from restituting the intestinal barrier, and fared worse than control animals (Liu et al. 2007).

While differences in dosing and bioavailability prevent direct extrapolation of these animal results to a human model, emerging evidence suggests the effects may be similar. First, humans metabolize quercetin in the hepatosplanchnic tissues (Rechner et al. 2002). Using a porcine model to simulate human gastrointestinal physiology, Biegler

et al. (2008) showed a single oral quercetin dose (25 mg/kg) significantly increased quercetin concentrations in liver, jejunum, and colon tissues. Second, bioavailability of ingested quercetin is moderate to high in human subjects (Hollman et al. 1995). Third, hepatosplanchnic tissues benefit most from the heat shock response following exercise/heat stress (Dong et al. 2005; Flanagan et al. 1995; Hung et al. 2005; Kluger et al. 1997; Kokura et al. 2007; Manzerra et al. 1997; Juel et al. 2008; Sepponen et al. 2006; Stojadinovic et al. 1995; Weshler et al. 1984). Taken together, these studies suggest quercetin may be sequestered in hepatosplancnic tissues in concentrations sufficient to diminish the preconditioning response. This may be a significant concern for military soldiers exposed to exercise/heat stress, as it may increase susceptibility to heat illness.

Problem Statement

Oral quercetin supplementation is being supplied to military soldiers experiencing oxidative stress, but the possibility of a suppressed heat shock response and increased susceptibility to heat illness have not been examined.

Purpose of Study

This study examined the effect of 1000 mg twice-daily quercetin supplementation on physiological adjustments to a 7 day heat acclimation (HA) protocol. Researchers hypothesized that while subjects in the quercetin supplemented condition would appear heat acclimated according to traditional measures (improved sweat rate, heart rate, and body temperature responses to exercise stress), the suppressed heat shock response would limit their protection against heat illness. To test this hypothesis subjects underwent HA

in both quercetin and placebo supplemented conditions. Intestinal permeability was measured with lactulose sugar probes in each condition, at baseline, and on the 1st and 7th days of HA. This allowed assessment of both transient and sustained quercetin dosing effects on intestinal permeability. Plasma quercetin was measured to verify that the quercetin was absorbed and increased in the circulation in the quercetin supplemented condition.

Quercetin is a potent antioxidant/anti-inflammatory agent (Boots et al. 2007). Endotoxin activation of inflammatory cytokines is a major cause of organ and tissue injury in heat illness. Thus, there is no guarantee a significant leak detected by lactulose sugar probes is physiologically relevant, as the beneficial effects of quercetin may buffer its' detrimental effects of endotoxin leakage. The inflammatory cytokines TNF-a and Il-6, and the anti-inflammatory cytokine Il-10 were assessed in plasma to examine the net inflammatory response. Because an insufficient cellular heat shock response is thought to drive the detrimental effects of quercetin, HSP72 was assessed in peripheral blood mononuclear cells (PBMC) with the assumption that changes in leukocytes are representative of changes in other heat-sensitive cells of the body.

Hypotheses

The following research hypotheses were tested in this study.

Hypothesis I. 2g daily quercetin supplementation will reduce thermotolerance at the level of the gastro-intestinal tissues, resulting in increased ($p < .05$) urinary lactulose excretion and endotoxin levels in plasma on days 1 and 7 of exercise/ heat stress.

Rationale. Suppression of the preconditioning response has been shown in isolated cells, tissues, and intact animals (Hosokowa et al. 1990; Dokladny et al. 2006; Nakada et al. 2005). Quercetin appears in high concentration in splanchnic tissues (Biegler et al. 1998). Quercetin mediated suppression of paracellular barrier conditioning following exercise/heat stress would allow lactulose and endotoxin increased access to circulation.

Hypothesis II. 2g daily quercetin supplementation will attenuate ($p < .05$) the expected increase in HSP72 content of peripheral blood mononuclear cells responding to 7 days of exercise/heat stress.

Rationale. Reduced activity of HSF-1 and associated gene products have been shown in tissue culture and animal models. While these effects have not been attributed to quercetin in a human model, suppressed leukocyte expression of HSF-1 and HSP72 following exhaustive treadmill exercise was shown in humans supplemented with α-tocopherol, a similar antioxidant (Fischer et al. 2006; Niess et al. 2002). Suppression of HSP72 is one mechanism thought to contribute to reduced epithelial barrier integrity in cultured monolayers exposed to heat stress under quercetin supplementation (Dokladny et al. 2006).

Hypothesis III. The increase ($p < .05$) in epithelial barrier permeability in subjects exercising under 2g/d quercetin supplementation will be accompanied by concomitant increases ($p < .05$) in inflammatory (TNF-a and Il-6) and anti-inflammatory (Il-10) cytokine levels in plasma.

Rationale. Quercetin mediated suppression of paracellular barrier conditioning following exercise/heat stress would allow endotoxin increased access to circulation. Endotoxin

stimulates an exaggerated immune response, characterized by increased inflammatory and anti-inflammatory cytokine concentrations in plasma (Bannerman et al. 2003).

Hypothesis IV. 2g daily quercetin supplementation will diminish subject's capacity to acclimate to 7 days of exercise/heat stress, resulting in reduced ($p < .05$) improvements in systemic markers of heat acclimation (core, mean skin, mean body temperatures; heart rate; physiological strain).

Rationale. While there is no published evidence that quercetin's mechanism of action interferes with these adaptations, animal models of proponolol-mediated HSP72 suppression (Maloyan et al. 2002) and studies on thermotolerance and impaired HSP72 response (Moran et al. 2006) suggest this may occur.

Scope of the Study

Eight healthy male subjects repeated two 7-day heat acclimation conditions. Subjects took quercetin in one condition, and placebo (powdered food coloring of similar color and texture) in the other. At least 3 months elapsed before subjects were allowed to repeat the study in the opposite condition. The order was balanced, quercetin supplementation was double blind, and the study was performed in winter months to minimize potential for natural heat acclimitization. Subjects were instructed to avoid caffeine, alcohol, anti-oxidant vitamins, and to stay well hydrated. They were also instructed to avoid exercise and heat exposure that was not directly outlined by the experiment. During the study period subjects ate packaged meals prepared by licensed dieticians to ensure adequate nutrition and to avoid high dietary quercetin intake. Average dietary quercetin intake by Westerners is 20 mg/d, and the upper limit in these

meals was set at 10mg/d. As subjects were provided with 2g quercetin per day in the supplemented condition, this represents a 200 fold increase over possible dietary inclusion.

Each condition began with a basal measurement of intestinal permeability, at rest, in non-exercise/heat stress conditions. This provided a control value for intestinal permeability for each subject, to which subsequent exercise/heat stress values were compared. This was followed by an acute thermal challenge, used as a baseline measurement of subject's capacity to work in the heat. After a 1-week washout to avoid carryover, each subject completed 7 days of exercise in a hot room (100 min/day). Subjects performed additional measures of intestinal permeability on days 1 and 7 of heat acclimation, allowing researchers to examine the effects of acute and chronic heat exposure on gastrointestinal barrier function, under normal conditions, and also under the influence of quercetin. The absolute exercise workload and ambient environmental conditions on day 6 of heat acclimation were identical to that of the acute thermal challenge, providing a posttest measure of each subjects' capacity to work in the heat (indicating acquisition of heat acclimated phenotype).

Assumptions

The following assumptions were made in this study.

1. Subjects did not perform external exercise during the data collection period.
2. Subjects consumed all foodstuffs provided by study dieticians, both inside and outside of the hospital

3. Subjects avoided consumption of outside foodstuffs, and were truthful in their responses on the dietary recall.
4. Subjects properly mixed all quercetin or placebo supplemented drinks, consumed all drinks in combination with their last daily meal, and avoided external dietary supplementation with quercetin or antioxidant vitamins.
5. The three month washout period between heat acclimations was sufficient to avoid any carryover effects. College-aged male subjects were an accurate representation of military soldiers and endurance athlete populations.
6. A laboratory exercise/heat stress test is representative of field conditions.
7. Differences in systemic markers of heat acclimated phenotype and cellular markers of acquired thermotolerance between quercetin and placebo supplemented conditions can be attributed to inhibition of the heat shock response, and not other properties inherent to quercetin, the subject, the heat, or the exercise.
8. While urinary lactulose excretion was measured under non-exercise/heat stress conditions (control), and then following acute and chronic exercise/heat stress, it is possible external influences (gastrointestinal transit, subject adherence to experimental protocol) may have influenced subject responses. We assume delimiting the subject population to Exercise Science graduate students, and further confining said subjects to the UNM General Clinical Research Center the night prior to each trial, minimized the likelihood this occurred.
9. HSP72 is an acceptable representation of other cells, including circulating leukocytes, fixed macrophages, and epithelial/endothelial cell barriers.

10. Increased concentrations of the plasma cytokines measured in this study (TNF-a, Il-6, Il-10) are largely influenced by gastrointestinal barrier leak, and not unduly influenced by exercise alone, heat stress alone, or subject-specific differences.
11. The plasma cytokines measured in this study accurately reflect the desired study outcomes. Meaning other cytokines, or other mediators altogether, would not have provided a more meaningful outcome.

Limitations

The following limitations were identified for this study.

1. The study group consisted of healthy males in the age range of 18 to 40 years old; therefore, study results can only be generalized to a healthy population of similar age and the same gender.
2. A single dose (2g) of daily quercetin supplementation was used in this study, leaving the possibility that non-significant findings may be related to dosing issues.
3. To ensure adequate experimental control, environmental temperatures, subject medical history, subject stress-exposure history, subject diet, subject hydration status, and subject external activities (exercise, heat, and UV exposure) were carefully reviewed/controlled prior to study onset, and during study participation. This level of experimental control is not representative of true conditions for either the military or endurance athlete populations of interest.

Significance of Study

This study identified whether quercetin's capacity to suppress the heat shock response resulted in increased gastrointestinal permeability when provided to subjects during repeat days of exercise in the heat. This study is unique, as both significant and non-significant findings would benefit the scientific community. Quercetin's thermo-sensitizing capacity in cell culture has never been analyzed in animals, much less humans. If quercetin were to cause significant gastrointestinal permeability and increase bacterial translocation, this information should be considered by the military oversight committees. We hope this would lead to re-examination of the proposed utility and safety of this supplement for soldiers experiencing heat stress. Non-significant findings would indicate that the highest dose fielded to-date does not put soldiers at increased risk of heat illness.

Definition of Terms

The terms in this study have been operationally defined as follows:

Bioavailability. Metabolic availability of an ingested substance.

Exertional heat stroke. Life threatening event characterized by core temperature >40ºC, sustained inflammatory response, disseminated intravascular coagulation, adult respiratory distress syndrome, and diffuse liver injury.

Flavanol. Subgroup of larger flavonoid family that uses the 3-hydroxyflavone backbone.

Gram negative bacteria. Pathogenic bacterial strain that stains negative for granules (synonymous with endotoxin). LPS is a cell wall constituent of gram negative bacteria.

Heat intolerance. Inability to tolerate physical activity in hot ambient conditions, evidenced by increased susceptibility to heat stroke.

Hepatosplanchnic tissues. Combination term for small intestine, large intestine, and liver tissues.

Occludin. 62 kDa tetra-span protein isoform vital to paracellular barrier integrity.

Reactive oxygen species. Molecule that contains an unpaired electron in outer orbital. Reactive oxygen species contribute to pathophysiology of exertional heat stroke.

Trimerization. An activated transcription factor co-localizes with two like factors, forming a trimer complex.

References

1. al-Mashhadani SA, Gader AG, al Harthi SS, Kangav D, Shaheen, FA, Bogus F. The coagulopathy of heat stroke: alterations in coagulation and fibrinolysis in heat stroke patients during the pilgrimage (Haj) to Makkah. Blood Coagul & Fibrin. 1994:5(5):731-736.

2. Amorim FT, Yamada PM, Robergs RA, Schneider SM, Moseley PL. The effect of the rate of heat storage on serum heat shock protein 72 in humans. Eur J Appl Physiol. 2008;104(6):965-72.

3. Armstrong LE, Casa DJ, Millard-Stafford M, Moran DS, Pyne SW, Roberts WO. American College of Sports Medicine position stand. Exertional heat illness during training and competition. Med Sci Sports Exercise. 2007;39(3):556 - 72.

4. Bachkosky J, Andrews M, Douglass R, Feigley J, Felton L, Fernandez F, Fratarangelo P, Johnson-Winegar A, Kohn R, Polmar N, Rumpf R, Sommerer J, Williamson W. Lightening the Load. 2007; Military report, unclassified:89 pages.

5. Baker JW, Deitch EA, Li M, Berg RD, Specian RD. Strenuous exercise causes systemic endotoxemia. J Appl Physiol. 1988;28(7):896-906.

6. Bannerman DD, Goldblum SE. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. Am J Physiol Lung Cell Mol Physiol. 2003;284(6):L899-914.

7. Bieger J, Cermak R, Blank R, de Boer VC, Hollman PC, Kamphues J, Wolffram S. Tissue distribution of quercetin in pigs after long-term dietary supplementation. J Nutr. 2008;138(8):1417-20.

8. Boots AW, Haenen GR, Bast A. Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol. 2008;585(2-3):325-37.

9. Bouchama A, Knochel JP. Heat stroke. N Engl J Med. 2002;346(25):1978-88.

10. Bouchama A, Parhar RS, el-Yazigi A, Sheth K, al-Sedairy S. Endotoxemia and release of tumor necrosis factor and interleukin 1 alpha in acute heatstroke. J Appl Physiol. 1991;70 (6):2640-2644.

11. Brown AS, Davis JM, Murphy EA, Carmichael MD, Carson JA, Ghaffar A, Mayer EP. Susceptibility to HSV-1 infection and exercise stress in female mice: Role of estrogen. J Appl Physiol. 2007;10(5):1592-7.

12. Camus G, Poortmans J, Nys M, Deby-Dupont G, Duchateau J, Deby C, Lamy M. Mild endotoxaemia and the inflammatory response induced by a marathon race. Clin Sci. 1997;92(4):415-22.

13. Carter R 3rd, Cheuvront SN, Williams JO, Kolka MA, Stephenson LA, Sawka MN, Amoroso PJ. Epidemiology of hospitalizations and deaths from heat illness in soldiers. Med Sci Sports Exercise. 2005;37(8):1338-44.

14. Cheuvront SN, Ely BR, Kenefick RW, Michniak-Kohn BB, Rood JC, Sawka MN. No effect of nutritional adenosine receptor antagonists on exercise performance in the heat. Am J Physiol Regul Integr Comp Physiol. 2009;296(2):R394-401.

15. Davis JM, Murphy EA, McClellan JL, Carmichael MD, Gangemi JD. Quercetin reduces susceptibility to influenza infection following stressful exercise. Am J Physiol Regul Integr Comp Physiol. 2008;295(2):R505-9.

16. Deaciuc IV, Fortunato F, D'Souza NB, Hill DB, Schmidt J, Lee EY, McClain CJ. Modulation of Caspase-3 Activity and Fas Ligand mRNA Expression in Rat Liver Cells In Vivo by Alcohol and Lipopolysaccharide. Alcohol Clin Exp Res. 1999;23(2):349-56.

17. Ding LA, Li JS, Li YS, Zhu NT, Liu FN, Tan L. Intestinal barrier damage caused by trauma and lipopolysaccharide. World J Gastroenterol. 2004;10(16):2373-8.

18. Dokladny K, Kozak A, Wachulec M, Wallen ES, Ménache MG, Kozak W, Kluger MJ, Moseley PL. Effect of heat stress on LPS-induced febrile response in D-galactosamine-sensitized rats. Am J Physiol Regul Integr Comp Physiol. 2001;280(2): R338-44.

19. Dokladny K, Moseley PL, Ma TY. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. Am J Physiol Gastrointest Liver Physiol. 2006;290(2):G204-12.

20. Dokladny K, Wharton W, Lobb R, Ma TY, Moseley PL. Induction of physiological thermotolerance in MDCK monolayers: contribution of heat shock protein 70. Cell Stress Chaperones. 2006;11(3):268-75.

21. Dokladny K, Ye D, Kennedy JC, Moseley PL, Ma TY. Cellular and molecular mechanisms of heat stress-induced up-regulation of occludin protein expression: Regulatory role of heat shock factor-1. Am J Pathol. 2008;172(3):659-70.

22. Dong HP, Chen HW, Hsu C, Chiu HY, Lin LC, Yang RC. Previous heat shock treatment attenuates lipopolysaccharide-induced hyporesponsiveness of platelets in rats. Shock. 2005;24(3):239-44.

23. End DW, Look RA, Shaffer NL, Balles EA, Persico FJ. Non-selective inhibition of mammalian protein kinases by flavinoids in vitro. Res Commun Chem Pathol Pharmacol. 1987;56(1):75-86.

24. Finaud J, Lac G, Filaire E. Oxidative stress: Relationship with exercise and training. Sports Med. 2006;36(4):327-58.

25. Fischer CP, Hiscock NJ, Basu S, Vessby B, Kallner A, Sjoberg LB, Febbraio MA, Pedersen BK. Vitamin E isoform-specific inhibition of the exercise-induced heat shock protein 72 expression in humans. J Appl Physiol. 2006;100:1679–87.

26. Flanagan SW, Ryan AJ, Gisolfi CV, Moseley PL. Tissue-specific HSP70 response in animals undergoing heat stress. Am J Physiol. 1995;268(1 Pt 2):R28-32.

27. Gaffin SL, Hubbard RW (2002) Pathophysiology of heatstroke. In: Pandolf KB, Burr RE (ed) Medical Aspects of Harsh Environments, Vol 1. The Office of the Surgeon General at TMM Publications, Washington DC.

28. Gathiram P, Wells MT, Brock-Utne JG, Gaffin SL (1987). Anti lipoplysaccharide improves survival in primates subjected to heat stroke. Circ Shock. 1987;23(3):157-64.

29. Government publication. Operational rations of the department of defense. NATICK PAM 30-25, 8th Edition, APRIL 2009;Approved for public release

30. Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R, Riess H. The cellular and molecular basis of hyperthermia. Crit Rev Oncol Hematol. 2002;43(1):33-56.

31. Hollman PCH, deVries JHM, van Leeuwen SD, Mengelers MJB, Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am J Clin Nutr. 1995;62:1276-92 69.

32. Horowitz M, Eli-Berchoer L, Wapinski I, Friedman N, Kodesh E. Stress-related genomic responses during the course of heat acclimation and its association with ischemic reperfusion cross-tolerance. 1: J Appl Physiol. 2004;97(4):1496-507.

33. Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, Sakai T, Nishino H, Aoike A, Kawai K, et al. Flavonoids inhibit the expression of heat shock proteins. Cell structure and function. 1990;15:393–401.

34. Huang L, Mivechi NF, Moskophidis D. Insights into regulation and function of the major stress-induced hsp70 molecular chaperone in vivo: analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. Mol Cell Biol. 2001;21(24):8575-91.

35. Hung CH, Chang NC, Cheng BC, Lin MT. Progressive exercise preconditioning protects against circulatory shock during experimental heatstroke. Shock. 2005;23(5):426-33.

36. Jaeschke H. Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. Am J Physiol. 1997:273(3):G602-611.

37. Jeukendrup AE, Vet-Joop K, Sturk A, Stegen JH, Senden J, Saris WH, Wagenmakers AJ. Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase reaction during and after a long-distance triathlon in highly trained men. Clin Sci. 2000;98(1):47-55.

38. Juel IS, Solligård E, Tvedt KE, Skogvoll E, Jynge P, Beisvag V, Erlandsen SE, Sandvik AK, Aadahl P, Grønbech JE.Post-ischaemic restituted intestinal mucosa is more resistant to further ischaemia than normal mucosa in the pig. Scand J Clin Lab Inv. 2008;68(2): 106-16.

39. Kluger MJ, Rudolph K, Soszynski D, Conn CA, Leon LR, Kozak W, Wallen ES, Moseley PL. Effect of heat stress on LPS-induced fever and tumor necrosis factor. Am J Physiol. 1997;273(3 Pt 2):R858-63.

40. Kokura S, Yoshida N, Okuda T, Nakabe N, Sakamoto N, Isozaki Y, Hattori T, Handa O, Takagi T, Naito Y, Yoshikawa T. Hyperthermia ameliorates 2,4,6-trinitrobenzene sulphonic acid-induced colitis in rats: The role of heat shock proteins. Int J Hyperthermia. 2007;23(1):17-28.

41. Kriegel KC, Wall PT, Gisolfi CV. Peripheral vascular responses to hyperthermia in the rat. J Appl Physiol. 1988;64: 2582 – 2588.

42. Lambert GP, Gisolfi CV, Berg DJ, Moseley PL, Oberley LW, Kregel KC. Selected contribution: Hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. J Appl Physiol. 2002;92(4):1750-61.

43. Lambert GP. Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: The ‘canary in the coal mine’ during exercise-heat stress? Med Sport Sci. Basel, Karger, 2008;53:61-73.

44. Li Z, Diehl AM. Innate immunity in the liver. Curr Opin Gastroenterol. 2003;19(6):565-71.

45. Lim CL, Mackinnon LT. The roles of exercise-induced immune system disturbances in the pathology of heat stroke: The dual pathway model of heat stroke. Sports Med. 2006;36(1):39-64.

46. Liu WL, Chen SJ, Chen Y, Sun LM, Zhang W, Zeng YM, Zhou TH, Si JM. Protective effects of heat shock protein70 induced by geranylgeranylacetone in atrophic gastritis in rats. Acta Pharmacol Sin. 2007;28(7):1001-6.

47. Lumlertgul D, Chuaychoo B, Thitiarchakul S, Srimahachota S, Sangchun K, Keoplung M. Heat stroke-induced multiple organ failure. Ren Fail. 1992;14(1):77-80.

48. Malm C. Susceptibility to infections in elite athletes: the S-curve. Scand J Med Sci Sports. 2006;16(1):4-6.

49. Maloyan A, Horowitz M. beta-Adrenergic signaling and thyroid hormones affect HSP72 expression during heat acclimation. *J Appl Physiol*. 2002;93(1):107-115.

50. Manzerra P, Rush, SJ, Brown IR. Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. Journal of Cellular Physiology 1997;170:130-137.

51. McClung, JP, Hasday JD, He J, Montain SJ, Cheuvront SN, Sawka MN, Singh I. Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. Am J Physiol Regul Integr Comp Physiol. 2008;294:R185–R191.

52. Mitchell D, Senay LC, Wyndham CH, van Rensburg AJ, Rogers GG, Strydom NB. Acclimatization in a hot, humid environment: energy exchange, body temperature, and sweating. J Appl Physiol. 1976;40(5):768-78.

53. Moran DS, Eli-Berchoer L, Heled Y, Mendel L, Schocina M, Horowitz M. Heat intolerance:Does gene transcription contribute? J Appl Physiol.2006;100(4):1370-6.

54. Moseley PL, Gapen C, Wallen ES, Walter ME, Peterson MW. Thermal stress induces epithelial permeability. Am J Physiol. 1994;267(2 Pt 1):C425-34.

55. Moseley PL, Gisolfi CV. New frontiers in thermoregulation and exercise. Sports Med. 1993;16(3):163-7.

56. Moseley PL. Heat shock proteins and heat adaptation of the whole organism. J Appl Physiol. 1997;83: 1413-17.

57. Moseley PL. Heat shock proteins and the inflammatory response. Ann N Y Acad Sci. 1998;856:206-13.

58. Murphy EA, Davis JM, Carmichael MD, Gangemi JD, Ghaffar A, Mayer EP. Exercise stress increases susceptibility to influenza infection. Brain Behav Immun. 2008; 22:1152-1155.

59. Nadel ER, Wenger CB, Ronberts MF, Stolwijk AJ, Cafarelli E Physiological defenses against hyperthermia of exercise. Ann. N.Y. Acad. Sci. 1977;301:98 – 109.

60. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappa beta system. Clin Vaccine Immunol. 2006;13(3):319-28.

61. Nakada J, Matsura T, Okazaki N, Nishida T, Togawa A, Minami Y, Inagaki Y, Ito H, Yamada K, Ishibe Y. Oral administration of geranylgeranylacetone improves survival rate in a rat endotoxin shock model: administration timing and heat shock protein 70 induction. Shock. 2005;24(5):482-7.

62. Nakatani Y, Fukui H, Kitano H, Nagamoto I, Tsujimoto T, Kuriyama S, Kikuchi E, Hoppou K, Tsujii T. Endotoxin clearance and its relation to hepatic and renal disturbances in rats with liver cirrhosis. Liver. 2001;21(1):64-70.

63. Nathan CF. Secretory products of macrophages. J Clin Invest. 1987;79(2):319-326.

64. Ng QY, Lee KW, Byrne C, Ho TF, Lim CL. Plasma endotoxin and immune responses during a 21-km road race under a warm and humid environment. Ann Acad Med Singapore. 2008;37(4):307-14.

65. Nieman DC, Henson DA, Gross SJ, Jenkins DP, Davis JM, Murphy EA, Carmichael MD, Dumke CL, Utter AC, McAnulty SR, McAnulty LS, Mayer EP. Quercetin reduces illness but not immune perturbations after intensive exercise. Med Sci Sports Exer. 2007;39(9):1561-69.

66. Nieman DC, Johanssen LM, Lee JW, Arabatzis K. Infectious episodes in runners before and after the Los Angeles Marathon. J Sports Med Phys Fitness. 1990;30(3):316-28.

67. Niess AM, Fehrenbach E, Schlotz E, Sommer M, Angres C, Schositsch KT, Battenfeld N, Golly IC, Biesalski HK, Northoff H, Dickhuth HH. Effects of RRR-a-tocopherol on leukocyte expression of HSP72 in response to exhaustive treadmill exercise. Int J Sports Med. 2002; 23:445–52.

68. Niessen CM. Tight junctions/adherens junctions: basic structure and function. J Invest Dermatol. 2007;127(11):2525-32.

69. Nolan JP. Endotoxin, reticuloendothelial function, and liver injury. Hepatology. 1981;1(5):458-65.

70. Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. J Appl Physiol. 1997;82(2):571-6.

71. Powers SK, Jackson MJ. Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. Physiol Rev. 2008;88(4):1243-76.

72. Rechner AR, Kuhnle G, Bremner P, Hubbard GP, Moore KP, Rice-Evans CA. The metabolic fate of dietary polyphenols in humans. Free Radic Biol Med. 2002;33(2):220-35.

73. Robertson MJ, Cameron C, Lazo S, Cochran KJ, Voss SD, Ritz J. Costimulation of human natural killer cell proliferation: role of accessory cytokines and cell contact-dependent signals. Nature Immunol. 1996;15(5):213-225.

74. Rowell LB, Blackmon JR, Martin RH, Mazzarella JA, Bruce RA. Hepatic clearance of indocyanine green in man under thermal and exercise stresses. J Appl Physiol. 1965;20(3):384-94.

75. Rowell LB. Human cardiovascular adjustments to exercise and thermal stress. Physiol Rev. 1974;54(1):75-159.

76. Sam AD 2nd, Sharma AC, Law WR, Ferguson JL. Splanchnic vascular control during sepsis and endotoxemia. Front Biosci. 1997;2:e72-92.

77. Selkirk GA, McLellan TM, Wright HE, Rhind SG. Mild endotoxemia, NF-kappaB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. Am J Physiol Regul Integr Comp Physiol. 2008;295(2):R611-23.

78. Sepponen K, Poso AR. The inducible form of heat shock protein 70 in the serum, colon, and small intestine of the pig: Comparison to conventional stress markers. Vet J. 2006;171(3):519-24.

79. Sonna LA, Gaffin SL, Pratt RE, Cullivan ML, Angel KC, Lilly CM. Effect of acute heat shock on gene expression by human peripheral blood mononuclear cells. J Appl Physiol. 2002;92(5):2208-20.

80. Stojadinovic A, Kiang J, Smallridge R, Galloway R, Shea-Donohue T. Induction of heat shock protein 72 protects against ischemia/reperfusion in rat small intestine. Gastroenterology. 1995;109(2):505-15.

81. Strieter RM, Lynch JP 3rd, Basha MA, Standiford TJ, Kasahara K, Kunkel SL. Host responses in mediating sepsis and adult respiratory distress syndrome. Semin Respir Infect. 1990;5(3):233-47.

82. Suffredini AF, Fromm RE, Parker MM, Brenner M, Kovacs JA, Wesley RA, Parrillo JE. The cardiovascular response of normal humans to the administration of endotoxin. N Engl J Med. 1989;321(5):280-7.

83. Sumagin R, Sarelius IH. TNF-alpha activation of arterioles and venules alters distribution and levels of ICAM-1 and affects leukocyte-endothelial cell interactions. Am J Physiol Heart Circ Physiol. 2006;291(5):H2116-2125.

84. Wang ZZ, Wang CL, Wu TC, Pan HN, Wang SK, Jiang JD. Am J Med. Autoantibody response to heat shock protein 70 in patients with heatstroke. 2001;111(8):654-7.

85. Weshler Z, Kapp DS, Lord PF, Hayes T. Development and decay of systemic thermotolerance in rats. Cancer Res. 1984;44(4):1347-51.

86. Wu C. Heat shock transcription factors: Structure and regulation. Annu Rev Cell Dev Biol. 1995;11:441-69.

87. Wu T, Chen S, Xiao C, Wang C, Pan Q, Wang Z, Xie M, Mao Z, Wu Y, Tanguay RM. Presence of antibody against the inducible Hsp71 in patients with acute heat-induced illness. Cell Stress Chaperones. 2001;6(2):113-20.

88. Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM. Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans. J Appl Physiol. 2007;103(4):1196-204.

89. Zambraski, EJ. Military Performance Division: Bioactive food compounds: Potential to improving soldier performance and mitigating injury. Unclassified Presentation. 2006. Available at *www.militaryfood.org/powerpoint/F06_ SESSIONV_ FOODCOM_POUNDS_Zambraski.pp*t.

CHAPTER II

REVIEW MANUSCRIPT

This chapter presents a review manuscript, entitled “Heat shock proteins and heat adaptation of the whole organism: An update” that will be submitted to the Journal of Applied Physiology. It is authored by Matthew Kuennen, Trevor Gillum, Karol Dokladny, Sue Schneider, and Pope Moseley. The manuscript follows the formatting and style guidelines of the journal. The references cited are provided at the end of the manuscript.

Title:
Heat shock proteins and heat adaptation of the whole organism: An update

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Running head:
Integrated thermotolerance and heat adaptation

Communication:
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Abstract:

Thermotolerance and acclimatization are two key adaptations to heat exposure that have traditionally been regarded as separate phenomena. In 1997 we proposed that these responses were integrated, and reviewed contributions of the heat shock response to heat adaptation of the whole organism. Research over the past 13 years has provided considerable evidence supporting this claim, in animals and humans, in both laboratory and natural settings. Insufficient activation of the heat shock response may also contribute to heat intolerance in animal and human populations; this concept is supported by recent laboratory studies reporting blockade of both thermotolerance and acclimation in animals and humans that were exposed to heat and exercise/heat stress under the influence of pharmacologic inhibitors of the heat shock response . While the mechanism behind this loss of acclimatory fitness is not well understood, current research suggests an insufficient heat shock response may contribute to greater exercise/heat stress induced disruptions of the gastrointestinal barrier, resulting in systemic inflammation and perturbed thermoregulatory responses. This evidence is reviewed.

Introduction:

Paragraph 1 In 1997 we reviewed contributions of the cellular stress response (called thermotolerance) to heat adaptation of the whole organism (called acclimatization). Despite the limited data available at that time, we speculated that "*heat acclimatization is the result of the organism's ability to dissipate heat more effectively as well as the organism's ability to either block or tolerate gut-associated endotoxin translocation, downregulate cytokine production, or develop an increased tolerance to cytokine exposure*" (66). In 2002 a colleague introduced the concept of "cross tolerance", providing evidence that the increase in heat shock protein expression following stress exposure, in conjunction with the ensuing cellular responses, confers tolerance for a variety of stressors at the level of the integrated organism. He concluded this review with the comment "*Despite the significant amount of progress that has been made regarding biochemical and structural features of HSP70, the mechanisms by which these proteins provide protection against cellular stress are still not thoroughly understood.*" (45).

Paragraph 2 Now, in 2010, the number of studies addressing thermotolerance and heat adaptation at the level of the integrated organism have increased exponentially. Therefore we now propose that our prior definition of thermotolerance as a "*cellular adaptation* caused by a *single, severe* but nonlethal *heat* exposure that allows the organism to *survive* a subsequent and *otherwise lethal* stress" is inherently flawed. To rectify this oversight, we have coined a new term, "integrated thermotolerance" which we define here as "an *integrated series of adaptations in cells, tissues, and the whole organism*, caused by one severe *or multiple moderate* exposures to *any stressor sufficient to activate the heat shock*

response, which allow the organism to *tolerate exposure to lesser disruptive stressors* and survive an otherwise lethal stress." In this paper we review evidence supporting integrated thermotolerance contributing to heat adaptation of the whole organism, in animals and humans, in laboratory and natural settings. Further, we provide evidence of an impaired heat shock response contributing to heat intolerance of the whole organism, in animals and humans, in laboratory and natural settings.

I. Tissue Responses to Thermal Challenge

Paragraph 3 Based on available literature, it is now clear that some of the symptoms associated with exertional heat illness in humans can be traced back to stress-induced disruptions of the gastrointestinal barrier (54). One major question has been the role of the heat shock proteins in preventing this response. Due to complexities associated with the integrated organism, it is sometimes cleaner to examine this question in tissue culture experiments. The following sections review evidence supporting contributions of the heat shock response to integrated thermotolerance in tissue culture experiments, as well as evidence supporting suppression of the heat shock response as a means to prevent integrated thermotolerance acquisition.

Evidence that the heat shock response confers integrated thermotolerance in confluent monolayer tissue models.

Paragraph 4 Both severe and physiologically relevant temperature exposures have been shown to compromise epithelial barrier integrity, and strong linear correlations have been noted between the loss of barrier integrity and severity of thermal exposure (15, 65). In

contrast, prior exposure to both modest (39°C) and severe (42°C) preconditioning heat stress increases: 1) HSP70 protein expression (15, 16, 65); 2) junctional localization of occludin, a tight junction protein (16, 17); and 3) the temperature required to elicit barrier disruption (65). The on/off kinetics of gain and loss of improved barrier function correlate linearly with HSP70 synthesis and decay (65). Genetically altering monolayers to constitutively over-express HSP70 confers identical protection (16), underscoring the contribution of this gene product to barrier integrity. Parallel findings have been reported in isolated gastrointestinal cells (71) and liposome models (98).

Suppressing or removing the heat shock response prevents acquisition of integrated thermotolerance in confluent monolayer tissue models.

Paragraph 5 Quercetin is a pharmacological HSF-1 inhibitor that has been reported to sensitize laboratory animals (68) and cancer patients (21) to severe thermal and LPS exposure. Adding quercetin to monolayer experiments: 1) increases the severity of heat-induced epithelial barrier disruption in unconditioned monolayers (15); 2) blunts the increase in HSP70 protein expression following preconditioning heat stress (15, 16); 3) reduces preconditioning-mediated increases in tight junction proteins (16, 17); and 4) removes the benefit of preconditioning heat stress on epithelial barrier permeability (15, 16). These results have been replicated using the protein-synthesis inhibitor cyclohexamide (16) and siRNA depletion of HSF-1 (17), supporting suppression of the heat shock response as the mechanism by which this loss of function occurs.

II. Whole Body Responses to Exertional Heat Stress

Overview of the heat shock response.

Paragraph 6 Do these results in monolayer experiments translate to humans experiencing exertional heat stress? Based on available data, we can now confidently state that exertional heat stress increases 1) large molecule permeability of the gastrointestinal barrier (74); 2) endotoxin translocation into circulation (24, 81); and 3) resulting inflammatory cytokine cascades (81, 82). The following paragraph contains an abbreviated description of how these reactions are mediated.

Paragraph 7 Exercise in hot ambient conditions requires increased perfusion to both exercising skeletal muscle and to the skin for thermoregulation. Blood is shunted away from splanchnic tissues to meet the increased perfusion need. The reduction in splanchnic perfusion has been reported to exceed 70% during severe exertional heat stress (78). The splanchnic circulation is particularly susceptible to reduced blood flow. The combination of hypoxic, ischemic, and thermal stressors reduces epithelial barrier integrity of gastrointestinal tissues (12, 45, 46, 48). The gastrointestinal tract is the major source of endogenous endotoxin, and epithelial cell damage allows endotoxin entry into the portal circulation. Fixed tissue macrophages in the liver (Kupfer cells) respond to circulating LPS by secreting pro-inflammatory cytokines (TNF-a, Il-6) that target LPS removal (39). Cytokine release by Kupfer cells activates circulating leukocytes and has a profound impact on surrounding tissues. These cytokines increase concentrations of adhesion molecules on the surface of leukocytes and endothelial cells (91), facilitating leukocyte rolling, firm adhesion (31), and subsequent migration into the interstitum. These

processes are important in the pathogenesis of microvascular injury. Neutrophils are the first responders to bacterial infection. Following activation by circulating inflammatory cytokines (Il-1b, Il-6, TNF-a) (77), neutrophils produce microbicidal agents that allow them to phagocotize LPS. Increased release of these agents is problematic, causing damage to epithelial and endothelial barriers in small intestine and liver tissues, and perpetuating the inflammatory response (2). Barrier damage facilitates increased LPS extravasiation from the gut, which can overwhelm the protective confines of the portal circulation and immunoregulatory mediators in the liver, resulting in LPS spillover into the systemic circulation (1). Resulting systemic inflammation and hypercoagulation states contribute to multiple organ dysfunction, and in severe cases, death (4, 56).

The heat shock response confers integrated thermotolerance in laboratory animal experiments.

Paragraph 8 Based on the above model of exertional heat illness, heatstroke pathology is caused by: 1)increased gastrointestinal barrier permeability; 2)extravasation of gut bacteria into the portal circulation; 3) targeted immune responses against gut bacteria in the liver, portal, and systemic circulations; and 4) cytokine-mediated chemotaxis of immunoregulatory mediators. From these observations, methods targeting epithelial/endothelial barrier integrity, gut bacteria, and cytokines should reduce susceptibility to heat illness. Heat shock preconditioning has been shown to target these weak links in the integrated organism. The following sections detail how these benefits are conferred.

Paragraph 9 Macrophages: Liver Kupfer cells represent 80-90% of fixed tissue macrophages in the reticuloendothelial system (69). Preconditioning macrophages at febrile-range temperatures (39.5°C) attenuates LPS stimulated TNF-a and Il-1b expression (9, 19). Macrophages that constitutively over-express HSP70 exhibit similar protection, in the absence of a preconditioning heat stress (13). LPS-induced NFKB activation, IKBA degradation, and resulting inflammatory cytokine (TNF-a and Il-6) production are attenuated in RAW 264.7 macrophages (84) and Kupfer cells of mice treated with sodium arsenite to induce HSP70 protein expression (92). HSF-1, the transcription factor that stimulates increased HSP70 expression, also acts directly to suppress inflammatory cytokine production by interacting directly with the TNF-a promoter region (85).

Paragraph 10 Liver: Preconditioning heat stress renders rats (42) resistant to endotoxin-induced mortality. The time course of this protection is positively correlated with the induction of HSP70 in the liver (22). Identical protection has been shown in rats using exercise as the preconditioning stimulus and induced heatstroke as the challenge variable (38). This protection is associated with 1) attenuated hepatocyte apoptosis (41); 2) accelerated recovery of transcriptional processes in hepatic tissues following LPS challenge (73); and 3) reductions in liver and plasma TNF-a and Il-6 concentrations (14, 17, 38, 42). Invoking the heat shock response via thermal preconditioning reduces LPS-induced NF-KB activation and IKBA degradation (75, 76), in a manner identical to that described for cultured macrophages above. Similar findings have been reported in mice

infected with an adenovirus vector that causes over-expression of HSP70 in liver tissues in the absence of preconditioning stress (17).

Paragraph 11 Gut: Prior exposure to ischemic preconditioning renders rats (62), dogs (20) and pigs (40) resistant to ischemia/reperfusion-induced oxidative injury, intestinal permeability, and systemic inflammatory responses. Protection against endotoxin-induced mortality is also related to induction of HSP70 in gut tissues (36). Preconditioning heat stress has been shown to prevent damage to intestinal mucosa, neutrophil infiltration, and repress inflammatory cytokine cascades following ischemia/reperfusion challenge (23, 88, 89). Preconditioning has been further shown to maintain high rates of leukocyte rolling in the face of ischemia/reperfusion stress, resulting in reduced leukocyte adherence to postcapillary venules and reduced migration of leukocytes into interstitial spaces of rat mesentery (8). The heat shock response also directly represses NK cell mediated cytotoxicity via reductions in perforin mRNA and protein expression (29). This reduction in perforin may serve to reduce epithelial and endothelial barrier damage associated with excessive perforin release. Heat preconditioning also has been shown to reverse sulfonic acid-induced colitis in rats, preventing the rise in neutrophil chemoattractant factors, TNF-a, and myleoperoxidase activity reported in sham treated animals (44).

Paragraph 12 Vasculature: Thermal preconditioning protects coronary arteries against ischemia/reperfusion-induced cytokine injury, and the time course of

HSP70 synthesis in myocardial tissues is inversely correlated with plasma Il-1b levels (27). Reductions in LPS-induced vascular permeability and TNF-a content of dermal tissues have also been reported in mice that were previously exposed to preconditioning thermal stress (90). In this study, the inhibition of vascular permeability correlated temporally with increased HSP70 content of dermal tissues. Transgenic HSP70 over-expression has been reported to protect against hyperthermia, circulatory shock, and cerebral ischemia in a mouse heatstroke model (50). Thermal preconditioning increases HSP70 in lung tissue and reduces LPS-induced myeloperoxidase activity and lung barrier permeability in rats (43).

Suppressing the heat shock response prevents acquisition of integrative thermotolerance in laboratory animal experiments.

Paragraph 13 From the above sections, it is clear that the heat shock response is the default pathway for multiple stress adaptations. Based on this observation, one can understand why it is difficult to block the heat shock response in the integrated organism. Attempts have been made to genetically alter mice to prevent stress-mediated HSP70 induction (37), but they have been largely unsuccessful. Further, because HSF-1, independent of HSP70, is known to confer protection against various physiological stressors, simply blocking HSP70 should not block the entire heat shock response. For this reason, a common approach used by investigators is to pharmacologically stimulate the heat shock response in an animal model, comparing this response to that of animals receiving both a pharmacologic stimulator and a pharmacologic repressor. The following paragraph details observed responses to pharmacologic-mediated HSP70 over-

expression. The next paragraph examines what happens when animals are simultaneously dosed with a pharmacologic HSP70 stimulator and repressor.

Paragraph 14 Geranylgeranylacetone (GGA) is a potent stimulator of HSP70 synthesis in respiratory, myocardial, hepatic, and renal tissues (30, 72, 95). It has been reported to provide protection against oxidant-induced injury in intestinal cell culture experiments (71) and NSAID-induced membrane permeability in a liposome model (98). These effects are identical to observations in cultured monolayers after a preconditioning thermal stress. Rodent models utilizing GGA to stimulate the heat shock response have reported restoration of gastrointestinal mucosal barrier integrity and repression of inflammatory cytokines in mice recovering from experimental atrophic gastritis (55). GGA has also been reported to repress LPS-induced inflammatory cytokine (TNF-a and Il-6) production and prevent LPS-induced mortality (68), similar to previous reports on mice following a preconditioning thermal stress (22).

Paragraph 15 Adding the HSF-1/HSP70 inhibitor quercetin to these rodent models increases atrophic gastritis-associated inflammation (55) and removes protection against LPS-induced mortality (68). Quercetin also has been reported to suppress liver HSP70 synthesis and prevent liver regeneration in mice recovering from partial hepatectomy (83). Quercetin also blocks sodium arsenite-induced HSP70 elevation in rat small intestine, preventing the reductions in ischemia/reperfusion-induced plasma TNF-a, neutrophil chemoattractant factor 1, and tissue injury that are normally associated with this preconditioning stimulus (96).

III. Laboratory Heat Acclimation

Paragraph 16 Repeated exposure to exercise, to heat stress, or to exercise-heat stress 1) improves the capacity of animals and humans to dissipate heat (58, 79,); 2) reduces animal mortality to experimental heatstroke (38); and 3) reduces incidence of exertional heat illness in humans (7). This protection is long lasting and results from improvements in sweating threshold and sensitivity, increased cutaneous blood flow, and increased plasma volume (67). The combination of these factors reduces cardiovascular and thermoregulatory strain (58). Heat acclimation also stimulates the heat shock response, but its significance in the whole body responses to heat exposure remains unclear. In this section we examine evidence that 1) heat acclimation stimulates the heat shock response; and 2) suppression of the heat shock response prevents humans and animals from acclimating to repeated heat exposure.

Laboratory heat acclimation activates the heat shock response and increases HSP70 in animals and humans.

Paragraph 17 Laboratory heat acclimation has been shown to increase HSP72 expression in cardiac tissues of mice (60), and in peripheral blood mononuclear cells (PBMC) (58, 63, 101) and serum (80 ,101) of humans. The practical implications of an increase in serum HSP70 are unclear. Higher constitutive HSP70 levels in PBMCs may contribute to reductions in inflammatory cytokine production (47), in a manner similar to animals in nature. It has been reported that increased constitutive HSP70 content in PBMCs (101) and myocardial tissues (33) after heat acclimation, result in reduced de novo HSP70 synthesis in response to a subsequent thermal challenge (63). However, this reduced HSP response to heat may have been due to a reduction in heat storage, as mice

and humans in these studies were heat acclimated using a traditional, constant work level heat acclimation protocol. Using a controlled hyperthermia method of heat acclimation, which requires subjects to sustain a core temperature >39°C for 50 minutes on each of 7 days of successive heat exposure, we have shown a similar HSP70 accumulation in PBMC on day 1 and day 7 of exercise/heat stress (47), despite an elevated baseline. Mouse models suggest the increase in constitutive baseline HSP70 protein levels may be retained for >1 month following heat acclimation (93). We have recently confirmed these findings in human subjects (47).

Paragraph 18 It has been speculated that thermotolerance (ability to avoid exertional heat illness) in heat acclimated human populations may result from heat shock-mediated improvements in gastrointestinal barrier function (49). Observations of: 1)improved epithelial barrier function in heat-preconditioned monolayer experiments (15, 16, 17, 65); 2) marathon runners with core temperatures >42°C exhibiting no outward symptoms of endotoxemia (61); and 3) reduced circulating endotoxin concentrations in trained versus untrained runners during exposure to identical exercise/heat stress (81) tend to support this opinion. However, caution is advised when interpreting the results of these human studies, as they relied on intact populations and did not measure heat acclimation-mediated changes in gastrointestinal barrier permeability directly. We recently examined gastrointestinal barrier responses to 7-days of heat acclimation exercise in humans, and were unable to detect any improvements in epithelial barrier function (47). Additional observations from this study are detailed in the following section.

Suppressing the heat shock response prevents animals and humans from acclimating to laboratory heat/exercise exposure.

Paragraph 19 A recent study examined the capacity of humans to acclimate to exercise/heat exposure under the influence of the HSF-1/HSP70 inhibitor quercetin (47). Subjects in this study were exposed to 7-days of heat acclimation exercise in normal dietary conditions, and again while ingesting 2 g/d quercetin supplementation. The condition order was counterbalanced, supplementation was double blind, and >3months elapsed between trials (washout). As compared to their individual responses under placebo supplementation, subjects acclimated while taking quercetin exhibited an attenuated cellular heat shock response to exercise, greater gastrointestinal barrier permeability, and increased concentrations of inflammatory cytokines in plasma. These findings in the exercising human mirror those in quercetin-supplemented cells (35), tissues (16, 17) and animals (55, 68) challenged with heat and LPS stressors. It was further noted that subjects under quercetin supplementation, subjects did not acquire the cardiovascular and thermoregulatory benefits traditionally associated with heat acclimation. This was an unexpected finding, and may possibly be explained by cytokine-mediated feedback to the central temperature controller (51).

Paragraph 20 Similar findings have been reported in a mouse model. Maloyan (2002) examined the ability of rats to acclimatize to 30 days of passive heat exposure under normal conditions, and also under the influence of beta-adrenergic blockade (blocks HSP70) (59). Rats that were acclimatized under HSP70 blockade exhibited decreased heat endurance when compared to their control counterparts (32). Further, while heat

acclimatization conferred complete protection against ischemia-induced infarct in rats acclimated under normal conditions, rats acclimated under HSP70 blockade were no more protected than control rats, who had received no preconditioning exposure (34).

Paragraph 21 From these studies we conclude that animals and humans that have been pharmacologically manipulated to suppress HSP70 induction receive little or no benefit from exposure to preconditioning heat stress.

IV. Natural Heat Acclimatization

Paragraph 22 The previous sections provided clear evidence that HSF-1 and HSP-70, both individually and together, are necessary for successful induction of integrated thermotolerance and acclimation in laboratory experiments. In this section we extend these observations to animals in nature. We focus on evidence that 1) the heat shock response contributes to natural heat acclimatization; and 2) an absent or insufficient heat shock response renders animals intolerant to natural thermal stress exposure.

The heat shock response contributes to natural heat acclimatization.

Paragraph 23 In nature, it has been established that thermotolerant organisms have higher HSP70 mRNA and protein content in cells and tissues under non-stress conditions and at normal physiological temperatures. In fact, this adaptation has been shown conserved across species of mussels (6), crabs (87), snails (94), fish (10, 11), ants (25), salamanders (70) and other lizards (97). The common link between these organisms is environmental; they all reside in environments that are prone to rapid temperature

changes. In light of this observation, higher constitutive HSP70 in these organisms has been speculated to confer protection against sudden, acute thermal exposure, when the rate of de novo HSP70 production would be too slow to offer protection. The desert ant species *Cataglyphis bombycina* is an excellent example of this fact. High constitutive levels of HSP70 allow *Cataglyphis* to forage at midday in the Saharra desert, tolerating body temperatures in excess of 55°C (25). This adaptation is vital to *Cataglyphis* survival, as it affords a slightly higher critical thermal maximum than *Acanthodactylus dumerili*, a predatory lizard species that is similarly thermally adapted (99). Further evidence of themotolerance contributing to selection bias in nature is provided by the blue mussel species *M. galloprovincialis*, which has migrated from the Mediterranean Sea to out-compete native mussel species (*M. traossulus*) along the California coastline. This takeover, likely resulting from differences in the critical thermal maximum for cardiac function of these two species (31°C and 26°C for *M. galloprovincialis* and *M. traossulus*, respectively), has resulted in complete replacement of the native species along the southern half of the California Coastline (5).

Paragraph 24 Parallel studies on human skin fibroblasts suggest similar adaptations occur in geographically diverse human populations. Lyhsako (1994) compared HSP70 responses of skin fibroblasts of ethnic Turkmen living in the desserts of central Asia to those of European Russians residing in temperate climates (57). While skin fibroblasts from heat acclimatized Turks exhibited robust HSP70 responses through 6 hours of exposure to 42.5°C heat stress, Russian fibroblasts exhibited similar responses through 4 hours of thermal exposure, but dropped to an almost undetectable level by the 6^{th} hour of

exposure. It should also be noted that Turkmen fibroblasts were remarkably resilient to heat shock-induced mortality, while Russian fibroblasts were remarkably susceptible.

An insufficient or absent heat shock response prevents natural heat acclimatization.

Paragraph 25 The pacific oyster *Crassostrea gigas* is particularly susceptible to thermal exposure in the period following the summer spawning season. Mass mortality, which can exceed 50% of the total observed population , is related to insufficient accumulation of HSP70 in gill tissues upon thermal stress exposure (53). There is also evidence of less thermotolerant species of land (97) and sea (100) dwellers disappearing from their natural habitats at temperatures that would require synthesis of HSP70. *H. Attentuae* is a hydra species with unstable HSP70 mRNA, which prevents it from accumulating HSP70 protein in response to thermal exposure (26). Acclimatizing this hydra species to non-lethal temperatures does not induce thermotolerance, but instead further sensitizes it to heat-induced mortality (3). It is interesting to note that the increased mortality reported in this hydra species following "preconditioning" thermal stress is similar to the loss of preconditioning benefits in laboratory animals under pharmacological HSP70 inhibition (14; 68).

Insufficient activation of the heat shock response renders humans intolerant to heat exposure in nature.

Paragraph 26 Recent research suggests that insufficient activation of the heat shock system may also contribute to heat intolerance in active military populations. These soldiers exhibit excessive metabolic heat storage and physiological strain in response to

exertional heat stress, and are at increased risk of exertional heat illness over the general population (18). When challenged with a standard heat tolerance tests (3.5mph, 40°C, 40% RH), heat intolerant individuals exhibited disruptions in the heat shock response at both the transcript (HSF-1) and protein (HSP70) levels (64). These authors suggested that the sluggish vasomotor responses exhibited by heat intolerant subjects may be a reflection of insufficient HSP70 accumulation in the central nervous system. This suggestion is supported by previous reports of HSP70-mediated potentiation of sympathetic and parasympathetic responses elicited by the nucleus tractus solitarius, which contributed to improved baroreceptor responsiveness and avoidance of heat stroke-mediated cerebral ischemia and hypotension (52). Further support is provided by observations of reduced HSP70 production in the ventral paraventricular and lateral magnocellular nuclei of aged animals exposed to thermal stress. It has been speculated that the attenuated heat shock response reported in aged animals (28) and humans (86) may contribute to their increased susceptibility to classic heatstroke (32). Continuing research in this area will likely add considerably to our understanding of heatstroke-related pathology.

Perspectives.

Paragraph 27 Based on recent studies, there is now sufficient data to support the inter-connection between changes that occur in the cell (thermotolerance) and changes that occur in the whole organism (heat acclimation). It is now clear that heat acclimation is associated with improved tolerance of gut-associated bacteria; however, whether or not heat acclimation confers direct benefits on gastrointestinal tissues remains to be seen.

There is also direct evidence of both down regulated cytokine production and improved cytokine tolerance in animals and humans responding to heat acclimation and other forms of preconditioning exposure. Further, these is evidence that pharmacologic suppression of the heat shock response, in addition to preventing acquisition of integrated thermotolerance, also limits acquisition of the heat acclimated phenotype. Studies examining the role of heat shock proteins in central nervous system adaptations to heat acclimation represent an exciting new area of research; one that will likely greatly advance our understanding of the integration between molecular biology and whole body responses to heat exposure.

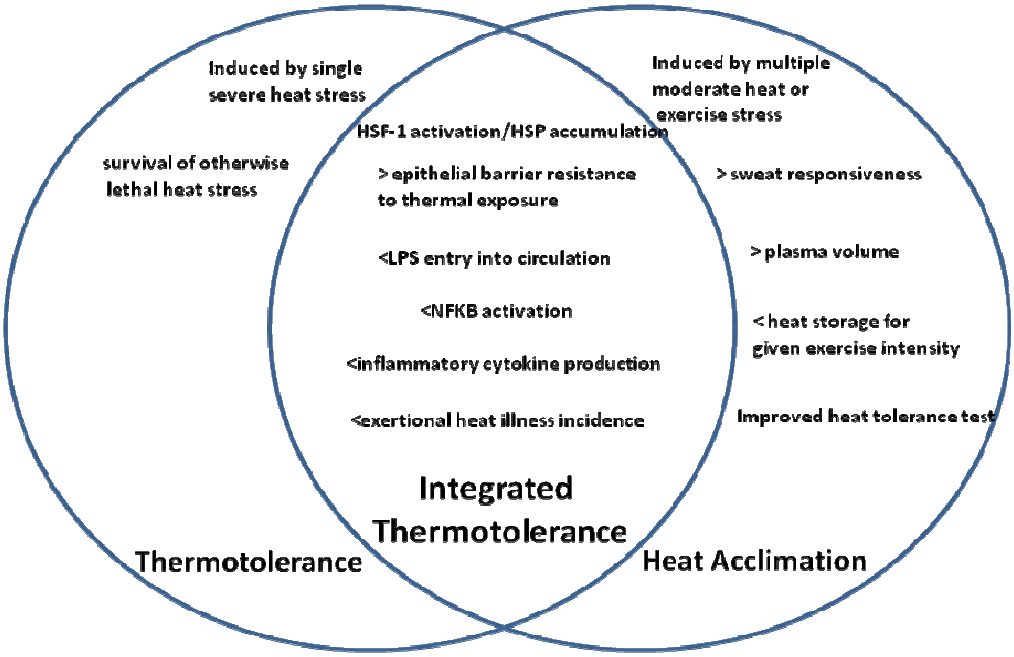

Figure 1: Thermotolerance and heat acclimation are related through the heat shock response. We have coined the term “integrated thermotolerance” to describe this relationship.

References.

1. Baker JW, Deitch EA, Li M, Berg RD, Specian RD. Strenuous exercise causes systemic endotoxemia. *J Appl Physiol* 28(7):896-906,1988.

2. Bannerman DD, Goldblum SE. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. *Am J Physiol Lung Cell Mol Physiol* 284(6):L899-914, 2003.

3. Bosch TC, Krylow SM, Bode HR, Steele RE. Thermotolerance and synthesis of heat shock proteins: These responses are present in Hydra attenuate but absent in Hydra oligactis. *Proc Natl Acad Sci USA* 85:7927–7931, 1988.

4. Bouchama A, Parhar RS, el-Yazigi A, Sheth K, al-Sedairy S. Endotoxemia and release of tumor necrosis factor and interleukin 1 alpha in acute heatstroke. *J Appl Physiol* 70(6):2640-2644, 1991.

5. Braby CE, Somero GN. Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus Mytilus). *J Exp Biol* 209:2554-2566, 2006.

6. Buckley BA, Owen ME, Hofmann GE. Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus Mytilus) changes as a function of thermal history. *J Exp Biol* 204:3571-3579, 2001.

7. Carter R 3rd, Cheuvront SN, Williams JO, Kolka MA, Stephenson LA, Sawka MN, Amoroso PJ. Epidemiology of hospitalizations and deaths from heat illness in soldiers. *Med Sci Sports Exercise* 37(8):1338-1344, 2005.

8. Chen G, Kelly C, Stokes K, Wang JH, Leahy A, Bouchier-Hayes D. Induction of heat shock protein 72kDa expression is associated with attenuation of ischaemia-reperfusion induced microvascular injury. *J Surg Res* 69(2):435-439, 1997.

9. Cooper ZA, Singh IS, Hasday JD. Febrile range temperature represses TNF-alpha gene expression in LPS-stimulated macrophages by selectively blocking recruitment of Sp1 to the TNF-alpha promoter. *Cell Stress Chaperones*. (March 10, 2010). [Epub ahead of print]

10. Dietz TJ, Somero GN. The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus Gillichthys). *Proc Natl Acad Sci USA* 89(8):3389–3393, 1992.

11. diIorio PJ, Holsinger K, Schultz RJ, Hightower LE. Quantitative evidence that both Hsc70 and Hsp70 contribute to thermal adaptation in hybrids of the livebearing fishes Poeciliopsis. *Cell Stress Chaperones* 1:139-147, 1996.

12. Ding LA, Li JS, Li YS, Zhu NT, Liu FN, Tan L. Intestinal barrier damage caused by trauma and lipopolysaccharide. *World J Gastroenterol* 10(16):2373-2378, 2004.

13. Ding XZ, Fernandez-Prada CM, Bhattacharjee AK, Hoover DL. Over-expression of hsp-70 inhibits bacterial lipopolysaccharide-induced production of cytokines in human monocyte-derived macrophages. *Cytokine*. 16(6):210-219, 2001.

14. Dokladny K, Kozak A, Wachulec M, Wallen ES, Ménache MG, Kozak W, Kluger MJ, Moseley PL. Effect of heat stress on LPS-induced febrile response in D-galactosamine-sensitized rats. *Am J Physiol Regul Integr Comp Physiol* 280(2):R338-R344, 2001.

15. Dokladny K, Moseley PL, Ma TY. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *Am J Physiol Gastrointest Liver Physiol* 290(2):G204-212, 2006.

16. Dokladny K, Wharton W, Lobb R, Ma TY, Moseley PL. Induction of physiological thermotolerance in MDCK monolayers: contribution of heat shock protein 70. *Cell Stress Chaperones* 11(3):268-275, 2006.

17. Dokladny K, Ye D, Kennedy JC, Moseley PL, Ma TY. Cellular and molecular mechanisms of heat stress-induced up-regulation of occludin protein expression: Regulatory role of heat shock factor-1. *Am J Pathol* 172(3):659-670. 2008.

18. Epstein Y. Heat intolerance: predisposing factor or residual injury? *Med Sci Sports Exercise* 22(1):29–35, 1990.

19. Fairchild KD, Viscardi RM, Hester L, Singh IS, Hasday JD. Effects of hypothermia and hyperthermia on cytokine production by cultured human mononuclear phagocytes from adults and newborns. *J Interferon Cytokine Res* 20(12):1049-1055, 2000.

20. Ferencz A, Szántó Z, Kalmár-Nagy K, Horváth OP, Rõth E. Mitigation of oxidative injury by classic and delayed ischemic preconditioning prior to small bowel autotransplantation. *Transplant Proc* 36(2):286-288, 2004.

21. Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, Baker J, Kerr DJ. Phase I Clinical Trial of the Flavonoid Quercetin: Pharmacokinetics *Physiol* 83:1413-1417, 1996.

22. Flanagan SW, Ryan AJ, Gisolfi CV, Moseley PL. Tissue-specific HSP70 response in animals undergoing heat stress. *Am J Physiol* 268(1 Pt 2):R28-R32, 1995.

23. Fleming SD, Starnes BW, Kiang JG, Stojadinovic A, Tsokos GC, Shea-Donohue T. Heat stress protection against mesenteric I/R-induced alterations in intestinal mucosa in rats. *J Appl Physiol* 92(6):2600-2607, 2002.

24. Gathiram P, Wells MT, Brock-Utne JG, Gaffin SL. Anti lipoplysaccharide improves survival in primates subjected to heat stroke. *Circ Shock* 23(3): 157-164, 1987.

25. Gehring WJ, Wehner R. Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara desert. *Proc Natl Acad Sci U S A* 92(7):2994-2998, 1995.

26. Gellner K, Praetzel G, Bosch TC. Cloning and expression of a heat inducible hsp70 gene in two species of Hydra which differ in their stress response. *Eur J Biochem* 210(3):683–691, 1992.

27. Grünenfelder J, Zünd G, Stucki V, Hoerstrup SP, Kadner A, Schoeberlein A, Turina M. Heat shock protein upregulation lowers cytokine levels after ischemia and reperfusion. *Eur Surg Res* 33(5-6):383-387, 2001.

28. Hall DM, Xu L, Drake VJ, Oberley LW, Oberley TD, Moseley PL, Kregel KC. Aging reduces adaptive capacity and stress protein expression in the liver after heat stress. *J Appl Physiol* 89: 749–759, 2000.

29. Harada H, Murakami T, Tea SS, Takeuchi A, Koga T, Okada S, Suico MA, Shuto T, Kai H. Heat shock suppresses human NK cell cytotoxicity via regulation of perforin. *Int J Hyperthermia* 23(8):657-665, 2007.

30. Hirakawa T, Rokutan K, Nikawa T, Kishi K. Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* 111(2):345-357, 1996.

31. Hopkins AM, Baird AW, Nusrat A. ICAM-1: targeted docking for exogenous as well as endogenous ligands. *Adv Drug Deliv Rev* 56(6):763-778, 2004.

32. Horowitz M. Heat acclimation and cross-tolerance against novel stressors: genomic-physiological linkage. *Prog Brain Res* 162:373-392, 2007.

33. Horowitz M, Maloyan A, Shlaier J. HSP 70 kDa dynamics in animals undergoing heat stress superimposed on heat acclimation. *Ann N Y Acad Sci* 813:617-619, 1997.

34. Horowitz M. Genomics and proteomics of heat acclimation. *Front Biosci (Schol Ed)* 2:1068-1080, 2010.

35. Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, Sakai T, Nishino H, Aoike A, Kawai K. Flavonoids inhibit the expression of heat shock proteins. *Cell Struct Funct* 15:393–401, 1990.

36. Hotchkiss R, Nunnally I, Lindquist S, Taulien J, Perdrizet G, Karl I. Hyperthermia protects mice against the lethal effects of endotoxin. *Am J Physiol* 265(6 Pt 2):R1447-R1457, 1993.

37. Huang L, Mivechi NF, Moskophidis D. Insights into regulation and function of the major stress-induced hsp70 molecular chaperone in vivo: analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. *Mol Cell Biol* 21(24):8575-8591, 2001.

38. Hung CH, Chang NC, Cheng BC, Lin MT. Progressive exercise preconditioning protects against circulatory shock during experimental heatstroke. *Shock* 23(5):426-433, 2005.

39. Jaeschke H. Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. *Am J Physiol* 273(3):G602-G611, 1997.

40. Juel IS, Solligård E, Tvedt KE, Skogvoll E, Jynge P, Beisvag V, Erlandsen SE, Sandvik AK, Aadahl P, Grønbech JE. Post-ischaemic restituted intestinal mucosa is more resistant to further ischaemia than normal mucosa in the pig. *Scand J Clin Lab Inv* 68(2):106-116, 2008.

41. Klosterhalfen B, Hauptmann S, Offner FA, Amo-Takyi B, Töns C, Winkeltau G, Affify M, Küpper W, Kirkpatrick CJ, Mittermayer C. Induction of heat shock protein 70 by zinc-bis-(DL-hydrogenaspartate) reduces cytokine liberation, apoptosis, and mortality rate in a rat model of LD100 endotoxemia. *Shock* 7(4):254-62, 1997.

42. Kluger MJ, Rudolph K, Soszynski D, Conn CA, Leon LR, Kozak W, Wallen ES, Moseley PL. Effect of heat stress on LPS-induced fever and tumor necrosis factor. *Am J Physiol* 273(3 Pt 2):R858-R863, 1997.

43. Koh Y, Lim CM, Kim MJ, Shim TS, Lee SD, Kim WS, Kim DS, Kim WD. Heat shock response decreases endotoxin-induced acute lung injury in rats. *Respirology* 4(4):325-330, 1999.

44. Kokura S, Yoshida N, Okuda T, Nakabe N, Sakamoto N, Isozaki Y, Hattori T, Handa O, Takagi T, Naito Y, Yoshikawa T. Hyperthermia ameliorates 2,4,6-trinitrobenzene sulphonic acid-induced colitis in rats: The role of heat shock proteins. *Int J Hyperthermia* 23(1):17-28, 2007.

45. Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 92(5):2177-2186, 2002.

46. Kriegel KC, Wall PT, Gisolfi CV. Peripheral vascular responses to hyperthermia in the rat. *J Appl Physiol* 64: 2582–2588, 1988.

47. Kuennen M, Gillum T, Dokladny K, Bedrick E, Schneider D, Moseley P. Schneider SM. Thermotolerance and heat acclimation share a common mechanism in humans. *Proc Natl Acad Sci USA [manuscript in preparation]*.

48. Lambert GP, Gisolfi CV, Berg DJ, Moseley PL, Oberley LW, Kregel KC. Selected contribution: Hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. *J Appl Physiol* 92(4):1750-1761, 2002.

49. Lambert GP. Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: The 'canary in the coal mine' during exercise-heat stress? *Med Sport Sci.* Basel, Karger, 53:61-73, 2008.

50. Lee WC, Wen HC, Chang CP, Chen MY, Lin MT. Heat shock protein 72 overexpression protects against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke. *J Appl Physiol* 100(6):2073-2082, 2006.

51. Leon LR. Invited review: Cytokine regulation of fever: studies using gene knockout mice. *J Appl Physiol* 92(6):2648-2655, 2002.

52. Li PL, Chao YM, Chan SH, Chan JY. Potentiation of baroreceptor reflex response by heat shock protein 70 in nucleus tractus solitarii confers cardiovascular protection during heatstroke. *Circulation*. 103(16):2114-2119, 2001.

53. Li Y, Qin JG, Abbott CA, Li X, Benkendorff K. Synergistic impacts of heat shock and spawning on the physiology and immune health of Crassostrea gigas: an explanation for summer mortality in Pacific oysters. *Am J Physiol Regul Integr Comp Physiol* 293(6):R2353-R2362, 2007.

54. Lim CL, Mackinnon LT. The roles of exercise-induced immune system disturbances in the pathology of heat stroke: The dual pathway model of heat stroke. *Sports Med* 36(1):39-64, 2006.

55. Liu WL, Chen SJ, Chen Y, Sun LM, Zhang W, Zeng YM, Zhou TH, Si JM. Protective effects of heat shock protein70 induced by geranylgeranylacetone in atrophic gastritis in rats. *Acta Pharmacol Sin* 28(7):1001-1006, 2007.

56. Lumlertgul D, Chuaychoo B, Thitiarchakul S, Srimahachota S, Sangchun K, Keoplung M. Heat stroke-induced multiple organ failure. *Ren Fail* 14(1):77-80, 1992.

57. Lyashko VN, Vikulova VK, Chernicov VG, Ivanov VI, Ulmasov KA, Zatsepina OG, Evgen'ev MB. Comparison of the heat shock response in ethnically and

ecologically different human populations. *Proc Natl Acad Sci USA* 91:12492–12495, 1994.

58. Magalhães FD, Amorim FT, Passos RL, Fonseca MA, Oliveira KP, Lima MR, Guimarães JB, Ferreira-Júnior JB, Martini AR, Lima NR, Soares DD, Oliveira EM, Rodrigues LO. Heat and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced increase in intracellular and plasma Hsp72 in humans. *Cell Stress Chaperones* (April 23, 2010) [Epub ahead of print]

59. Maloyan A, Horowitz M. Beta-adrenergic signaling and thyroid hormones affect HSP72 expression during heat acclimation. *J Appl Physiol* 93(1):107-115, 2002.

60. Maloyan A, Palmon A, Horowitz M. Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. *Am J Physiol* 276(5 Pt 2):R1506-1515, 1999.

61. Maron MB, Wagner JA, Horvath SM. Thermoregulatory responses during competitive marathon running. *J Appl Physiol* 42(6):909–914, 1977.

62. McCallion K, Wattanasirichaigoon S, Gardiner KR, Fink MP. Ischemic preconditioning ameliorates ischemia- and reperfusion-induced intestinal epithelial hyperpermeability in rats. *Shock* 14(4):429-434, 2000.

63. McClung, JP, Hasday JD, He J, Montain SJ, Cheuvront SN, Sawka MN, Singh I. Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. *Am J Physiol Regul Integr Comp Physiol* 294:R185–R191, 2008.

64. Moran DS, Eli-Berchoer L, Heled Y, Mendel L, Schocina M, Horowitz M. Heat intolerance: does gene transcription contribute? *J Appl Physiol* 100(4):1370-1376, 2006.

65. Moseley PL, Gapen C, Wallen ES, Walter ME, Peterson MW. Thermal stress induces epithelial permeability. *Am J Physiol* 267(2 Pt 1):C425-C434, 1994.

66. Moseley PL. Heat shock proteins and heat adaptation of the whole organism. *J Appl Physiol* 83: 1413-1417, 1997.

67. Nadel ER, Wenger CB, Ronberts MF, Stolwijk AJ, Cafarelli E. Physiological defenses against hyperthermia of exercise. *Ann. N.Y. Acad. Sci* 301:98–109, 1977.

68. Nakada J, Matsura T, Okazaki N, Nishida T, Togawa A, Minami Y, Inagaki Y, Ito H, Yamada K, Ishibe Y. Oral administration of geranylgeranylacetone improves survival rate in a rat endotoxin shock model: administration timing and heat shock protein 70 induction. *Shock* 24(5):482-7, 2005.

69. Nathan CF. Secretory products of macrophages. *J Clin Invest* 79(2):319-326, 1987.

70. Near JC, Easton DP, Rutledge PS, Dickinson DP, Spotila JS. Heat shock protein 70 gene expression in intact salamanders Eurycea bislineata in response to calibrated heat shocks and to high temperatures encountered in the field. *J Exp Zool* 256:303–314, 1990.

71. Ohkawara T, Takeda H, Nishiwaki M, Nishihira J, Asaka M. Protective effects of heat shock protein 70 induced by geranylgeranylacetone on oxidative injury in rat intestinal epithelial cells. *Scand J Gastroenterol* 41(3):312-317, 2006.

72. Otaka M, Odashima M, Tamaki K, Watanabe S. Expression and function of stress (heat shock) proteins in GI tract. *Int J Hyperthermia* 25(8):634-640, 2009.

73. Paidas CN, Mooney ML, Theodorakis NG, De Maio A. Accelerated recovery after endotoxic challenge in heat shock-pretreated mice. *Am J Physiol Regul Integr Comp Physiol* 282(5):R1374-R1381, 2002.

74. Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. *J Appl Physiol* 82(2):571-576, 1997.

75. Pritts TA, Wang Q, Sun X, Fischer DR, Hungness ES, Fischer JE, Wong HR, Hasselgren PO. The stress response decreases NF-kappaB activation in liver of endotoxemic mice. *Shock* 18(1):33-37, 2002.

76. Pritts TA, Wang Q, Sun X, Moon MR, Fischer DR, Fischer JE, Wong HR, Hasselgren PO. Induction of the stress response in vivo decreases nuclear factor-kappa B activity in jejunal mucosa of endotoxemic mice. *Arch Surg* 135(7):860-866, 2000.

77. Robertson MJ, Cameron C, Lazo S, Cochran KJ, Voss SD, Ritz J. Costimulation of human natural killer cell proliferation: role of accessory cytokines and cell contact-dependent signals. *Nature Immunol* 15(5):213-225, 1996.

78. Rowell LB, Blackmon JR, Martin RH, Mazzarella JA, Bruce RA. Hepatic clearance of indocyanine green in man under thermal and exercise stresses. *J Appl Physiol* 20(3):384-394, 1965.

79. Sakurada S, Hales JR. A role for gastrointestinal endotoxins in enhancement of heat tolerance by physical fitness. *J Appl Physiol* 84(1):207-214, 1998.

80. Sandström ME, Siegler JC, Lovell RJ, Madden LA, McNaughton L. The effect of 15 consecutive days of heat-exercise acclimation on heat shock protein 70. *Cell Stress Chaperones* 13(2):169-175, 2008.

81. Selkirk GA, McLellan TM, Wright HE, Rhind SG. Expression of intracellular cytokines, HSP72, and apoptosis in monocyte subsets during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 296(3):R575-R586, 2009.

82. Selkirk GA, McLellan TM, Wright HE, Rhind SG. Mild endotoxemia, NF-kappaB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 295(2):R611-23, 2008.

83. Shi Q, Dong Z, Wei H. The involvement of heat shock proteins in murine liver regeneration. *Cell Mol Immunol* 4(1):53-57, 2007.

84. Shi Y, Tu Z, Tang D, Zhang H, Liu M, Wang K, Calderwood SK, Xiao X. The inhibition of LPS-induced production of inflammatory cytokines by HSP70 involves inactivation of the NF-kappaB pathway but not the MAPK pathways. *Shock* 26(3):277-284, 2006.

85. Singh IS, He JR, Calderwood S, Hasday JD. A high affinity HSF-1 binding site in the 5'-untranslated region of the murine tumor necrosis factor-alpha gene is a transcriptional repressor. *J Biol Chem* 277(7):4981-4988, 2002.

86. Singh R, Kølvraa S, Bross P, Jensen UB, Gregersen N, Tan Q, Knudsen C, Rattan SI. Reduced heat shock response in human mononuclear cells during aging and its association with polymorphisms in HSP70 genes. *Cell Stress Chaperones* 11(3):208-215, 2006.

87. Stillman JH, Tagmount A. Seasonal and latitudinal acclimatization of cardiac transcriptome responses to thermal stress in porcelain crabs, Petrolisthes cinctipes. *Mol Ecol* 18(20):4206-4226, 2009.

88. Stojadinovic A, Kiang J, Goldhill J, Matin D, Smallridge R, Galloway R, Shea-Donohue T. Induction of the heat shock response prevents tissue injury during acute inflammation of the rat ileum. *Crit Care Med* 25(2):309-317, 1997.

89. Stojadinovic A, Kiang J, Smallridge R, Galloway R, Shea-Donohue T. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology* 109(2):505-515, 1995.

90. Suganuma T, Irie K, Fujii E, Yoshioka T, Muraki T. Effect of heat stress on lipopolysaccharide-induced vascular permeability change in mice. *J Pharmacol Exp Ther* 303(2):656-663, 2002.

91. Sumagin R, Sarelius IH. TNF-alpha activation of arterioles and venules alters distribution and levels of ICAM-1 and affects leukocyte-endothelial cell interactions. *Am J Physiol Heart Circ Physiol* 291(5):H2116-2125, 2006.

92. Sun D, Chen D, Du B, Pan J. Heat shock response inhibits NF-kappaB activation and cytokine production in murine Kupffer cells. *J Surg Res* 129(1):114-121, 2005.

93. Tetievsky A, Cohen O, Eli-Berchoer L, Gerstenblith G, Stern MD, Wapinski I, Friedman N, Horowitz M. Physiological and molecular evidence of heat acclimation memory: a lesson from thermal responses and ischemic cross-tolerance in the heart. *Physiol Genomics* 34(1):78-87, 2008.

94. Tomanek L, Somero GN. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus Tegula) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J Exp Biol* 202 (Pt 21):2925-2936, 1999.

95. Tomisato W, Tsutsumi S, Tsuchiya T, Mizushima T. Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. *Biol Pharm Bull* 24(8):887-891, 2001.

96. Tsuruma T, Yagihashi A, Watanabe N, Yajima T, Kameshima H, Araya J, Hirata K. Heat-shock protein-73 protects against small intestinal warm ischemia-reperfusion injury in the rat. *Surgery* 125(4):385-395, 1999.

97. Ulmasov KA, Shammakov S, Karaev K, Evgen'Ev MB. Heat shock proteins and thermoresistance in lizards. *Proc Natl Acad Sci USA* 89(5):1666–1670, 1992.

98. Ushijima H, Tanaka K, Takeda M, Katsu T, Mima S, Mizushima T. Geranylgeranylacetone protects membranes against nonsteroidal anti-inflammatory drugs. *Mol Pharmacol* 68(4):1156-1161, 2005.

99. Wehner R, Marsh AC, Wehner S. Desert ants on a thermal tightrope. *Nature* 357:586-587, 1992.

100. Welch PS, Loomis HA. A limno-logical study of Hydra oligactis in Douglas Lake, Michigan. *Trans Am Microsc Soc* 43:203-235, 1924.

101. Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM. Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans. *J Appl Physiol* 103(4):1196-1204, 2007.

CHAPTER III

RESEARCH MANUSCRIPT

This chapter presents a research manuscript, entitled “Thermotolerance and Heat Acclimation share a common mechanism in humans”. This manuscript will be submitted to the Proceedings of the National Academy of Sciences. It is authored by Matthew Kuennen, Trevor Gillum, Karol Dokladny, Edward Bedrick, Sue Schneider, and Pope Moseley. The manuscript follows the formatting and style guidelines of the journal. The references cited are provided at the end of the manuscript.

Title:
Thermotolerance and heat acclimation share a common mechanism in humans

Running Title:
Thermotolerance and heat acclimation are related through the cellular stress response

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ABSTRACT.

Thermotolerance and heat acclimation are key adaptation processes that have been hitherto viewed as separate physiologic phenomena. Here we show these processes share a common basis, as both are governed by the heat shock response. We evaluated the effects of a standard laboratory heat shock response-inhibitor (QUERCETIN; 2000mg/d) on established markers of thermotolerance [gastrointestinal barrier permeability, plasma TNF-a, Il-6, and Il-10 concentrations; leukocyte HSP70 content(HSP70)] and heat acclimation [reduced body temperatures, heart rate, and physiologic strain in response to exercise/heat stress] in male subjects (n=8) completing a 7-day heat acclimation protocol. These same subjects completed an identical protocol under placebo supplementation (PLACEBO). QUERCETIN increased gastrointestinal barrier permeability and TNF-a on the 1st day of exercise/heat stress, no differences in these variables were reported in PLACEBO. Exercise HSP70 responses were increased and plasma cytokines (Il-6 and Il-10) were decreased on the 7th day of heat acclimation in PLACEBO; with concomitant reductions in exercise body temperatures, heart rate, and physiologic strain. In contrast, gastrointestinal barrier permeability remained elevated, HSP70 was not increased, Il-6 and Il-10 were not decreased, and exercise body temperatures were not reduced on the 7th day of heat acclimation in QUERCETIN. While exercise heart rate and physiologic strain were reduced in the QUERCETIN group, this occurred much later in exercise than with PLACEBO. Consistent with the concept that thermotolerance and heat acclimation are related through the heat shock response, repeated exercise/heat stress reduces circulating cytokine concentrations and increases cytoprotective HSP70, contributing to reductions

in both cellular and systemic markers of heat strain. Exercising under the influence of a heat shock response-inhibitor prevents both cellular and systemic heat adaptations.

INTRODUCTION.

Paragraph 1. Prior exposure to a single preconditioning heat stress allows cells (1), tissues (2), and animals (3) to survive an otherwise lethal heat shock. This protection, termed thermotolerance, is synonymous with the heat shock response. While the major function of the heat shock response is protein management (4), the heat shock response has also been shown to enhance epithelial barrier resistance to heat exposure (5, 6, 7) and, in animals, to reduce inflammatory cytokine production through inhibition of NFKB (8). Together these improvements limit stress-mediated inflammation by preventing endotoxin translocation into the portal circulation and limiting the systemic inflammatory response.

Paragraph 2. Repeated exposure to exercise, to heat stress, or to exercise-heat stress improves the capacity of animals and humans to dissipate heat (9, 10) and reduces incidence of exertional heat illness (11, 12). This protection entails a set of major physiological and metabolic adaptations and has been termed heat acclimation. Acclimatization in nature and acclimation in laboratory settings is long lasting and results from improvements in sweat sensitivity and rate, cutaneous vasodilaton, and increased plasma volume (13). Heat acclimation also invokes the heat shock response (14), but the role of the heat shock response in acclimatization is largely ignored, since the heat shock response is considered to be an adaptation primarily conferring thermotolerance. We have

proposed that the cellular mechanisms that convey acquired thermotolerance in animals and humans might also contribute to the systemic adaptations associated with the heat acclimated phenotype (15). The evidence across animal phyla, including ants (16), lizards (17), hydra (18), and other ocean dwellers (19, 20) suggest contributions of the heat shock response to natural heat acclimatization. Here we show through HSF-1 inhibition, that blunting the cellular heat shock response in exercising humans increases both intestinal permeability and inflammatory cytokine response from exercise. Surprisingly, HSF-1 inhibition rendered human subjects both heat intolerant and blocked their exercise acclimation, uncovering a previously unappreciated link between thermotolerance and the acclimatized phenotype in humans.

RESULTS.

Paragraph 3. **Baseline Characteristics.** Eight men [mean(SEM), age: 28(1.6) yr, body mass: 77.0(3.5) kg, height: 178.4(2.5) cm, body fat: 5.9(0.7) %, maximal oxygen uptake 55.6(2.3) $ml \cdot kg^{-1} \cdot min^{-1}$] participated in this study. Participants were healthy, physically active, and did not disclose history of heat illness or gastrointestinal barrier dysfunction. Individual subjects completed 7 days of heat acclimation exercise in either QUERCETIN or PLACEBO supplemented conditions, allowed 95(9) days for condition washout, then repeated HA in the opposite condition. Condition order was counterbalanced and supplementation was double-blind. Data were collected during winter and spring months (October – March) to minimize effects of natural heat acclimatization.

Paragraph 4. **Thermotolerance (1-day).** The ability of the heat shock response to confer benefits on gastrointestinal barrier function and inflammatory cytokine responses in

cellular (5, 6, 7) and animal models (8, 21) is well established; however, these beneficial adaptations have not yet been examined in a human exercise model. For this reason we measured subjects' gastrointestinal barrier and cytokine responses to an acute bout of exercise/heat stress under normal physiologic conditions (PLACEBO) and also under the influence of the HSF-1 inhibitor quercetin (QUERCETIN).

Paragraph 5. We used the synthetic disaccharide lactulose (242kDa) to assess exercise-induced alterations in GI barrier function. We also assessed plasma endotoxin levels, to determine if alterations in GI barrier function contributed to increased endotoxin transit into circulation. Because TNF-a, Il-6, and Il-10 are known responders to endotoxin exposure and correlate strongly with the end order dysfunctions that comprise human exertional heat illness (22, 23), we measured these cytokines in plasma. In addition, we measured heat shock protein 70 (HSP70) concentrations in peripheral blood mononuclear cells (PBMCs) responding to exercise stress.

Paragraph 6. Under PLACEBO conditions, urinary lactulose excretion and plasma endotoxin levels were not increased on day 1($p= 0.480$ and 0.224; respectively), suggesting that this acute bout of exercise/heat stress was insufficient stimulus to cause a detectable increase in gastrointestinal permeability. In agreement with a prior publication from our laboratory (14), we also did not detect an increase in subjects plasma TNF-a concentrations with exercise ($p= 0.269$). Plasma Il-6 ($p= 0.000$) and Il-10 ($p= 0.001$) concentrations showed the expected increases with exercise, with Il-6 being increased immediately post exercise ($p= 0.000$), 2 hours post exercise ($p= 0.000$), and 4 hours post

exercise (p=0.000); and Il-10 being increased immediately post exercise (p= 0.009) and 2 hours post exercise (p=0.008). We did not detect any increases in PBMC HSP70 content with exercise (p= 0.327).

Paragraph 7. Exposure to the HSF-1 inhibitor (QUERCETIN) significantly altered the exercise response compared to placebo exposed subjects. Quercetin exposure resulted in an increase in both urinary lactulose excretion (p= 0.032) and plasma TNF-a concentrations (p= 0.015) with exercise. Plasma endotoxin levels also tended (p=0.124) to increase with exercise; this non-significant finding may be explained by a competent reticuloendothelial system filtering endotoxin that had crossed the gastrointestinal barrier, before it reached the systemic circulation. Il-6 (p= 0.000) and Il-10 (p= 0.001) exercise responses were similar to that reported in PLACEBO, with Il-6 being increased immediately post exercise (p= 0.000), 2 hours post exercise (p= 0.000), and 4 hours post exercise (p=0.025); and Il-10 being increased immediately post exercise (p= 0.001). We did not detect an increase in PBMC HSP70 content with exercise (p= 0.144). We speculate that the increased TNF-a plasma concentration under QUERCETIN might reflect increased production by liver macrophages responding to endotoxin spillover, consistent with the inflammatory model of heat stress (15).

Paragraph 8. **Acquired Thermotolerance (7-day).** After the effects of HSF-1 inhibition on human cellular, tissue, and plasma responses to an acute bout of exercise/heat stress had been determined, we next sought to determine what effect repeated bouts of exercise/heat stress would have on these responses. To examine these effects we had

subjects perform 7 consecutive days of exercise/heat stress in the presence (QUERCETIN) or absence (PLACEBO) of HSF-1 inhibition.

Paragraph 9. In PLACEBO, urinary lactulose excretion (p= 0.230), plasma endotoxin (p=0.297) and plasma TNF-a concentrations (p= 0.085) were not affected by the repeated exercise/heat stress. The plasma Il-6 (p= 0.000) response to exercise remained fairly consistent, with plasma Il-6 concentrations again being increased immediately post (p= 0.000) and 2 hours post exercise (p= 0.026). However, unlike that reported for acute exercise/heat exposure on day 1, Il-6 had returned to baseline by 4 hours post exercise (p= 0.072). Also in contrast to our findings on day 1, we did not detect an increase in Il-10 with exercise following repeated exercise/heat stress (p=0.069). In contrast to our findings for PBMC HSP70 content on day 1 (NS), we detected an increase in HSP70 with exercise following repeated exercise/heat stress (p= 0.049), at 4 hours post exercise (p=0.011). This was an expected finding, and is in agreement with our laboratory's previous observations in subjects completing a 10-day heat acclimation protocol (14).

Paragraph 10. QUERCETIN supplementation resulted in persistent, exercise induced increases in urinary lactulose excretion (p= 0.021), indicating that exercise conditioning under HSF-1 inhibition could not overcome this defect. Plasma endotoxin levels also rose with exercise (p=0.0316), suggesting that the impairment in gastrointestinal barrier function resulting from multiple days of exercise/heat stress was severe enough to exceed the endotoxin clearing capacity of the reticuloendothelial system. As such, these data are quite consistent with our previous reports on the inhibitory effect of quercetin on

epithelial barrier conditioning in vitro (5, 6, 7). They also support reports by other labs of attenuated gastrointestinal barrier restitution in vivo (24). While gastrointestinal permeability remained, we did not find a persistent increase in plasma TNF-a at rest or with exercise (p= 0.569) in these subjects. Thus, there was a decline in post-exercise TNF-a between days 1 and 7 of the exercise protocol. Similar to that reported for the same subjects under PLACEBO supplementation, Il-6 (p= 0.000) remained affected by exercise, with plasma concentrations being increased immediately post exercise (p= 0.000) and 2 hours post exercise (p=0.000). However, unlike that reported on day 7 for PLACEBO supplementation, plasma Il-6 concentrations remained elevated 4 hours post exercise (p=0.045). We also noted differences in plasma Il-10 responses to exercise/heat stress on day 7 of PLACEBO and QUERCETIN supplementation; in the former there was no increase with exercise (p=0.069), while in the latter the increase (p=0.043) with exercise was retained (immediately post exercise; p=0.051). In further contrast to what we reported for these subjects under PLACEBO supplementation, we did not detect an increase in HSP70 with exercise (p= 0.188).

Paragraph 11. Insufficiencies in gastrointestinal barrier function following both acute and chronic exposure to exercise/heat stress; in conjunction with increased endotoxin appearance in circulation; concomitant increases in inflammatory cytokine production (TNF-a); and diminished HSP70 production in leukocytes responding to chronic exercise, heat, and endotoxin stresses suggest that thermotolerance was dramatically altered by HSF-1 inhibition. It is also interesting to note that Il-6 and Il-10 exercise responses were attenuated following exposure to chronic exercise/heat stress under PLACEBO, but not

QUERCETIN supplementation. As Il-6 is an inflammatory cytokine associated with gastrointestinal barrier disruption (25) and Il-10 is an anti-inflammatory cytokine associated with improvements in gastrointestinal barrier function (26), and both are known responders to endotoxin stimulation (23), these differences are likely artifact resulting from sustained gastrointestinal barrier dysfunction. Il-10 is also a repressor of TNF-a response; suggesting that maintenance of high Il-10 levels with chronic exercise/heat stress might explain the reduction in TNF-a from day 1 to day 7 of exercise/heat stress under QUERCETIN supplementation. Alternatively, as quercetin has been previously shown to reduce NF-KB activity in macrophages responding to LPS exposure (27), this may also reflect a direct action of dietary quercetin supplementation. The increase in exercise HSP70 levels in PLACEBO, and lack thereof in QUERCETIN, suggests that repeated dosing with the HSF-1 inhibitor quercetin may have contributed to a diminished heat shock response. An attenuated heat shock response in the face of increased stimulus for HSP70 induction (> circulating endotoxin and inflammatory cytokines) is intriguing.

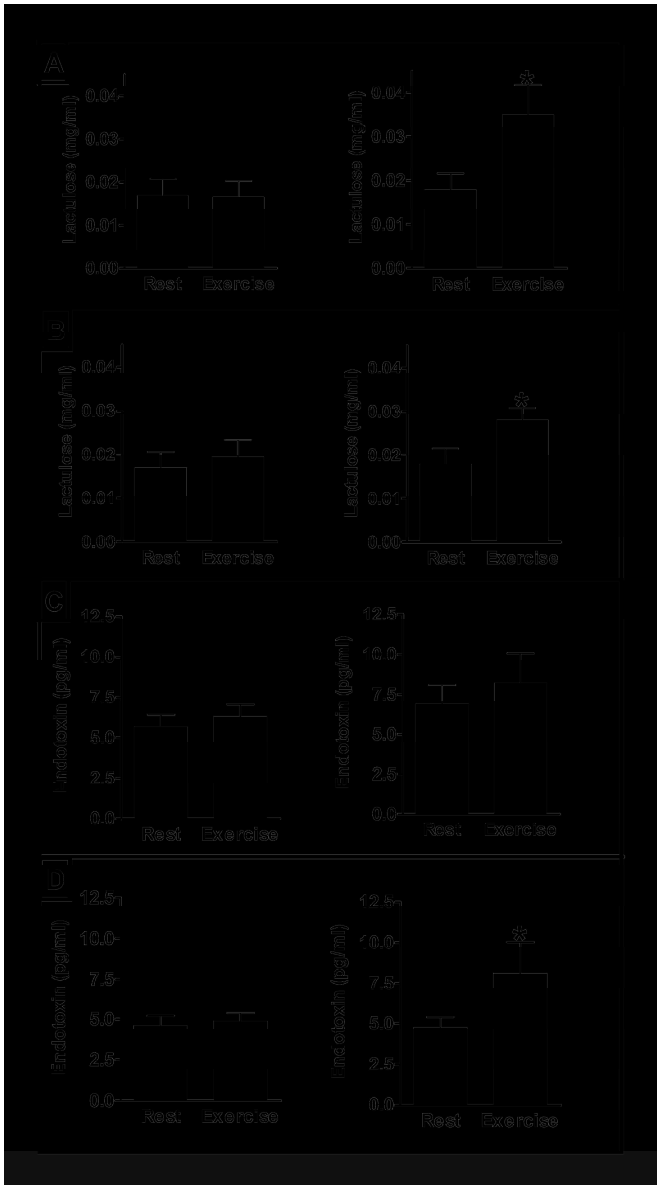

Fig. 1. The HSF-1 inhibitor quercetin alters well established markers of thermotolerance in humans responding to 7 days of repeated exercise/heat stress. (A) urinary lactulose excretion in non-exercise/heat stress conditions (Rest) and on the 1st day of exercise/heat stress under placebo (left panel) and quercetin (right panel) conditions. (B) urinary lactulose excretion in non-exercise/heat stress conditions (Rest) and on the 7th day of exercise/heat stress under placebo (left panel) and quercetin (right panel) conditions. (C) plasma endotoxin at rest (rest) and immediately following exercise on the first day of exercise/heat stress under placebo (left panel) and quercetin (right panel) conditions. (D) plasma endotoxin at rest (rest) and immediately following exercise on the seventh day of exercise/heat stress under placebo (left panel) and quercetin (right panel) conditions. *Increased from pre-exercise value, $p < 0.05$. Data are mean ± SEM, n=8 for all panels.

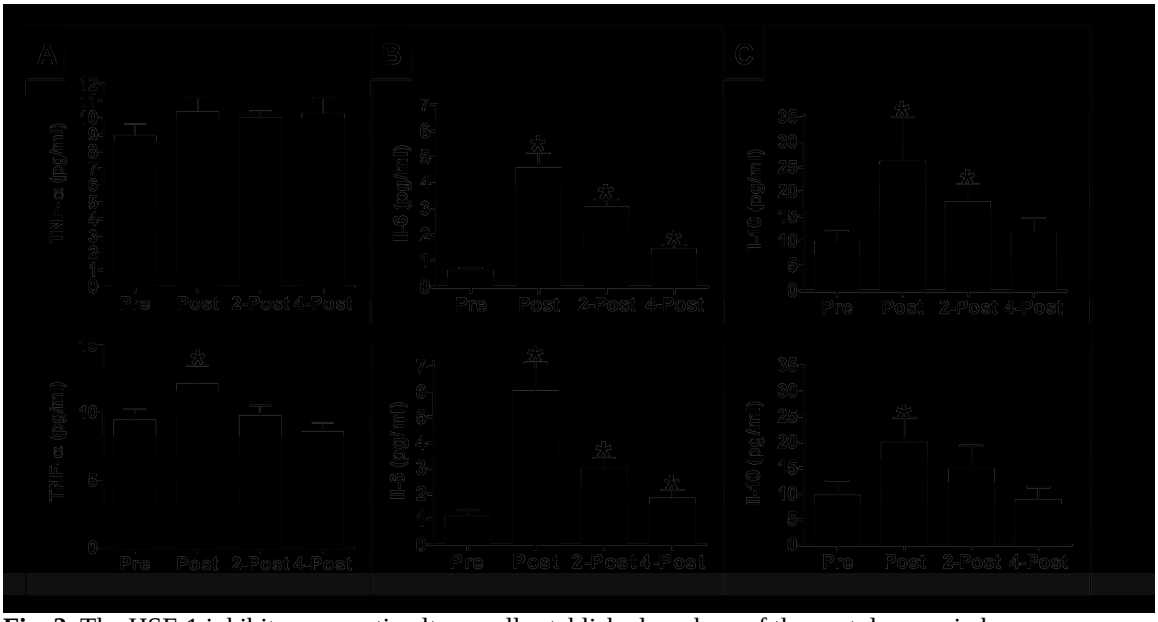

Fig. 2. The HSF-1 inhibitor quercetin alters well established markers of thermotolerance in humans responding to an acute bout of exercise/heat stress. Data were collected on the first day of heat acclimation exercise. (A) plasma TNF-a; (B) plasma Il-6; and (C) plasma Il-10 before exercise (Pre), after exercise (Post), two hours after exercise (2-Post) and 4 hours after exercise (4-post) under placebo (top of panel) and quercetin (bottom of panel) conditions.*Increased from pre-exercise value, p<.05. Data are mean ± SEM, n=8 for all panels.

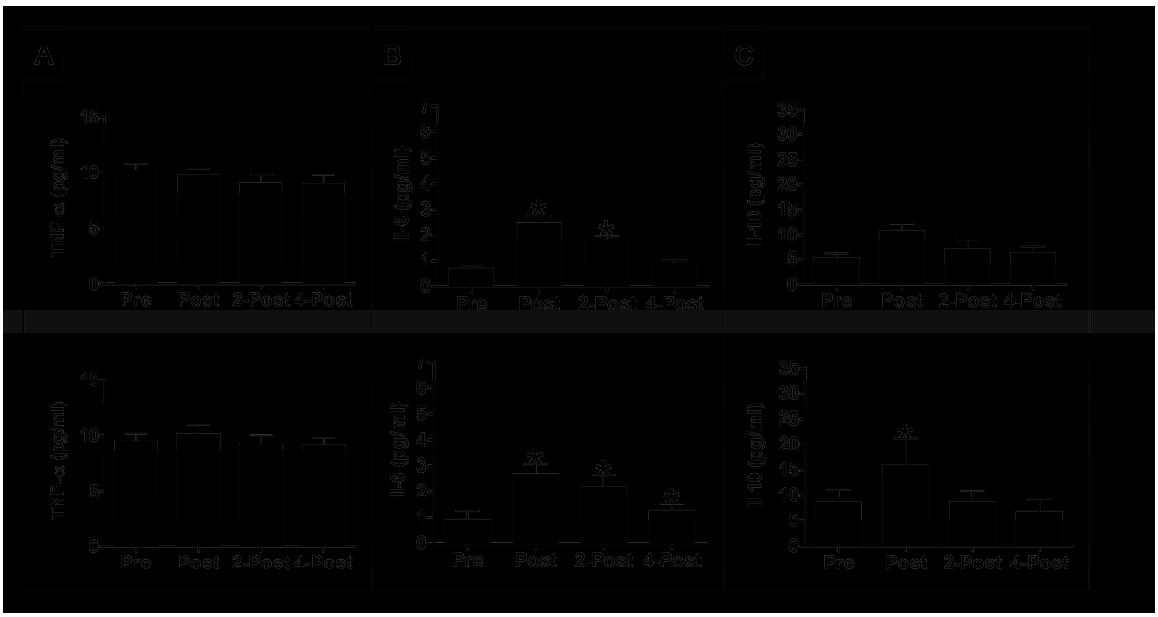

Fig. 3. The HSF-1 inhibitor quercetin alters well established markers of thermotolerance in humans responding to 7 days of repeated exercise/heat stress. Data were collected on the 7th day of heat acclimation exercise. (A) plasma TNF-a; (B) plasma Il-6; and (C) plasma Il-10 before exercise (Pre), after exercise (Post), two hours after exercise (2-Post) and 4 hours after exercise (4-post) under placebo (top of panel) and quercetin (bottom of panel) conditions. *Increased from pre-exercise value, p< 0.05. Data are mean ± SEM, n=8 for all panels.

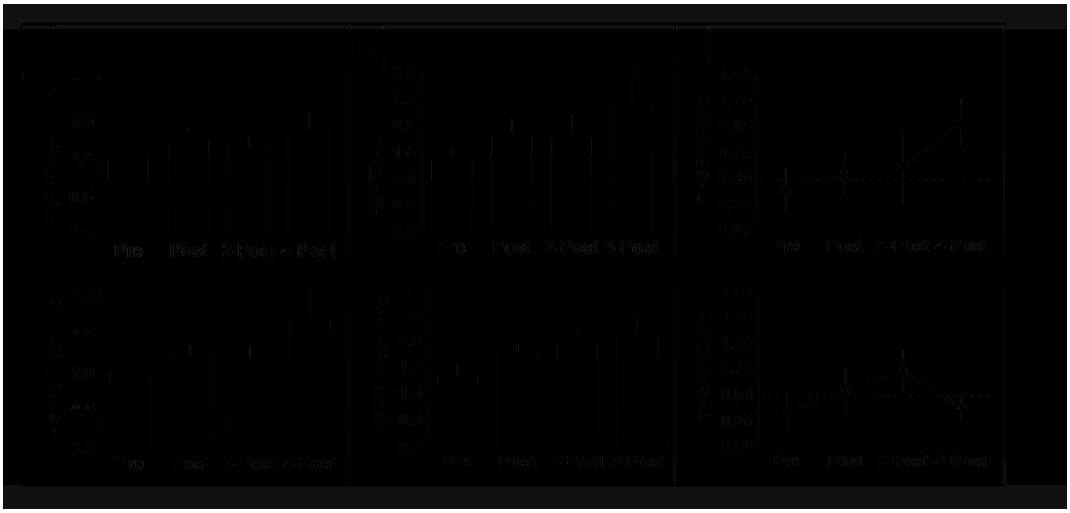

Fig. 4. The HSF-1 inhibitor quercetin alters production of stress-inducible heat shock protein 70 (HSP70) in response to exercise/heat stress. HSP70 protein content of peripheral blood mononuclear cells (PBMC) responding to (A) acute and (B) chronic exercise/heat stress. Measurements were taken before exercise (Pre), after exercise (Post), two hours after exercise (2-Post) and 4 hours after exercise (4-post) under placebo (top of panel) and quercetin (bottom of panel) conditions. (C) HSP70 protein content of PBMC was increased in response to chronic exercise/heat stress under placebo, but not quercetin supplemented conditions. Data represents change score, derived from subtracting HSP70 response on day 1 from response on day 7, at each individual time point. *Increased from pre-exercise value, $p<.05$. Data are mean ± SEM, n=8 for all panels.

Paragraph 12. **Acquisition of the Heat Acclimated Phenotype.** Given the clear differences in exercise associated gut permeability to lactulose, cytokine production, and HSP70 in PBMC between QUERCETIN and PLACEBO exposed subjects, we next examined the physiologic profiles of the 2 groups following a standardized heat acclimation protocol. In essence, we sought to determine the effect of HSF-1 inhibition, which altered markers of thermotolerance, on subjects' ability to achieve the heat acclimated phenotype. Heat acclimation is known to stimulate multiple systemic adaptations, including reductions in the rate of rise of core, skin, and mean body temperatures with exercise, exercise heart rate response, and physiological strain, as well as increases in plasma volume and whole body sweat responses (13). We quantified these same improvements in the present study by having subjects perform an exercise bout at

the beginning and end of the 7 day heat acclimation protocol. Exercise workload (45 min at 50% VO2max) and ambient environmental conditions (46.5°C, 20%RH) were matched between bouts (additional data on conditional equality available in METHODS).

Paragraph 13. Each of core temperature (p= 0.022), mean skin temperature (p= 0.001), and mean body temperature (p= 0.002) were reduced after 7 days of heat acclimation under PLACEBO supplementation. Core temperature was reduced from 40 - 45; mean skin temperature was reduced from 35 - 45; and mean body temperature was reduced from 30 - 45 minutes of exercise, respectively. Heart rate (p= 0.010) and physiological strain (p= 0.016) responses to exercise were also improved, with heart rate being reduced from 5 - 45 minutes of exercise, and physiological strain being reduced from 10 - 45 minutes of exercise. We also noted significant increases in whole body sweat rate (from 31±1.6 ml/min to 34.3±2.2 ml/min; p= 0.044) and plasma volume expansion (16±4%).

Paragraph 14. **Quercetin blocked the ability of subjects to heat acclimate through exercise.** Contrary to our findings for the same subjects under PLACEBO supplementation, we did not detect any improvements in core temperature (p= 0.130), mean skin temperature (p= 0.974), or mean body temperature (p= 0.109) responses to exercise/heat stress with QUERCETIN. Further, while overall improvements in heart rate (p= 0.020) and physiologic strain (p= 0.017) responses to exercise/heat stress were detected in the QUERCETIN condition, reductions in these responses came much later in exercise. As compared to the PLACEBO condition, where improvements in heart rate and physiologic strain were evident at 5 and 10 minutes of exercise, respectively; under

QUERCETIN improvements were not detected until 20 and 25 minutes of exercise had elapsed. While thermoregulatory responses in subjects under QUERCETIN supplementation were clearly compromised, these differences cannot be explained by differences in subjects' ability to sweat (which increased from 30.3±3.4 ml/min to 35.2±3.7 ml/min; p= 0.002) or to expand their plasma volume (plasma volume expansion = 16±5%), which were identical (p= 0.486 and 0.535; respectively) between conditions.

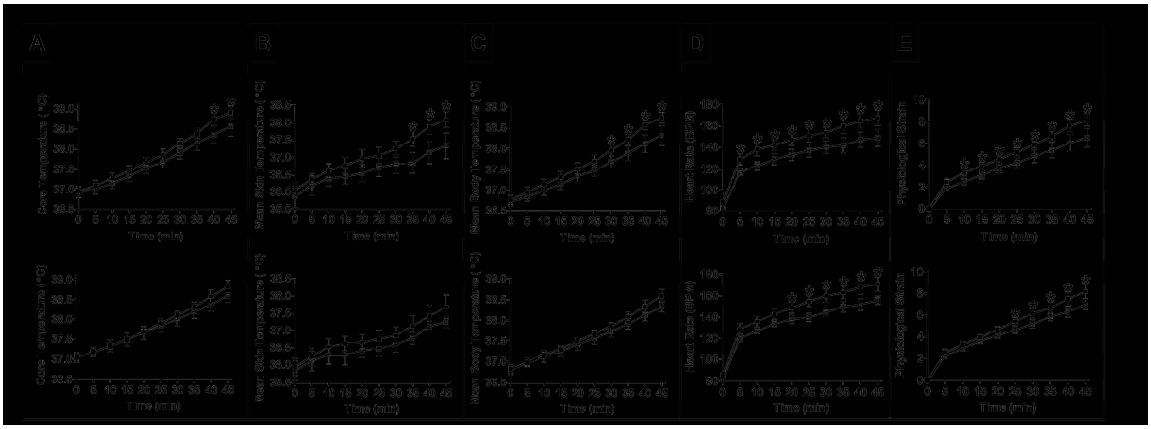

Fig. 5. The HSF-1 inhibitor quercetin alters well established systemic adaptations that characterize the heat acclimated phenotype. (A) Core temperature; (B) mean skin temperature; (C) mean body temperature; (D) heart rate; and (E) physiological strain responses (scored 0-10, where 0 = no strain and 10 = high strain) to a matched exercise bout performed at the start (dark squares) and end (light squares) of the 7-day heat acclimation protocol under placebo (top of panel) and quercetin (bottom of panel) conditions. *Improved exercise response, $p < 0.05$. Data are mean ± SEM, n=8 for all panels.

DISCUSSION.

Paragraph 15.In the present study we examined the effect of a standard laboratory HSF-1 inhibitor, quercetin, on well described markers of thermotolerance and heat acclimation in humans. We found that oral quercetin supplementation at 2 g/d was sufficient to disrupt the normal cellular accumulation of HSP70 in PBMC. The blunted HSP70 response was associated with impairments in gastrointestinal barrier function and

inflammatory/anti-inflammatory cytokine profiles. These findings in the exercising human mirror those in cells (28), tissues (6, 7) and animals (24, 29). While this is the first clear example of this effect in humans, the major finding of this study was the ability of HSF-1 inhibition to impede acquisition of the acclimated phenotype. This study provides the clearest support to date for the link between thermotolerance and heat acclimation.

Paragraph 16. **HSF-1 inhibition reduces acquisition of heat acclimated phenotype**. The ability of HSF-1 inhibition to confer detriments on systemic adaptations to chronic exercise/heat stress becomes more intriguing when one considers that all the changes in physiology associated with heat acclimation are designed to limit the rise in body temperature for a given amount of work in the heat. How then, could HSF-1 inhibition confer this loss of acclamatory fitness? Referencing classic studies of both natural acclimatization and laboratory heat acclimation, we note multiple instances where active heat exposure is reported to increase animal (30) and human (31, 32) capacity to tolerate higher internal temperatures before cessation of work. This is not part of the set of adaptations we usually associate with either acclimatization or acclimation, and suggests a more basic cellular mechanism.

Paragraph 17. The present study is the first to demonstrate a link between thermotolerance and acclimation in humans through inhibition of the heat shock system. However, the association between thermotolerance and adaptation has been suggested in prior reports which took advantage of natural differences between species. Perhaps the best example of such adaptations is the Saharan ant *Cataglyphis bombycina*, which shows

elevated levels of HSP70 at low ambient temperatures (25°C). This adaptation has been suggested to contribute to *C. bombycina* capacity to forage at midday, tolerating surface temperatures 15°C higher than all other desert ant species (16). This adaptation is vital to *C. bombycina* survival, as the associated increase in CTM (53-55°C) gives it a slight competitive advantage over *Acanthodactylus dumerili*, a predatory lizard species with similar thermal adaptation (33). There is also evidence of elevated HSP70 levels at normal ambient temperatures in lizards (34) and multiple insect species (35, 36) that reside in environments prone to rapid temperature change. This high constitutive HSP70 expression has been suggested to allow these organisms to perform work at elevated temperatures, where *de novo* HSP70 synthesis would be too slow to confer benefit (15). Benefits of this adaptation are apparent when one examines *Hydra attenuate*, a hydra species that has naturally lost the ability to accumulate HSP70 in response to thermal exposure. Acclimating this hydra species does not induce thermotolerance, rather, it increases mortality to subsequent thermal exposure (18). In all of these studies, the natural experiment suggested that improved heat tolerance, as measured by work in the heat, was associated with an elevated HSP response. Likewise, an evolved decrease in HSP response predicted less capability to perform work in heat and heat intolerance.

Paragraph 18.Intriguingly, a similar repression of the heat shock response has been reported in heat intolerant military personnel, and has been suggested to explain the excessive metabolic heat storage and physiological strain these soldiers experience in response to exertional heat stress (37). It should also be noted that these soldiers are at significantly increased risk of developing exertional heat illness than the general

population (37, 38). Reduced acclimatory capacity has also been reported in rats acclimatized under HSP70 blockade (39). Like that reported by heat intolerant military personnel, and for otherwise healthy subjects in the present study, rats that were acclimated under HSP70 blockade exhibited decreased heat endurance and increased tissue damage in response to subsequent thermal challenge (40).

Paragraph 19. **Conclusions.** In this study we reported that supplementation with an HSF-1 inhibitor prior to a single bout of exercise heat stress increased gastrointestinal barrier permeability and inflammatory cytokine responses. Following 7 days of repeated exercise/heat stress the increase in gastrointestinal barrier permeability and altered exercise cytokine responses remained. More importantly, the ability of repeated exercise/heat stress to confer the heat acclimated phenotype was diminished in these subjects. These data provide evidence of considerable overlap in human thermotolerance and heat acclimation responses, and for the first time link benefits of each through activation of the heat shock response.

MATERIALS & METHODS.

Paragraph 20. The present study was approved by the ethics committee of the University of New Mexico, Albuquerque, USA. All study participants gave written informed consent prior to study participation. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Paragraph 21. Experimental Design. Eight subjects completed 7 days of heat acclimation (HA) exercise in both PLACEBO and QUERCETIN supplemented conditions. Each subject began the study by providing baseline measures of fitness (maximal oxygen consumption and body composition). They next completed a baseline test of gastrointestinal barrier permeability, in non-exercise/heat stress conditions. On the following day subjects performed a standardized heat tolerance test to provide baseline measurements of their capacity to tolerate exercise heat stress. After a 7-day washout, subjects began the heat acclimation protocol. Additional measures of gastrointestinal barrier permeability, cytokines, and HSP70 were performed on the 1st and 7th days of heat acclimation exercise, allowing researchers to examine subjects' acute and acquired thermotolerance responses. A second heat tolerance test was performed on the 6th day of heat acclimation exercise, allowing researchers to examine subjects' ability to acclimate to repeated exercise/heat exposure. Figure 6 provides a schematic of the study design. These measures are described in greater detail in the following sections.

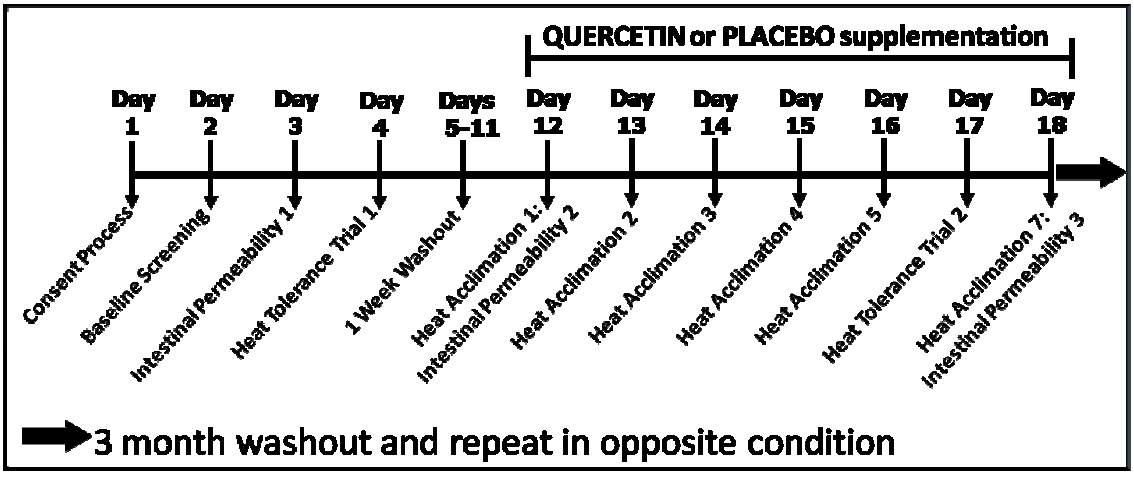

Fig. 6. Study Schematic.

Paragraph 22. **Baseline Measures of Fitness.** Each subject performed a continuous graded treadmill test in a temperate room (22ºC to 24ºC, 30% to 40% RH) to determine VO_2pk through open circuit spirometry. VO_2pk was defined as the highest 30-second value when two of the following criteria were met: (a) a plateau (change in VO_2 < 150 $ml \cdot min^{-1}$) with increase in workload, (b) a maximal respiratory exchange ratio greater than 1.1, and (c) heart rate (HR) greater than 95% of the age-predicted maximum (220 − age). Percent body fat was calculated from the average of 3 skinfold sites (41).

Paragraph 23. **Gastrointestinal Barrier Permeability.** Following an overnight fast subjects ingested 10g lactulose (Kristalose, Cumberland Pharmaceuticals) dissolved in 50 ml dH_2O. Subjects collected their urine into sterile containers for 8 hours post ingestion, at which time 10 ml was aliquoted and stored at -80°C for subsequent analysis of urinary lactulose excretion.

Paragraph 24. **Standardized Heat Tolerance Tests.** Subjects began each heat tolerance test by providing a urine sample, from which hydration was assessed via urine osmolality. They next inserted a calibrated rectal thermistor (YSI Precision 4400 Series, Yellow Springs Inc) 12 cm into the rectum. Following insertion subjects provided a nude body mass to 0.1 kg, then donned athletic shorts, socks, and shoes and entered the environmental chamber. Subjects were next fitted with a telemetric heart rate (HR) transmitter strap and watch (S810i series, Polar). Uncovered skin thermistors (Grant Instruments Ltd) were then attached to the upper arm, upper thigh, chest, and calf with

elastic straps. Heart rate, core, and skin temperatures were recorded at 5 minute intervals during exercise.

Paragraph 25. Each subject completed 45 minutes of exercise at a workload corresponding to 50% of his measured VO_2max. Subjects drank ad libitum during exercise. Following exercise termination subjects immediately left the environmental chamber, toweled dry, then provided a post-exercise nude body mass for calculation of sweat losses and urine sample for hydration assessment. Differences in whole body responses to exercise in the heat (core temperature, mean skin temperature, mean body temperature, heart rate, physiological strain, plasma volume, and whole body sweat rate) were calculated as previously described (14, 42). Great care was taken to minimize the potential influence of extraneous variables (ambient environment, exercise characteristics, and subject hydration status) on experimental design (Table 1).

	Ambient Environment		Exercise Response		Hydration Status		
	Dry Bulb Temperature (°C)	Relative Humidity (%)	Oxygen Uptake (ml/kg/min)	Exercise Workload (m/s)	H_2O Intake (L)	Urine Osmolality (mOsm/kg)	
						Pre Exercise	Post Exercise
HT1	**47.0(.4)**	**19.7(1.3)**	**28.2(1.0)**	**2.11(.15)**	**1.0(.13)**	**487(71)**	**392(87)**
HT2	**46.8(.4)**	**21.0(1.6)**	**27.3(1.5)**	**2.11(.15)**	**0.88(.12)**	**440(68)**	**482(89)**
HT1	**46.2(.4)**	**22.3(1.3)**	**28.4(1.3)**	**2.11(.15)**	**0.94(.11)**	**475(81)**	**436(85)**
HT2	**46.0(.4)**	**21.2(1.3)**	**27.6(1.6)**	**2.11(.15)**	**0.88(.23)**	**511(86)**	**560(77)**

Table 1. Equality of Heat Tolerance Tests. There were no differences in the ambient environment, subject exercise responses, or hydration status between the 1st (HT1) and 2nd (HT2) heat tolerance tests in either PLACEBO supplemented (shaded) or QUERCETIN supplemented (unshaded) conditions. Data are mean(SEM).

Paragraph 26. **Heat Acclimation Protocol.** Subjects exercised in the environmental chamber to increase their core temperature to $\geq$ 39°C in 50 minutes of exercise. They then rested in the environmental chamber for 10 minutes, followed immediately by

another 50 minute exercise bout. This method of controlled hyperthermia allowed subjects to maintain core temperature above 39°C for the entire second 50 min exercise bout, providing for a greater and more sustained increase in core temperature over traditional protocols (10). Workload (speed/ %grade) was recorded every five minutes and oxygen consumption was recorded every 15 minutes during exercise. Additional measures of oxygen consumption were taken 5 minutes after each workload transition. Exercise termination criteria included 1) completing the full 100 minutes of exercise, 2) core temperature $\geq$ 39.5 ^{o}C, 3) HR $\geq$ 98% of HRmax, or 4) subject request. Preparatory and exercise procedures were otherwise identical to those described previously for the heat tolerance tests. Additional measures of gastrointestinal barrier permeability and blood samples for thermotolerance variables were taken on days 1 and 7 of heat acclimation. Equality of mean core temperature (2nd 50min exercise bout), peak core temperature, and mean exercise workloads are depicted in Table 2. Blood sampling and assay techniques are described in further detail in the following sections.

	Mean Core Temperature (°C)	**Peak Core Temperature (°C)**	**Mean Exercise Workload (m/s)**
Day 1	**39.02 (0.04)**	**39.33 (0.12)**	**1.75 (0.06)**
Day 7	**39.02 (0.04)**	**39.35 (0.09)**	**1.80 (0.06)**
Day 1	**38.93 (0.04)**	**39.28 (0.14)**	**1.77 (0.07)**
Day 7	**39.12 (0.03)**	**39.42 (0.06)**	**1.85 (0.07)**

Table 2. Equality of Heat Acclimation Days 1 and 7. Mean and peak core temperatures, as well as mean exercise workloads are depicted for the 1st and 7th days of heat acclimation in PLACEBO supplemented (shaded) and QUERCETIN supplemented conditions (unshaded) conditions. Data are mean(SEM).

Paragraph 27. **Blood sampling procedure.** Posture-controlled blood samples were collected without stasis from each subject before (pre), after (post), 2 hours post (2post) and 4 hours post (4post) exercise on the 1st and 7th days of HA exercise via venipuncture

of an antecubital vein. These samples were used to assess cytokine and HSP70 responses to exercise. Additional posture-controlled blood samples were taken on each heat tolerance test, prior to exercise. These samples were used to assess heat acclimation-induced changes in hematocrit and hemoglobin, from which plasma volume expansion was calculated with a standardized formula (43).

Paragraph 28. **Endotoxin.** Plasma endotoxin was assessed with a limulus amebocyte lysate chromogenic endpoint assay from Cell Sciences (HIT302, Canton, MA, USA) sensitive to 1.4 pg/ml. Samples were diluted 1:3 in endotoxin free water, then heated at 75°C for 10 min. Following heating samples and controls were run in duplicate and assessed relative to manufacturer provided standards. Endotoxin concentration was taken as the average of sample absorbance after the control background had been removed.

Paragraph 29. **Inflammatory/anti-inflammatory cytokines.** Serum TNF-a was assessed with a solid phase chemiluminescent immunometric assay (Immulite 1000 TNF-a, Siemens Medical Solutions Diagnostics) sensitive to 1.7 pg/ml. Serum IL-6 was assessed with an ELISA (Quantikine HS, R&D Systems) sensitive to 0.039 pg/ml. Plasma Il-10 was assessed with an EIA (Titerzyme EIA, Assay Designs) sensitive to 3.75 pg/ml. All cytokines and standards were measured in duplicate and performed according to manufacturer instructions.

Paragraph 30. **HSP70.** PBMCs were isolated from whole blood and analyzed for HSP70 protein content as described previously (14), with these modifications. The membrane

was cut longitudinally after blocking. Primary monoclonal (SPA-812, Assay Designs) and secondary polyclonal (81-6120, Invitrogen) antibodies were applied to the upper half of the membrane for HSP70 detection. The lower half of the membrane was treated with primary monoclonal (A5441, Sigma) and secondary polyclonal (61-0120, Invitrogen) antibodies for B-actin detection. HSP70 was quantified relative to B-actin to control for differences in gel loading.

Paragraph 31. **Urinary lactulose excretion.** Lactulose was quantified with an EIA (K-FRUGL, Megazyme), with some deviations from manufacturer's instructions. The supplied glucose/fructose (0.2 mg/ml) standard was serially diluted 1:2. 55 µl of blank, standard, or urine were added to 96 well microtiter plates, followed by 55ul of TAE, 10ul of B-galactosidase, 10µl imidizole buffer, and 10 µl β – NAD^+/ATP solution. The plate was mixed and allowed to incubate for 3 minutes at 37°C, then read at 340nm to measure background absorbance. The plate was next incubated at 37°C for 2 hours to allow b-galactosidase conversion of lactulose into free glucose and fructose. After incubation hexokinase + G-6-P dehydrogenase solution was diluted 1:5 in TAE buffer, and 10ul was added to all occupied wells. The plate was then mixed and incubated at 37°C for 5 minutes. Absorbance (340nm) was measured to determine free glucose concentration. PGI was then diluted 1:5 in .5XTAE buffer, and 10ul was added to the plate. The plate was then mixed, incubated at 37°C (5min), and read (340nm) to determine free fructose concentration. Urinary lactulose concentration was quantified as the difference between the A2 and A3 readings after background (A1) had been removed.

Paragraph 32. **Statistical Analyses.** All statistical analyses were performed with STATISTICA, version 7.1 (StatSoft, Tulsa, OK, USA). Variables were tested for normal distribution using the Kolmogorov-Smirnov test. Nonnormally distributed variables were log transformed to approximate a normal distribution before applying a t-test or repeated measures analysis. The repeated factors assumption of sphericity was tested with Mauchly's sphericity test. When necessary, a Greenhouse-Geisser correction was applied to the F-ratio to correct for sphericity violations. All data represent mean (SEM) for n=8. Statistical significance was set at $p \leq 0.05$.

Paragraph 33. **Gastrointestinal Barrier Permeability:** Alterations from basal gastrointestinal barrier function (lactulose and endotoxin) due to exercise/heat stress on day 1 and day 7 of HA were assessed via paired t-tests. The relationship between lactulose and endotoxin readings was examined via linear regression.

Paragraph 34. **Molecular Responses to HA Exercise:** Exercise alterations in TNF-a, Il-6, Il-10, and HSP70 were assessed via one-way RM ANOVA (time). Dunnet's test was used to assess significant differences from baseline.

Paragraph 35. **Whole Body Responses to HA Exercise:** Alterations in core temperature, mean skin temperature, mean body temperature, heart rate, and physiologic strain responses to exercise on day 1 and day 7 of HA were assessed via two-way RM ANOVAs (day * time). Tukeys post hoc tests were used to assess significant main effects

and interactions, where appropriate. Differences in plasma volume and whole body sweat rate from day 1 to day 7 of HA were examined via paired t-tests.

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REFERENCES.

1. Li GC (1985) Elevated levels of 70,000 dalton heat shock protein in transiently thermotolerant Chinese hamster fibroblasts and in their stable heat resistant variants. *Int J Radiat Oncol Biol Phys* 11:165–177.

2. Moseley PL, Gapen C, Wallen ES, Walter ME, Peterson MW (1994) Thermal stress induces epithelial permeability. *Am J Physiol* 267:C425–C434.

3. Weshler Z, Kapp DS, Lord PF, Hayes T (1984) Development and decay of systemic thermotolerance in rats. *Cancer Res* 44:1347-1351.

4. Mizzen LA, Welch WJ (1988) Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J Cell Biol* 106:1105-1116.

5. Dokladny K, Wharton W, Lobb R, Ma TY, Moseley PL (2006) Induction of physiological thermotolerance in MDCK monolayers: contribution of heat shock protein 70. *Cell Stress Chaperones* 11:268-275.

6. Dokladny K, Moseley PL, Ma TY (2006) Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *Am J Physiol Gastrointest Liver Physiol* 290:G204-G212.

7. Dokladny K, Ye D, Kennedy JC, Moseley PL, Ma TY (2008) Cellular and molecular mechanisms of heat stress-induced up-regulation of occludin protein expression: Regulatory role of heat shock factor-1. *Am J Pathol* 172:659-670.

8. Dokladny K, Lobb R, Wharton W, Ma TY, Moseley PL (2010) LPS-induced cytokine levels are repressed by elevated expression of HSP70 in rats: possible role of NF-kappaB. Cell Stress Chaperones. 15:153-163.

9. Sakurada S, Hales JR (1998) A role for gastrointestinal endotoxins in enhancement of heat tolerance by physical fitness. *J Appl Physiol* 84:207-214.

10. Magalhães FD, et al. (2010) Heat and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced increase in intracellular and plasma Hsp72 in humans. Cell Stress Chaperones. [Epub ahead of print]

11. Hung CH, Chang NC, Cheng BC, Lin MT (2005) Progressive exercise preconditioning protects against circulatory shock during experimental heatstroke. *Shock* 23:426-433.

12. Carter R 3rd, et al. (2005) Epidemiology of hospitalizations and deaths from heat illness in soldiers. *Med Sci Sports Exercise* 37:1338-1344.

13. Nadel ER, Pandolf KB, Roberts MF, Stolwijk JAJ (1974) Mechanisms of thermal acclimation to exercise and heat. *J Appl Physiol* 37:515–520.

14. Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM (2007) Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans. *J Appl Physiol* 103:1196-1204.

15. Moseley PL (1997) Heat shock proteins and heat adaptation of the whole organism. *J Appl Physiol* 83:1413-1417.

16. Gehring WJ, Wehner R (1995) Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proc Natl Acad Sci USA* 92:2994–2998.

17. Ulmasov KA, Shammakov S, Karaev K, Evgen'Ev MB (1992) Heat shock proteins and thermoresistance in lizards. *Proc Natl Acad Sci USA* 89:1666–1670.

18. Bosch TC, Krylow SM, Bode HR, Steele RE (1988) Thermotolerance and synthesis of heat shock proteins: These responses are present in *Hydra attenuate* but absent in *Hydra oligactis*. *Proc Natl Acad Sci USA* 85:7927–7931.

19. Buckley BA, Owen ME, Hofmann GE (2001) Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus Mytilus) changes as a function of thermal history. *J Exp Biol* 204:3571-3579.

20. diIorio PJ, Holsinger K, Schultz RJ, Hightower LE (1996) Quantitative evidence that both Hsc70 and Hsp70 contribute to thermal adaptation in hybrids of the livebearing fishes Poeciliopsis. *Cell Stress Chaperones* 1:139-147.

21. Dokladny K, et al. (2001) Effect of heat stress on LPS-induced febrile response in D-galactosamine-sensitized rats. *Am J Physiol Regul Integr Comp Physiol* 280:R338-R344.

22. Bouchama A, al-Sedairy S, Siddiqui S, Shail E, Rezeig M (1993) Elevated pyrogenic cytokines in heatstroke. *Chest* 104:1498-1502.

23. Lim CL, Mackinnon LT (2006) The roles of exercise-induced immune system disturbances in the pathology of heat stroke: The dual pathway model of heat stroke. *Sports Med* 36:39-64.

24. Liu WL, et al. (2007) Protective effects of heat shock protein70 induced by geranylgeranylacetone in atrophic gastritis in rats. *Acta Pharmacol Sin*. 28:1001-1006.

25. Tazuke Y, Drongowoski RA, Teitelbaum DH, Coran AG (2003) Interleukin-6 changes tight junction permeability and intracellular phospholipid content in a human enterocyte cell culture model. *Pediatr Surg Int* 19:321-325.

26. Madsen KSA, Lewis MM, Tavernini J, Hibbard R, Fedorak N (1997) Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. *Gastroenterology* 113:151-159.

27. Nair MP, et al. (2006) The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappa beta system. *Clin Vaccine Immunol* 13:319-328.

28. Hosokawa N, et al. (1990) Flavonoids inhibit the expression of heat shock proteins. *Cell Struct Funct* 15:393-401.

29. Nakada J, et al. (2005) Oral administration of geranylgeranylacetone improves survival rate in a rat endotoxin shock model: administration timing and heat shock protein 70 induction. *Shock* 24:482-487.

30. Fruth JM, Gisolfi CV (1983) Work-heat tolerance in endurance- trained rats. *J Appl Physiol* 54:249–253.

31. Maron MB, Wagner JA, Horvath SM (1977) Thermoregulatory responses during competitive marathon running. *J Appl Physiol* 42:909–914.

32. Selkirk GA, McLellan TM, Wright HE, Rhind SG (2008) Mild endotoxemia, NF-kappaB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 295:R611-R623.

33. Wehner R, Marsh AC, Wehner S. Desert ants on a thermal tightrope. *Nature* 357:586-587, 1992.

34. Ulmasov HA, Karaev KK, Lyashko VN, Evgen'ev MB (1993) Heat-shock response in camel (Camelus dromedarius) blood cells and adaptation to hyperthermia. *Comp Biochem Physiol B* 106:867-872.

35. Evgen'ev MB, Garbuz DG, Shilova VY, Zatsepina OG (2007) Molecular mechanisms underlying thermal adaptation of xeric animals. *J Biosci* 32:489-499.

36. Rinehart JP, et al. (2006) Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proc Natl Acad Sci U S A* 103:14223-14227.

37. Moran DS, et al. (2006) Heat intolerance: does gene transcription contribute? *J Appl Physiol* 100:1370-1376.

38. Epstein Y (1990) Heat intolerance: predisposing factor or residual injury? *Med Sci Sports Exercise* 22:29–35.

39. Maloyan A, Horowitz M (2002) beta-Adrenergic signaling and thyroid hormones affect HSP72 expression during heat acclimation. *J Appl Physiol* 93(1):107-115.

40. Horowitz M (2007) Heat acclimation and cross-tolerance against novel stressors: genomic-physiological linkage. *Prog Brain Res* 162:373-392.

41. Jackson AS, Pollock ML (1978) Generalized equations for predicting body density of men. *Br J Nutr* 40:497-504.

42. Kuennen MR, Gillum TL, Amorim FT, Kwon YS, Schneider SM (2010) Palm cooling to reduce heat strain in subjects during simulated armoured vehicle transport. *Eur J Appl Physiol* 108:1217-1223.

43. Dill DB, Costill DL (1974) Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37:247-248.

CHAPTER IV

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The review manuscript titled “Heat shock proteins and heat adaptation of the whole organism: An update” reviewed current evidence supporting contributions of the heat shock response to heat adaptation of the whole organism. In addition, the review manuscript examined evidence of natural and artificial suppression of the heat shock response contributing to heat maladaptations of the whole organism. A systematic process was used to detail these contributions at the level of the isolated cell, confluent monolayer, isolated tissue, integrated animal, and integrated human.

The research manuscript titled “Thermotolerance and heat acclimation are related through the cellular stress response” provided evidence of pharmacological (quercetin) repression of the heat shock response contributing to increased gastrointestinal barrier permeability and inflammatory cytokine responses in human subjects during exertional heat stress. While this was the first demonstration of these findings in a human model, they were expected, and in agreement with prior work performed in cell culture, tissue, and animal models. An unexpected and intriguing finding was the diminished capacity of subjects under quercetin supplementation to acclimate to exercise/heat exposure. This was evidenced by non-improvement in core, skin, and mean body temperature, as well as reduced improvement in heart rate and physiological strain responses to a matched exercise bout performed at the beginning and end of the heat acclimation protocol. It is important to note that other adaptations typically associated with heat acclimation, namely plasma volume expansion and an increased sweat rate, were not altered by

quercetin supplementation. A reduced acclimation capacity in the face of normal mechanisms for heat dissipation suggests this response may be centrally mediated, but this statement is speculatory and beyond the scope of the current study. As it stands, this study provides the clearest support to date for the link between thermotolerance and heat acclimation.

Conclusions

The significant findings presented in the research manuscript were: a)Acute exercise/heat stress caused increased gut permeability and plasma TNF-a concentrations (post) in subjects under quercetin, but not placebo supplementation; b)Acute exercise/heat stress caused increased Il-6(post;2post;4post) and Il-10(post;2post) plasma concentrations, but did not alter the HSP70 content of peripheral blood mononuclear cells. This response was identical in quercetin and placebo supplemented subjects; c) Chronic exercise/heat stress caused increased gut permeability in subjects under quercetin, but not placebo supplementation; d)The increase in Il-6 with exercise was retained in placebo(post; 2post) and quercetin(post;2post;4post) supplemented subjects following chronic exercise/heat stress exposure; e)The increase in TNF-a noted in quercetin supplemented subjects following acute exercise/heat stress was not retained following chronic exposure to exercise/heat stress. TNF-a was also not increased in placebo supplemented subjects following chronic exercise/heat stress exposure; f) Il-10 remained increased by exercise(post) in quercetin, but not placebo supplemented subjects following chronic exercise/heat stress exposure; g)HSP70 was increased with exercise(4post) in subjects under placebo, but not quercetin supplementation, indicating

the expected rise in HSP70 following heat acclimation was retained in placebo, but absent under quercetin supplementation; h) Core, skin, and mean body temperature responses to a heat tolerance test were improved in subjects under placebo, but not quercetin supplementation; i) Heart rate and physiological strain responses to a heat tolerance test were improved in subjects under both placebo and quercetin supplementation. However, improvements were noted earlier in exercise under placebo supplementation; j) plasma volume was expanded and sweat rate was improved by heat acclimation. Quercetin supplementation did not influence these variables.

These were also significant findings that were not presented in the research manuscript. These included: a) plasma cytokine concentrations (TNF-a, Il-6, and Il-10) are reduced by heat acclimation. Because heat acclimation was accomplished by a controlled hyperthermia technique, which maintained subject core temperature >39°C for a minimum of 50 minutes on each day of exercise/heat stress, these results are not a reflection of reduced exercise/heat stress that have been noted in traditional heat acclimation models; b) using this controlled hyperthermia technique, we reported an increase in PBMC HSP70 content with exercise on the 7^{th} day of heat acclimation. This contrasts previous work performed in our lab, where an increase in basal HSP70 on day 6 and day 10 of heat acclimation were reported to limit HSP70 increases with exercise. This is likely due to the use of controlled hyperthermia in the present study, versus traditional heat acclimation in our prior research.

Recommendations

1. This study was originally designed to examine whether contraindications existed for the current practice of large-dose dietary quercetin supplementation in military soldiers and endurance athletes. The possibility that dietary quercetin supplementation may suppress the heat shock response and confer negative consequences on human gastrointestinal tissues during exercise heat stress was based on a small number of cell culture studies, and one animal model of LPS-induced sepsis. There were also a small number of animal studies supporting antioxidant supplementation as reducing stress-induction of heat shock protein-70 and conferring negative consequences, but these were far from conclusive. At the time we knew the study would benefit from prior testing in an animal model, but there were insufficient time and resources to accomplish this. Based on the results of this study in a human population, it is suggested a similar study be performed in an animal population, under more controlled conditions.

2. The mechanism by which a functional heat shock response suppresses gastrointestinal barrier dysfunction is not well understood. Human models limit capacity to examine heat stress-induced alterations in gut tissues. Such studies should be performed in animals, and should examine the transcription factors HSF-1 and NF-KB. It is also recommended that the effects of quercetin supplementation be tested in this model.

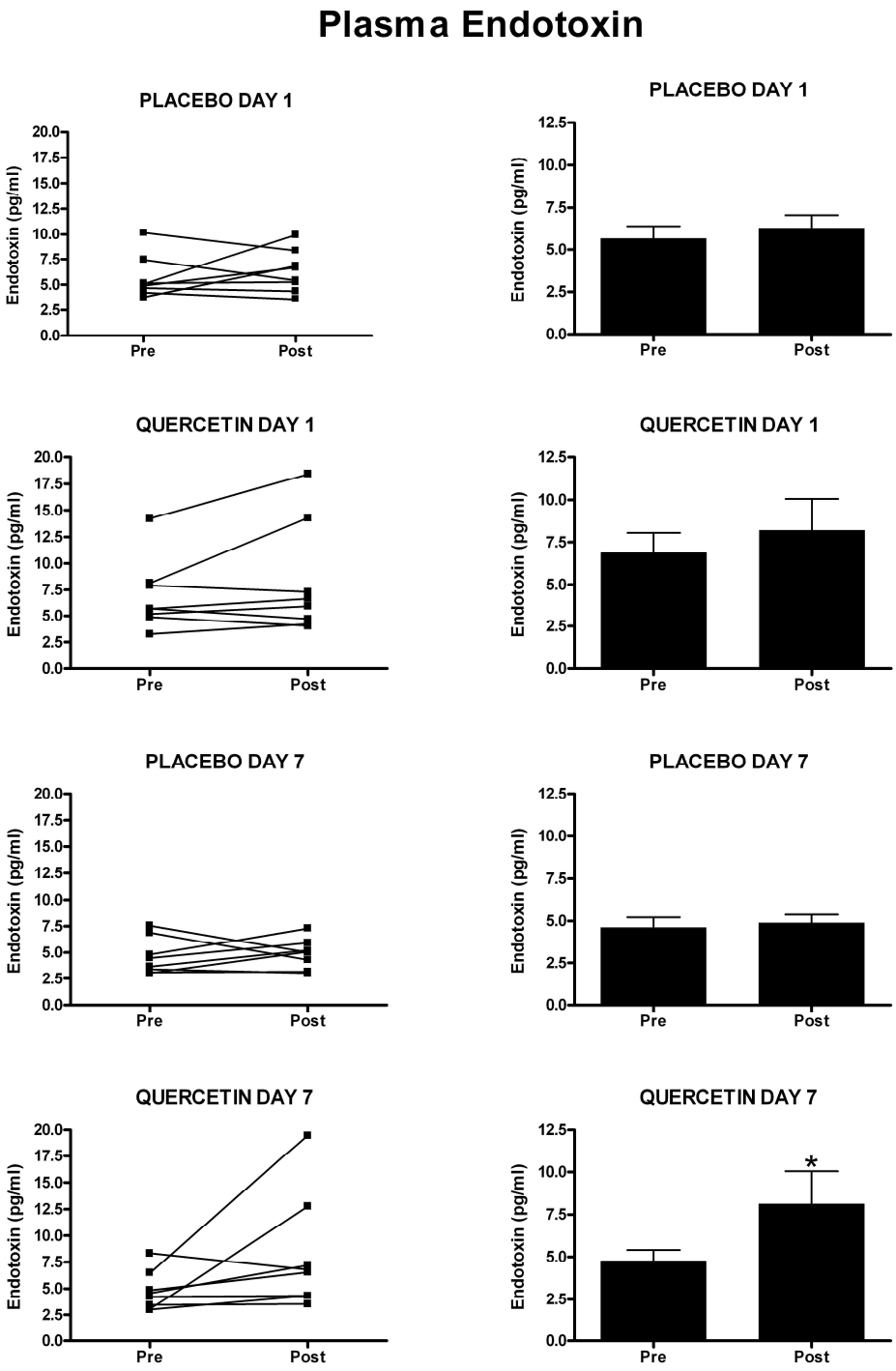
3. We began this study with some assumptions. We assumed we would note a robust disruption of gastrointestinal barrier permeability with acute exercise/heat stress. We further assumed this permeability would be reduced by acclimating subjects to exercise heat exposure. Neither effect was shown in subjects under placebo supplementation. Questions remain as to whether this result was due to insufficient method development on our part, or whether gastrointestinal barrier integrity is truly not improved with heat acclimation. Further research should be performed to examine this question.

4. The outcome variables tested in this study were sufficient to answer basic questions about quercetin-induced gastrointestinal barrier permeability. Now that these questions have been answered, further research should examine the effect of quercetin-mediated gastrointestinal barrier dysfunction on other cytokines (Il-8), acute phase proteins (CRP), and coagulation factors (complement) of interest.

APPENDICES

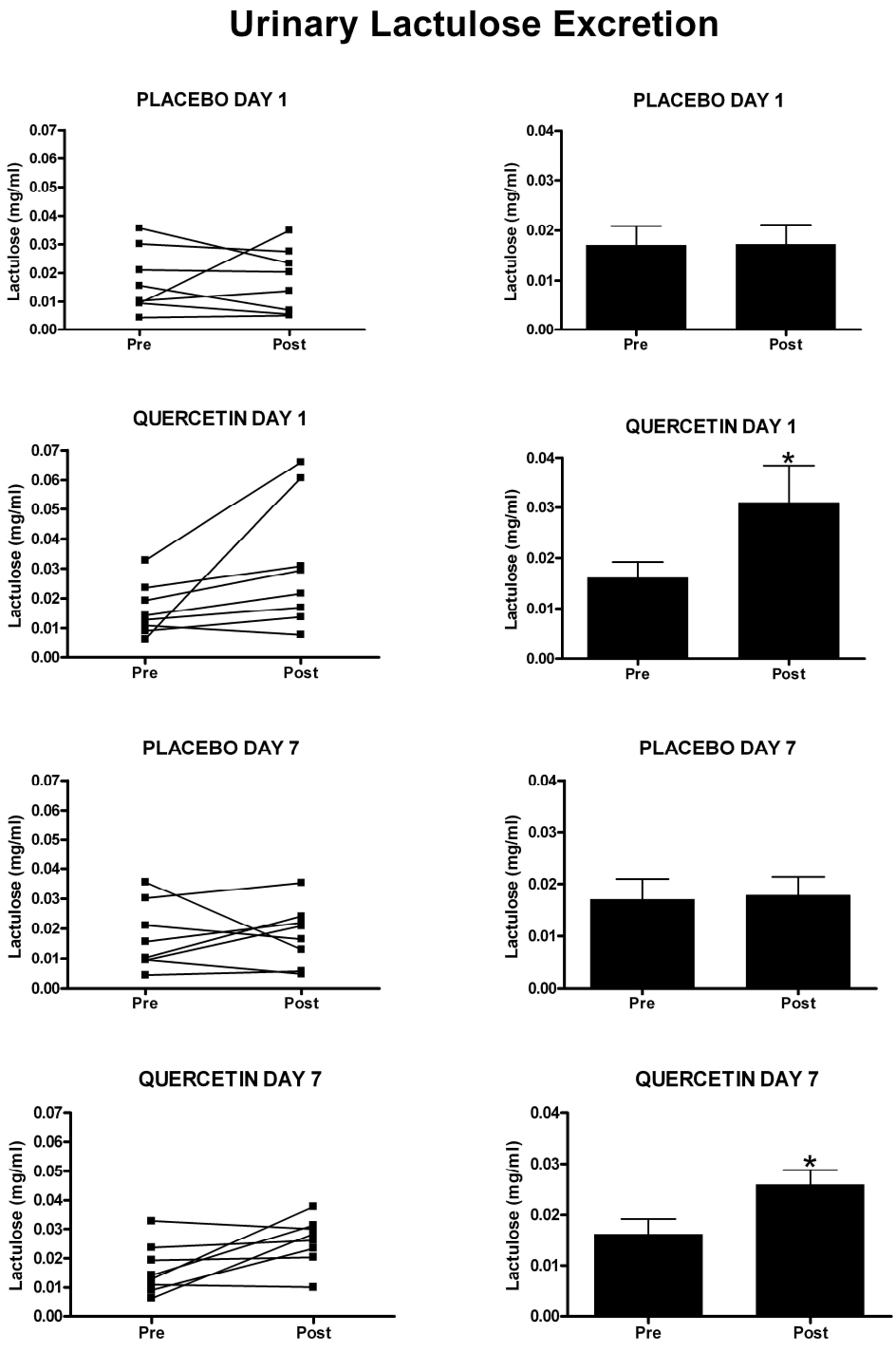
A. Supplemental Data: Plasma Endotoxin
B. Supplemental Data: Urinary Lactulose Excretion
C. Supplemental Data: Regression of Lactulose on Endotoxin
D. Supplemental Data: Core Temperature
E. Supplemental Data: Extracellular HSP70
F. Supplemental Data: Interleukin 1b
G. Supplemental Data: Glutathione Peroxidase
H. Informed Consent
I. HIPAA
J. Data Safety and Monitoring Plan
K. Physician's Order Sheet
L. Nursing Order Sheet
M. Clinical & Translational Science Center: Request for Admission
N. Food Intolerance Questionnaire
O. Dietary Instructions
P. Study Diet: Flowsheet
Q. Quercetin/Placebo Mixing Instructions
R. Core Lab: Blood/Urine Collection Procedure
S. Health History/Physical Activity Questionnaire
T. Preliminary Testing: Data Sheet
U. Heat Acclimation: Data Sheet
V. Poster: Presented NM Legislature, Santa Fe, NM
W. Poster: Presented Experimental Biology, Anaheim, CA
X. Study Budget

APPENDIX A



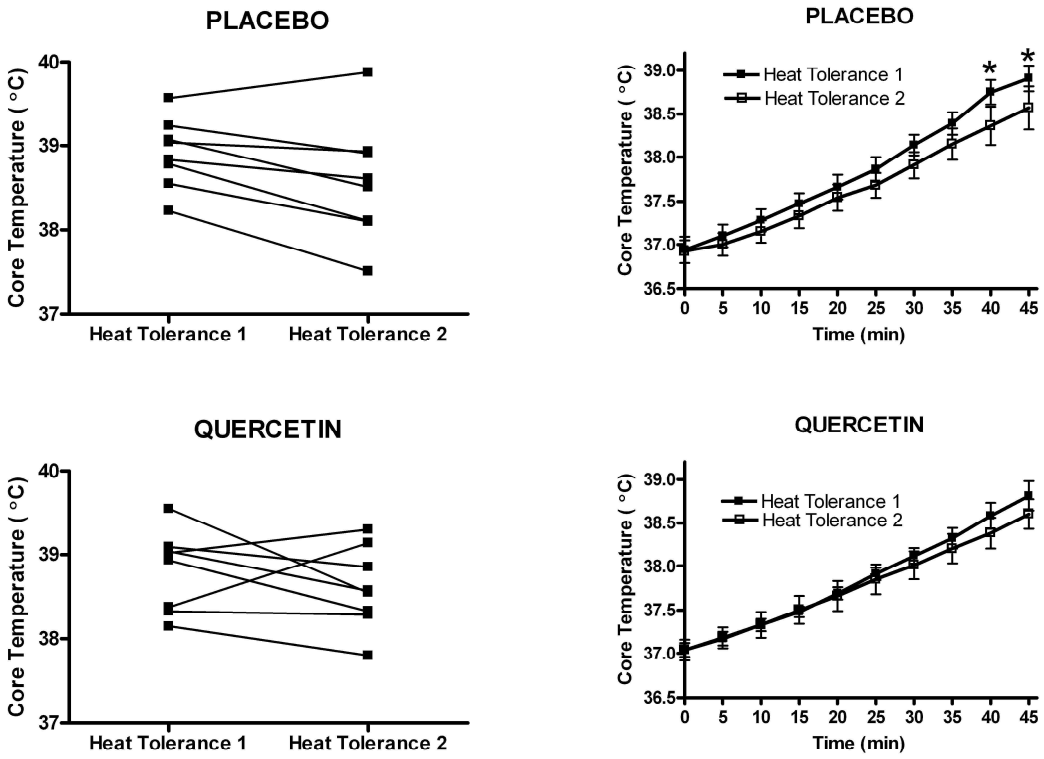
Supplemental Figure 1: Plasma Endotoxin. Data are presented individually (left) and together (right). *significant at $p < 0.05$.

APPENDIX B



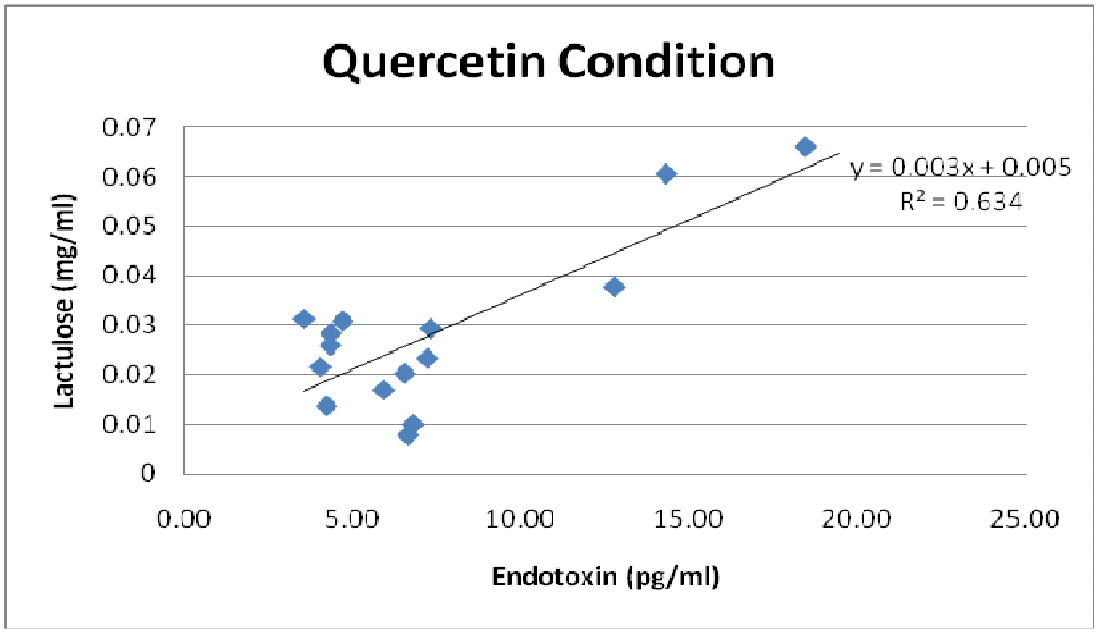
Supplemental Figure 2: Urinary Lactulose Excretion. Data are presented individually (left) and together (right). *significant at $p < 0.05$.

APPENDIX C

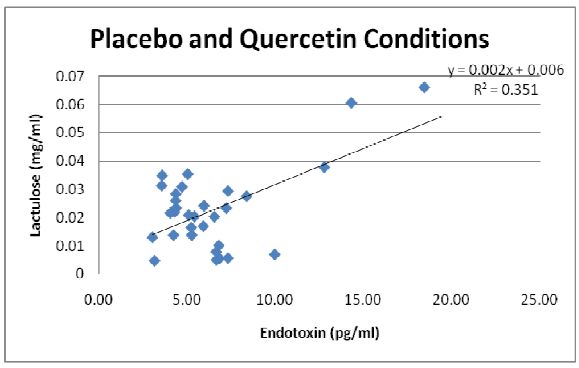


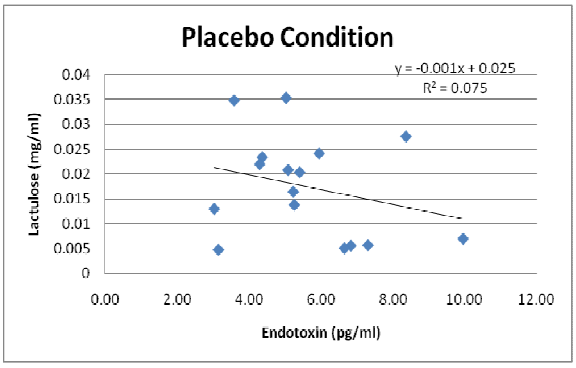
Supplemental Figure 3: Core Temperature. Final (45 minute) core temperature values for individual subjects are depicted (left) as well as core temperature values for the entire 45 minute exercise bout (right). *significant at $p < 0.05$.

APPENDIX D



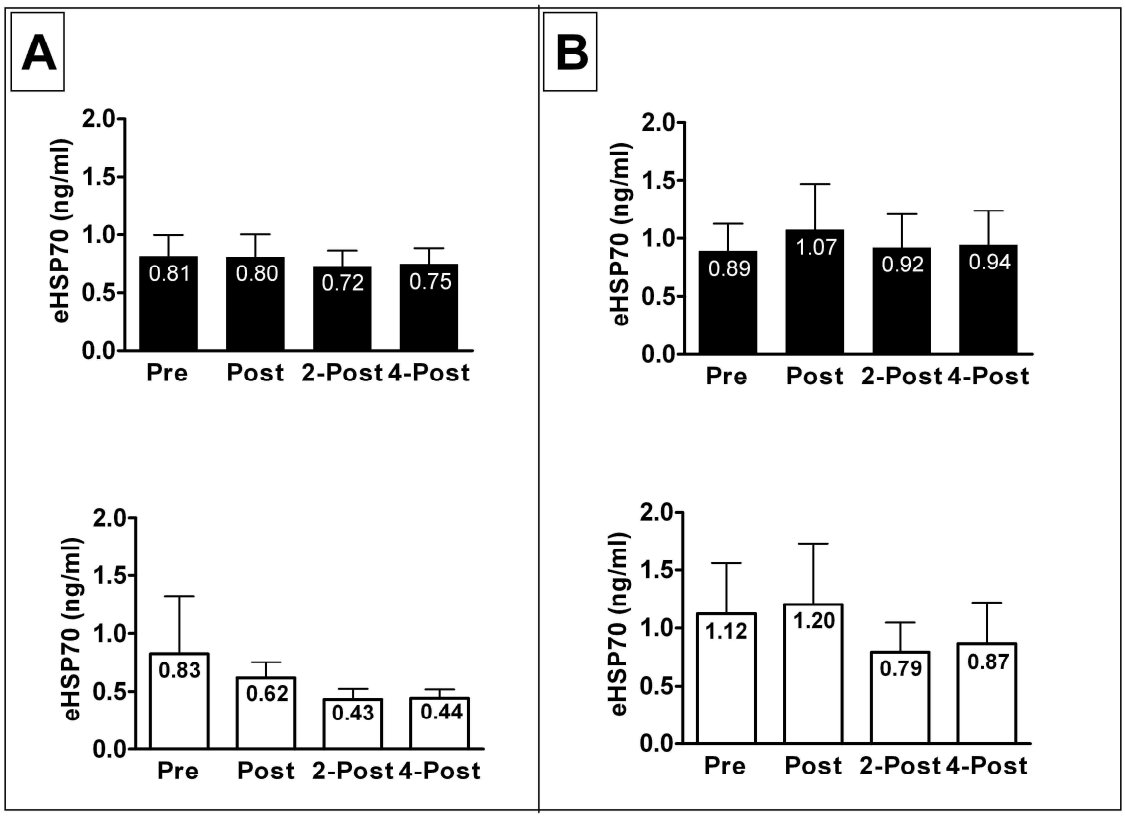
Supplemental Figure 4. The regression of systemic endotoxin vs urinary lactulose was highly significant (r^2=0.634), supporting lactulose as a useful marker of gastrointestinal barrier permeability.





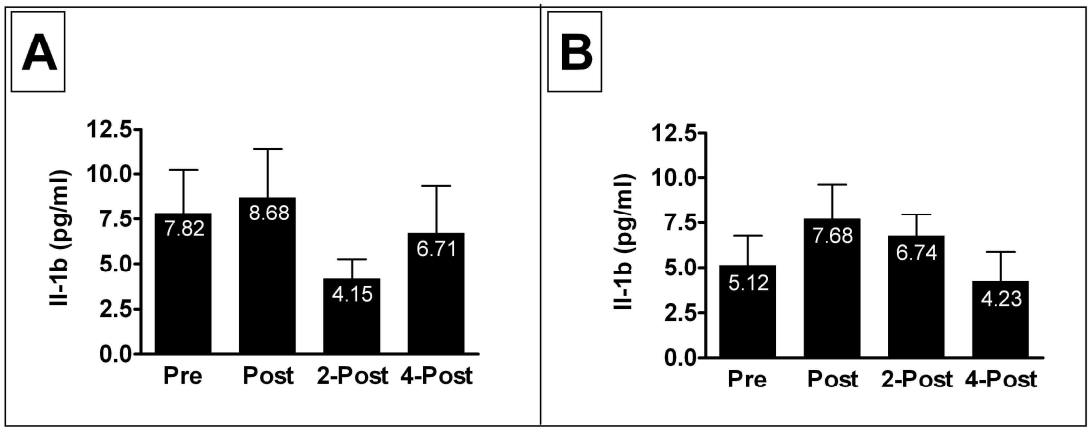
Supplemental Figures 5 and 6 Care should be taken not to apply linear regression to non-significant findings. Figure 5 (left) represents the regression of lactulose on endotoxin for the combination of placebo and quercetin supplemented conditions. Note that r2 has been reduced to 0.351. Figure 6 (right) represents the regression of lactulose on endotoxin for placebo supplemented conditions, Note that barrier leak was not shown with either of these variables in the placebo condition, r2= -0.075, and the relationship in inverted.

APPENDIX E



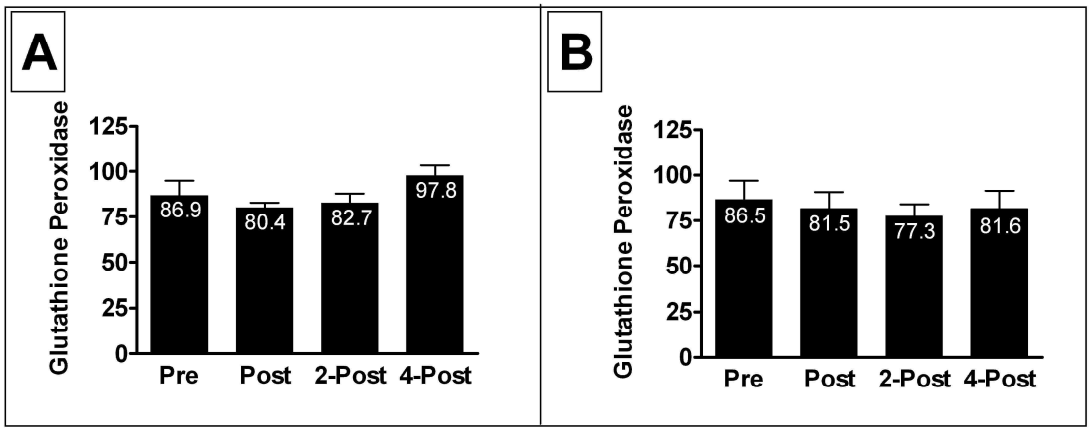
Supplemental Figure 7. Extracellular HSP70 was measured in plasma of subjects responding to (A) acute and (B) chronic exercise/heat stress. Measurements were taken before exercise (Pre), after exercise (Post), two hours after exercise (2-Post) and 4 hours after exercise (4-post) under placebo (top of panel) and quercetin (bottom of panel) conditions. (C) Data are mean ± SEM, n=8 for all panels. This figure is provided only to depict data collected with this dissertation, not for interpretation purposes. The large standard error associated with this measurement leaves considerable doubt as to the validity of this assay.

APPENDIX F



Supplemental Figure 8. Interleukin 1b was measured in plasma of subjects responding to (A) acute and (B) chronic exercise/heat stress. Measurements were taken before exercise (Pre), after exercise (Post), two hours after exercise (2-Post) and 4 hours after exercise (4-post) Data are mean ± SEM, n=6 for both panels. 3 subjects exercised under placebo supplementation, and 3 subjects exercised under quercetin supplementation. This figure is provided only to depict data collected with this dissertation, not for interpretation purposes. The large standard error associated with this measurement leaves considerable doubt as to the validity of this assay.

APPENDIX G



Supplemental Figure 7. Glutathione peroxidase activity of subjects responding to (A) acute and (B) chronic exercise/heat stress. Measurements were taken before exercise (Pre), after exercise (Post), two hours after exercise (2-Post) and 4 hours after exercise (4-post) Data are mean ± SEM, n=4 for both panels. 2 subjects exercised under placebo supplementation, and 2 subjects exercised under quercetin supplementation. This figure is provided only to depict data collected with this dissertation, not for interpretation purposes. The UNM General Clinical Research Center, which funded this assay as part of an NIH-appropriated grant, has ceased to exist. These data may be made available to the investigator at a later date.

APPENDIX H

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

Effect of Combined Exercise, Heat, and Quercetin Supplementation on Whole Body Stress Response

You are being asked to participate in a research study performed by Dr. Pope Moseley, Principal Investigator, and his associates from the Department of Internal Medicine and the Department of Health Exercise and Sports Sciences. This research is testing whether a nutritional supplement called quercetin increases heat stress (reduced tolerance to heat) during exercise.

Quercetin is found in common foods like apples, onions, berries, and leafy green vegetables. In addition to dietary sources of quercetin, it is also sold in health food stores as a popular over the counter supplement. Athletes take it to reduce their exercise stress, but we think it may affect their tolerance to heat. What we're interested in is how supplementing the diet with quercetin effects heat shock protein (HSP) levels during repeated exercise in the heat. HSP are proteins your body makes in response to stress. They play a role in protecting cells and the intestine lining against heat stress.
You are being asked to participate in this study because you are healthy, between the ages of 18-39 years, and do not have a previous history of heat related injury or other serious disease that may prevent you from being able to exercise in the heat. As many as 11 people 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 immediate 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:

Screening process & paperwork (Approximately 3 hours over TWO DAYS)

Study Day 1 - You will be asked to sign this informed consent, and a Health Insurance Portability and Accountability Act (HIPAA) form. You will complete a health history/physical activity questionnaire and a Heat Shock Protein Questionnaire.

Study Day 2 - Your blood pressure, height and weight will be measured. We will also draw a blood sample of 1 teaspoon of blood from an arm vein to measure your glucose and cholesterol. You should not have eaten for at least 12 hours prior to the blood draw. After this sample is processed the results will be sent to the investigators, who will provide you with a copy.

- To determine the percentage of fat in your body, three skinfolds will be measured, by lightly pinching your skin over the chest, abdomen & thigh.
- To determine your physical fitness, you will perform a maximal exercise test on a treadmill. The test will last between 8 and 12 minutes, depending on your current exercise level. During the test we will take breath samples, which allow us to calculate the maximal amount of oxygen your body is using. To collect your breath samples, you will breathe through a sterile, rubber mouthpiece and wear a noseclip. This may cause you to experience some discomfort (dry mouth) and feel confined. You will need to have at least average aerobic fitness to qualify for this study.

Exercise and Heat Testing (OVERVIEW)
- You will be asked to participate in TWO EXERCISE AND HEAT TESTING CONDITIONS, separated by at least 1 month. During one condition you will be given quecetin supplements twice a day for 7 days

(powder taken in a glass of TANG, a flavored children's beverage) and during the other condition, you will be given a placebo (like a sugar pill) that is taken the same way. These two exercise and heat testing conditions will occur randomly, meaning you have a 50/50 chance of starting the study in either test. Neither you nor the investigator will know which condition you are in. Each condition will include 8 bouts of exercise in the heat (lasting between 30 to 100 minutes). On Study Days 3, 12, and 18 you will go the General Clinical Research Center (GCRC) at UNM hospital (5^{th} Floor), where you will stay in a private room overnight.

In both study conditions the GCRC kitchen will provide you with all meals and snacks, which you will substitute for what you eat normally. You will begin eating these meals/snacks three days before starting each study condition, and will continue eating them until that condition is finished. To make sure you like all the foods included in the meals we will have you fill out a sheet that identifies foods you like and don't like. We will take you to the UNM hospital and show you where to pick these meals up. The hospital would like you to take enough meals for 2 days, which means you will need to have a good sized refrigerator to keep them in. If you live in the dorms or do not have access to a large refrigerator let us know, and we will make alternative arrangements. We are giving you these meals so we can make sure you are not eating large amounts of quercetin. It is very important that you eat all of the meals that are provided to you and nothing else during the experiment. These meals will be big, and probably contain more calories than you are used to eating, so you should not be hungry.

If you forget and eat something that was not part of these meals, you need to let us know. To aid you in this we will give you a diet log, which you will use to write down everything you eat and drink each day. If we find that you are repeatedly eating things that are not part of the diet we may, at our discretion, remove you from the study.

<u>EXERCISE AND HEAT TESTING CONDITION 1 (~84 hrs)</u>

Study Day 3 - You will report to the GCRC at 5:00pm. When you arrive you will receive a controlled diet and spend the night. The next morning before breakfast you will be asked to drink a cup of sugar water. We will draw a blood sample (~ 1 tablespoon), collect all of your urine, and you will be monitored for the next 8 hours. Once you have finished this 8 hour monitoring period you will be able to go home.

Study Day 4 - You will report back to the Exercise Physiology Lab in Johnson Center the next morning to begin the daily exercise portion of the study in the heat. During this visit, you will eat breakfast, have another blood sample (~ 1 tablespoon) drawn, and a urine sample taken.

You will next be asked to insert a small, flexible probe 4 inches into your rectum so we can measure your body temperature during the exercise. You will then exercise for 30 minutes on a treadmill at a moderate to high intensity. The room you are exercising in is hot (42-44ºC; 107 – 111 ºF, 30-45% relative humidity) because we are trying to increase your body temperature. At the end of the exercise you will stand for 5 minutes so we can draw another 1 tablespoon of your blood. We will monitor you for 1 hour to make sure you do not have a bad reaction to exercising in the heat before being allowed to go home. You will have a break on Study Days 3 – 5 and will eat your own diet during this time. On Study Day 6 you will pick up your meals at the GCRC kitchen to prepare for the next exercise testing.

Study Days 12-18 - You will be asked to come back to the Exercise Physiology Laboratory 7 more times over the next 10 days to perform more exercise in the heat. Like before, we will sample your urine on all of these days. Just like before, we'll draw another tablespoon of your blood before and after exercise on these days, except for on Study Days 13 and 15. On these days we'll give your arms a break. This is also when supplementation of quercetin or placebo begins. You will be given a sports bottle that contains one of these supplements, mixed with TANG.

The only difference between the exercise on these days and that on Study Day 4 is you'll be working at a lower intensity, for a longer period of time. So, this exercise will be divided into (2) 50 minute bouts separated by a 30 minute break. After you finish, we will then provide you with a supplement that you will

take home. You will need to drink it right before your last meal of the day. Once we're sure you understand these instructions and you have no other questions, you can go home.
On Days 12 and 18, we will need additional intestinal measurements. So, the evening before these sessions you will report to the GCRC at 5:00pm. You will be admitted, provided meals, and spend the night, waking up the next morning and drinking the sugar water just like you did in the first visit. This is where things will change a little. You will be provided with the supplement in addition to your breakfast. You will then be transported to Johnson Center for the exercise session. At the end of exercise you will not go home. Instead, you will go back to the GCRC for a few hours to finish our measurements. We'll also take some more blood (2 tablespoons). When you finish this day you will have successfully finished the 1st study condition. Your next appointment, where you will begin the 2nd condition, will be in about 1 month.

EXERCISE AND HEAT TESTING CONDITION 2 (~84 hrs):

After that 1 month has passed you will repeat the entire experiment in the opposite condition. So, if you got the quercetin supplement in the 1st condition, you will get the powdered food coloring in this one, and vice versa. When you finish this condition we will give you a survey to see if you can guess which condition you got the supplement, and which condition you got the powdered food coloring.

Measurements made during testing:

1. Heart rate using a telemetric heart watch monitor which consists of a transmitter belt worn around the chest and a receiver watch worn on your wrist.

2. Bodyweight using a scale. You will measure your own nude body weight before and after each exercise session in a private room. On study days 12 – 18 the exercise session is divided into 2 bouts. We will need another nude bodyweight between these exercise bouts to make sure we give you the right amount of fluids to drink. Like before you will measure your own nude bodyweight, but this time researchers will remain in the hot room to assist you in case of emergency.

3. Skin temperature using 4 skin thermistors (small pieces of metal used to measure temperature) attached to you by elastic straps and tape at the upper arm, chest, upper leg, and calf.

4. Core temperature using a rectal thermometer inserted about 4 inches beyond the external anal sphincter. The thermometer is flexible and about half the width of a pencil. You will be instructed on how to insert the thermometer, and a lubricant will be provided to increase comfort.

5. Urine samples (1 tablespoon) will be taken before and after the exercise challenge to check your hydration. We will also collect all of your urine after you drink the sugar water during the GCRC stays.

6. Blood samples will be drawn from a vein in your arm with a needle and syringe on days where we only need 2 samples (2 tablespoons). To avoid having to stick you with needles more then 2 times per day, a catheter will be inserted into a vein in your arm on study days 12 and 18, because we need 4 samples (4 tablespoons) on those days. The total number of needle sticks you will receive over the course of each Exercise and Heat testing condition will be 12.

Since you are repeating this study in both conditions, approximately 34 tablespoons (around 2 cups) of your blood will be drawn over the 2.5 months you are participating in the study: 1 teaspoon for screening and 17 tablespoons (around 1 cup) during each of the Exercise and Heat Testing Conditions.

We know that this is a lot to review at one time. To help you understand what we will be doing in this study, we have attached a study calendar to the end of this form. It lists all tests we will perform, and which tests you can expect each day.

How long will I be in this study?

Participation in this study will require you to be in our laboratory for approximately 177 hours over a period of about 11 weeks. This time will include about 3 hours for the preliminary meetings and screening and 87 hours in each of the (2) study conditions; 21 hours on each of the 3 days where you are admitted to the hospital, and 4 hours per day on the other 6 days of exercise in the heat. Only ~27 of these hours will actually be involved with exercise, the rest of this time you will be staying at the hospital, eating the provided breakfast, performing pretest procedures, or resting. Remember, the (2) study conditions are separated by a 1 month break. Please, only agree to participate in this study if you think you will be able to commit to participating in both conditions.

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.

During the maximal treadmill exercise test you may experience extreme or accelerated 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. In rare instances maximal exercise can cause musculoskeletal soreness and injury, and very rarely heart attack or death. Because you are young and healthy, your risk of these things during exercise testing is very low. Investigators will monitor you during exercise, and will stop you if you show any signs of problem.

During the exercise in the heat you may experience the same risks listed for the maximal exercise test, and in extremely rare instances, heat exhaustion, heat stroke, or death. The conditions (environmental heat, exercise) involved in this study pose a small risk of heat illness. This is unlikely as you will be screened prior to study participation, and investigators will monitor your body temperatures and heart rate while you exercise. Heat illness usually does not occur at core temperatures below 41^{o}C (about 106°F), and we will stop you if you reach 39.5°C (103°F). There may also be a risk of reduced male fertility for up to a month following prolonged heat exposure.

Minor pain and discomfort may occur when inserting a needle or catheter to obtain blood, and there is a slight risk of bruising or infection at the site of sampling. Sterile equipment and standard procedures will be used to minimize these risks. You may feel some local discomfort, including slight pressure when you first insert the rectal thermistor we will use to measure your body temperature. This discomfort will diminish over time. When using these thermistors in other studies, subjects typically reported no remaining sensation 5 to 10 minutes after insertion.

We are testing the nutritional supplement, quercetin, because we think it may cause slight changes in gut permeability. Exercise, elevations of body temperature, fasting, and other states can cause the intestinal barrier to be leaky. Research by a number of scientists has focused on the potential impact of this leak to allow products in the intestine to transfer into the bloodstream. Much of this work has been done in tissue culture cells. And while exercise itself may cause this leak, we believe it is important to understand whether supplements like quercetin can make this leak greater. We do not anticipate that this should be a problem for you, as the procedures in this study should cause only minor increases in gut permeability. The sugar water we will use to study these effects does not pose any additional risk to you.

There will be some invasion of privacy in this study. A loss of privacy may be associated with the nude weight measurements and the rectal temperature probe. Some private medical information (from the medical history questions) will be obtained. There will also be some inconvenience to you. During both conditions you will be asked to restrict your level of exertion and to eat the diet we provide you.

As with any research, there may be unforeseeable risks. There may be risks associated with stress, emotional distress, inconvenience and possible loss of privacy. Every effort will be made to minimize any risk that may occur. Emergency equipment (defibrillator and crash cart) are available in the Exercise Physiology Laboratory and emergency procedures are established to call for help (in UNM Hospital

doctors are on site, and in the Exercise Physiology Laboratory a doctor is in an adjacent building about 2 minutes away – in the Student Health Center).

For more information about risks and side effects, contact the Principal Investigator, Dr. Pope Moseley, M.D. at 272-6314, who will discuss your concerns. You can also contact the medical monitor for the Exercise Physiology Laboratory, Dr. Akshay Sood, M.D, at 272-8700.

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 supplementing with quercertin prevents people from getting the full benefit of repeated exercise in the heat. This is an important question, because many people use quercetin, or products like quercetin, in combination with exercise in hot conditions. If quercetin is found to reduce this benefit, this would help us design future studies on antioxidants and heat stress. This could also be useful to the military, as they might choose to avoid supplementing with quercetin and other antioxidants when preparing soldiers for desert combat.

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 assigned a study number and all data collected in this study will be designated by this code to provide some confidentiality for the data records and computer files. Only the consent forms and medical history information will include your name. This information will be stored in a locked file cabinet separate from the site where the study data are stored. Only investigators in this study will have access to the study code to be able to link your data to your name. This study code will be destroyed 1 year after the study is published. The remaining data will be shredded 5 years after the study is published.

Information contained in your study records may be used only 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, again separate from the data or the code key that links the codes and names.

What are the costs of taking part in this study?

You will not be charged for any study procedures.

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?

In return for your time and the inconvenience of participating in this study, you will be paid up to a maximum of $600 after you have completed both study conditions. If you do not complete the study, you will be paid $100 for each overnight stay at GCRC that you have already completed. You will be mailed a check approximately (3) weeks after completing each study condition. Compensation over $600 will be reported to the IRS.

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 are a student, your participation in this study will not influence your future grades or any other interactions with the faculty. If you choose to withdraw from this study please contact Matthew Kuennen at 505-314-4400 as soon as possible.

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. Pope Moseley or his associates will be glad to answer them at 505-277-5248, Monday – Friday 8am – 5 pm. If you need to contact someone after business hours or on weekends, please call Matt Kuennen, the study coordinator's cell phone at 505-314-4400. 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 subject (type or print)	Signature of Subject Date

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

_____	_____/ _____
Name of Investigator/ Research Team Member	Signature of Investigator/ Research Team Member Date

APPENDIX I

UNIVERSITY OF NEW MEXICO HEALTH SCIENCES CENTER
HIPAA[1] AUTHORIZATION TO USE AND DISCLOSE
PROTECTED HEALTH INFORMATION FOR RESEARCH PURPOSES

Title of Study: Effect of Combined Exercise, Heat, and Quercetin Supplementation on Whole Body Stress Response

Principal Investigator: **Dr. Pope Moseley, Ph.D.**
UNMHSC Department: **Department of Internal Medicine**
Mailing Address: **MSC 10 5550**
Co-Investigators: **Dr. Burke Gurney, Dr. Cliff Qualls, Dr. Karol Dokladny, Dr. Suzanne Schneider, Kevin Christmas, Matthew Kuennen, Trevor Gillum, Michelle Kulovitz**

Sponsor: GCRC
What is the purpose of this form? You have been asked to take part in a research study. The consent form for this study describes your participation, and that information still applies. This extra form is required by the federal Health Insurance Portability and Accountability Act (HIPAA). The purpose of this form is to get your permission (authorization) to use health information about you that is created by or used in connection with this research.

1. **What if I don't want my personal health information (PHI) to be used in this research study?** You do not have to give this permission. Your decision not to sign this form will not change your ability to get health care outside of this research study. However, if you do not sign, then you will not be allowed to participate in the study.

2. **What PHI am I allowing to be used for this research?** We will be using the results of your cholesterol screening and self reported medical history to determine if you qualify for this study. If you qualify, we will also take blood samples to examine heat shock protein and cytokine expression at rest and in response to exercise in the heat. Information gathered from these samples will not be included in your permanent health record.

3. **Where will researchers go to find my PHI?** The records we will need to see for this study will include the blood results sent to us from your screening blood sample and the medical history questionnaire you filled out at the beginning of this study. We will not need to access any of your medical records from your doctor or from the HRRC records.

4. **Who will be allowed to use my information for this research and why?** The researchers named above and their staff will be allowed to see and use your health information for this research study. It may be used to check on your progress during the study, or analyze it along with information from other study participants. Sometimes research information is shared with collaborators or other institutions. Your records may also be reviewed by representatives of the research sponsor or funding agency, the Food and Drug Administration (FDA) to check for quality, safety or effectiveness, or the Human Research Review Committee (HRRC) for the purposes of oversight and subject safety and compliance with human research regulations.

5. **Will my information be used in any other way?** Your information used under this permission may be subject to re-disclosure outside of the research study and be no longer protected under certain

[1] HIPAA is the Health Insurance Portability and Accountability Act of 1996, a federal law related to privacy of health information.

circumstances such as required reporting of abuse or neglect, required reporting for law enforcement purposes, and for health oversight activities and public health purposes.

6. **What if I change my mind after I give this permission?** You can change your mind and withdraw this permission at any time by sending a written notice to the Principal Investigator at the mailing address listed at the top of this form to inform the researcher of your decision. If you withdraw this permission, the researcher may only use and share your information that has already been collected for this study. No additional health information about you will be collected by or given to the researcher for the purposes of this study.

7. **What are the privacy protections for my PHI used in this research study?** HIPAA regulations apply to personal health information in the records of health care providers and other groups that share such information. There are some differences in how these regulations apply to research, as opposed to regular health care. One difference is that you may not be able to look at your own records that relate to this research study. These records may include your medical record, which you may not be able to look at until the study is over. The HIPAA privacy protections may no longer apply once your PHI has been shared with others who may be involved in this research.

8. **How long does this permission allow my PHI to be used?** If you decide to be in this research study, your permission to access and use your health information in this study may not expire, unless you revoke or cancel it. Otherwise, we will use your information as long as it is needed for the duration of the study.

I am the research participant or the personal representative authorized to act on behalf of the participant. By signing this form, I am giving permission for my personal health information to be used in research as described above. I will be given a copy of this authorization form after I have signed it.

_____	_____	_____
Name of Research Subject	Signature of Subject/Legal Representative	Date

_____

Describe authority of legal representative

_____	_____	_____
Name of Person Obtaining Authorization	Signature	Date

APPENDIX J

UNIVERSITY OF NEW MEXICO HEALTH SCIENCES CENTER
GENERAL CLINICAL RESEARCH CENTER
DATA AND SAFETY MONITORING PLAN

DOUBLE CLICK ON LINKS TO ACTIVATE THEM
Detailed instructions for completing the DSMP. Please contact the GCRC Research Subject Advocate at 272-6817 or email at creich@salud.unm.edu with any additional questions.

General Information		
HRRC #:	08-606	
Principal Investigator/ *PI Signature/Date:	Dr. Pope Moseley, M.D.	
PI contact information Phone/Fax/Email/MS Code:	272-6314/ pmoseley@salud.unm.edu /10-5550	
Protocol Title:	**Effect of Combined Exercise, Heat, and Quercetin Supplementation on Whole Body Stress Response**	
Co-Investigators:	Dr. Burke Gurney, Dr. Cliff Qualls, Dr. Karol Dokladny, Dr. Suzanne Schneider, Kevin Christmas, Matthew Kuennen, Trevor Gillum	
Study Contact/Phone/Email: (if different from PI)	Matthew Kuennen/505-314-4400/mkuennen@unm.edu	
FOR GCRC USE ONLY		
GAC Approval Date	**Final Risk Determination**	**RSA Office Approval Date**
Research Subject Advocate Signature		

**** PI signature/date is required***

STEP I: PROJECT OVERVIEW

Provide a brief summary (less than 250 words) of the proposed research

I A. Background: The human body produces substantial heat during exercise. Strenuous exercise in the heat can cause a significant increase in core temperature. In extremely severe circumstances, the combination of this heat production and insufficient venting to the environment can cause heat illness. One strategy to avoid heat illness is performance of repeated exercise (6 to 10 days) in hot ambient conditions, preconditioning the body to avoid future problems. This process is called heat acclimation (HA). One hallmark of heat acclimation is an improved sweating response, increasing the body's capacity to cool. Another is an increase in blood volume, which may help maintain intestinal blood flow during heat exposure. A third hallmark is the induction of a family of stress proteins that are integral for cellular integrity, commonly referred to as "heat shock proteins" (HSP). Following significant whole body heat stress, lack of induction of HSP_{72}, the most heat responsive family member, causes a significant reduction in thermotolerance.

Quercetin, an antioxidant found in apples, onions, and leafy greens, has been proposed for use as a nutritional supplement to prevent inflammatory cascades that result from oxidant stresses. It has caught the attention of the U.S. military, with reports indicating it may become a standard component of soldier's meals. However, we believe a potential oversight may exist. Quercetin is used in cellular experiments to block HSP_{72} synthesis, shown in our lab and others to reduce heat tolerance following a preconditioning stress. It appears that quercetin, while beneficial in combating oxidative stress, may actually undermine the utility of HA.

I B. Rationale for the Project: The US military has shown that strenuous exercise increases susceptibility to viral infection. Quercetin supplementation has been shown to reduce this susceptibility in both animal and human models. According to reports from the Department of Defense, further testing of quercetin has been authorized, with the possibility of incorporation into standard meals. A powerful antioxidant, quercetin has been suggested to act by suppressing HSP_{72}, which are vital for viral replication and transfer. Unfortunately, while vital in viral infection, HSP_{72} also protect against thermal injury.

Multiple studies have shown that moderate, repeated increases in body temperature cause HSP_{72} to be upregulated in intestinal tissues. This offers protection against heat illness by inactivating gut released bacteria before they infiltrate the circulation. Occludin, an intestinal protein vital to tight junction integrity, is also increased with repeated heat exposures, providing a significant barrier to bacterial passage. Using a cellular monolayer to mimic intestinal tissue, our laboratory showed an increase in occludin and HSP_{72} following a preconditioning heat stress. These cellular adjustments were sufficient to combat an excessive increase in permeability when the monolayer was challenged with an additional heat stress. When these same monolayers were cultured with quercetin, HSP_{72} and occludin were not induced, and permeability was significantly increased. If this were to translate to a human supplementation model, it would indicate that quercetin may block the preconditioning effect of prior heat stress.

I C. Study Aim(s): Our goal is to determine if, and to what extent, dietary supplementation with quercetin affects the heat shock protein response following 7 days of heat acclimation. We believe that athletes and others who use quercetin in combination with work in hot environments may be blocking specific adaptations that provide protection during repeated heat stress. We hypothesize that supplementing subjects with quercetin while they exercise to achieve heat acclimation will significantly suppress HSP_{72} levels, which may negate HSP_{72} ability to reduce intestinal permeability following heat acclimation.

I D. Design ***(include things like whether the study is investigator initiated, multi-center, pilot study, blinded, randomized, involves placebo, phase of study, prevention study):***

Overall Protocol

This investigator initiated study will involve a repeated-measures design (**2 conditions**) in which subjects will perform exercise in the heat. Each condition will begin with an **Intestinal Permeability Measurement (IP1)**, where subjects will be admitted to the General Clinical Research Center (GCRC), drinking a test solution and collecting their urine for 8 hours to measure intestinal integrity prior to heat stress. This will be followed by a **Heat Tolerance Trial (HT1)**, used as a baseline measurement of subject's capacity to work in the heat. Following HT1 each subject will perform **7 Days of Heat Acclimation (HA).** On days 1 and 7 of HA subjects will follow exercise by performing additional **Intestinal Permeability Measurements**, providing pre **(IP2)** and post acclimation **(IP3)** measurements of intestinal permeability. Day 6 of HA will provide a posttest measurement of subjects' capacity to work in the heat, and will be synonymous with **Heat Tolerance Trial 2 (HT2).**

Subjects will take quercetin during the seven days of HA in one condition, and placebo in the other. At least 1 month must elapse before a subject will be allowed to repeat the opposite condition. The order will be balanced (half will take quercetin first, the other half placebo). This will be a double blinded study. Neither the investigators nor the subjects will know whether a HA involves quercetin or placebo.

Due to the possibility of outside factors causing variations in intestinal permeability over the course of the study, **IP1** will always be used as a baseline condition, with **IP2** and **IP3** being scored relative (as percent change) to the first. Subjects will never be supplemented in **IP1**, as this might affect basal measurements of permeability. However, because quercetin supplementation may only exert a transient effect on intestinal permeability, subjects will always be supplemented (with either placebo or quercetin, according to condition) in **IP2** and **IP3**.

In all conditions, subjects will be required to follow guidelines on diet and exercise. They will be instructed to avoid caffeine, alcohol, and to stay well hydrated. They will be instructed to avoid exercise that is not directly outlined by the experiment (see attached). Their meals will be provided by the GCRC to ensure adequate nutrition and to avoid high dietary levels of quercetin. We are currently working with BioNutrition on this menu. To ensure compliance with this diet, subjects will fill out a food diary (see attached). The specific protocols for baseline screening, risk factor screening, IPs, HTs and the 7 days of HA are detailed below, followed by a description of data management procedures. It should be noted that all specific times of day are for illustrative purposes only, and may be adjusted per subject request (i.e. subject who is not a morning person).

Baseline Screening:

Subject will fill out a short questionnaire (see attached) that examines daily activities which may influence HSP expression that would be independent of the experimental protocol. Subjects who perform 1 or less of the activities on the screening questionnaire will move on to Risk Factor Screening.

Risk Factor Screening:

Prior to testing, young (between 18-39 years of age) healthy male subjects will be screened for cardiovascular risk factors. This includes one blood draw for the determination of lipid profile and glucose, which will be disposed after the analyses. Positive 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*** (>140/90 mmHg) or on antihypertensive medication

4. ***Impaired fasting glucose*** (>110 mg/dl)

5. ***Dyslipidemia*** (LDL>130 mg/dl or HDL<40 mg/dl, total serum cholesterol >200 mg/dl or on lipid lowering medication)

If subjects have an HDL >60 mg/dl, this is a negative risk factor and may negate a positive risk factor. Subjects who have more then 1 risk factor will be excluded from the study and no further action will be taken. They will be informed of the screening results, and will be provided with original reports of their results. Subjects will also have the option of having these reports sent directly to their personal doctor.

6. ***Low VO2 peak***: Each subject will perform a treadmill ergometer stress test in a thermoneutral room (19-22°C, 30%RH) to determine VO_2peak (peak oxygen consumption), a measure of cardiopulmonary fitness. The protocol for this test consists of a subject selected running speed and grade, used to produce a peak value within 8-12 minutes. Heart rate will be measured with a Polar heart rate monitor. This test will be conducted by a trained exercise physiologist and will be terminated if contraindications to exercise appear (American College of Sports Medicine Guidelines for Exercise Testing & Prescriptions, 2006), or at the subjects' request. The most immediate termination criteria include chest pain, symptoms of light-headedness, ataxia or failure of heart rate to increase with an increase in exercise intensity. Subjects with VO2 peak <40ml/kg/min will be assumed to live a sedentary lifestyle, which will be counted as a risk factor.

7. ***Impaired Body Composition***: Weight and height will be measured with an accurate scale (±50 g) and a stadiometer, respectively. BMI will be calculated from weight and height. Skin folds will be used to assess body fat percentage using 3 sites (chest, abdomen, thigh). Population specific equations will be used to calculate body fat from body density. Overfatness (BMI>30 kg/m^2 and/or body fat > 25%) will be counted as a risk factor.

Experimental Conditions:

INTESTINAL PERMEABILITY MEASUREMENT 1 (IP1): Subjects will report to the GCRC by 1700 hours on the evening prior to the permeability measurement. They will consume a standardized meal and snack before retiring for the evening. All meals eaten by subjects throughout the duration of the study will be provided by GCRC, and will be standardized for caloric content according to current dietary reference intakes (see attached for calculations used for individual subjects).

The next morning at 0600 subjects will consume a test solution containing 10 g lactulose (Sigma Aldrich, 61630) and 5 g d-mannitol (Sigma Aldrich, M9546) dissolved in 50 ml of distilled water. Following ingestion ALL urine will be collected for 8 hours, with urine collected during each 2 hour period (e.g. 0 – 2 hours, 2-4 hours, etc) being combined into individual, sealed flasks. 2 ml from each flask will be stored at –20ºC for future assessment of lactulose and mannitol content. The ratio of lactulose to mannitol in collected urine will serve as a marker of intestinal permeability, with the different collection time points assessing the time course of this response.

At 0800 subjects will consume a standardized breakfast (30% fat, 30% carbohydrate, 40% protein).

At 1000, 4 hours after ingestion of the test solution, a 20 ml blood sample from an antecubital vein will also be collected, allowing for measurement of HSP from peripheral blood mononuclear cells (PBMCs), and serum levels of HSP, the inflammatory cytokines TNF-a and Il-6, and the anti-inflammatory cytokine Il-10.

At 1400 subjects will finish with urine collection and be allowed to leave the GCRC, returning to the Exercise Physiology Laboratory the next morning to perform the first heat tolerance test (HT1).

HEAT TOLERANCE TEST 1 (HT1): Arriving at the lab at 0800, subjects will consume a standardized breakfast (30% fat, 30% carbohydrate, 40% protein).

At 0900 subjects will void, providing a urine sample for measurement of hydration. Subjects will then weigh nude, followed by insertion of a flexible thermistor 12 cm beyond the external anal sphincter for continuous monitoring of core temperature. Researchers will then attach 4 skin thermistors to subjects with elastic straps and tape, at the upper arm, chest, upper leg, and calf, to measure skin temperature. Subjects will then stand for 5-10 minutes, allowing these temperatures to stabilize.

At 0950 20 ml of blood (**pre HT sample**) will be drawn from an antecubital vein, allowing for measurement of pre-exercise HSP from peripheral blood mononuclear cells (PBMCs), and serum levels of HSP, the inflammatory cytokines TNF-a and Il-6, and the anti-inflammatory cytokine Il-10.

At 1000 subjects will begin the heat tolerance test, which will consist of 30 minutes of treadmill jogging at 70% of VO2pk. Immediately following exercise subjects will remain standing, and 20 ml of blood (**post HT sample**) will be drawn from an antecubital vein. This blood will be assayed for all the same components as the pre HT sample. Subjects will then leave the climactic chamber and void into a calibrated beaker, providing a post exercise urine sample for analysis of hydration. This will also allow measurement of urine volume, avoiding error when measuring post exercise body weight. Subjects will then completely disrobe, towel dry, and provide a nude weight.

After completing HT1, subjects will wait for one week prior to continuing with the experiment, providing sufficient time for washout of any transient heat exposure effects. After 1 week the subject will begin the seven day HA protocol.

SEVEN DAY HA PROTOCOL: Subjects will perform 7 heat exercise bouts within 10 calendar days. These bouts will be performed at the same time of day for each individual subject, in either the quercetin supplemented or placebo condition. These conditions will be counterbalanced to avoid order effects, and at least 1 month will elapse before subjects are allowed to complete the opposite condition. To further control the study, both researchers and subjects will be blinded as to what condition the subject is performing. We are currently working with Nancy Morgan to determine if the UNMH Pharmacy can dispense the supplement/placebo. If this is the case Biostatistics will generate a randomization table to determine supplement order in the repeated trials, distributing all supplementation to subjects. If Pharmacy cannot dispense the supplement, we have recruited someone from our laboratory who is not directly involved in this study (Dr. Burke Gurney). He will be responsible for providing subjects supplementation, and will be the only one aware of which supplement the subject is receiving. Quercetin is an antioxidant found in high concentration in apples, onions, and leafy greens. The quercetin we propose to use in this study is a food grade high purity material, produced by Quercegen Inc. under license for E. Merck. All quercetin from this company comes with a high performance liquid chromatography (HPLC) tracing to show purity (see attached for sample tracing). Purity is assessed in each container individually, so although this tracing is for the exact quercetin aglycone powder we propose to use (QU995, Quercegen Pharma), we will receive a separate tracing upon purchase. Quercetin can be purchased over the counter from health food stores and does not require dispersal by a pharmacist.

In the supplemented condition subjects will receive Tang powder and 1000 mg pure quercetin aglycone powder, while in the placebo condition subjects will receive Tang powder in combination with 1000 mg powdered food coloring. In both conditions, the Tang powder and supplement (quercetin or placebo) will be combined with 16oz of water. Subjects will consume the supplement two times each day, immediately prior to their first and last daily meals. The dosage of this supplement (30 mg/kg/d) was chosen based on quercetin's bioavailability and a similar study where subjects exercised in the heat. Subjects achieved plasma levels of quercetin around 2.5 uM, and we desire to replicate this dosage. Other than subjects receiving quercetin or placebo, all other aspects between the (2) seven day heat acclimation conditions will be identical. However, the protocol for individual **HA Exercise Bouts** within each condition will vary slightly, with these variations detailed in the following sections. To aid interpretation of study design, a pictorial depiction has been provided (see attached).

Protocol for HA Exercise Bout Day 1 + Intestinal Permeability Measurement 2 (IP2): Subjects will report to the GCRC by 1700 hours on the evening prior to the first day of HA. They will consume a standardized meal and snack before retiring for the evening.

The next morning at 0600 subjects will consume a test solution containing 10 g lactulose (Sigma Aldrich, 61630) and 5 g d-mannitol (Sigma Aldrich, M9546) dissolved in 50 ml of distilled water. Following ingestion ALL urine will be collected for 10 hours, following the same protocol as discussed previously.

At 0800 subjects will consume the supplement or placebo beverage, immediately prior to a standardized breakfast (30% fat, 30% carbohydrate, 40% protein).

At 0830 subjects will be transported from the GCRC to the Exercise Physiology Lab in Johnson Center. Once there they will void, providing a urine sample for measurement of hydration. Subjects will then weigh nude, followed by insertion of a flexible thermistor 12 cm beyond the external anal sphincter for continuous monitoring of core temperature. Researchers will then attach 4 skin thermistors to subjects with elastic straps and tape, at the upper arm, chest, upper leg, and calf, to measure skin temperature. Subjects will then stand for 5-10 minutes, allowing these temperatures to stabilize.

At 0950, ~4 hours after ingestion of the test solution, a 20 ml blood sample from an antecubital vein will be collected (**pre Exercise sample**), allowing for measurement of HSP from peripheral blood mononuclear cells (PBMCs), and serum levels of quercetin, HSP, the inflammatory cytokines TNF-a and Il-6, and the anti-inflammatory cytokine Il-10.

At 1000 the subject will begin the exercise portion of the challenge, which will consist of 100 minutes of treadmill jogging at a workload (~50% VO2pk) designed to increase core temperature to 39ºC. Subjects, clothed in shorts and a t-shirt, will exercise for 50 minutes in a climactic chamber maintained at 43ºC, 40% relative humidity, then rest for 30 minutes. During the rest period, while still within the climactic chamber, subjects will strip naked, towel dry, and gather nude body weight. Subjects will then be provided with distilled water at a volume equal to weight loss. After consuming the water at a rate of 20% total volume every 5 minutes, subjects will resume exercise, finishing the final 50 minutes of jogging in a manner identical to the first.

At ~1200, immediately following exercise subjects will remain standing, and 20 ml of blood (**post Exercise sample**) will be drawn from an antecubital vein. This blood will be assayed for all the same components as the pre Exercise sample. Subjects will then void to provide a post test urine sample for assessment of exercise induced dehydration. After voiding subjects will strip naked, towel dry, and gather nude bodyweight. Upon showering subjects will be transported back to the GCRC to complete **IP2**

At 1230, upon arrival at the GCRC, subjects will be seated comfortably, and urine collection will continue.

At 1300 a blood sample from an antecubital vein will be collected (**1 hour Post Exercise sample**)
This blood will be assayed for all the same components as the pre and post Exercise samples.

At 1400 a blood sample from an antecubital vein will be collected (**2 hour Post Exercise sample**) This blood will be assayed for all the same components as the pre and post Exercise samples.

Subjects will then be released GCRC, returning to the Exercise Physiology Laboratory the next morning to continue with the second day of HA. The protocol for the HA exercise bouts will be nearly identical from the 2nd through the 5th days of HA. The only difference will be the pre and post exercise blood draws, which will not be performed on days 2 and 4.

Protocol for HA Exercise Bout Days 2-5: Subjects will consume the supplement or placebo beverage at 0800, immediately prior to a standardized breakfast (30% fat, 30% carbohydrate, 40% protein).

At 0900 subjects will void, providing a urine sample for measurement of hydration. Subjects will then weigh nude, followed by insertion of a flexible thermistor 12 cm beyond the external anal sphincter for continuous monitoring of core temperature. Researchers will then attach 4 skin thermistors to subjects with elastic straps and tape, at the upper arm, chest, upper leg, and calf, to measure skin temperature. Subjects will then stand for 5-10 minutes, allowing these temperatures to stabilize.

At 0950 20 ml of blood (**pre Exercise sample**) will be drawn from an antecubital vein, allowing for measurement of HSP from peripheral blood mononuclear cells (PBMCs), and serum levels of quercetin, HSP, the inflammatory cytokines TNF-a and Il-6, and the anti-inflammatory cytokine Il-10.

At 1000 subjects will begin exercise, which will consist of 100 total minutes of treadmill jogging, divided into two 50 minute bouts. Subjects, clothed in shorts and a t-shirt, will jog for 50 minutes in a climactic chamber maintained at 43ºC, 40% relative humidity. The goal of this exercise is to increase core temperature to 39ºC. As

subjects become heat acclimated (over the course of 7 days of HA) the workload will become easier and core temperature will not rise as much, causing researchers to increase the workload. For example, on the first day if workload was set at 50% VO2pk, it may be necessary for researchers to increase this workload to 55% VO2pk by the end of HA. After finishing the first jogging bout, the subject will remain inside the climactic chamber and rest for 30 minutes. During this rest period subjects will completely disrobe, towel dry, and re-weigh. Subjects will then be provided a commercially available children's drink (TANG: mixed per manufacturers instructions) at a volume equal to weight loss. After consuming the rehydration drink at a rate of 20% total volume every 5 minutes, subjects will complete the final 50 minutes of treadmill exercise, requiring adjustments to workload in a manner similar to the first.

At ~1200, immediately following exercise subjects will remain standing, and 20 ml of blood (**post Exercise sample**) will be drawn from an antecubital vein. This blood will be assayed for all the same components as the pre HT sample. Subjects will then leave the climactic chamber and void into a calibrated beaker, providing a post exercise urine sample for analysis of hydration. This will also allow measurement of urine volume, avoiding error when measuring post exercise body weight. Subjects will then completely disrobe, towel dry, and provide a nude weight.

After these measurements are taken subjects will be provided with an additional bottle of the supplement or placebo powder (depending on condition), which they will mix with 16oz of water and consume immediately prior to their last meal of the day. When subjects report back to the Exercise Physiology Laboratory each morning for the HA exercise bouts, they will return the sports bottle to improve compliance with the supplementation protocol.

***Protocol for HA Exercise Bout Day 6 (Equivalent to* HEAT TOLERANCE TEST 2(HT2)):** This test will be identical to HT1. After subjects finish with posttest measurements they will be provided with an additional bottle of the supplement or placebo powder (depending on condition), which they will mix with 16oz of water and consume immediately prior to their last meal of the day. When subjects report back to the Exercise Physiology Laboratory the next morning for the final HA exercise bout in this condition, they will return the sports bottle to improve compliance with the supplementation protocol.

Protocol for HA Exercise Bout Day 7: This test will be identical to HA Exercise Bout Day 1, and will include the third intestinal permeability measurement (**IP3**). After completing this 7th day of HA subjects will leave the lab, not reporting back to complete the second HA condition for at least (1) calendar month. After completing the second condition subjects will answer a short questionnaire to determine in which condition they thought they were supplemented with quercetin (see attached).

I E. Study Population *(include characteristics and number of subjects to be enrolled):* We will recruit up to 11 physically fit male subjects, hoping that at least 8 subjects will complete the 2 conditions. We will collect data until 8 subjects have completed all runs and their data have been assessed. In previous studies in our laboratory involving HSP, exercise, and protocol adherence 2-3 subjects usually are either disqualified or cannot complete all the trials for scheduling or technical reasons. All subjects will be screened for cardiovascular risk factors, and only will be included if they have less than two CAD risk factors. Subjects must also have a VO_2max > 40 ml/kg/min ml/kg/min, as well as a body fat percentage < 25%. These criteria help to insure uniformity of the subjects and that they have a fitness level sufficient to complete the exercise procedures.

I F. Outcome Measures:
Outcome Measures of IP1: Mannitol is a small molecule, which will be able to pass through the cells of the small intestine, while lactulose is a much larger molecule, requiring passage between cells, through tight junctions. Due to their different molecular sizes, these two sugar molecules have significantly different absorptive capacities. This test will provide basal levels of HSP, cytokines, serum endotoxin, glutathione peroxidase, and intestinal permeability.

Outcome Measures of the HT1 and HT2: Subjects core and skin temperatures at the end of each HT will be compared within conditions to see if subjects exercise core temperature response was reduced following HA. This difference in peak core and skin temperatures between HT1 and HT2 will also be compared between the supplemented and placebo conditions, to determine if quercetin supplementation influenced this response.

Collected blood will be layered over Histopaque and centrifuged, causing the blood to separate into erythrocytes, PBMCs, and plasma. Erythrocytes will be disposed of according to OSHA regulations. PBMCs will be extracted from separated blood, washed twice, and stored at –80°C. 4 ml of plasma will be aliquoted and stored at –80°C. After a sufficient number of subjects have been completed, the stored PBMC samples will be assayed for HSP, and the stored plasma samples will be assayed for HSP, TNF-a, Il-6, and Il-10. For each subject we will plot the differences in HSP, TNF-a, Il-6, and Il-10 between the two HTs. These measurements will also be compared

between the supplemented and placebo conditions for each individual subject, to determine if either condition influenced response. Plasma quercetin values will also be assessed.

Outcome Measures of the 7 days of HA and IP2 and IP3: Workload (treadmill km/hr) will be assessed across days of HA to determine if work performance in the heat was significantly improved. Day 6 of HA will not be included in these comparisons, as the workload on this trial was set at 50% VO2pk. This trial is equivalent to the second HT and has been included above.

Collected blood will be layered over Histopaque and centrifuged, causing the blood to separate into erythrocytes, PBMCs, and plasma. Erythrocytes will be disposed of according to OSHA regulations. PBMCs will be extracted from separated blood, washed twice, and stored at –80°C. 4 ml of plasma will be aliquoted and stored at –80°C. After a sufficient number of subjects have been completed, the stored PBMC samples will be assayed for HSP, and the stored plasma samples will be assayed for HSP, endotoxin, TNF-a, Il-6, and Il-10. Plasma quercetin levels and glutathione peroxidase will also be assessed. For each subject we will plot the differences between HSP, serum endotoxin, TNF-a, Il-6, and Il-10 across the 7 days of HA. We will also plot these variables for each individual subject across the supplemented and placebo conditions, to see if the supplement caused any significant differences. Again, day 6 of HA will not be included in these comparisons, because the set workload would confuse blood measurements. Because these blood samples are equivalent to the second HT, they been included above.

In each condition we will also examine the ratio of lactulose/d-mannitol in the urine collected during the GCRC confinement following days 1 and 7 of HA, converting these ratios to a change score by setting the pre heat acclimation permeability measurements to a value of 1.0. By comparing these ratios we will be able to see if either condition resulted in a significant difference in intestinal permeability. By comparing these differences between the two conditions, we will be able to see if quercetin supplementation had any effect on intestinal permeability when compared to placebo.

I G. Purpose: ☐ Therapeutic ☒ Physiologic ☐ Epidemiologic ☐ Diagnostic ☐ Genetic testing

STEP II: CLASSIFICATION OF RISK

II A. STUDY POPULATION(S)

Check all characteristics that apply to all study groups (i.e., experimental groups and control groups) **Use "Other Population Characteristics" to specify characteristics not listed here.

Study Population(s)		
Healthy Adult Populations	**Vulnerable Populations**	**Seriously Ill/High Risk Populations**
☒ Over age 18	☐ Children	☐ Premature infants/VLBW/ELBW
☒ Healthy volunteers	☐ Cognitively impaired	☐ Patients in ICU
☒ Not pregnant	☐ Economically or educationally disadvantaged	☐ High risk (> 5%) of serious adverse events from underlying condition Specify Condition:
	☒ Students/staff/employees	☐ Cancer
	☐ Pregnant women/Fetuses	☐ Immune-compromised
	☐ Prisoners	☐ End-Stage Renal Disease
	☐ Targeted or limited to specific ethnic group(s)	☐ Attribution of adverse events may be difficult

***Other Population Characteristics*:**

II B. RESEARCH PROCEDURES AND INTERVENTIONS

Enter each procedure or intervention that will be performed during the research as well as the level of risk associated with it **(Use Procedure/Intervention Risk Classification List as a reference guide)**

Research Procedures and Interventions	
Procedure being done for research purposes	**Risk level for subject (mark appropriate column)**

	Minimal/Low	Moderate	High
Treadmill Exercise Pretesting (x1)	☒	☐	☐
Anthropometric/body measurements(x1)	☒	☐	☐
Treadmill Exercise in the heat (x8 each condition	☐	☒	☐
Rectal thermistor to monitor core temperature	☒	☐	☐
Quercetin/Placebo	☒	☐	☐
Questionnaires of non-sensitive nature	☒	☐	☐
Blood draw -35 Blood Samples = 685ml, over 11+ weeks	☒	☐	☐
Mannitol/lactulose ingestion and 8 hour urine collection (x3 each condition)	☒	☐	☐
Controlled diet	☒	☐	☐

II C. OVERALL RISK CLASSIFICATION

Indicate your overall assessment of risk classification by placing a check mark next to 'Low', 'Moderate', or 'High'. Describe any factors that affect your determination of the overall risk, taking in to consideration the highest marked risk for the study population, procedure(s), or intervention(s).

Overall Risk Classification			
PI's Overall Assessment of Risk Level:	☐ Minimal Risk ☒ Low Risk ☒ Moderate Risk ☐ High Risk	**RSA's Overall Assessment of Risk Level:** ***(To be completed by RSA)***	☐ Minimal Risk ☐ Low Risk ☐ Moderate Risk ☐ High Risk

PI rationale for Overall Risk Classification (between low and moderate):
Exercise tests are performed during this study, including a maximal symptom-limited test during screening to assess fitness level and to prescribe the exercise setting (50% VO2pk) used during exercise in the heat. This risk will be minimized by carefully screening of subjects (must have less than 1 CAD risk factor), recruiting men less than 40 years of age that are healthy and active, and monitoring of heart rate, symptoms and blood pressure. Exercise testing has a very low risk of death (<0.01%) and complications of the heart (<0.01%) in patients suspected of cardiovascular disease. The risk for healthy, young subjects recruited in this study would be even lower. Exercise tests will be performed by experienced and qualified exercise physiologists.

During exercise in the heat, the risks include those mentioned above, and in rare instances, heat exhaustion, heat stroke, or death. The conditions (environmental heat, exercise) involved in this study pose a small risk of heat illness. This is unlikely as the subjects are monitored continuously and heat illness usually does not occur at core temperatures below 41°C. Subjects core temperature will be monitored continuously during exercise in the heat. Subjects will be removed from heat if core temperature exceeds 40°C. Heart rate will also be monitored continuously and subjects will be removed from the heat chamber immediately if HR exceeds 98% predicted HRmax. Subject discomfort also is examined during heat exposures and subjects will be removed if they experience any of the signs (confusion, ataxia) or symptoms (severe nausea, hypotension) of heat illness. Using similar termination criteria in previous studies in our laboratory, no subjects experienced severe heat illness or exhaustion and all immediately recovered upon leaving the heat chamber.

In the event heat illness does occur, all researchers are trained on response. A large cooler filled with ice water will always be kept immediately outside the climactic chamber. At either subject request or researcher's decision, subjects will be removed from the chamber and placed on their back with the legs elevated. Towels will be wetted with this ice water and placed on subject's forehead, neck, and arms. The subject's hand will be placed in the ice water filled cooler. Recent reviews by the American College of Sports Medicine note cold water immersion as the gold standard for prevention of heat illness.

Venous blood draws pose a risk of local infection (<0.1%), hematoma (<0.2%), fainting (<0.01%) and light-headedness (<0.1%). To minimize risk of hematoma and infection, on days where more then 2 needle sticks would be required of subjects, a venous catheter will be inserted to avoid repeat sticks. Further minimizing risk of hematoma or infection, the blood drawn in this study will be performed by persons trained in phlebotomy.

The Exercise Physiology Laboratory has emergency equipment (crash cart, oxygen, AED) and the personnel who work in this laboratory are trained to recognize problems during exercise testing (CPR trained exercise physiologists). Emergency procedures are practiced to immediately call a physician (2 min away in the Student Health Center), call dial 911 to activate the EMT system (5 minutes away on other side of the Johnson Field).

Subjects could then be transported to the UNM hospital (10 min away).

The risk of compromising subject confidentiality has also been addressed. To minimize this risk, all data collected in this study will be stored on data sheets (with subject codes only) or in password protected computers: again without identifiers. A code key that attaches subjects to their codes will be kept in a separate locked file cabinet, and only accessible by the study coordinator, Matthew Kuennen. Only investigators in this study have access to the key for the locked file cabinet where the data are stored or the password to obtain access to the computer files. Consent forms, blood screening results and medical history/activity questionnaires and the subject code key are kept in a locked file cabinet in the office of Dr. Suzanne Schneider, Johnson Center Room B145. Data will be kept for 5 years after publication of the data, then shredded. Data from subjects who are screened out or who do not complete the study will be destroyed as soon as the data are published (in case we later decide to use some of the data). Blood products and collected urine will be discarded immediately after analysis.

This study will help determine if quercetin is decreasing HSP_{72} synthesis during repeated exercise in the heat. HSP_{72} works to prevent cellular aggregation, protein denaturation, and increase the likelihood of survival in the face of multiple stressors. If HSP_{72} synthesis is suppressed, and is found to reduce acclimatory adaptations, this information can be utilized by firefighters, military, summer sport athletes, and anyone else who lives or works in a hot environment, to avoid increasing their susceptibility to heat illness. If HSP synthesis is not decreased, or if HSP synthesis is decreased but there is no effect on acclimatory responses, this research would still benefit society, as answering this one question would likely spawn many more.

The risks in this study are minimized because of the stringent screening and monitoring procedures. It is considered reasonable in relation to the anticipated benefits. Prior research in this area has not indicated any increase in risk resulting from quercetin supplementation, exercise in the heat, or the combination thereof. Our study proposes to use a protocol that is much less stringent then research that has been performed previously. **Specifically:**

1. We propose to supplement subjects with 1000mg quercetin aglycone twice daily. Other studies in the literature have administered a much larger amount (4000mg) as a single dose, without subjects reporting any ill effect.

2. Quercetin has been examined in combination with repeated, strenuous exercise. Nieman and others (2007) examined the effect of 3 weeks of quercetin supplementation on 3 consecutive days of intense cycling (3 hours per day at 57% maximal workload). The supplement used by Nieman's group (QU995; Quercegen Pharma) is not only the same supplement we propose to use, but was also administered the same way were propose (in combination with Tang). Subjects did not report any ill effect of quercetin supplementation.

3. Quercetin has also been examined in combination with strenuous exercise in the heat. Cheuvront and others (2008) examined the same dosage of quercetin we propose, supplied by the same company we propose, and had subjects exercise in the same ambient conditions we propose. They also did not report any problems with quercetin itself, or with heat illness. In a separate experiment, Nieman and others (2007) examined the effect of 3 weeks of quercetin supplementation on performance in the Western States Endurance Run. This 160 km race, in which ambient temperatures regularly exceed 38°C, is commonly referred to as the most demanding race in America. Average completion times are in excess of 25 hours. No incidence of heat related illness was reported.

RSA rationale for Overall Risk Classification: (***To be completed by RSA***)
This research is studying the use of a nutritional supplement, quercetin vs. placebo in healthy adult males. It is unclear whether there is a risk for septicemia in subjects if intestinal permeability is increased, and if so, what type of monitoring would be required to assess for early signs of this complication. An appropriate plan is in place should a medical emergency occur during exercise testing. This study is initially assessed as moderate risk and will require annual monitoring by an independent monitor for safety. If any problems are encountered (heat illness or AE's from quercetin), the monitoring frequency may need to be re-evaluated.

STEP III: ADVERSE EVENTS

III A. ANTICIPATED ADVERSE EVENTS

Indicate any anticipated adverse events that could potentially occur during participation in the research study and up to 30 days after completion of study participation. Please indicate whether each event is: (a) serious, (b) research related or (c) related to the participant's underlying condition.

Anticipated Adverse Events			
Adverse Event	**Serious Adverse Event?**	**Related to the Research?**	**Related to the Underlying Condition?**
Risks of blood draws: local infection, pain, hematoma, fainting, and light-headedness .	☐Yes ☒No	☒	☐
musculoskeletal injury or a cardiovascular event during exercise testing	☒Yes ☐No	☒	☐
fatigue, nausea, fever, dizziness, fainting, breathlessness/dyspnea, or psychological stress (ie., panic) during exercise testing	☐Yes ☒No	☒	☐
Breach of confidentiality	☐Yes ☒No	☒	☐
Risk of mild heat illness: fatigue, nausea, fever, tachycardia, rash, muscle cramping, mild inflammation, dizziness, fainting, breathlessness/dyspnea, psychological stress	☒Yes ☐No	☒	☐
Skin irritation from EKG leads	☐Yes ☒No	☒	☐
Discomfort from rectal thermistor	☐Yes ☒No	☒	☐
Risks of quercetin: GI discomfort, increased intestinal permeability, limited bacterial leak, mild inflammation, fever	☐Yes ☒No	☒	☐

III B. MONITORING ADVERSE EVENTS

Place a check mark next to each method that will be used to monitor for adverse events. Use "Other Monitoring Procedures" to indicate monitoring procedures not included here. Please indicate the frequency with which each monitoring method will be performed (e.g., each visit, continuously, hourly, daily, weekly, monthly, annually, and so on).

Monitoring Adverse Events					
Subject Report		**Frequency**	**Safety Labs**		**Frequency**
☒	Spontaneous subject report	PRN	☐	Chemistry panel	
☐	Subject diary		☐	Pregnancy test	
☒	Subject interview	After Exercise	☐	CBC w/differential	
☐	Other: (specify):		☐	Other: (specify):	
Periodic Observations		**Frequency**	**Continuous Monitoring**		**Frequency**
☒	Vital signs		☒	Heart rate by EKG	During each bout of exercise testing
☐	ECG		☐	Respiration rate	
☐	Medical record review		☐	Oxygen saturation	
☐	Follow-up outpatient visit		☒	Blood pressure	End of each Exercise challenge
☐	Chest X-ray		☒	Other: (specify): core and skin temperatures	continuous during exercise
☐	Other (specify):		**Other Monitoring Procedures**		**Frequency**
Study Visit Monitoring Personnel		**Frequency**	☐		
☒	Principal Investigator	PRN	☐		
☒	Co-Investigator (specify) Dr. Karol Dokladny, Dr. Suzanne Schneider, Kevin Christmas, Trevor Gillum	continuous during exercise, q2h during GCRC admission	☐		
☒	Study Coordinator(specify)Matthew Kuennen	continuous during exercise, q2h during	☐		

		GCRC admission			
☒	GCRC Inpatient Nurses	Hourly	☐		
☐	GCRC Outpatient Nurses		☐		
☐	GCRC Scatterbed Nurses		☐		
☐	Other (specify):		☐		

III C. GRADING ADVERSE EVENTS

There are several standardized scales designed for disease-specific trials to grade AEs by severity. These scales may also be applicable to other studies, but may not be appropriate for all studies. In the event that a standardized scale is not appropriate for a particular study, the PI should indicate a more appropriate grading scale that will be used to evaluate the severity of adverse events. Check the grading scale that will be used to grade the severity of any adverse events.

Grading Adverse Events	
☒	**Adverse Events in Adults** Common Terminology Criteria for Adverse Events (CTCAE, v 3.0) - Cancer Therapy Evaluation Program (CTEP) CTCAE v.3.0
☐	**Adverse Events in Newborns Under 3 Months of Age** NIH National Institute of Allergy and Infectious Diseases, Division of AIDS Toxicity Tables Pediatric Toxicity Table < 3 months.pdf
☐	**Adverse Events in Children and Newborns Over 3 Months of Age)** NIH National Institute of Allergy and Infectious Diseases, Division of AIDS Toxicity Tables Pediatric Toxicity Table > 3 months.pdf
☐	**Other standardized grading scale. Specify (or attach paper/electronic copy):**

III D. REPORTING ADVERSE EVENTS

Place a check mark in all appropriate columns to indicate all entities to which AE reports will be submitted.

Reporting Adverse Events			
☒	GCRC RSA Office	☐	NIH Sponsor Institute (specify):
☒	HRRC	☐	Indian Health Service (IHS) IRB
☐	Other Sponsor (specify):	☐	Navajo IRB
☐	FDA	☐	Other Tribal IRB or Council (specify):
☒	Independent Monitor	☐	VA Research and Development Committee
☐	Data and Safety Monitoring Board/Committee	☐	Other (specify):

STEP IV: SAFETY AND EFFICACY REVIEW

IV A. DATA FOR INTERIM SAFETY AND EFFICACY REVIEW

Annual Review of the following data are **REQUIRED** by HRRC and GCRC: 1) The number of participants **screened**, 2) The number of participants **enrolled**, 3) The number of participants that withdraw or are **withdrawn**, 4) **Adverse events**, and 5) **Protocol violations/deviations**.

In the table below, indicate what data will be submitted for interim safety and efficacy review (check all that apply):

Data for Interim Safety and Efficacy Review			
Interim Safety Review		Interim Efficacy Review	
☒	Safety lab/data	☐	Primary outcome indicators (specify):
☒	Research-related adverse events	☐	Secondary outcome indicators (specify):
☐	Additional safety data (specify)		

IV B. FREQUENCY OF INTERIM SAFETY AND EFFICACY REVIEW

Indicate the time intervals when interim safety/efficacy review will occur. If different data will be reviewed at different intervals, please provide brief details of the schedule.

Frequency of Interim Safety and Efficacy Review	
☒ Every 12 months (At the time of HRRC renewal)	☐ After every _____ subjects enrolled
☐ Every 6 months – starting date:	☐ After subjects, then (specify)
☐ Every 3 months - starting date:	☐ Other (specify):

IV C. CRITERIA FOR PROTOCOL REVISION OR STUDY SUSPENSION

Specify any safety and efficacy outcomes defined in the protocol which would indicate a need to amend the protocol or to discontinue one or more arms of the protocol (i.e. unanticipated events, early demonstration of efficacy, inferiority, or futility where the results will not confirm nor reject the hypothesis, etc.)?
(NOTE: This does not refer to withdrawing single subjects)

Criteria For Study Suspension
None

IV D. SAFETY AND EFFICACY REVIEW PERSONNEL

Indicate who will review the study (check all that apply) and what role they will have in the review (e.g., review AE reports, analyze safety data, interim efficacy analysis).

Safety and Efficacy Review Personnel				
Reviewer(s)	Review enrollment data	Review individual AE reports	Periodic safety analysis	Periodic efficacy analysis
GCRC Research Subject Advocate	☒	☒	☐	☐
HRRC	☒	☒	☐	☐
Principal Investigator	☒	☒	☒	☐
Committee made up of PI/Co-investigators	☒	☒	☒	☐
Independent Monitor (Name) Chris McGrew, MD Email Address: CMcgrew@salud.unm.edu	☐	☐	☐	☐
DSMB or DSMC (Independent, Non-Independent, or GCRC) (*Attach DSMB Roster and Charter**)	☐	☐	☐	☐
Other (specify):	☐	☐	☐	☐

*A DSMB ***Roster*** is a list of DSMB members including each member's name, credentials, affiliations, and roles on the DSMB. A DSMB ***Charter***, also called a Constitution or Standard Operating Procedures is a description of specific activities, procedures, methods, and decision criteria that the DSMB will use in it's review of safety and efficacy data. If necessary, a GCRC DSMB may be created to review the research when data and safety monitoring by a board/committee is required and is not available from a sponsoring organization.

DSMB ROSTER *(may attach separate list)*

APPENDIX K

Physician Order Sheet
HRRC/CTSC 08-606: Effect of Combined Exercise, Heat, and Quercetin Supplementation on Whole Body Stress Response

PI and Physician: Pope Moseley, M.D. Phone: 272-6314, Lab Phone: 272-5882
Study Coordinator: Matt Kuennen. Phone: 314-4400, Lab Phone: 277-2658

Subject Name: _____ Date: _____

Admit to 5E: Private Room **Attending**: Pope Moseley, MD

Estimated length of stay: 14 hours

Diagnoses: HRRC 08-606: Effect of Combined Exercise, Heat, and Quercetin Supplementation on Whole Body Stress Response.

Condition: Study involves young, healthy patients who will perform daily exercise in a hot room to acclimate to desert heat. Testing if nutritional supplement quercetin has any effect on heat acclimation.

Vital Signs: Day 1 on Admit: BP, Pulse, Temp, Weight

Activities: Day 1: check in, vital signs, supper, pm snack *Provide patient 4 large brown urine collection containers for mannitol/lactulose urine sugar test on day 2

Day 2: discharge

Diet: CRC Metabolic Kitchen has orders for low quercetin meals. Ensure subjects eat all food, drink all fluids provided.

Diet Restrictions: No caffeine after 2100 day 1
Begin overnight fast 2300 day 1
No food between 0700 and 1500 day 2 (water ad-lib)
No caffeine until 1500 day 2

Meds: Pt may bring prescription meds from home and self administer

Labs: Investigator provides patient mannitol/lactulose sugars dispensed in distilled water at 0700 day 2. Immediately after consumption begin 8 hour urine collection. Aliquots given to Core Lab, who has orders for processing.

Discharge Date: _____ Discharge Time:___0700_____

If any problems, concerns, or questions please notify Matt Kuennen: 314-4400; 277-2658.

APPENDIX L

Flowsheet for Protocol #08-606 "Effect of Combined Exercise, Heat, and Quercetin Supplementation on Whole Body Stress Response"

Principle Investigator: Pope Moseley, MD (Phone 272-6314)
Co-Investigators: Dr. Burke Gurney, Dr. Cliff Qualls, Dr. Karol Dokladny, Dr. Suzanne Schneider, Kevin Christmas, Matthew Kuennen, Trevor Gillum

For study questions please contact Matt Kuennen, study coordinator:
work: 277-2658; 277-1419 cell: 314-4400 email:mkuennen@unm.edu

Admission: coordinated by Matt Kuennen

Patient Study Number_____ Date _____ Admission # _____

Time	Study Date	Party Responsible	Procedures	Please initial when complete
	1___	not applicable	NONE	
1700	2 ___	Inpatient	Admit patient to Private Room 5E Vital signs on admission BP _____/_____ Pulse_____ Temp_____ Weight_____	
1720	2	Inpatient	Provide patient supper MUST EAT/DRINK ALL PROVIDED	
2000	2	Inpatient	Provide patent evening snack MUST EAT/DRINK ALL PROVIDED	
2100	2	Inpatient	NO CAFFEINE AFTER 2100	
2300	2	Inpatient	PATIENT BEGIN OVERNIGHT FAST	
0645	3__	Inpatient	Pick up 5g MANNITOL/ 10g LACTULOSE aliquots and mixing cup from 5E medicine storage:	
0650	3	Inpatient	Mix mannitol and lactulose per instructions provided by Pharmacy	
0700	3	Inpatient	Provide patient w/ man/lac beverage: MUST DRINK ENTIRE SOLUTION	8 hr fast
0700	3	Inpatient	-All urine produced by patient must be collected for 8 hours (0700 – 1500hrs) -Urine collected into 2 hr aliquots (e.g. 0-2 hrs; >2-4 hrs; >4-6 hrs; >6-8 hrs) -Provide patient with sterile urine collection container for 0-2 hr urine collection	
0715	3	Inpatient	-22 ml blood draw from antecubital vein -Use 18 gauge needle, draw slowly -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top]	15 min post man/lac

			-Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	
0715	3	Core Lab	-Separate blood according to provided “Blood Collection Protocol” -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
0750	3	BioNutrition	Reconstitute 50g Our Family Orange Breakfast Drink powder with 16.9 oz bottled H2O	
0800	3	BioNutrition	-Provide beverage to patient -MUST DRINK ENTIRE BEVERAGE	1 hr post man/lac
0900	3	Inpatient	-Provide patient with new urine container for >2-4 hr urine collection -Walk 0-2 hr urine container to Core lab immediately	
0900	3	Core Lab	-Record urine volume in 0-2 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1100	3	Inpatient	-Provide patient with new urine container for >4-6 hr urine collection -Walk >2-4 hr urine container to Core lab immediately	
1100	3	Core Lab	-Record urine volume in >2-4 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1300	3	Inpatient	-Provide patient with new urine container for >6-8 hr urine collection -Walk >4-6 hr urine container to Core lab immediately	
1300	3	Core Lab	-Record urine volume in >4-6 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1500	3	Inpatient	-D/C urine collection -Walk >6-8 hr urine container to Core lab immediately	
1500	3	Core Lab	-Record urine volume in >6-8 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1500	3	BioNutrition	-Provide patient with late lunch -Provide patient with supper, evening snack, and 2 bottled H2O (given as pack meals)	

1530	3	Inpatient	D/C patient to home	
0750	4___	BioNutrition	Reconstitute 50g Our Family Orange Breakfast Drink powder with 16.9 oz bottled H2O	
0800	4	BioNutrition	-Have patient eat standardized breakfast/ drink beverage in Outpatient office -MUST EAT/DRINK ALL PROVIDED	
0830	4	Investigator	-Pick up 2 each of: 10 ml EDTA vacutainer, 8 ml SST vacutainer, 5 ml sodium heparin vacutainer, 4 ml EDTA vacutainer -Walk patient to Johnson Center for exercise	
0900	4	Investigator	-22 ml blood draw from antecubital vein -Use 18 gauge needle, draw slowly -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top]	
0930	4	Investigator	-22 ml blood draw from antecubital vein -Use 18 gauge needle, draw slowly -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top]	
0945	4	Investigator	Deliver blood samples to Core lab	
0945	4	Core Lab	-Separate blood according to provided “Blood Collection Protocol” -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
	5___	not applicable	NONE	
	6___	not applicable	NONE	
	7___	not applicable	NONE	
	8___	not applicable	NONE	
0800	9___	BioNutrition	-Have patient eat standardized breakfast in Outpatient office -Instruct patient to eat/drink all provided -Weigh-back any leftovers	
0830	9	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, supper, evening snack, and 5 bottled H2O (given as pack meals) -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
0800	10__	BioNutrition	-Have patient eat standardized breakfast in Outpatient office	

			-Instruct patient to eat/drink all provided -Weigh-back any leftovers	
0830	10	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, supper, evening snack, and 5 bottled H2O (given as pack meals) -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
0800	11__	BioNutrition	-Have patient eat standardized breakfast in Outpatient office -Instruct patient to eat/drink all provided -Weigh-back any leftovers	
0830	11	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, and 3 bottled H2O (given as pack meals) -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover that evening for Weigh-back	
1700	11	Inpatient	Admit patient to Private Room 5E Vital signs on admission BP _____/_____ Pulse_____ Temp_____ Weight_____	
1720	11	Inpatient	Provide patient supper MUST EAT/DRINK ALL PROVIDED	
2000	11	Inpatient	Provide patent evening snack MUST EAT/DRINK ALL PROVIDED	
2100	11	Inpatient	NO CAFFEINE AFTER 2100	
2300	11	Inpatient	PATIENT BEGINS OVERNIGHT FAST	
0645	12__	Inpatient	Pick up 5g MANNITOL/ 10g LACTULOSE aliquots and mixing cup from 5E medicine storage:	
0650	12	Inpatient	Mix mannitol and lactulose per instructions provided by Pharmacy	
0700	12	Inpatient	Provide patient w/ man/lac beverage: MUST DRINK ENTIRE SOLUTION	8 hr fast
0700	12	Inpatient	-All urine produced by patient must be collected for 8 hours (0700 – 1500hrs) -Urine collected into 2 hr aliquots (e.g. 0-2 hrs; >2-4 hrs; >4-6 hrs; >6-8 hrs) -Provide patient with sterile urine collection container for 0-2 hr urine collection	

0715	12	Inpatient	-Set patient with 18 gauge venous catheter in LEFT antecubital fossa -Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	15 min post man/lac
0715	12	Core Lab	-Separate blood according to provided "Blood Collection Protocol" -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
0720	12	Inpatient	-Make catheter suitable for transport to Johnson Center -Make 0-2hr urine container suitable for transport to Johnson Center -Provide patient with an extra urine container	
0725	12	BioNutrition	Provide INVESTIGATOR with 1g quercetin or placebo aliquot, 50g Our Family Orange Breakfast Drink powder aliquot, a 16.9 oz bottled H2O, and a sports bottle	
0730	12	Investigator	TEMPORARY D/C patient to Johnson Center Exercise Physiology Lab	
0745	12	Investigator	Prepare patient for exercise	
0755	12	Investigator	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	12	Investigator	-Provide supplement beverage to patient -MUST DRINK ENTIRE BEVERAGE	1 hr post man/lac
0800	12	Investigator	Have patient begin exercise challenge	
0850	12	Investigator	Have patient discontinue exercise: 1st bout is complete	
0855	12	Investigator	Begin rehydrating patient with dH2O equal to weight loss	
0900	12	Investigator	-Provide patient with new urine container for >2-4 hr urine collection -Walk 0-2 hr urine container from Johnson Center to Core Lab	
0910	12	Core Lab	-Record urine volume in 0-2 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
0920	12	Investigator	Rehydration complete: Have patient perform 2nd exercise bout	

1010	12	Investigator	Have patient discontinue exercise: 2nd exercise bout complete -Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	3 hr 10 min post man/lac

1025	12	Core Lab	-Separate blood according to provided "Blood Collection Protocol" -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1045	12	Investigator	-Make catheter and >2-4 hr urine container suitable for transport to CTSC	
1050	12	Investigator	Return patient to CTSC	
1100	12	Investigator	-Provide patient with new urine container for >4-6 hr urine collection -Walk >2-4 hr urine container to Core Lab immediately	
1100	12	Core Lab	-Record urine volume in >2-4 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1210	12	Inpatient	-Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	5 hr 10 min post man/lac 2 hr post exercise
1210	12	Core Lab	-Separate blood according to provided "Blood Collection Protocol" -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1300	12	Inpatient	-Provide patient with new urine container for >6-8 hr urine collection -Walk >4-6 hr urine container to Core Lab immediately	
1300	12	Core Lab	-Record urine volume in >4-6 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	

1410	12	Inpatient	-Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	7 hr 10 min post 4 hr post exercise
1410	12	Core Lab	-Separate blood according to provided "Blood Collection Protocol" -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1500	12	Inpatient	-D/C urine collection -Walk >6-8 hr urine container to Core lab immediately	8 hr post man/lac
1500	12	Core Lab	-Record urine volume in >6-8 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1500	12	BioNutrition	-Provide patient with late lunch	
1520	12	BioNutrition	-Provide patient with supper, evening snack, and 2 bottled H2O (given as pack meals) -Provide patient with SECOND DOSE of quercetin/placebo (1g), 50g Our Family Orange Breakfast Drink, a 16.9oz bottled water, and a sports bottle -Ensure patient understands how to mix supplement/drink powder/water properly -Instruct patient to take supplement with supper -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
1530	12	Inpatient	D/C patient to home	
0750	13 __	BioNutrition	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	13	BioNutrition	-Have patient eat standardized breakfast/ drink supplement beverage in Outpatient office -MUST EAT/DRINK ALL PROVIDED	
0825	13	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, supper, evening snack, and 5 bottled H2O (given as pack meals) -Provide patient with SECOND DOSE of quercetin/placebo (1g), 50g Our Family Orange Breakfast Drink, a 16.9oz bottled water, and a sports bottle -Instruct patient to take supplement with supper	

			-Ensure patient understands how to mix supplement/drink powder/water properly -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
0750	14__	BioNutrition	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	14	BioNutrition	-Have patient eat standardized breakfast/ drink supplement beverage in Outpatient office -MUST EAT/DRINK ALL PROVIDED	
0825	14	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, supper, evening snack, and 5 bottled H2O (given as pack meals) -Provide patient with SECOND DOSE of quercetin/placebo (1g), 50g Our Family Orange Breakfast Drink, a 16.9oz bottled water, and a sports bottle -Instruct patient to take supplement with supper -Ensure patient understands how to mix supplement/drink powder/water properly -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
0750	15__	BioNutrition	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	15	BioNutrition	-Have patient eat standardized breakfast/ drink supplement beverage in Outpatient office -MUST EAT/DRINK ALL PROVIDED	
0825	15	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, supper, evening snack, and 5 bottled H2O (given as pack meals) -Provide patient with SECOND DOSE of quercetin/placebo (1g), 50g Our Family Orange Breakfast Drink, a 16.9oz bottled water, and a sports bottle -Instruct patient to take supplement with supper -Ensure patient understands how to mix supplement/drink powder/water properly -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
0750	16__	BioNutrition	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	16	BioNutrition	-Have patient eat standardized breakfast/ drink supplement beverage in Outpatient office -MUST EAT/DRINK ALL PROVIDED	
0825	16	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, supper, evening snack, and 5	

			bottled H2O (given as pack meals) -Provide patient with SECOND DOSE of quercetin/placebo (1g), 50g Our Family Orange Breakfast Drink, a 16.9oz bottled water, and a sports bottle -Instruct patient to take supplement with supper -Ensure patient understands how to mix supplement/drink powder/water properly -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover following am for Weigh-back	
0750	17__	BioNutrition	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	17	BioNutrition	-Have patient eat standardized breakfast/ drink supplement beverage in Outpatient office -MUST EAT/DRINK ALL PROVIDED	
0810	17	Investigator	-Pick up 2 each of: 10 ml EDTA vacutainer, 8 ml SST vacutainer, 5 ml sodium heparin vacutainer, 4 ml EDTA vacutainer	
0820	17	BioNutrition	-Provide patient with morning snack, lunch, afternoon snack, and 3 bottled H2O (given as pack meals) -Instruct patient to eat/drink all provided -Instruct patient to bring back any leftover that evening for Weigh-back	
0900	17	Investigator	-Draw 22 ml blood from antecub -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top]	
0930	17	Investigator	-Draw 22 ml blood from antecub -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top]	
0945	17	Investigator	Deliver blood samples to Core lab	
0945	17	Core Lab	-Separate blood according to provided “Blood Collection Protocol” -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1700	17	Inpatient	Admit patient to Private Room 5E	

1710	17	Inpatient	Vital signs on admission BP _____/_____ Pulse_____ Temp_____ Weight_____	
1720	17	BioNutrition?	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
1725	17	BioNutrition?	Have patient eat standardized supper/ drink supplement beverage MUST EAT/DRINK ALL PROVIDED	
2000	17	Inpatient	Provide patient with evening snack MUST EAT/DRINK ALL PROVIDED	
2100	17	Inpatient	NO CAFFEINE AFTER 2100	
2300	17	Inpatient	PATIENT BEGINS OVERNIGHT FAST	
0645	18__	Inpatient	Pick up 5g MANNITOL/ 10g LACTULOSE aliquots and mixing cup from 5E medicine storage:	
0650	18	Inpatient	Mix mannitol and lactulose per instructions provided by Pharmacy	
0700	18	Inpatient	Provide patient w/ man/lac beverage: MUST DRINK ENTIRE SOLUTION	
0700	18	Inpatient	-All urine produced by patient must be collected for 8 hours (0700 – 1500hrs) -Urine collected into 2 hr aliquots (e.g. 0-2 hrs; >2-4 hrs; >4-6 hrs; >6-8 hrs) -Provide patient with sterile urine collection container for 0-2 hr urine collection	
0715	18	Inpatient	-Set patient with 18 gauge venous catheter in LEFT antecubital fossa -Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	
0715	18	Core Lab	-Separate blood according to provided “Blood Collection Protocol” -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
0720	18	Inpatient	-Make catheter suitable for transport to Johnson Center -Make 0-2hr urine container suitable for transport to Johnson Center -Provide patient with an extra urine container	

0725	18	BioNutrition	Provide INVESTIGATOR with 1g quercetin or placebo aliquot, 50g Our Family Orange Breakfast Drink powder aliquot, a 16.9 oz bottled H2O, and a sports bottle	
0730	18	Investigator	TEMPORARY D/C patient to Johnson Center Exercise Physiology Lab	
0745	18	Investigator	Prepare patient for exercise	
0755	18	Investigator	Mix 1g quercetin or placebo with 50g Our Family Orange Breakfast Drink powder and reconstitute with 16.9 oz bottled H2O	
0800	18	Investigator	-Provide supplement beverage to patient -MUST DRINK ENTIRE BEVERAGE	
0800	18	Investigator	Have patient begin exercise challenge	
0850	18	Investigator	Have patient d/c exercise: 1st bout is complete	
0855	18	Investigator	Begin rehydrating patient with dH2O equal to weight loss	
0900	18	Investigator	-Provide patient with new urine container for >2-4 hr urine collection -Walk 0-2 hr urine container from Johnson Center to Core Lab	
0910	18	Core Lab	-Record urine volume in 0-2 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
0920	18	Investigator	Rehydration complete: Have patient perform 2nd exercise bout	
1010	18	Investigator	Have patient discontinue exercise: 2nd exercise bout complete -Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top]	
1015	18	Investigator	Walk blood samples from Johnson Center to Core Lab	
1025	18	Core Lab	-Separate blood according to provided “Blood Collection Protocol” -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1045	18	Investigator	-Make catheter and >2-4 hr urine container suitable for transport to CTSC	
1050	18	Investigator	Return patient to CTSC	
1100	18	Investigator	-Provide patient with new urine container for >4-6 hr urine collection -Walk >2-4 hr urine container to Core Lab immediately	
1100	18	Core Lab	-Record urine volume in >2-4 hr urine	

			container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1210	18	Inpatient	-Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	
1210	18	Core Lab	-Separate blood according to provided "Blood Collection Protocol" -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1300	18	Inpatient	-Provide patient with new urine container for >6-8 hr urine collection -Walk >4-6 hr urine container to Core Lab immediately	
1300	18	Core Lab	-Record urine volume in >4-6 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1410	18	Inpatient	-Draw 22 ml blood -Dispense 10 ml blood into 10 ml EDTA vacutainer [purple top] -Dispense 7 ml blood into 8 ml SST vacutainer [red top] -Dispense 4 ml blood into 5 ml sodium heparin vacutainer [green top] -Dispense 1 ml blood into 4 ml EDTA vacutainer [purple top] -Walk blood samples to Core lab immediately	
1410	18	Core Lab	-Separate blood according to provided "Blood Collection Protocol" -Assay blood for TNF-a, Il-6, and glutathione peroxidase	
1500	18	Inpatient	-D/C urine collection -Walk >6-8 hr urine container to Core lab immediately	
1500	18	Core Lab	-Record urine volume in >6-8 hr urine container -Aliquot 6 ml sample into sterile container -Store urine aliquot at -20°C -Discard remaining urine	
1500	18	BioNutrition	-Provide patient with CELEBRATION MEAL	
1530	18	Inpatient	D/C patient to home	

***After minimum of 3 month washout patient repeats entire study**

APPENDIX M

Print Form

THE UNIVERSITY of NEW MEXICO

CTSC REQUEST FOR ADMISSION

Protocol #
Date of Admission Time Today's Date

Name of Participant: , ,
Last First MI
Address: City , State Zip

Telephone (Home/Cellular): (Work Phone):

Sex: Date of Birth: UNMH#:
Weight: Height:

Race: □ American Indian or Alaska Native □ Asian □ Black or African American
□ Native Hawaiian or Other Pacific Islander □ White □ More than one Race

Ethnicity: (must check one) □ Hispanic/Latino □ Not Hispanic or Latino

Principal Investigator (Print & Sign)
Print Sign

Admitting Physician: Attending Physician:
Primary Diagnosis: Special Procedure:
Signed Consent Must be: □ Attached □ On File Orders Attached: □ Yes □ No
Number of Hospitalization Days:
Room: Preference: □ Private □ Semi Private
Hospital Status (must check one):
□ "A" Study Pay (Grant) □ "B" Insurance/Patient Pay □ "D" Drug Study (Industry Pay)

Diet Orders:

DIETARY INTAKE CHECKLIST

Limitations	Check if Present	Comments
Diabetic	☐	
Sodium Restriction	☐	
Fat Restriction	☐	
Lactose Intolerance	☐	
Vegetarian	☐	

APPENDIX N

Q STUDY (08-606) FOOD ITEMS

In order to fulfill the dietary restrictions of this research protocol while still meeting your nutritional needs, you must be willing to eat eggs, cheese, beef, pork, poultry, butter, and milk, and one or more of the fruits and juices listed below. You must also be willing to eat bell peppers, zucchini, mushrooms, broccoli, cauliflower, carrots and green salad. We want to accommodate your preferences as best we can, but please be aware that the less you are willing to eat (the more dislikes you have), the more monotonous your diet will be. If you would like to speak with the study dietitians directly, please call Rosemary at 272-5501 or Christine at 272-0196.

Pt ID # _____ **Age** _____ **Height (inches)** _____ **Weight (lbs)** _____

Please circle the food items that you are unwilling to eat:

Juices
- Orange
- Pineapple
- Grape

Fruit
- Oranges
- Kiwi
- Honeydew
- Cantaloupe
- Apricots
- Peaches
- Bananas
- Pineapple

Breakfast items:
- Scrambled Eggs
- Bacon
- Sausage
- Hash browns
- French Toast
- Blueberry Pancakes
- Streusel Coffee Cake
- Pumpkin Muffin
- Flour Tortilla
- Ham Frittata
  - Spinach
  - Bell peppers
- Vegetable Cheese Omelet
  - Bell peppers
  - Mushrooms
- White toast
- Wheat toast

Side Dishes
- White rice
- Spaghetti
- Rotini
- Broccoli
- Cauliflower
- Carrots
- Dinner Roll
- Garden Salad
  - Lettuce
  - Radishes
  - Carrots
  - Tomatoes
- Spanish rice
- Tomato soup
- Vegetable soup
- Tortilla chips

Entrees
- Chicken Salad Sandwich
- Turkey & Cheese Sandwich
- Ham & Cheese Sandwich
- Chicken Fajitas
- Meatloaf
- Spaghetti with Meat Sauce
- East Indian Chicken Curry
- Chicken Parmesan
- Beef & Cheese Soft Tacos
- Chicken Tetrazzini (mushrooms, pasta & creamy sauce)
- Chicken Stir-fry (zucchini, broccoli, carrot, bell pepper)
- Beef Stir-fry (zucchini, broccoli, carrot, bell pepper)
- Beef Lasagna
- Beef Stroganoff

Desserts
- Chocolate Cake
- Banana Bread
- Cream Cheese Brownie
- Oatmeal Cake
- Vanilla Pudding
- Butter Cookies
- Chocolate Chip Cookie Bar

Snack Items
- Peach Yogurt
- Vanilla Yogurt
- Jello®
- Rice Krispie Treat®
- Club Crackers
- Pretzels
- Graham Crackers
- Cheese Stick

Other
- 1% Milk
- Whole Milk
- Butter
- Margarine
- Ranch Dressing
- House Vinaigrette
- Pico de Gallo
- Coffee
- Decaf Coffee
- Soft drink, regular, caffeinated
- Soft drink, regular, caffeine free
- Soft drink, diet, caffeinated
- Soft drink, diet, caffeine free

Please FAX to: 272-0266

Placebo Condition	**Intake (Kcal/d)**	**CHO (g/d)**	**FAT (g/d)**	**PRO (g/d)**	**Q (g/d)**
Days 9-11	3628.4 ± 47.5	447.6 ± 7.3	133.8 ± 2.1	176.9 ± 3.2	6.9 ± 0.3
Days 12-18	3756.1 ± 61.4	476.0 ± 11.0	136.6 ± 2.3	172.9 ± 2.6	8.0 ± 0.2

Quercetin Condition	**Intake (Kcal/d)**	**CHO (g/d)**	**FAT (g/d)**	**PRO (g/d)**	**Q (g/d)**
Days 9-11	3636.1 ± 51.1	446.0 ± 8.7	135.0 ± 1.9	177.3 ± 3.3	6.8 ± 0.3
Days 12-18	3713.3 ± 50.3	465.2 ± 8.2	136.4 ± 2.2	174.4 ± 2.7	8.0 ± 0.2

Dietary Intake for Placebo and Quercetin Supplemented Conditions

APPENDIX O

UNIVERSITY OF NEW MEXICO
CLINICAL & TRANSLATIONAL SCIENCE CENTER – BIONUTRITION UNIT

DIETARY INSTRUCTIONS FOR PROTOCOL 08-606

As a part of this research study, the Metabolic Kitchen has prepared all your meals and snacks. Please eat these bagged meals and snacks on this day:

Mon Tues Wed Thurs Fri Sat Sun

Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31

WE ASK THAT YOU DO THE FOLLOWING:

1. You MUST keep your meals refrigerated until you are ready to eat them.

2. Your main dishes are in microwave safe containers. If you use a conventional oven to heat your main dishes, please transfer the food into an oven safe dish.

3. Please eat ONLY the foods and beverages provided to you, however you may have all the tap or plain bottled water you want in addition to what is provided.

4. Please make every effort to eat ALL the food and beverages that are provided daily. It is important that you eat each meal's foods at the meal specified (such as eat all your dinner food at dinner time).

5. Please return ALL empty containers of foods and beverages to the metabolic kitchen tomorrow morning when you come in to eat your breakfast. Please also bring back any food and beverages that you did not eat. This is extremely important.

6. If you should eat or drink something that was not provided to you (other than water as mentioned above), please record the item name, amount consumed, and name of restaurant (if applicable) on the attached form. This is extremely important.

If you have any questions please call the Metabolic Kitchen at 272-2601. Thank you!

CLINICAL AND TRANSLATIONAL SCIENCE CENTER
Additional Food & Beverage Intake Form - Protocol No: 08-606

Participant ID #: _____ Date of Intake: __ __ / __ __ / __ __ Day: Sun Mon Tues Wed Thurs Fri Sat

If you ate or drank anything today that was not provided to you (other than plain water) please record it here. Be sure to include brand names, weights and/or measured amounts. If you ate at a restaurant, please provide the name of the restaurant. Bring this form to the Metabolic Kitchen in the morning. Thank you.

Place	Amount	Food Description	Office Use Only

APPENDIX P

08-606 PATIENT DAILY LOG SHEET FOR MEAL TYPE & COMPOSITION - ARMS 1 & 2

1. Low Q = low quercetin, low lectin (no legumes/nuts) meals. Daily meals will be calculated to meet "high" calories using the following equation: TEE = 864 – (9.72 x age in years) + 1.54 x (14.2 x wt in kg + 503 x ht in m)
2. Certain specified meals will meet requirements of 40% fat, 30% protein, 30% CHO.
3. The inpatient standardized breakfast will be identical for each subject. Use specific spreadsheet for this meal.

Patient ID _____

Day	Date	Meals		Type of meal
Day 1	_____	No Meals		
Day 2	_____	Admission to GCRC	5:20 PM Supper	low Q, high cal
			8:00 HS snack	low Q, high cal (no caffeine after 9:00 PM)
Day 3	_____	8:00 Orange Drink Plain		
		3:00 PM Lunch		low Q, high cal
		Packed out Dinner & HS snack		low Q, high cal
Day 4	_____	8:00 AM Orange Drink Plain on breakfast tray		
		8:00 AM Breakfast at GCRC		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
Day 5	_____	No meals		
Day 6	_____	No meals		
Day 7	_____	No meals		
Day 8	_____	No meals		
Day 9	_____	8:00 AM Breakfast at GCRC		low Q, high cal
		Packed out Lunch, Dinner & 3 snacks		low Q, high cal
Day 10	_____	8:00 AM Breakfast at GCRC		low Q, high cal
		Packed out Lunch, Dinner & 3 snacks		low Q, high cal
Day 11	_____	8:00 AM Breakfast at GCRC		low Q, high cal
		Packed out Lunch, AM, PM snacks		low Q, high cal
		Admission to GCRC	5:20 PM Supper	low Q, high cal
			8:00 PM HS snack	low Q, high cal (no caffeine after 9:00 PM)
Day 12	_____	7:25 AM Drug Bag to investigator		
		3:00 PM Lunch		low Q, high cal
		3:25 PM Discharged with:		
		Drug Bag		
		Packed out Dinner		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out HS snack		low Q, high cal, no caffeinated beverages
Day 13	_____	7:50 AM mix contents of Drug Bag and serve with breakfast tray		
		8:00 AM Breakfast at GCRC		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out Lunch, AM, PM snacks		low Q, high cal
		Packed out Drug Bag		
		Packed out Dinner		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out HS snack		low Q, high cal, no caffeinated beverages
Day 14	_____	7:50 AM mix contents of Drug Bag and serve with breakfast tray		
		8:00 AM Breakfast at GCRC		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out Lunch, AM, PM snacks		low Q, high cal
		Packed out Drug Bag		
		Packed out Dinner		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out HS snack		low Q, high cal, no caffeinated beverages
Day 15	_____	7:50 AM mix contents of Drug Bag and serve with breakfast tray		
		8:00 AM Breakfast at GCRC		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out Lunch, AM, PM snacks		low Q, high cal
		Packed out Drug Bag		
		Packed out Dinner		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out HS snack		low Q, high cal, no caffeinated beverages
Day 16	_____	7:50 AM mix contents of Drug Bag and serve with breakfast tray		
		8:00 AM Breakfast at GCRC		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out Lunch, AM, PM snacks		low Q, high cal
		Packed out Drug Bag		
		Packed out Dinner		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out HS snack		low Q, high cal, no caffeinated beverages
Day 17	_____	7:50 AM mix contents of Drug Bag and serve with breakfast tray		
		8:00 AM Breakfast at GCRC		low Q, 40% fat, 30% pro, 30% CHO, no caffeinated beverages
		Packed out Lunch, AM, PM snacks		low Q, high cal
		2:30 PM Put Drug Bag on top of employee refrigerator		
		2:30 PM Place clear tub for weigh backs in office area and affix laminated sign		
		Admission to GCRC	5:20 PM Dinner	low Q, 40% fat, 30% pro, 30% CHO
			8:00PM HS snack	low Q, high cal (no caffeine after 9:00 PM)
Day 18	_____	7:25 AM Drug Bag to investigator		
		3:00 PM Regular Lunch		
		Discharged		

RSW 3/5/09, 3/26/09

APPENDIX Q

08-606 Mixing Instructions: Placebo/Quercetin Supplement

Items Needed:	Item Abbreviated Name:
Brown glass bottle w/ 1g placebo/quercetin powder	Brown bottle
50 g Our Family Orange Breakfast drink mix	Orange mix
16.9 oz bottled water	Bottled water
Mixing bottle (clear plastic, large opening)	Clear bottle
Waxed paper square	Waxed paper

1. Fold waxed paper lengthwise to create a crease. Unfold waxed paper and place on counter.

2. Remove cap from clear bottle. Place clear bottle on top of waxed paper.

3. Remove cap from brown bottle. Turn brown bottle upside down over clear bottle. (mouth of clear bottle is big enough that brown bottle should fit inside).

4. Carefully tap bottom of brown bottle to transfer placebo/quercetin powder to clear bottle. Put cap back on brown bottle and place on waxed paper.

5. Fill clear bottle to 8oz line with bottled water. Put cap back on clear bottle. Shake well, for at least 1 minute. Shake longer if you still see floating powder chunks.

6. There is still some powder left in brown bottle. Take cap off brown bottle. Fill brown bottle ½ full with bottled water. Put cap back on brown bottle. Mix brown bottle well, for about 1 minute.

7. Transfer fluid from brown bottle to clear bottle

8. Repeat steps 6 and 7 until all powder has been removed from brown bottle.

9. Add the rest of the bottled water to the clear bottle.

10. Add the orange mix to the clear bottle.

11. Cap the clear bottle. Mix clear bottle well, for about 1 minute. Mix longer if you still see floating powder chunks.

12. Serve the drink.

*Drink to be served at room temperature. Please do not refrigerate drug bag.

APPENDIX R

Core Lab: Blood Collection Protocol

From the 8 ml SST tube (contains 7 ml):
1. Il-1b –MOSELEY [500 ul]
2. TNF-a –CORE LAB [750 ul]
3. Il-6 –CORE LAB [750 ul]

From the 5 ml Na Heparin plasma tube (contains 4 ml):
1. Quercetin – MOSELEY [1.5 ml]

From the 4 ml EDTA plasma tube (contains 1 ml):
1. Glutathione peroxidase –CORE LAB

***you can spin these as needed to get samples of interest. The 10 ml EDTA plasma tube discussed below cannot be spun. It will be processed with histopaque 1077 b/c we need to isolate PBMCs.**

From the 10 ml EDTA plasma tube (contains 10 ml):
1. Il-10 – MOSELEY [1 ml]
2. Endotoxin – MOSELEY [1 ml]
3. Extracellular HSP72 – MOSELEY [1 ml]

PBMCs isolated from 10 ml EDTA plasma sample:
1. HSP72 – MOSELEY [aliquot ½ cell pellet into 1.7ml mini-eppendorf]
2. HSF-1 – MOSELEY [aliquot ½ cell pellet into 1.7ml mini-eppendorf]

***see page 2 for PBMC isolation procedure**

Standard Label Template

Please label each sample with:
1. Date sample collected
2. What sample is (PBMC or PLASMA)
3. What sample collected for (e.g. HSP72, Il-1b, etc)
4. Subject number

APPENDIX S

HEALTH HISTORY AND PHYSICAL ACTIVITY QUESTIONNAIRE

Subject Code: ________________________________ 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 ____	Heat illness ____	Blackouts _____
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 treadmill 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

Blood Results:

Total Cholesterol __________________   LDL________________   HDL_________________

Fasting blood glucose _________________

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.

4. Dyslipidemia: LDL>130 mg/dl or HDL<40 mg/dl, or on lipid lowering medication, or total serum cholesterol >200 mg/dl.

5. Impaired fasting glucose > 100 mg/dl.

6. BMI>30 kg/m2

7. Sedentary lifestyle: persons not meeting ACSM requirement for active lifestyle.

APPENDIX T

Data Sheet for Screening

Subject Code:________________________ Date______/______/______ Time: _________ am/pm

P_B (mmHg)__________Temp (ºC) ________________ Humidity (%) ______________

Age__________ 220-Age__________ Gender____

Height (in)_______ Height (cm)________ Weight (kg) ________ BMI (kg/m^2) __________

♦♦♦

SKINFOLDS (mm)

	1	2	3	AVG	
CHEST	_______	_______	_______	_______	Db ______
ABDOMEN	_______	_______	_______	_______	%BF ______
THIGH	_______	_______	_______	_______	

♦♦♦

Treadmill PROTOCOL

Time	Workload	VO2 (m/kg/min)	HR (BPM)	Blood Pressure (mmHg)	RPE/symptoms

Time at termination:
Reason for termination:
VO_2max (ml/kg/min):_________

APPENDIX U

Subject Number_____ **Date_____**

Condition (circle one) HT1 IP2 HA2 HA3 HA4 HA5 HT2 IP3

Pre BW: _____ **H2O drank during test _____**
Post BW: _____

Bout 1

Time	HR	Db	WBGT	GT	WB	Tcore	Tarm	Tthigh	Tcalf	Tchest	Work	VO2
0												
5												
10												
15												
20												
25												
30												
35												
40												
45												
50												

Bout 2

Time	HR	Db	WBGT	GT	WB	Tcore	Tarm	Tthigh	Tcalf	Tchest	Work	VO2
0												
5												
10												
15												
20												
25												
30												
35												
40												
45												
50												

Hydration Status

Time	Urine	Hematocrit	Hemoglobin
Pre		/ / /	/ /
Post		/ / /	/ /

Notes:

Supplies ready for bloods? _____
Radio? _____
Squirrel logging at start of test? _____
Disarm Squirrel before unplugging? _____

APPENDIX V

Effect of Combined Heat Acclimation and Quercetin Supplementation on Gut Permeability

Matthew Kuennen, Trevor Gillum, Kevin Christmas, Michelle Kulovitz, Karol Dokladny, Sue Schneider, Pope Moseley
University of New Mexico, Albuquerque, NM

Introduction:

Exertional heatstroke is a life threatening condition characterized by a core temperature >40°C, central nervous system dysfunction, altered serum enzymes, and a sustained inflammatory response (Bouchama 2002). Military soldiers and endurance athletes represent two at-risk populations (Smith III). Heat acclimation, accomplished via 8-12 days of exercise in the heat, is an established method for reducing heatstroke risk. Cell culture and animal models suggest that HA may improve gut barrier morphology, preventing bacteria from leaving the gut, entering the bloodstream, and stimulating inflammatory responses that drive heatstroke pathology (Dokladny 2006; Sakurada 1998). This has not been examined in a human population.

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a popular dietary supplement that is heavily marketed to enhance athletic fit (is antioxidant, anti-inflammatory, antinociceptive, and anti-viral properties. A limited number of studies have reported enhanced exercise performance following 1-2 weeks of high dose quercetin supplementation (MacRae 2006; Nieman 2010). The U.S. military is particularly interested in quercetin. In early 2009 quercetin was added to Nutritionally Optimized First Strike Rations® for provision during high stress military deployments (Govt publication 2009).

However, quercetin has also been shown to be a potent suppressor of the heat shock response (HSR) (Hosokawa 1990), an evolutionarily conserved mechanism that allows organisms to adapt to sublethal heat stress exposure (Lindquist 1986). Using cell culture models to simulate intact gut epithelium, our lab has shown quercetin prevents the normal restitution of gut barrier integrity following sub-lethal thermal stress exposure (Dokladny 2006a, 2006b, 2008). Others have reported a suppressed heat shock response in rodents receiving oral quercetin dosing. In this study animals exhibited increased inflammatory cytokine levels and mortality from gut bacterial challenge (Nakada 2005). In a separate experiment orally dosed quercetin reduced rodents ability to reinitiate a damaged gastric barrier (Liu 2007). The unifying link between all of these studies is a suppression of the stress inducible heat shock protein 72 (HSP72), which is known to afford protection at multiple levels. Collectively, these studies suggest that dietary quercetin supplementation by military soldiers during desert deployments may suppress HSP72 and limit protection normally afforded by a standardized heat acclimation protocol.

Purpose:

The purpose of this study is to determine whether heat acclimation reduces stress-induced gut permeability in a human model. A secondary purpose is to determine whether inflammatory and anti-inflammatory cytokine responses are reduced following a heat acclimation protocol. We will also determine what effect large dose quercetin supplementation has on the primary and secondary study purposes.

Hypotheses:

We hypothesize that heat acclimation will reduce stress-induced gut permeability, resulting in ameliorated inflammatory and anti-inflammatory cytokine responses. We further hypothesize quercetin will diminish the reduction in stress-induced gut permeability, resulting in sustained inflammatory and anti-inflammatory cytokine responses that drive heatstroke pathology.

Methods:

Study Design: Eight subjects will complete 7 days of heat acclimation exercise (HA) in both quercetin supplemented (2g/d) and placebo conditions (counter-balanced, double-blind). Gut permeability will be assessed at baseline, and on the first and last days of HA. Figure 1 provides a schematic of the overall study design.

Assays to be employed: Sugar probes and plasma endotoxin levels will be used to assess gut permeability. Plasma concentrations of inflammatory (TNF-a, Il-1b, Il-6) and anti-inflammatory cytokines (Il-10) will also be measured. HSP72 will be assessed in monocytes (immune cells) and plasma.

Figure 1. Study Experimental Design

Results:

Age (yrs)	Height (cm)	Weight (kg)	Body Fat (%)
30 ± 2	177 ± 3	77 ± 5	10 ± 1

Table 1. Subject Characteristics. Data are mean ± SE, n = 5.

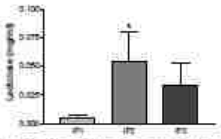


Figure 2. Lactulose (disaccharide) in urine collected at rest (IP1) and after exercising to 39°C on the first (IP2) and seventh (IP3) days of heat acclimation. *different from IP1 (p<0.05). Data are mean ± SE, n = 5.

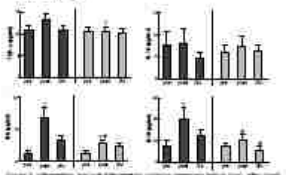


Figure 3. Inflammatory and anti-inflammatory cytokine responses before (pre), after (post) and 1 hours after (2hr) exercising to a core temperature of 39°C on day 1 (dark bars) and day 7 (light bars) of a heat acclimation protocol. *different from respective control condition (p<0.05). #different between conditions (p<0.05). †different between conditions (p<0.05). Data are mean ± SE, n = 5 for all figures.

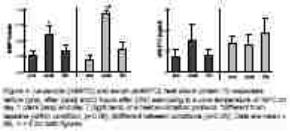


Figure 4. Leukocyte (CD45+) and monocyte (CD45+CD14+) heat shock protein 72 expression before (pre), after (post) and 2 hours after (2hr) exercising to a core temperature of 39°C on day 1 (dark bars) and day 7 (light bars) of a heat acclimation protocol. *different from respective control condition (p<0.05). #different between conditions (p<0.05). †different between conditions (p<0.05). Data are mean ± SE, n = 5 for all figures.

Conclusion:

Two subjects have completed the study, the other six have completed heat acclimation in one of two study conditions. Data has been presented for 5 subjects. Data currently support heat acclimation's ability to reduce stress-induced gut permeability and inflammatory/anti-inflammatory cytokine cascades. The study is double blind, so the effect of quercetin on this model has not yet been examined.

References:

Bouchama A et al. Heat stroke. N Engl J Med. 2002;346(25):1978-88.

Cuttre R et al. Epidemiology of hospitalizations and deaths from heat illness in soldiers. Med Sci Sports Exerc. 2005

Dokladny K et al. Induction of physiological thermotolerance in MDCK monolayers: contribution of heat shock protein 70. Cell Stress Chaperones. 2006

Dokladny K et al. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. Am J Physiol Gastrointest Liver Physiol. 2006

Dokladny K et al. Cellular and molecular mechanisms of heat stress-induced up-regulation of occludin protein expression: Regulatory role of heat shock factor-1. Am J Pathol. 2008

Government publication. Operational ration of the department of defense. NATICK PAM 30-23, 8th Edition, APRIL 2009. Approved for public release

Hosokawa N et al. Flavonoids inhibit the expression of heat shock proteins. Cell Struct Funct. 1990

Lindquist S. The heat-shock response. Annu Rev Biochem. 1986

Liu WL et al. Protective effects of heat shock protein70 induced by geranylgeranylacetone in atrophic gastritis in rats. Acta Pharmacol Sin. 2007;28(7):1001-6.

MacRae HSH et al. Dietary antioxidant supplementation combined with quercetin improves cycling time trial performance. Int J Sport Nut Exer Metab. 2006

Nakada J et al. Oral administration of geranylgeranylacetone improves survival rate in a rat endotoxin shock model: administration timing and heat shock protein 70 induction. Shock. 2005

Nieman DC et al. Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. Med Sci Sports Exerc. 2010

Sakurada S et al. A role for gastrointestinal endotoxin in enhancement of heat tolerance by physical fitness. J Appl Physiol. 1998

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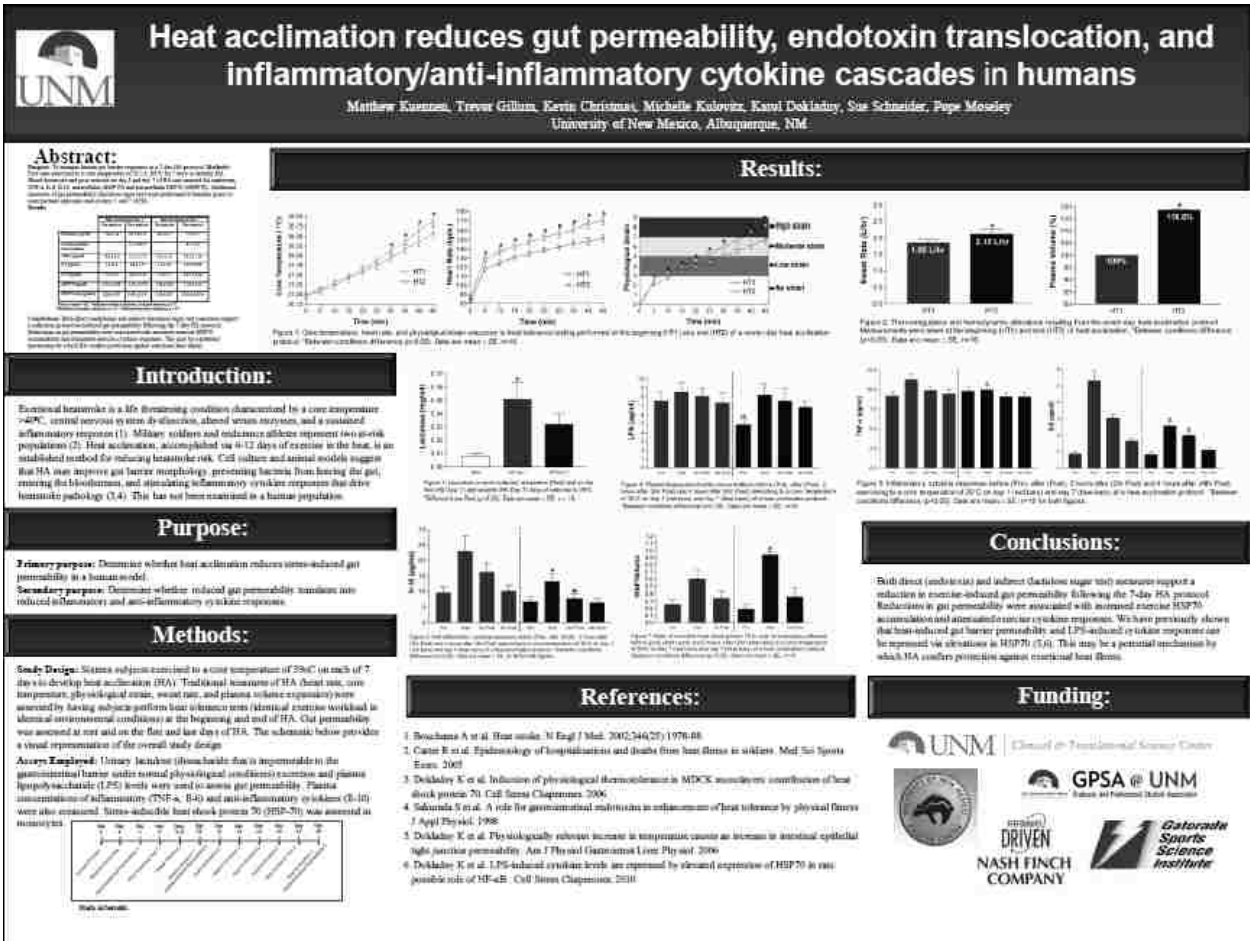
*This research is a collaborative effort between the Chairman of the UNM Department of Internal Medicine and the UNM Department of Health, Exercise and Sport Sciences.

APPENDIX W


Heat acclimation reduces gut permeability, endotoxin translocation, and inflammatory/anti-inflammatory cytokine cascades in humans
Matthew Kuennen, Trevor Gillum, Kevin Christmas, Michelle Kolovitz, Kanvi Dokladny, Sue Schneider, Pope Moseley
University of New Mexico, Albuquerque, NM
Abstract:
Results:
Introduction:
Purpose:
Methods:
Conclusions:
References:
Funding:
UNM
GPSA @ UNM
DRIVEN
NASH FINCH COMPANY
Gatorade Sports Science Institute

APPENDIX X

HRRC/CTSC 08-606: Study Budget

Item Description (manufacturer, product #, where produced):	Cost:	**Funded by:**
Lactulose (Quintron, QT02500-10-5, Milwaukee, WI, USA)	$187	GCRC 09
Mannitol (Spectrum, MA165-500GM, Gardena, CA, USA)	$146	GCRC 09
TNF-α assay (CTSC core lab, std procedure)	$2080	GCRC 09
Il-6 assay (CTSC core lab, std procedure)	$4160	GCRC 09
Glutathione peroxidase assay (CTSC core lab, std procedure)	$1456	GCRC 09
Participant Meals	-	GCRC 09
Participant Reimbursement	$4800	GCRC 09
Cell Separation (Sigma Aldrich, Histopaque 1077, St. Louis, MO, USA)	$900	GCRC 09
Placebo (powdered food coloring purchased at Royal Icing)	$240	GCRC 09
UNM Pharmacy Dispensing Fees	$1764	GCRC 09
B-glucuronidase/arylsulfatase kit (Roche Diagnostics, 10127698001, Indianapolis, IN, USA)	$440	Gatorade 09
SPE cartridges from Waters (WAT094225, Milford, MA)	$555	Gatorade 09
High performance liquid chromatography (UNM Mass Spec charges $100/hr user fee)	$950 $2500	RPT 09 GRD 2010
Mannitol kit (Megazyme International, KFRUGL, Bray, County Wicklow, Ireland)	$495	SRAC 09
Nuclear Extraction Kit (Active Motif, 40010, Carlsbad, CA, USA)	$560	P Moseley
B-Actin 1° antibody (Sigma Aldrich, A3853, St. Louis, MO, USA)	$300	P Moseley
HSF-1 1°antibody (Assay Designs, SPA-950F, Ann Arbor, MI, USA)	$340	P Moseley
2° antibody (Assay Designs, SAB-300J, Ann Arbor, MI, USA)	$200	P Moseley
Vacutainer tubes (BD, 366450, Franklin Lakes, NJ USA)	$170	S Schneider
Our Family drink mix (Nash Finch, Northfield, IL, USA)	$240	donation
Quercetin (Nutravail Technologies, QU995, Chantilly, VA, USA)	$1000	donation
eHSP ELISA kit (Assay Designs, EKS-715, Ann Arbor, MI, USA)	$1725	GRD 08/09
Endotoxin kit (Cell Sciences, HIT302, Canton, MA, USA)	$1800	Gatorade 09 GRD 08/09
Il-1b EIA kit. (Assay Designs, 900-029, Ann Arbor, MI, USA)	$1700	Gatorade 09 GRD 08/09
Il-10 EIA kit (Assay Designs, 900-036, Ann Arbor, MI, USA)	$2490	Gatorade 09 GRD 08/09
Mannitol Dehydrogenase (Megazyme International, MDH, Bray County, Wicklow, Ireland)	$300	Gatorade 09 H. Lin
B-glucuronidase/arylsulfatase kit (Roche Diagnostics, 10127698001, Indianapolis, IN, USA)	$440	GRD 09/10
SPE cartridges (Waters, WAT094225, Milford, MA, USA)	$555	GRD 09/10
Pipettors and tips (VWR Interantional, various #, Chicago, Il, USA)	$1000	RPT 10
Total Funding Required:	**$33493**	

REFERENCES

1. al-Mashhadani SA, Gader AG, al Harthi SS, Kangav D, Shaheen, FA, Bogus F. The coagulopathy of heat stroke: alterations in coagulation and fibrinolysis in heat stroke patients during the pilgrimage (Haj) to Makkah. *Blood Coagul Fibrinolysis* 5(5):731-736, 1994.

2. Amorim FT, Yamada PM, Robergs RA, Schneider SM, Moseley PL. The effect of the rate of heat storage on serum heat shock protein 72 in humans. *Eur J Appl Physiol* 104(6):965-972, 2008.

3. Armstrong LE, Casa DJ, Millard-Stafford M, Moran DS, Pyne SW, Roberts WO. American College of Sports Medicine position stand. Exertional heat illness during training and competition. *Med Sci Sports Exercise* 39(3):556–572, 2007.

4. Bachkosky J, Andrews M, Douglass R, Feigley J, Felton L, Fernandez F, Fratarangelo P, Johnson-Winegar A, Kohn R, Polmar N, Rumpf R, Sommerer J, Williamson W. Lightening the Load. 2007; *Military report*, unclassified:89 pages.

5. Baker JW, Deitch EA, Li M, Berg RD, Specian RD. Strenuous exercise causes systemic endotoxemia. *J Appl Physiol* 28(7):896-906,1988.

6. Bannerman DD, Goldblum SE. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. *Am J Physiol Lung Cell Mol Physiol* 284(6):L899-914, 2003.

7. Bieger J, Cermak R, Blank R, de Boer VC, Hollman PC, Kamphues J, Wolffram S. Tissue distribution of quercetin in pigs after long-term dietary supplementation. *J Nutr* 138(8):1417-20, 2008.

8. Boots AW, Haenen GR, Bast A. Health effects of quercetin: from antioxidant to nutraceutical. *Eur J Pharmacol* 585(2-3):325-37, 2008.

9. Bosch TC, Krylow SM, Bode HR, Steele RE. Thermotolerance and synthesis of heat shock proteins: These responses are present in Hydra attenuate but absent in Hydra oligactis. *Proc Natl Acad Sci USA* 85:7927–7931, 1988.

10. Bouchama A, al-Sedairy S, Siddiqui S, Shail E, Rezeig M. Elevated pyrogenic cytokines in heatstroke. *Chest* 104:1498-1502, 1993.

11. Bouchama A, Knochel JP. Heat stroke. *N Engl J Med* 346(25):1978-88, 2002.

12. Bouchama A, Parhar RS, el-Yazigi A, Sheth K, al-Sedairy S. Endotoxemia and release of tumor necrosis factor and interleukin 1 alpha in acute heatstroke. *J Appl Physiol* 70(6):2640-2644, 1991.

13. Braby CE, Somero GN. Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus Mytilus). *J Exp Biol* 209:2554-2566, 2006.

14. Brown AS, Davis JM, Murphy EA, Carmichael MD, Carson JA, Ghaffar A, Mayer EP. Susceptibility to HSV-1 infection and exercise stress in female mice: Role of estrogen. *J Appl Physiol* 10(5):1592-1597, 2007.

15. Buckley BA, Owen ME, Hofmann GE. Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus Mytilus) changes as a function of thermal history. *J Exp Biol* 204(Pt 20):3571-3579, 2001.

16. Camus G, Poortmans J, Nys M, Deby-Dupont G, Duchateau J, Deby C, Lamy M. Mild endotoxaemia and the inflammatory response induced by a marathon race. *Clin Sci* 92(4):415-422, 1997.

17. Carter R 3rd, Cheuvront SN, Williams JO, Kolka MA, Stephenson LA, Sawka MN, Amoroso PJ. Epidemiology of hospitalizations and deaths from heat illness in soldiers. *Med Sci Sports Exercise* 37(8):1338-1344, 2005.

18. Chen G, Kelly C, Stokes K, Wang JH, Leahy A, Bouchier-Hayes D. Induction of heat shock protein 72kDa expression is associated with attenuation of ischaemia-reperfusion induced microvascular injury. *J Surg Res* 69(2):435-439, 1997.

19. Cheuvront SN, Ely BR, Kenefick RW, Michniak-Kohn BB, Rood JC, Sawka MN. No effect of nutritional adenosine receptor antagonists on exercise performance in the heat. *Am J Physiol Regul Integr Comp Physiol* 296(2):R394-401, 2009.

20. Cooper ZA, Singh IS, Hasday JD. Febrile range temperature represses TNF-alpha gene expression in LPS-stimulated macrophages by selectively blocking recruitment of Sp1 to the TNF-alpha promoter. *Cell Stress Chaperones*. (March 10, 2010). [Epub ahead of print]

21. Davis JM, Murphy EA, McClellan JL, Carmichael MD, Gangemi JD. Quercetin reduces susceptibility to influenza infection following stressful exercise. *Am J Physiol Regul Integr Comp Physiol* 295(2):R505-R509, 2008.

22. Deaciuc IV, Fortunato F, D'Souza NB, Hill DB, Schmidt J, Lee EY, McClain CJ. Modulation of Caspase-3 Activity and Fas Ligand mRNA Expression in Rat Liver Cells In Vivo by Alcohol and Lipopolysaccharide. *Alcohol Clin Exp Res* 23(2):349-356, 1999.

23. Dietz TJ, Somero GN. The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus Gillichthys). *Proc Natl Acad Sci USA* 89(8):3389–3393, 1992.

24. diIorio PJ, Holsinger K, Schultz RJ, Hightower LE. Quantitative evidence that both Hsc70 and Hsp70 contribute to thermal adaptation in hybrids of the livebearing fishes Poeciliopsis. *Cell Stress Chaperones* 1:139-147, 1996.

25. Dill DB, Costill DL. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37(2):247-248, 1974.

26. Ding LA, Li JS, Li YS, Zhu NT, Liu FN, Tan L. Intestinal barrier damage caused by trauma and lipopolysaccharide. *World J Gastroenterol* 10(16):2373-2378, 2004.

27. Ding XZ, Fernandez-Prada CM, Bhattacharjee AK, Hoover DL. Over-expression of hsp-70 inhibits bacterial lipopolysaccharide-induced production of cytokines in human monocyte-derived macrophages. *Cytokine*. 16(6):210-219, 2001.

28. Dokladny K, Kozak A, Wachulec M, Wallen ES, Ménache MG, Kozak W, Kluger MJ, Moseley PL. Effect of heat stress on LPS-induced febrile response in D-galactosamine-sensitized rats. *Am J Physiol Regul Integr Comp Physiol* 280(2):R338-R344, 2001.

29. Dokladny K, Lobb R, Wharton W, Ma TY, Moseley PL. LPS-induced cytokine levels are repressed by elevated expression of HSP70 in rats: possible role of NF-kappaB. *Cell Stress Chaperones* 15(2):153-163, 2010.

30. Dokladny K, Moseley PL, Ma TY. Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *Am J Physiol Gastrointest Liver Physiol* 290(2):G204-212, 2006.

31. Dokladny K, Wharton W, Lobb R, Ma TY, Moseley PL. Induction of physiological thermotolerance in MDCK monolayers: contribution of heat shock protein 70. *Cell Stress Chaperones* 11(3):268-275, 2006.

32. Dokladny K, Ye D, Kennedy JC, Moseley PL, Ma TY. Cellular and molecular mechanisms of heat stress-induced up-regulation of occludin protein expression: Regulatory role of heat shock factor-1. *Am J Pathol* 172(3):659-670. 2008.

33. Dong HP, Chen HW, Hsu C, Chiu HY, Lin LC, Yang RC. Previous heat shock treatment attenuates lipopolysaccharide-induced hyporesponsiveness of platelets in rats. *Shock* 24(3):239-244, 2005.

34. End DW, Look RA, Shaffer NL, Balles EA, Persico FJ. Non-selective inhibition of mammalian protein kinases by flavinoids in vitro. *Res Commun Chem Pathol Pharmacol* 56(1):75-86, 1987.

35. Epstein Y. Heat intolerance: predisposing factor or residual injury? *Med Sci Sports Exercise* 22(1):29–35, 1990.

36. Evgen'ev MB, Garbuz DG, Shilova VY, Zatsepina OG (2007) Molecular mechanisms underlying thermal adaptation of xeric animals. *J Biosci* 32(3):489-499.

37. Fairchild KD, Viscardi RM, Hester L, Singh IS, Hasday JD. Effects of hypothermia and hyperthermia on cytokine production by cultured human mononuclear phagocytes from adults and newborns. *J Interferon Cytokine Res* 20(12):1049-1055, 2000.

38. Feder ME, Krebs RA. Natural and genetic engineering of thermotolerance in *Drosophila melanogaster*. *Am Zool* 38(2):503–517, 1998.

39. Ferencz A, Szántó Z, Kalmár-Nagy K, Horváth OP, Rõth E. Mitigation of oxidative injury by classic and delayed ischemic preconditioning prior to small bowel autotransplantation. *Transplant Proc* 36(2):286-288, 2004.

40. Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, Baker J, Kerr DJ. Phase I Clinical Trial of the Flavonoid Quercetin: Pharmacokinetics *Physiol* 83:1413-1417, 1996.

41. Finaud J, Lac G, Filaire E. Oxidative stress: Relationship with exercise and training. *Sports Med* 36(4):327-358, 2006.

42. Fischer CP, Hiscock NJ, Basu S, Vessby B, Kallner A, Sjoberg LB, Febbraio MA, Pedersen BK. Vitamin E isoform-specific inhibition of the exercise-induced heat shock protein 72 expression in humans. *J Appl Physiol* 100:1679–1687, 2006.

43. Flanagan SW, Ryan AJ, Gisolfi CV, Moseley PL. Tissue-specific HSP70 response in animals undergoing heat stress. *Am J Physiol* 268(1 Pt 2):R28-R32, 1995.

44. Fleming SD, Starnes BW, Kiang JG, Stojadinovic A, Tsokos GC, Shea-Donohue T. Heat stress protection against mesenteric I/R-induced alterations in intestinal mucosa in rats. *J Appl Physiol* 92(6):2600-2607, 2002.

45. Fruth JM, Gisolfi CV. Work-heat tolerance in endurance- trained rats. *J Appl Physiol* 54(1):249–253, 1983.

46. Gaffin SL, Hubbard RW. Pathophysiology of heatstroke. In: Pandolf KB, Burr RE (ed) Medical Aspects of Harsh Environments, Vol 1. The Office of the Surgeon General at TMM Publications, Washington DC, 2002.

47. Gathiram P, Wells MT, Brock-Utne JG, Gaffin SL. Anti lipoplysaccharide improves survival in primates subjected to heat stroke. *Circ Shock* 23(3): 157-164, 1987.

48. Gehring WJ, Wehner R. Heat shock protein synthesis and thermotolerance in Cataglyphis, an ant from the Sahara desert. *Proc Natl Acad Sci U S A* 92(7):2994-2998, 1995.

49. Gellner K, Praetzel G, Bosch TC. Cloning and expression of a heat inducible hsp70 gene in two species of Hydra which differ in their stress response. *Eur J Biochem* 210(3):683–691, 1992.

50. Government publication. Operational rations of the department of defense. NATICK PAM 30-25, 8th Edition, Approved for public release, April 2009.

51. Grünenfelder J, Zünd G, Stucki V, Hoerstrup SP, Kadner A, Schoeberlein A, Turina M. Heat shock protein upregulation lowers cytokine levels after ischemia and reperfusion. *Eur Surg Res* 33(5-6):383-387, 2001.

52. Hall DM, Xu L, Drake VJ, Oberley LW, Oberley TD, Moseley PL, Kregel KC. Aging reduces adaptive capacity and stress protein expression in the liver after heat stress. *J Appl Physiol* 89: 749–759, 2000.

53. Harada H, Murakami T, Tea SS, Takeuchi A, Koga T, Okada S, Suico MA, Shuto T, Kai H. Heat shock suppresses human NK cell cytotoxicity via regulation of perforin. *Int J Hyperthermia* 23(8):657-665, 2007.

54. Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R, Riess H. The cellular and molecular basis of hyperthermia. *Crit Rev Oncol Hematol* 43(1):33-56, 2002.

55. Hirakawa T, Rokutan K, Nikawa T, Kishi K. Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* 111(2):345-357, 1996.

56. Hollman PCH, deVries JHM, van Leeuwen SD, Mengelers MJB, Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* 62:1276-92 69, 1995.

57. Hopkins AM, Baird AW, Nusrat A. ICAM-1: targeted docking for exogenous as well as endogenous ligands. *Adv Drug Deliv Rev* 56(6):763-778, 2004.

58. Horowitz M. Heat acclimation and cross-tolerance against novel stressors: genomic-physiological linkage. *Prog Brain Res* 162:373-392, 2007.

59. Horowitz M, Eli-Berchoer L, Wapinski I, Friedman N, Kodesh E. Stress-related genomic responses during the course of heat acclimation and its association with ischemic reperfusion cross-tolerance. *J Appl Physiol* 97(4):1496-1507, 2004.

60. Horowitz M, Maloyan A, Shlaier J. HSP 70 kDa dynamics in animals undergoing heat stress superimposed on heat acclimation. *Ann N Y Acad Sci* 813:617-619, 1997.

61. Horowitz M. Genomics and proteomics of heat acclimation. *Front Biosci (Schol Ed)* 2:1068-1080, 2010.

62. Horowitz M. Heat acclimation and cross-tolerance against novel stressors: genomic-physiological linkage. *Prog Brain Res* 162:373-392, 2007.

63. Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, Sakai T, Nishino H, Aoike A, Kawai K. Flavonoids inhibit the expression of heat shock proteins. *Cell Struct Funct* 15:393–401, 1990.

64. Hotchkiss R, Nunnally I, Lindquist S, Taulien J, Perdrizet G, Karl I. Hyperthermia protects mice against the lethal effects of endotoxin. *Am J Physiol* 265(6 Pt 2):R1447-R1457, 1993.

65. Huang L, Mivechi NF, Moskophidis D. Insights into regulation and function of the major stress-induced hsp70 molecular chaperone in vivo: analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. *Mol Cell Biol* 21(24):8575-8591, 2001.

66. Hung CH, Chang NC, Cheng BC, Lin MT. Progressive exercise preconditioning protects against circulatory shock during experimental heatstroke. *Shock* 23(5):426-433, 2005.

67. Jackson AS, Pollock ML. Generalized equations for predicting body density of men. *Br J Nutr* 40(3):497-504, 1978.

68. Jaeschke H. Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. *Am J Physiol* 273(3):G602-G611, 1997.

69. Jeukendrup AE, Vet-Joop K, Sturk A, Stegen JH, Senden J, Saris WH, Wagenmakers AJ. Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase reaction during and after a long-distance triathlon in highly trained men. *Clin Sci* 98(1):47-55, 2000.

70. Juel IS, Solligård E, Tvedt KE, Skogvoll E, Jynge P, Beisvag V, Erlandsen SE, Sandvik AK, Aadahl P, Grønbech JE. Post-ischaemic restituted intestinal mucosa is more resistant to further ischaemia than normal mucosa in the pig. *Scand J Clin Lab Inv* 68(2):106-116, 2008.

71. Klosterhalfen B, Hauptmann S, Offner FA, Amo-Takyi B, Töns C, Winkeltau G, Affify M, Küpper W, Kirkpatrick CJ, Mittermayer C. Induction of heat shock protein 70 by zinc-bis-(DL-hydrogenaspartate) reduces cytokine liberation,

apoptosis, and mortality rate in a rat model of LD100 endotoxemia. *Shock* 7(4):254-62, 1997.

72. Kluger MJ, Rudolph K, Soszynski D, Conn CA, Leon LR, Kozak W, Wallen ES, Moseley PL. Effect of heat stress on LPS-induced fever and tumor necrosis factor. *Am J Physiol* 273(3 Pt 2):R858-R863, 1997.

73. Koh Y, Lim CM, Kim MJ, Shim TS, Lee SD, Kim WS, Kim DS, Kim WD. Heat shock response decreases endotoxin-induced acute lung injury in rats. *Respirology* 4(4):325-330, 1999.

74. Kokura S, Yoshida N, Okuda T, Nakabe N, Sakamoto N, Isozaki Y, Hattori T, Handa O, Takagi T, Naito Y, Yoshikawa T. Hyperthermia ameliorates 2,4,6-trinitrobenzene sulphonic acid-induced colitis in rats: The role of heat shock proteins. *Int J Hyperthermia* 23(1):17-28, 2007.

75. Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 92(5):2177-2186, 2002.

76. Kriegel KC, Wall PT, Gisolfi CV Peripheral vascular responses to hyperthermia in the rat. *J Appl Physiol* 64: 2582–2588, 1988.

77. Kuennen MR, Gillum TL, Amorim FT, Kwon YS, Schneider SM. Palm cooling to reduce heat strain in subjects during simulated armoured vehicle transport. *Eur J Appl Physiol* 108(6):1217-1223, 2010.

78. Lambert GP, Gisolfi CV, Berg DJ, Moseley PL, Oberley LW, Kregel KC. Selected contribution: Hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. *J Appl Physiol* 92(4):1750-1761, 2002.

79. Lambert GP. Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: The ‘canary in the coal mine’ during exercise-heat stress? *Med Sport Sci.* Basel, Karger, 53:61-73, 2008.

80. Lee WC, Wen HC, Chang CP, Chen MY, Lin MT. Heat shock protein 72 overexpression protects against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke. *J Appl Physiol* 100(6):2073-2082, 2006.

81. Leon LR. Invited review: Cytokine regulation of fever: studies using gene knockout mice. *J Appl Physiol* 92(6):2648-2655, 2002.

82. Li GC. Elevated levels of 70,000 dalton heat shock protein in transiently thermotolerant Chinese hamster fibroblasts and in their stable heat resistant variants. *Int J Radiat Oncol Biol Phys* 11(1):165–177, 1985.

83. Li PL, Chao YM, Chan SH, Chan JY. Potentiation of baroreceptor reflex response by heat shock protein 70 in nucleus tractus solitarii confers cardiovascular protection during heatstroke. *Circulation*. 103(16):2114-2119, 2001.

84. Li Y, Qin JG, Abbott CA, Li X, Benkendorff K. Synergistic impacts of heat shock and spawning on the physiology and immune health of Crassostrea gigas: an explanation for summer mortality in Pacific oysters. *Am J Physiol Regul Integr Comp Physiol* 293(6):R2353-R2362, 2007.

85. Li Z, Diehl AM. Innate immunity in the liver. *Curr Opin Gastroenterol* 19(6):565-71, 2003.

86. Lim CL, Mackinnon LT. The roles of exercise-induced immune system disturbances in the pathology of heat stroke: The dual pathway model of heat stroke. *Sports Med* 36(1):39-64, 2006.

87. Liu WL, Chen SJ, Chen Y, Sun LM, Zhang W, Zeng YM, Zhou TH, Si JM. Protective effects of heat shock protein70 induced by geranylgeranylacetone in atrophic gastritis in rats. *Acta Pharmacol Sin* 28(7):1001-1006, 2007.

88. Lumlertgul D, Chuaychoo B, Thitiarchakul S, Srimahachota S, Sangchun K, Keoplung M. Heat stroke-induced multiple organ failure. *Ren Fail* 14(1):77-80, 1992.

89. Lyashko VN, Vikulova VK, Chernicov VG, Ivanov VI, Ulmasov KA, Zatsepina OG, Evgen'ev MB. Comparison of the heat shock response in ethnically and ecologically different human populations. *Proc Natl Acad Sci USA* 91:12492–12495, 1994.

90. Madsen KSA, Lewis MM, Tavernini J, Hibbard R, Fedorak N. Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. *Gastroenterology* 113(1):151-159, 1997.

91. Magalhães FD, Amorim FT, Passos RL, Fonseca MA, Oliveira KP, Lima MR, Guimarães JB, Ferreira-Júnior JB, Martini AR, Lima NR, Soares DD, Oliveira EM, Rodrigues LO. Heat and exercise acclimation increases intracellular levels of Hsp72 and inhibits exercise-induced increase in intracellular and plasma Hsp72 in humans. *Cell Stress Chaperones* (April 23, 2010) [Epub ahead of print]

92. Malm C. Susceptibility to infections in elite athletes: the S-curve. *Scand J Med Sci Sports* 16(1):4-6, 2006.

93. Maloyan A, Horowitz M. Beta-adrenergic signaling and thyroid hormones affect HSP72 expression during heat acclimation. *J Appl Physiol* 93(1):107-115, 2002.

94. Maloyan A, Palmon A, Horowitz M. Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. *Am J Physiol* 276(5 Pt 2):R1506-1515, 1999.

95. Manzerra P, Rush, SJ, Brown IR. Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. *J Cell Physiol* 170:130-137, 1997.

96. Maron MB, Wagner JA, Horvath SM. Thermoregulatory responses during competitive marathon running. *J Appl Physiol* 42(6):909–914, 1977.

97. McCallion K, Wattanasirichaigoon S, Gardiner KR, Fink MP. Ischemic preconditioning ameliorates ischemia- and reperfusion-induced intestinal epithelial hyperpermeability in rats. *Shock* 14(4):429-434, 2000.

98. McClung, JP, Hasday JD, He J, Montain SJ, Cheuvront SN, Sawka MN, Singh I. Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. *Am J Physiol Regul Integr Comp Physiol* 294:R185–R191, 2008.

99. Mitchell D, Senay LC, Wyndham CH, van Rensburg AJ, Rogers GG, Strydom NB. Acclimatization in a hot, humid environment: energy exchange, body temperature, and sweating. *J Appl Physiol* 40(5):768-78, 1976.

100. Mizzen LA, Welch WJ. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J Cell Biol* 106(4):1105-1116, 1988.

101. Moran DS, Eli-Berchoer L, Heled Y, Mendel L, Schocina M, Horowitz M. Heat intolerance: does gene transcription contribute? *J Appl Physiol* 100(4):1370-1376, 2006.

102. Moseley PL, Gapen C, Wallen ES, Walter ME, Peterson MW. Thermal stress induces epithelial permeability. *Am J Physiol* 267(2 Pt 1):C425-C434, 1994.

103. Moseley PL, Gisolfi CV. New frontiers in thermoregulation and exercise. *Sports Med* 16(3):163-7, 1993.

104. Moseley PL. Heat shock proteins and heat adaptation of the whole organism. *J Appl Physiol* 83: 1413-1417, 1997.

105. Moseley PL. Heat shock proteins and the inflammatory response. *Ann N Y Acad Sci* 856:206-213, 1998.

106. Murphy EA, Davis JM, Carmichael MD, Gangemi JD, Ghaffar A, Mayer EP. Exercise stress increases susceptibility to influenza infection. *Brain Behav Immun* 22:1152-1155, 2008.

107. Nadel ER, Pandolf KB, Roberts MF, Stolwijk JAJ. Mechanisms of thermal acclimation to exercise and heat. *J Appl Physiol* 37(4):515–520, 1974.

108. Nadel ER, Wenger CB, Ronberts MF, Stolwijk AJ, Cafarelli E. Physiological defenses against hyperthermia of exercise. *Ann. N.Y. Acad. Sci* 301:98–109, 1977.

109. Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappa beta system. *Clin Vaccine Immunol* 13(3):319-328, 2006.

110. Nakada J, Matsura T, Okazaki N, Nishida T, Togawa A, Minami Y, Inagaki Y, Ito H, Yamada K, Ishibe Y. Oral administration of geranylgeranylacetone improves survival rate in a rat endotoxin shock model: administration timing and heat shock protein 70 induction. *Shock* 24(5):482-487, 2005.

111. Nakatani Y, Fukui H, Kitano H, Nagamoto I, Tsujimoto T, Kuriyama S, Kikuchi E, Hoppou K, Tsujii T. Endotoxin clearance and its relation to hepatic and renal disturbances in rats with liver cirrhosis. *Liver* 21(1):64-70, 2001.

112. Nathan CF. Secretory products of macrophages. *J Clin Invest* 79(2):319-326, 1987.

113. Near JC, Easton DP, Rutledge PS, Dickinson DP, Spotila JS. Heat shock protein 70 gene expression in intact salamanders Eurycea bislineata in response to calibrated heat shocks and to high temperatures encountered in the field. *J Exp Zool* 256:303–314, 1990.

114. Ng QY, Lee KW, Byrne C, Ho TF, Lim CL. Plasma endotoxin and immune responses during a 21-km road race under a warm and humid environment. *Ann Acad Med Singapore* 37(4):307-314, 2008.

115. Nieman DC, Henson DA, Gross SJ, Jenkins DP, Davis JM, Murphy EA, Carmichael MD, Dumke CL, Utter AC, McAnulty SR, McAnulty LS, Mayer EP. Quercetin reduces illness but not immune perturbations after intensive exercise. *Med Sci Sports Exercise* 39(9):1561-1569, 2007.

116. Nieman DC, Johanssen LM, Lee JW, Arabatzis K. Infectious episodes in runners before and after the Los Angeles Marathon. *J Sports Med Phys Fitness* 30(3):316-328, 1990.

117. Niess AM, Fehrenbach E, Schlotz E, Sommer M, Angres C, Schositsch KT, Battenfeld N, Golly IC, Biesalski HK, Northoff H, Dickhuth HH. Effects of RRR-a-tocopherol on leukocyte expression of HSP72 in response to exhaustive treadmill exercise. *Int J Sports Med* 23:445–452, 2002.

118. Niessen CM. Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol* 127(11):2525-2532, 2007.

119. Nolan JP. Endotoxin, reticuloendothelial function, and liver injury. *Hepatology* 1(5):458-65, 1981.

120. Ohkawara T, Takeda H, Nishiwaki M, Nishihira J, Asaka M. Protective effects of heat shock protein 70 induced by geranylgeranylacetone on oxidative injury in rat intestinal epithelial cells. *Scand J Gastroenterol* 41(3):312-317, 2006.

121. Otaka M, Odashima M, Tamaki K, Watanabe S. Expression and function of stress (heat shock) proteins in GI tract. *Int J Hyperthermia* 25(8):634-640, 2009.

122. Paidas CN, Mooney ML, Theodorakis NG, De Maio A. Accelerated recovery after endotoxic challenge in heat shock-pretreated mice. *Am J Physiol Regul Integr Comp Physiol* 282(5):R1374-R1381, 2002.

123. Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. *J Appl Physiol* 82(2):571-576, 1997.

124. Powers SK, Jackson MJ. Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. *Physiol Rev* 88(4):1243-1276, 2008.

125. Pritts TA, Wang Q, Sun X, Fischer DR, Hungness ES, Fischer JE, Wong HR, Hasselgren PO. The stress response decreases NF-kappaB activation in liver of endotoxemic mice. *Shock* 18(1):33-37, 2002.

126. Pritts TA, Wang Q, Sun X, Moon MR, Fischer DR, Fischer JE, Wong HR, Hasselgren PO. Induction of the stress response in vivo decreases nuclear factor-kappa B activity in jejunal mucosa of endotoxemic mice. *Arch Surg* 135(7):860-866, 2000.

127. Rechner AR, Kuhnle G, Bremner P, Hubbard GP, Moore KP, Rice-Evans CA. The metabolic fate of dietary polyphenols in humans. *Free Radic Biol Med* 33(2):220-35, 2002.

128. Rinehart JP, Hayward SA, Elnitsky MA, Sandro LH, Lee RE Jr, Denlinger DL. Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. *Proc Natl Acad Sci U S A* 103(38):14223-14227, 2006.

129. Robertson MJ, Cameron C, Lazo S, Cochran KJ, Voss SD, Ritz J. Costimulation of human natural killer cell proliferation: role of accessory cytokines and cell contact-dependent signals. *Nature Immunol* 15(5):213-225, 1996.

130. Rowell LB, Blackmon JR, Martin RH, Mazzarella JA, Bruce RA. Hepatic clearance of indocyanine green in man under thermal and exercise stresses. *J Appl Physiol* 20(3):384-394, 1965.

131. Rowell LB. Human cardiovascular adjustments to exercise and thermal stress. *Physiol Rev* 54(1):75-159, 1974.

132. Sakurada S, Hales JR. A role for gastrointestinal endotoxins in enhancement of heat tolerance by physical fitness. *J Appl Physiol* 84(1):207-214, 1998.

133. Sam AD 2nd, Sharma AC, Law WR, Ferguson JL. Splanchnic vascular control during sepsis and endotoxemia. *Front Biosci* 2:e72-92, 1997.

134. Sandström ME, Siegler JC, Lovell RJ, Madden LA, McNaughton L. The effect of 15 consecutive days of heat-exercise acclimation on heat shock protein 70. *Cell Stress Chaperones* 13(2):169-175, 2008.

135. Selkirk GA, McLellan TM, Wright HE, Rhind SG. Expression of intracellular cytokines, HSP72, and apoptosis in monocyte subsets during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 296(3):R575-R586, 2009.

136. Selkirk GA, McLellan TM, Wright HE, Rhind SG. Mild endotoxemia, NF-kappaB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 295(2):R611-23, 2008.

137. Sepponen K, Poso AR. The inducible form of heat shock protein 70 in the serum, colon, and small intestine of the pig: Comparison to conventional stress markers. *Vet J* 171(3):519-524, 2006.

138. Shi Q, Dong Z, Wei H. The involvement of heat shock proteins in murine liver regeneration. *Cell Mol Immunol* 4(1):53-57, 2007.

139. Shi Y, Tu Z, Tang D, Zhang H, Liu M, Wang K, Calderwood SK, Xiao X. The inhibition of LPS-induced production of inflammatory cytokines by HSP70

involves inactivation of the NF-kappaB pathway but not the MAPK pathways. *Shock* 26(3):277-284, 2006.

140. Singh IS, He JR, Calderwood S, Hasday JD. A high affinity HSF-1 binding site in the 5'-untranslated region of the murine tumor necrosis factor-alpha gene is a transcriptional repressor. *J Biol Chem* 277(7):4981-4988, 2002.

141. Singh R, Kølvraa S, Bross P, Jensen UB, Gregersen N, Tan Q, Knudsen C, Rattan SI. Reduced heat shock response in human mononuclear cells during aging and its association with polymorphisms in HSP70 genes. *Cell Stress Chaperones* 11(3):208-215, 2006.

142. Sonna LA, Gaffin SL, Pratt RE, Cullivan ML, Angel KC, Lilly CM. Effect of acute heat shock on gene expression by human peripheral blood mononuclear cells. *J Appl Physiol* 92(5):2208-2220, 2002.

143. Stillman JH, Tagmount A. Seasonal and latitudinal acclimatization of cardiac transcriptome responses to thermal stress in porcelain crabs, Petrolisthes cinctipes. *Mol Ecol* 18(20):4206-4226, 2009.

144. Stojadinovic A, Kiang J, Goldhill J, Matin D, Smallridge R, Galloway R, Shea-Donohue T. Induction of the heat shock response prevents tissue injury during acute inflammation of the rat ileum. *Crit Care Med* 25(2):309-317, 1997.

145. Stojadinovic A, Kiang J, Smallridge R, Galloway R, Shea-Donohue T. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology* 109(2):505-515, 1995.

146. Strieter RM, Lynch JP 3rd, Basha MA, Standiford TJ, Kasahara K, Kunkel SL. Host responses in mediating sepsis and adult respiratory distress syndrome. *Semin Respir Infect* 5(3):233-247, 1990.

147. Suffredini AF, Fromm RE, Parker MM, Brenner M, Kovacs JA, Wesley RA, Parrillo JE. The cardiovascular response of normal humans to the administration of endotoxin. *N Engl J Med* 321(5):280-287, 1989.

148. Suganuma T, Irie K, Fujii E, Yoshioka T, Muraki T. Effect of heat stress on lipopolysaccharide-induced vascular permeability change in mice. *J Pharmacol Exp Ther* 303(2):656-663, 2002.

149. Sumagin R, Sarelius IH. TNF-alpha activation of arterioles and venules alters distribution and levels of ICAM-1 and affects leukocyte-endothelial cell interactions. *Am J Physiol Heart Circ Physiol* 291(5):H2116-2125, 2006.

150. Sun D, Chen D, Du B, Pan J. Heat shock response inhibits NF-kappaB activation and cytokine production in murine Kupffer cells. *J Surg Res* 129(1):114-121, 2005.

151. Tazuke Y, Drongowoski RA, Teitelbaum DH, Coran AG. Interleukin-6 changes tight junction permeability and intracellular phospholipid content in a human enterocyte cell culture model. *Pediatr Surg Int* 19(5):321-325, 2003.

152. Tetievsky A, Cohen O, Eli-Berchoer L, Gerstenblith G, Stern MD, Wapinski I, Friedman N, Horowitz M. Physiological and molecular evidence of heat acclimation memory: a lesson from thermal responses and ischemic cross-tolerance in the heart. *Physiol Genomics* 34(1):78-87, 2008.

153. Tomanek L, Somero GN. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus Tegula) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J Exp Biol* 202 (Pt 21):2925-2936, 1999.

154. Tomisato W, Tsutsumi S, Tsuchiya T, Mizushima T. Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. *Biol Pharm Bull* 24(8):887-891, 2001.

155. Tsuruma T, Yagihashi A, Watanabe N, Yajima T, Kameshima H, Araya J, Hirata K. Heat-shock protein-73 protects against small intestinal warm ischemia-reperfusion injury in the rat. *Surgery* 125(4):385-395, 1999.

156. Ulmasov HA, Karaev KK, Lyashko VN, Evgen'ev MB. Heat-shock response in camel (Camelus dromedarius) blood cells and adaptation to hyperthermia. *Comp Biochem Physiol B* 106(4):867-872, 1993.

157. Ulmasov KA, Shammakov S, Karaev K, Evgen'Ev MB. Heat shock proteins and thermoresistance in lizards. *Proc Natl Acad Sci USA* 89(5):1666–1670, 1992.

158. Ushijima H, Tanaka K, Takeda M, Katsu T, Mima S, Mizushima T. Geranylgeranylacetone protects membranes against nonsteroidal anti-inflammatory drugs. *Mol Pharmacol* 68(4):1156-1161, 2005.

159. Wang ZZ, Wang CL, Wu TC, Pan HN, Wang SK, Jiang JD. Am J Med. Autoantibody response to heat shock protein 70 in patients with heatstroke. *Am J Med* 111(8):654-657, 2001.

160. Wehner R, Marsh AC, Wehner S. Desert ants on a thermal tightrope. *Nature* 357:586-587, 1992.

161. Welch PS, Loomis HA. A limno-logical study of Hydra oligactis in Douglas Lake, Michigan. *Trans Am Microsc Soc* 43:203-235, 1924.

162. Weshler Z, Kapp DS, Lord PF, Hayes T. Development and decay of systemic thermotolerance in rats. *Cancer Res* 44(4):1347-1351, 1984.

163. Wu C. Heat shock transcription factors: Structure and regulation. Annu Rev Cell Dev Biol 11:441-469, 1995.

164. Wu T, Chen S, Xiao C, Wang C, Pan Q, Wang Z, Xie M, Mao Z, Wu Y, Tanguay RM. Presence of antibody against the inducible Hsp71 in patients with acute heat-induced illness. *Cell Stress Chaperones* 6(2):113-120, 2001.

165. Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM. Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans. *J Appl Physiol* 103(4):1196-1204, 2007.

166. Zambraski, EJ. Military Performance Division: Bioactive food compounds: Potential to improving soldier performance and mitigating injury. Unclassified Presentation. 2006. Available at www.militaryfood.org/ powerpoint/F06_SESSIONV_ FOODCOM_POUNDS_Zambraski.ppt.