

**SYSTEMIC INFLAMMATORY  
RESPONSE TO CONSECUTIVE DAYS OF EXERCISE IN THE HEAT**

by

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## Abstract

**PURPOSE:** The purpose of this study was to determine the LPS-mediated SIR to acute bouts of exercise in the heat and any adaptive response that occurs during consecutive days of exercise in the heat. **METHODS:** 8 healthy males (Age =  $24 \pm 3$  yrs,  $VO_{2max} = 55.3 \pm 3.6$  ml\*kg<sup>-1</sup>\*min<sup>-1</sup>) were asked to run (~78%  $VO_{2max}$ ) in a hot environment on 5 consecutive days. Participants ran each day until core temperature ( $T_c$ ) was elevated 2°C above rest or volitional exhaustion. Blood samples were obtained pre-exercise, post-exercise, 1 h post-exercise, and 3 h post-exercise on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day of exercise. **RESULTS:** Resting  $T_c$  ( $P < 0.05$ ) was significantly decreased on Days 4 and 5 as compared to Day 1. Heart rate (resting and exercise) and Physiological Strain Index were not significantly altered by 5 consecutive days of exercise in the heat. Exercise time was not significantly different among days. Plasma [IL-6] was significantly elevated post-exercise each day (14.8 pg/ml vs. 10.6 pg/ml) as compared to resting values. No significant difference in the IL-6 response was detected among days. Plasma [TNF- $\alpha$ ] was not significantly elevated above rest after exercise on any day though mean plasma concentrations were significantly lower on Day 5 at 1h (3.1 pg/ml vs. 4.7 pg/ml;  $P = 0.01$ ) and 3 h (3.5 pg/ml vs. 4.7 pg/ml;  $P = 0.05$ ) post exercise as compared to Day 1. Plasma [IL-10] was significantly (0.9 pg/ml vs 0.5 pg/ml;  $P < .01$ ) elevated from baseline at 1 h post exercise with no difference among days. Plasma LPS activity significantly increased post-exercise (1.4 EU/ml vs. 0.9 EU/ml;  $P < 0.01$ ) each day as compared to rest. No difference was detected among days for LPS activity. **CONCLUSION:** These

data indicate that consecutive days of exercise in the heat results in a less pro-inflammatory (TNF- $\alpha$ ) response to exercise and this adaptation may be a result of an increasing LPS tolerance.

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## List of Abbreviations

APR.....	Acute Phase Response
Caco-2.....	Human colonic epithelial cells
CHO.....	Carbohydrate
GI.....	Gastrointestinal
HSF-1.....	Heat Shock Factor -1
HSP-70.....	Heat Shock Protein-70
IL-1 $\beta$ .....	Interleukin-1 Beta
IL-10.....	Interleukin-10
IL-6.....	Interleukin-6
IL-1ra.....	Interleukin-1 receptor antagonist
HR.....	Heart rate
LPS.....	Lipopolysaccharide/Endotoxin
MDCK.....	Madin-Darby Canine Kidney epithelial cells
mRNA.....	Messenger ribonucleic acid
NAC.....	N-acetylcysteine
NO.....	Nitric Oxide
NSAIDS.....	Non-steroidal anti-inflammatory drugs
PBMC.....	Peripheral blood mononuclear cells
PSI.....	Physiological Strain Index

RH.....	Relative humidity
RNS.....	Reactive Nitrogen Species
ROS.....	Reactive Oxygen Species
SBF.....	Splanchnic flow
SIR.....	Systemic Inflammatory Response
SIRS.....	Systemic Inflammatory Response Syndrome
sTNFr.....	Soluble Tumor Necrosis Factor receptor
T <sub>c</sub> .....	Core temperature
TER.....	Trans-epithelial resistance
TJ.....	Tight junction
TNF- $\alpha$ .....	Tumor Necrosis Factor – alpha
VO <sub>2</sub> .....	Volume of oxygen consumption per minute
VO <sub>2max</sub> .....	Maximal Volume of Oxygen Consumption per minute

## **CHAPTER I**

### **INTRODUCTION**

High environmental temperatures present a natural hazard to occupational workers, athletes, and military personnel who are required to perform strenuous work under thermal stress. Between 1979 and 2002 the United States recorded 4,780 deaths associated with heat related complications; however these numbers are probably underestimated due to complications with diagnosis (19, 20). Over the past decade 118 fatalities per year were attributed to hot environmental temperatures (164). Heat stroke is the most debilitating of heat related illness and results from exposure to high environmental temperatures (referred to as classical heat stroke) or from strenuous exercise (referred to as exertional heat stroke). Both situations result in the elevation of core body temperatures  $>40^{\circ}\text{C}$  and central nervous system (CNS) abnormalities (e.g. convulsion and delirium). A more recent definition of heat stroke pathogenesis has been expanded to include the combined effects of the systemic inflammatory response syndrome (SIRS), coagulation abnormalities, and heat cytotoxicity (6).

Elevations in core temperature (hyperthermia) result when the body absorbs or produces more heat than it dissipates. Hyperthermia occurs as a result of increased rate of metabolic heat production, such as during strenuous bouts of exercise, or when environmental temperatures are greater than skin temperatures and heat is gained from the environment. Increased core temperatures promote dilation of the peripheral vasculature (71) to increase blood flow to the skin to promote heat dissipation.

Subsequently, vasoconstriction of the splanchnic vasculature decreases blood flow to splanchnic tissue (stomach, spleen, liver, intestines) (54). The blood flow reduction to the splanchnic tissue can result in an ischemic and hypoxic environment which promotes oxidative and nitrosative damage to the enterocytes, leading to decreased intestinal function (56, 75, 123). During an acute bout of exercise, blood is redistributed to both more metabolically active tissue (i.e. force-producing skeletal muscle) and to the peripheral vasculature to promote heat loss while blood flow to the splanchnic tissue is subsequently decreased (152). The reduction in blood flow to splanchnic tissue is linearly related to exercise intensity and the ambient environmental temperature (47, 179). Increased gastrointestinal permeability is well demonstrated after strenuous exercise over both short and long durations (1, 15, 67, 124), during passive heat stress (44, 56, 75), and in heat stroke (8). These prior studies suggest a link between elevated core body temperatures, induced by environment or exertion, and an increase in gastrointestinal (GI) permeability. Repeated exposure to exercise in high environmental temperatures induces physiological adaptations (e.g. expanded blood volume) that allow for greater perfusion of splanchnic tissue in subsequent bouts of exercise and a greater heat tolerance (92, 163, 191). This adaptive process is termed acclimation when conditions are artificially created under laboratory control and acclimatization when conditions are environmentally induced.

Millions of bacteria, critical to the proper functioning of the GI tract, are found and maintained throughout the lumen of the gastrointestinal (GI) system. These bacteria are prevented from entering the systemic circulation by the GI barrier, which represents the natural boundary between the GI lumen and the systemic circulation. Damage, and resultant decreased function, of this barrier leads to an increase in intestinal permeability

which allows for an increase in non-mediated diffusion of molecules from the GI lumen to the systemic circulation (182). Lipopolysaccharide (LPS; endotoxin) is a large lipid and polysaccharide containing molecule associated with the outer membrane of Gram-negative bacteria in the GI lumen. Increased intestinal permeability allows for the translocation of LPS from the GI lumen into the systemic circulation where it is a potent stimulator of the systemic inflammatory response (SIR) (144, 177, 185).

When local and systemic perturbations (such as bacterial infection) to immunological homeostasis occur, the first immunological reactions represent a non-specific response referred to as the SIR. In this response, immunomodulating proteins, referred to as cytokines, are secreted from various tissues. The first cytokines to be secreted into the circulation are TNF- $\alpha$  and IL-1 $\beta$ , which stimulates the production of IL-6 from immunocompetent cells. Both TNF- $\alpha$  and IL-1 $\beta$  are pyrogenic cytokines, which elevate body temperature and activate the immune cells. When elevated in blood plasma, IL-6 decreases the secretion of TNF- $\alpha$  and IL-1 $\beta$  and promotes the release of anti-inflammatory proteins and cytokines.

Exercise is known to cause a SIR that differs from bacterial infection (131). With exercise, IL-6 is increased in the plasma to a far greater extent than other acute phase cytokines, which is thought to be the reason for the anti-inflammatory effect of exercise (119). However, IL-6 and the SIR to exercise is modality, intensity, and duration dependent (135). The largest increases of plasma [IL-6] following an acute bout of exercise are seen after long duration (>2 h) (103, 105, 118, 119) of continuous exercise. Cycling exercise appears to elicit a much smaller increase in circulating IL-6 than a bout of running (38). Furthermore, exercise in hot, as compared to cool, environmental temperatures has been shown to increase the pro-inflammatory response to a bout of

exercise (14). It is thought that the reason for an increased pro-inflammatory response to strenuous exercise in hot environments is due to increased bacterial translocation into the systemic circulation caused by increased GI permeability associated with heavy ( $> 80\%$   $\text{VO}_{2\text{max}}$ ) (1, 124) and long duration ( $> 1$  h) (11) exercise.

Increased bacterial translocation, and the resultant inflammatory response, is suspected to cause the SIRS that has been described as a key factor in the pathogenesis of heat stroke (6, 78). Patients admitted to the hospital following heat stroke present with elevated levels of IL-6, TNF- $\alpha$ , and LPS (5, 8).

A wide body of information concerning the cytokine response to different exercise modalities, durations, and intensities (67, 103-105, 118, 119, 121, 122, 133-136, 138) in humans is available. However, information about the time course of the cytokine response to exercise in hot environmental temperatures as compared to cool environmental temperatures is sparse. Furthermore evidence of the time course of the cytokine response to repeated bouts of exercise in hot environmental temperatures is lacking. Lastly, controlled studies comparing both the plasma LPS response and SIR to exercise are lacking. Thus the purpose of this study was to determine the plasma LPS response and SIR to acute bouts of exercise in the heat and any adaptive response that occurs during consecutive days of exercise in the heat. We hypothesize the LPS response to an acute bout of exercise will be significantly decreased on Day 5 of exercise in the heat as compared to Day 1. Subsequently the SIR to exercise on Day 5 will result in a blunted IL-6 response and a less pro-inflammatory (TNF- $\alpha$ ) response as compared to Day 1.

## **CHAPTER II**

### **REVIEW OF LITERATURE**

#### **Introduction**

The review of literature provides a brief summary of cardiovascular responses to exercise, exercise in the heat, and cardiovascular adaptation to repeated bouts of exercise in the heat. Next, an overview of the gastrointestinal barrier, gastrointestinal barrier function, and causes of gastrointestinal barrier dysfunction in the heat and during exercise will be covered. A brief overview of the SIRS to bacterial infection and the SIRS to acute bouts of exercise in cool and hot environmental temperatures will be covered next. Finally, a brief overview on the SIRS role in the pathogenesis of heat illness and heat stroke will be discussed.

#### **Acute and Chronic Adaptations to Exercise in the Heat**

An acute bout of exercise results in an increased cardiac output ( $Q$ ), heart rate (HR), stroke volume (SV), and oxygen consumption rate ( $\dot{V}O_2$ ) (150). The magnitude of the increase in these variables, except SV, is linearly proportional to the exercise intensity. To maintain an adequate  $O_2$  supply to the most metabolically active tissue (i.e. force-producing skeletal muscle) blood flow is partitioned to that tissue from less metabolically active (i.e. splanchnic) tissue. Excess heat generated from elevated rates of metabolism increase body temperature proportional to relative work rates (157). To prevent detrimental heat gain from metabolic heat production, blood is also redistributed to the dilated peripheral vasculature to promote increased convective heat loss from deep

tissue to the periphery while at the same time, the evaporation of sweat decreases skin temperatures and cools blood in the skin microcirculation. In general, the circulatory changes that occur during an acute exercise bout must adequately vasoconstrict vasculature supplying tissues with lower metabolic rates so that blood flow to force-producing skeletal muscle and skin is sufficient to meet metabolic demands and prevent large gains in heat without compromising O<sub>2</sub> supply to the heart and brain.

Exercise in hot environments presents a challenge to thermoregulation due to a decrease in the thermal gradient between the skin and the surrounding environment, thus decreasing heat transfer from the skin to the environment. For a given exercise intensity, exercise in the heat requires a greater perfusion of cutaneous blood vessels to aid heat transfer from the core as compared to thermoneutral temperatures (2). It is now widely accepted that heat stress, exercise, and combined exercise and heat stress result in an increased skin blood flow (SBF) due to cutaneous vasodilation, and that splanchnic, and other less metabolically active tissue (adipose, nonworking skeletal muscle, and visceral organs) receive less blood supply (2, 71, 87, 95, 101, 145, 151-153, 157, 158). However, exercise in high environmental temperatures may result in a competition for blood flow between force-producing skeletal muscle and the skin at high exercise intensities (2, 53).

Heat acclimation is the process of repeated exposures to hot environments and the reduction of the stress imposed on the body by these environments that leads to an improved heat and exercise tolerance (128, 159). The main physiological adaptations that occur during acclimation to exercise in hot environments include hypervolemia, increased sweating rate, decreased core temperature (T<sub>c</sub>) for a given exercise intensity, decreased HR for a given exercise intensity, more dilute sweat, and a decreased T<sub>c</sub> at the

onset of sweating at a lower  $T_c$  (48, 96, 100, 166). Increased tolerance for exercise in the heat occur by the 2<sup>nd</sup> day when time to exhaustion is significantly increased (126). The increased ability to dissipate heat allows for greater perfusion of splanchnic, and other tissue, and results in less GI barrier dysfunction. This has been suggested to be one of the main mechanisms for the increase in heat and exercise tolerance in trained and acclimated individuals (156). However, to the authors knowledge no studies analyzing changes in LPS tolerance during the acclimation process are currently available.

A majority of adaptations (~80%) are observed in the first 7-10 days of exercise in the heat and the acclimation process is virtually complete in 21 days (35, 148). Experimental procedures to induce heat acclimation in humans have often consisted of low to moderate (40-50%  $VO_{2max}$ ) exercise intensities for 60-100 min per day (24, 45, 46, 125, 127, 142, 162). However, research findings indicate that heat acclimation can be achieved in highly trained individuals using higher exercise intensities (60-75%  $VO_{2max}$ ) with a reduced total amount of time required to achieve acclimation (5-8 days) (42, 43, 65, 154). Thus, the rapidity of acclimation to exercise in the heat is influenced by prior training status and the intensity and duration of the exercise that is performed in the heat.

### **Gastrointestinal Function and Permeability**

The GI barrier represents the natural barrier between the contents of the GI lumen and the splanchnic circulation. The epithelial lining of the GI tract consists of tightly packed columnar cells called enterocytes that are selectively permeable to nutrients, ions, and water. On the apical membrane of the enterocytes are millions of tiny microvilli, which increase the surface area for absorption by 10-fold in some regions of the GI tract (51). Transcellular movement of nutrients, ions, and solutes across enterocytes occurs through diffusion and active transport via specific pumps and channels. Paracellular

movement of molecules across the barrier occurs in tightly regulated junctions between the enterocytes (21). These tight junctions (TJ) are formed by contact of adjacent enterocytes in which multiple families of scaffolding and membrane spanning proteins (zonula occludens, claudins, occludin, and cadherins) “seal” the space and selectively regulate translocation of molecules based on molecular weight (21, 108). Molecules with a molecular mass greater than 180 Da move through the TJs into the mucosal layer (61).

Integrity of the GI barrier is important to not only prevent translocation of exogenous bacteria and toxins, but to prevent translocation of naturally occurring bacteria associated with the GI lumen. More than 500 bacterial species, both gram negative and gram positive, are known to populate the intestinal microflora with up to  $10^{12}$  colony forming units (CFU) per gram of intestines (111). LPS is a large lipid and polysaccharide containing molecule associated with the outer membrane of Gram-negative bacteria in the GI lumen. Increased intestinal permeability allows for the translocation of LPS from the GI lumen into the systemic circulation.

At rest GI permeability is present, even in healthy individuals. In the absence of disease or physiological stress the liver removes this LPS from the portal circulation preventing its accumulation in the general circulation. However, when physiological stresses such as exercise, hyperthermia, burns, or hemorrhage are imposed on the body, GI barrier dysfunction has been shown to occur (1, 27, 55, 75, 124). The mechanisms behind barrier dysfunction during physiological stresses are most likely multifactorial and include, but are not limited to, the cytotoxic effects of heat (hyperthermia), hypoxia induced by prolonged splanchnic ischemia, oxidative and nitrosative stress resulting from ischemia and resultant hypoxia, and high levels of circulating pro-inflammatory cytokines.

Effects of Hyperthermia On Epithelial Permeability In Vitro. Epithelial

permeability is assessed in cell culture using multiple cell lines including, but not limited to, human colonic epithelial cells (Caco-2), Madin-Darby Canine Kidney epithelial cells (MDCK), and T84 human epithelial cells. When grown to confluence these cell lines form enterocytes complete with tight junctions and a bipolar membrane that form a resistance to similar to the GI barrier. Measuring the transepithelial resistance (TER) provides a measurement of the total permeability of the membrane while measuring the rate of flux of large molecules (e.g. inulin) provides a measure of paracellular permeability. The aim of the following section is to provide an overview of the effects of hyperthermia on epithelial permeability *in vitro*.

Mosley et al. (94) exposed MDCK monolayers to physiologically relevant temperature increases (37°C-42°C) which resulted in significantly increased epithelial permeability, as measured by transepithelial resistance. Results indicate increased permeability during thermal stress was not accompanied by an increase in cell death. Permeability of mannitol (molecular mass = 182.172 kD) during thermal stress increased approximately 50-fold in monolayers exposed to 41.3°C as compared to monolayers exposed to 37°C indicating a significant increase in paracellular permeability. Interestingly, cells that received a 90 min heat exposure (41°C) 48 h prior to experimentation maintained epithelial permeability until bathing solution reached 39.4°C as compared to 38.3°C in unconditioned cells. This increased thermal tolerance was associated with an elevated level of the ubiquitously expressed cytoprotective protein, Heat Shock Protein 70 (HSP 70). HSP 70 is a cytoprotective protein associated with increased thermotolerance (183). A series (33) of experiments using the same cell line confirmed the adaptive and protective heat shock response to prior heat conditioning.

Briefly, quercetin was used during prior heat conditioning to block heat shock transcription factor 1 (HSF-1) activity, thus preventing the protective heat shock protein response. Results showed a decreased recovery rate of TER from thermal stress as compared to unconditioned cells. Similar results have been observed upon exposure of the T84 human epithelial cell line to thermal stress (194). Preconditioning these cells with thermal stress (39°C) for 1 h significantly decreased the decline in TER when cells were exposed to 43°C for 1 h as compared to unconditioned cells.

Utilizing cultured Caco-2 intestinal epithelial cells in a monolayer, Dokladny et al. (32) also provided evidence that physiologically relevant temperature increases (37°-41°C) decreased TER and significantly increased inulin flux indicating increased TJ permeability. Analysis of heat induced TER and paracellular permeability revealed an inverse linear relationship indicating a correlation ( $r = 0.96$ ) between an increase in paracellular permeability and increased epithelial permeability due to heat stress. In this study heat exposure also increased activity of HSF-1, expression of HSP 70, and expression of the tight junction protein, occludin. Blockade of HSF-1 activity with quercetin prevented increases in HSP 70 and occludin protein expression, ablated tight junction localization of occludin, and resulted in further decreases in TER and increases in TJ permeability.

Exposing the MDKC cell line to thermal stress (45°C) increased free radical production in epithelial cells as assessed through paramagnetic resonance spin trapping (40). Further studies (165) in Caco-2 cell monolayers provided evidence that the highly reactive oxygen species, hydrogen peroxide ( $H_2O_2$ ) induced a fall in TER and increased TJ permeability of the monolayer. Subsequently, ZO-1 and occludin, both TJ proteins, were found to not be localized to intercellular junctions.

Lambert et al. (74) exposed everted rat intestinal segments to incubation media for 120 min and showed significantly increased rates of FITC-dextran (FD-4; 4,000 Da) transport across the membrane of duodenal, jejunal, ileal, and colonic tissues in 42°C bathing solutions as compared to 37°C incubation mediums. Interestingly no evidence of lipid peroxidation, a measure of oxidative lipid damage, was found in either treatment. Furthermore, the use of antioxidant enzyme-mimetics, Tempol and Ebselen, as well as the Nitric Oxide Synthase (NOS) inhibitor, L-NAME, had no significant effect on the temperature induced increase in FD-4 transport. Similar results were found using similar methodology by Oliver et al. (113). The antioxidants, tiron and trolox, had no effect on the rate of temperature induced FD-4 transport; however the antioxidant N-acetylcysteine (NAC) prevented the temperature induced increase. Protein carbonyls, a measure of oxidative protein damage, were significantly increased after 90 min of 42°C incubation, while incubation of intestinal segments with NAC completely blunted temperature induced protein damage. In theory, the use of antioxidants should mitigate temperature induced increases in intestinal epithelial barrier permeability if free radical production during hyperthermia is a causative factor in barrier dysfunction. However, the use of everted intestinal tissue *in vitro* would isolate the effects of temperature on barrier dysfunction. The vasoconstriction, reduction of blood flow, and resultant hypoxia associated with hyperthermia *in vivo* would not be present in the isolated tissue bathed in media containing supraphysiological concentrations of oxygen and metabolites. Results from these isolated tissue experiments should be interpreted cautiously when applied to intact tissue experiments.

The studies discussed above (32, 33, 40, 74, 94, 113, 165, 183, 194) provide evidence that physiologically relevant increases in temperature increase epithelial and TJ

permeability in cell culture and isolated intestinal tissue *in vitro*. Furthermore, conditioning of epithelial cells to thermal stress mitigates temperature induced epithelial and TJ permeability indicating an adaptive response. This adaptive response is accompanied by increases in the ubiquitously expressed cytoprotective protein, HSP-70. Blockade of transcription factors for HSP-70 during conditioning or thermal stress prevents upregulation of protein and induces further increases in epithelial permeability. Thermal stress also increases production of free radicals in epithelial cells which may contribute to temperature induced increases in epithelial permeability.

Assessment of GI Permeability In Vivo. Hall et al. (55) found that portal blood of conscious, hyperthermic ( $T_c$  41.5°C) rats contained elevated levels of metal binding proteins and the reactive nitrogen species (RNS), nitric oxide (NO) when passively heated. Observatory in nature, these findings provided evidence that free radical production during hyperthermia may play a role in the loss of portal vasoconstriction during severe heat stress. Hypothesizing that splanchnic tissue hypoxia may be the cause of increased portal vein NO and metal binding proteins during heat stress, Hall et al. (54) then injected the hypoxic cell marker [ $^3\text{H}$ ]misonidazole into the carotid artery of rats while exposed to ambient temperatures of 40°C.  $T_c$  of the rats reached 41.5°C and biopsies of the liver and intestines showed 80% and 29% increases in [ $^3\text{H}$ ]misonidazole retention in these cells, respectively. This was accompanied by hyperthermic rats having increased splanchnic  $\text{O}_2$  consumption and significantly decreased liver glycogen content as compared to normothermic controls. Findings of this study support the hypothesis that splanchnic tissue undergoes hypoxia during hyperthermia.

In the last of these eloquent studies by Hall et al. (56) the mechanisms of GI barrier dysfunction during hyperthermia were addressed. Using similar methods as the

previous studies it was found that portal vein blood contained significantly elevated bacterial endotoxin concentrations during hyperthermia as compared to normothermia. Arterial blood concentrations of bacterial endotoxin were not significantly altered during hyperthermia, indicating that the liver was successful in removal of endotoxins during heat stress. During experimentation various antioxidant enzyme agonist, antagonists, and mimetics were injected into the arterial circulation to assess mechanisms of free radical production and its effects on GI barrier function during heat stress. Results indicated that the use of the NOS inhibitor, L-NAME, attenuated splanchnic blood flow (SBF) and portal vein content of nitrogen radical while increasing portal vein oxygen radical and bacterial endotoxin content as compared to hyperthermic controls. The use of the xanthine oxidase competitive antagonist allopurinol did not alter SBF or portal vein nitrogen radical content while significantly decreasing portal vein oxygen radical and bacterial endotoxin content. Results of these three (54-56) studies provide evidence that passive hyperthermia results in splanchnic tissue hypoxia (as measured by [<sup>3</sup>H]misonidazole retention) and increased GI bacterial translocation into the portal circulation. Resultant oxygen radical production during allopurinol treatment may be responsible for hyperthermia induced GI barrier dysfunction. Nitrogen radical production during L-NAME experiments may play a critical role in the maintenance of GI barrier integrity by preventing further decreases in SBF during hyperthermia.

Lambert et al. (75) provided evidence of temperature dependent increases in intestinal permeability to FD-4 during passive hyperthermia in an anesthetized unconscious rat. Plasma was analyzed for FD-4, injected distal to the pyloric sphincter prior to heat stress, after rats were heated to a peak T<sub>c</sub> of 41°C, 41.5°C, and 42°C. Only rats heated to 42°C had significantly elevated plasma [FD-4] despite temperature

dependent increases in all experiments. Histological evidence of intestinal damage due to hyperthermia was also provided by this study; transmission electron micrographs of small intestinal epithelial cells from heat stressed rats showed apparent damage to the microvilli on two adjacent enterocytes as compared to non-heat stressed control rats. Light micrographs of jejunal tissue provided evidence of sloughing of villus tip membranes as well as increased distance between villus tip membranes. Oliver et al. (113) provided similar results with FD-4 transport *in vivo* transport and histological evidence in heat stressed rats. Briefly, rats were heated to a  $T_c$  of 39.5°C over 1 h and then heated at 0.5°C/30 min until  $T_c$  reached 41°C, 41.5°C, or 42°C. Plasma was analyzed for [FD-4] after 30 min of recovery in rats surviving and results showed significantly elevated levels in hyperthermic rats as compared to time matched normothermic (37°C) controls.

The studies (54-56, 75, 113) discussed indicate that passive hyperthermia: 1) decreases SBF and results in splanchnic tissue hypoxia as measured by [<sup>3</sup>H]misonidazole retention ; 2) increases splanchnic tissue production of RNS/ROS; 3) increases GI permeability to bacterial endotoxin and FD-4; 4) results in histological damage to enterocytes and villus tips throughout the intestines. Furthermore, NOS activity and NO production during hyperthermia may play a critical role in maintenance of SBF and subsequently GI barrier maintenance. Oxygen radical production, via xanthine oxidase, during hyperthermia may play a critical role in the oxidant damage to enterocytes that may result in barrier dysfunction.

Assessment of GI Permeability in Exercising Humans. GI barrier permeability testing in humans can be accomplished through the use of orally ingested non-metabolized carbohydrate (CHO) probes. These probes are water soluble, not degraded in the small intestines, and have a limited ability to cross a healthy epithelium (e.g GI

barrier) (62). This method utilizes both mono and disaccharides to account for differences in gastric emptying, transit, and renal clearance and is expressed as a ratio of disaccharide-to-monosaccharide recovered (91). The disaccharides (i.e. lactulose) are absorbed into the portal circulation through paracellular absorption while the smaller monosaccharides (i.e. rhamnose) are absorbed through the transcellular pathway. An increase in intestinal permeability will result in increased urinary excretion of the probes with an increase in the disaccharide-to-monosaccharide ratio, expressed as a percent recovery of the ingested amount (124). This method of GI permeability assessment has been used in cystic fibrosis, celiac disease, and Crohn's disease patients (49, 66, 77, 189) and also after exercise (112, 124). Another method of determining GI barrier dysfunction and permeability is through measurement of plasma levels of endotoxin/LPS. While this method is not a direct measurement of permeability of the GI barrier, it is assumed that elevated levels of circulating endotoxin represent an increased permeability of the GI barrier to luminal contents, specifically LPS. Increased levels of plasma LPS may also indicate a decreased clearance by hepatic reticuloendothelial cells.

Increased levels of plasma endotoxin occur in individuals after endurance events (triathlons, half-marathons, marathons, ultra-marathons) (4, 11, 15, 67, 99, 105). In the study by Camus et al (15) 18 male marathoners had plasma [LPS] ranging from 5-13 pg/ml at the end of the race with one participant having a plasma [LPS] of 72 pg/ml. These results are in agreement with those Jeukendrup et al. (67) and Nieman et al. (105). In the study by Jeukendrup et al. (67) 18 of 29 randomly sampled participants in a long-distance triathlon presented with mild-endotoxemia (5-15 pg/ml) while Nieman et al. (105) found an average of 0.4-1.2 pg/ml in individuals finishing an ultramarathon. In similar studies, Brock –Utne et al. (11) randomly sampled 89 exhausted (requiring

medical attention) runners, and found 72 had plasma [LPS] above 100 pg/ml (endotoxemic). Two of these participants had plasma [LPS] above the lethal human dose of 1000 pg/ml. Bosenberg et al. (4) found plasma [LPS] of 294 pg/ml in 18 ultradistance triathlon competitors. As noted in the discussion by Jeukendrup et al. (67) the conflicting values found in their study and the studies by Camus et al. (15), and Nieman et al. (105) as compared to those of Brock-Utne et al. (11) and Bosenberg et al. (4) may be the result of the chemical assays used. In the latter studies (4, 11) it is not certain if the assays utilized for the determination of plasma [LPS] were specific for LPS which may be the reason for the high values observed.

Only the study by Ng and colleagues (99) compared  $T_c$  of the endurance runners completing a 21-km road race to the plasma [LPS] of these participants. Results indicated there was no association between plasma [LPS] concentrations (2.5 pg/ml) and  $T_c$  post-race. The authors speculated that the increase in plasma endotoxin concentration was more likely caused by decreased clearance of endotoxin by the liver and antibodies in the blood as opposed to an increase in translocation due to GI barrier dysfunction caused by hyperthermia (99). Regardless of the lack of correlation to  $T_c$ , this study provided evidence similar to those mentioned above that long duration endurance events result in significantly elevated plasma [LPS] in endurance-trained individuals.

Ashton et al. (1) found that subjects completing a progressive incremental cycling test to exhaustion (~15 minutes) experienced significantly increased plasma levels of endotoxin. Supplementation with the antioxidant ascorbic acid prevented the exercise induced endotoxemia and decreased the concentration of the RNS byproduct, nitrite. Interestingly there was a strong positive correlation ( $r = 0.76$ ,  $P = 0.004$ ) between exercise intensity and plasma endotoxin levels. This is in agreement with the study of

Pals et al. (124), which utilized urinary excretion of orally ingested probes to assess GI permeability at different exercise intensities. In this study one hour of exercise in a cool, dry environment (22 °C & 50% RH) at 80% of  $VO_{2max}$  resulted in a significant increase in small intestines permeability as compared to rest, 40%, and 60% of  $VO_{2max}$ . Exercise intensities below 80% resulted in nonsignificant increases in small intestines permeability as compared to rest.

A study by Kuennen et al. (72) found no significant change in urinary excretion of lactulose (molecular mass = 342.296 Da) after walking (45 min at 50%  $VO_{2max}$ ) in a hot environment (46.5°C, 20% RH) as compared to urinary excretion under non-stress conditions. No significant changes were found in plasma [LPS] as compared to resting after this heat stress trial. The lack of finding significant changes in two measures of GI permeability may be explained by the intensity of exercise ( $\sim 27.8 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) utilized during the heat stress trial. As mentioned before, the study of Pals et al. (124) showed an exercise intensity dependent relationship with changes in urinary excretion of CHO probes. Participants in the study by Kuennen et al. (72) then exercised in the heat for 7 days (100 min/day) and did not have significantly altered measures of GI permeability on the final day of exercise.

Selkirk et al. (161) showed a  $T_c$  dependent increase in plasma [LPS] in humans walking in 40°C and 30% RH. Trained ( $VO_{2peak} > 65 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) and untrained ( $VO_{2peak} < 50 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) individuals walked for 3 and 2 h respectively with blood sampled every 0.5°C increase in  $T_c$ . Plasma [LPS] in the trained subjects ranged from 3.8 pg/ml at rest to 16.5 pg/ml at exhaustion while untrained ranged from 3.8 pg/ml at rest to 34 pg/ml at exhaustion.

Plasma endotoxin concentrations are a result of the balance between translocation from the GI lumen and clearance by reticuloendothelial cells of the liver and immunoglobulin in the blood. Thus plasma [LPS] increases associated with exercise most likely reflect both an increase in translocation from the GI lumen due to barrier dysfunction and a decrease in removal due to decreased splanchnic blood flow. Regardless of the exact mechanism/cause, evidence indicates that strenuous exercise over long durations (>1 hr) at low-to-moderate (50-65%  $\text{VO}_{2\text{max}}$ ) and short (<20 min) strenuous (high intensity) exercise cause an increase in plasma [LPS] at the cessation of exercise. Evidence from direct evaluation of the GI permeability, through the use of orally ingested carbohydrate probes, suggests an intensity dependent increase in GI permeability.

### **Acute Phase Response and Exercise**

When local and systemic perturbations to immunological homeostasis occur, such as during bacterial infection and traumatic tissue damage, the first immunological response represents a non-specific innate release of immunomodulating proteins referred to as cytokines (30, 50). The cytokines released during this response may act as promoters of negative growth factors on cells, pro-inflammatory mediators, or anti-inflammatory mediators (50). The cytokines Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and Interleukin-1 (specifically IL-1 $\beta$ ) are first released from activated macrophages and synergistically promote fever, muscle catabolism, activation of white blood cells, the production of Nitric Oxide (NO), prostaglandin synthesis, and activation of the coagulation cascade (31). Because of these actions, and their influence on the specific immune response, these cytokines are considered pro-inflammatory cytokines.

Increased levels of circulating pro-inflammatory cytokines promote the synthesis and release of IL-6 (160). Despite little evidence to support it, IL-6 was assigned a pro-inflammatory status (31) in early studies. However, more recent evidence suggests that IL-6 acts as an anti-inflammatory cytokine by preventing further pro-inflammatory cytokine release (160), promoting release of IL-10 (114), and promoting release of soluble IL-1 receptor antagonist (IL-1ra) and soluble TNF- $\alpha$  receptor (sTNFr) (180). The sTNFr binds to TNF- $\alpha$  in the circulation and prevents interaction with surface bound TNF- $\alpha$  receptors while IL-1ra binds to surface IL-1 receptors with no signal transduction. These actions are considered to be anti-inflammatory as they prevent the pro-inflammatory actions of the TNF- $\alpha$  and IL-1 $\beta$ . Thus IL-6 acts as regulator of the systemic inflammatory response as its actions work to balance control of pro- and anti-inflammatory cytokine release (192).

IL-10 is the an anti-inflammatory cytokine of the non-specific immune response (114). Release of IL-10 will decrease pro-inflammatory (TNF- $\alpha$ ) cytokine synthesis by inhibition of NF- $\kappa$ B in LPS stimulated monocytes (22, 116) and to decrease surface expression of the TNF- $\alpha$  receptors (114). IL-10 also acts to decrease synthesis and secretion of IL-6 from activated macrophages (25).

Exercise Induced SIR. Cannon et al. (16) provided the first evidence that exercise resulted in increased elevations of a pyrogenic protein(s). Exercise induces an inflammatory response and this inflammatory response differs from that of bacterial infection. In response to exercise, IL-6 increases in the circulation to a greater extent than any other acute phase cytokine and active skeletal muscle is the site of its synthesis and secretion (38, 131). The exercise induced SIR is widely studied with the utilization of multiple study designs such as knee extensor exercise, marathon running, cycling and

running exercise while immersed in water, and shorter endurance events while running or cycling. While a significant amount of research exists on the SIR to exercise and exercise training, the following review will limit its scope to studies of acute bouts (i.e. 1 day) of exercise, studies comparing exercise in ambient/cool and hot environments, or studies examining the SIR to acclimation of exercise in the heat in healthy (i.e. no chronic disease present) human or animal models.

IL-6. Originally believed to be the result of skeletal muscle damage caused by contraction (13), findings from multiple studies indicate that active skeletal muscle, as opposed to white blood cells, is responsible for the increased plasma [IL-6] during exercise. Ostrokwi et al.(121) provided evidence that muscle tissue collected from participants completing a marathon contained significantly elevated levels of IL-6 mRNA as compared to resting samples. Analysis of plasma [IL-6] from these participants indicated nearly a 100-fold increase as compared to resting samples. Both concentric and eccentric contraction induced IL-6 mRNA increases in rats (68). Starkie et al. (169) also provided evidence that monocytes separated from whole blood following 2 h of cycling (70%  $VO_{2peak}$ ) resulted in significantly increased IL-6 positive monocytes as compared to resting. However, stimulation of these monocytes *in vitro* with LPS showed that monocytes did not spontaneously produce more IL-6 post exercise as compared to resting despite a 2-fold increase in plasma [IL-6] following exercise. The authors to speculate that monocytes were not the source of elevated plasma [IL-6] following exercise (140). This is in agreement with an earlier study by Ullum et al. (184) where peripheral blood mononuclear (PBMC) cells did not contain significantly elevated levels of pre-IL6 mRNA immediately following 2-h of cycling at ~75%  $VO_{2max}$ . Starkie et al. (172) observed similar findings in monocytes from runners following a marathon in which

plasma [IL-6] increased 120 fold. Spontaneous IL-6 production from stimulated monocytes *in vitro* was no different following the marathon as compared to pre-race samples, indicating an alternate site of IL-6 release during exercise.

Contracting skeletal muscle is a site of IL-6 mRNA and protein release (60, 70). Keller et al. (70) found that IL-6 mRNA was increased after 30 min of exercise as compared to resting samples. Steensberg et al. (176) provided evidence that IL-6 release from active muscle could account for exercise/contraction induced plasma [IL-6] increases during 5-h of knee extensor exercise. These findings were reproduced during 180 min of knee extensor exercise (175). Investigations utilizing microdialysis have found 5-100x greater interstitial [IL-6] as compared to simultaneous measurement of plasma [IL-6] (76, 149) indicating that muscle is a source of IL-6 release. Multiple studies indicate that plasma [IL-6] and intramuscular mRNA increases associated with contractions are dependent on muscle glycogen content (70, 86, 173). Authors suggested a glucoregulatory role for IL-6 during exercise (176). However, further investigations into contracting muscle's role in plasma [IL-6] revealed that Steensberg et al. (176) may have overestimated the net release of IL-6 from contracting muscle (38). It has been further shown that IL-6 may be released from peritendinous tissue (76) and the brain (109) during exercise. Furthermore, use of vitamin C and E supplementation still resulted in significantly elevated plasma [IL-6] despite blunted muscle IL-6 release indicating that contracting muscle is not the sole source of elevated plasma IL-6 during exercise. However, plasma [IL-6] was significantly lower during the vitamin supplementation trials as compared to placebo supplementation, indicating that contracting skeletal muscle is a primary source of IL-6 during exercise (39).

These studies(38, 39, 60, 68, 70, 76, 86, 109, 121, 140, 149, 169, 172, 173, 176, 184) provide evidence that 1) exercise/contractions increase skeletal muscle IL-6 mRNA content in both human and rat studies; 2) isolated monocytes do not spontaneously produce more IL-6 following exercise and most likely are not the source of exercise-induced plasma [IL-6] increases; 3) the majority of the exercise/contraction induced increase in plasma [IL-6] can be accounted for by release of IL-6 from active skeletal muscle and; 4) tissues such as the brain and tendons may also be possible sites of IL-6 release during exercise.

Since contracting muscle is the primary site of IL-6 released during exercise; the intensity, duration, and modality of exercise will determine the magnitude of increase. Exercise utilizing smaller muscle mass (e.g. arm cranking) may not elicit as great a response as exercise utilizing larger muscle masses (e.g. running). Fischer et al. (39), using the results of 67 studies, determined that the duration of the exercise, regardless of modality, could account for 51% of the variation in plasma IL-6 responses. This relationship becomes more pronounced when exercise intensity is taken into consideration, since exercise intensity, regardless of modality, influences the muscle mass involved in force production.

Plasma from individuals completing a 6-h run (average 65.1 km covered) contained approximately a 4-fold increase (average 71.5 pg/ml) in [IL-6] as compared to pre-exercise levels. However, the assay utilized in this study was later found to be non-specific for IL-6, so these results may indicate the total concentration of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  which may explain the high resting plasma [IL-6] (average 18.5 pg/ml) (34). Nielsen et al. (102) found as great as 2-fold increase in plasma [IL-6] following 6-repeated bouts (separated by 4 h) of maximal 2000m rowing over 2 days (3 per day).

Plasma [IL-6] remained elevated 1-h after the end of the 2<sup>nd</sup> and 3<sup>rd</sup> bout each day (102). Ostrowki and colleagues have provided multiple lines of evidence of increased plasma [IL-6] following marathon (~100-fold increase) (120) and after 2.5 h of treadmill running at 75%  $\text{VO}_{2\text{max}}$  (~25-fold increase) (118). Plasma [IL-6] remained elevated for 4 h after the marathon and 5 hours after the 2.5-hour run with peak [IL-6] found immediately post exercise in both cases. Pooled data from three separate marathons indicate a negative correlation ( $r=-0.30$ ,  $P < 0.05$ ) between running time and peak plasma [IL-6] and a positive correlation ( $r=0.32$ ,  $P < 0.05$ ) between running intensity and peak plasma [IL-6] (122). Nieman et al. (104) found a 125-fold increase in plasma [IL-6] after 160-km ultramarathon. Interestingly, participants who used non-steroidal anti-inflammatory drugs (NSAIDs) had a significantly greater increase in plasma [IL-6] as compared to participants who did not use NSAIDs (104). In a double-blind randomized design, subjects running (~75%  $\text{VO}_{2\text{peak}}$ ) for 2.5 h resulted in significantly attenuated plasma [IL-6] increases post exercise when a carbohydrate beverage was consumed with exercise as compared to ingestion of a non-energy containing placebo beverage. Plasma [IL-6] only remained elevated 1 h after the cessation of exercise in the placebo group (97). Attenuation of plasma [IL-6] increases during running and cycling exercise by ingestion of carbohydrate containing beverages observed in studies by Starkie et al. (170) and Nieman et al. (106) during cycling at 60% of maximal wattage and by Li et al. (80) after two 90 m cycling bouts at 60%  $\text{VO}_{2\text{max}}$ . The study by Starkie et al. (170) also revealed a modality dependent increase in plasma [IL-6] by having subjects perform both cycling and running exercise at the modality specific lactate threshold. Running elicited a far greater increase in plasma [IL-6] post exercise as compared to resting conditions when compared to cycling (170). This modality specific response indicates why cycling elicited

such a small increase in study by Ullum et al. (184), which exhibited a 1.6-fold increase in plasma [IL-6] after 1 h of cycling at 75%  $\text{VO}_{2\text{max}}$ . Plasma [IL-6] in these subjects returned to pre-exercise levels by 1 h post exercise (184).

Selkirk et al. (161) provided evidence that exercise induced increases in plasma [IL-6] rise in a  $T_c$  dependent manner during exercise performed in hot (40°C & 30% RH) environmental temperatures. Interestingly, a study designed to prevent exercise induced  $T_c$  increases by water immersion during treadmill exercise completely abolished exercise induced plasma [IL-6] increases found during exercise in hot (39°C) environmental temperatures (147). Direct comparison of exercise induced [IL-6] increases in hot as compared to cool/thermoneutral environments was studied in cyclist completing 1.5 h of exercise at 70%  $\text{VO}_{2\text{max}}$ . Significantly greater increases in plasma [IL-6] (1.5 fold vs. <1-fold) was found after 45 min of exercise and at exercise termination (3.5-fold vs. 1-fold) in hot (35° C) as compared to cool (15°) temperatures (171). Individuals completing 90 min of cycling at 60%  $\text{VO}_{2\text{max}}$  had significantly greater plasma [IL-6] increases from resting values when cycling in 32°C (~9.25-fold) temperatures vs. 18°C (~5.4-fold) (129). Running for 60 minutes at 60%  $\text{VO}_{2\text{max}}$  did not induce significantly different plasma [IL-6] increases when performed in 28°C and 50% RH vs. 18°C and 50% RH despite significantly larger increases in  $T_c$  during exercise in a hot environment (106). In the study by Brenner et al. (10), 1 h of cycling at 55%  $\text{VO}_{2\text{peak}}$  in 18°C water prevented exercise induced increases in  $T_c$  and produced significantly lower plasma [IL-6] post exercise compared to exercise in 35°C water. These finding were reproduced in a similar study (73) in which participants performed treadmill exercise for 2 h at 58%  $\text{VO}_{2\text{max}}$  while immersed in hot (36°C) or cool (23°C) water. Exercise in cool water prevented core temperature increases and induced significantly lower serum [IL-6] (~3-fold

increase from rest) increases at exercise termination as compared to hot water (~7-fold increase from rest) (73). These (10, 52, 73, 106, 129, 147, 161, 171) studies provide direct evidence that the SIR to exercise in hot environmental temperatures results in significantly elevated plasma [IL-6] as compared to exercise in cool environmental temperatures.

Hailes et al. (52) found a 2-fold increase in plasma [IL-6] in individuals cycling at 70% of  $VO_{2peak}$  in a 38°C & 40% RH environment until core temperatures reached 39.5°C. After five consecutive days of this exercise plasma [IL-6] only increased 1.2 fold despite significantly greater exercise time indicating adaptive response to repeated bouts of exercise in the heat (52). Similar results were found by Kuennen et al. (72) in individuals walking for 7 days in hot (46.5° & 20% RH) conditions. Plasma [IL-6] increased 7.1-fold as compared to resting on the first day of exercise in the heat and only 3.5-fold as compared to resting on the 7<sup>th</sup> day.

These studies (10, 13, 34, 38, 39, 52, 60, 68, 70, 72, 73, 76, 80, 81, 86, 99, 102-106, 109, 118-122, 129, 147, 149, 169-173, 175, 176, 184) provide evidence that plasma [IL-6] increases with exercise are 1) duration dependent; 2) intensity dependent; 3) modality dependent; 4) are attenuated by ingestion of carbohydrate beverages; 5) may remain significantly elevated post exercise; the duration of which is dependent on the magnitude of increase during exercise; 6) may be associated with rises in core temperature during exercise and thus altered by high environmental temperatures compared to cooler environmental temperatures and; 7) adaptation of the IL-6 response to exercise may occur in as little as 5 days due to repeated bouts of exercise in the heat.

TNF- $\alpha$ . Ostrowski et al. (121) provided evidence that individuals completing a marathon had significantly increased plasma [TNF- $\alpha$ ] immediately post-race (2.5-fold)

and 2 h post-race (1.6-fold) as compared to resting samples. TNF- $\alpha$  mRNA was not detected in muscle tissue or blood mononuclear cells at any time during this study indicating that neither muscle or blood mononuclear cells were responsible for exercise induced increases of circulating TNF- $\alpha$ . In a more detailed follow up to their original study, Ostrowski et al. (119) observed individuals completing a marathon had a significant 2.5 fold increase in plasma [TNF- $\alpha$ ] following a marathon. [TNF- $\alpha$ ] remained significantly elevated for 3 h after completion of the race in these individuals. The evidence showing unaltered TNF- $\alpha$  mRNA in blood mononuclear cells following the marathon is in agreement with Starkie et al. (172) which showed a 4.5-fold increase in plasma [TNF- $\alpha$ ] immediately following a marathon and remained significantly elevated (4.2-fold greater than rest) 2 h post marathon. Spontaneous production of TNF- $\alpha$  from LPS stimulated monocytes following the race was no different than resting samples. Similar results have been shown after 1 h of cycling at 75% of  $VO_{2max}$  and 2 h of cycling at 70% of  $VO_{2max}$ (169, 184). During 3 h combined cycling and walking exercise at 60-65%  $VO_{2max}$  plasma [TNF- $\alpha$ ] was significantly increased after 60 minutes of exercise, 180 minutes of exercise, and remained elevated 24 h (93) post-exercise.

Steensberg et al. (175) found that 180 minutes of knee extensor exercise at 55% maximal workload did not significantly alter muscle TNF- $\alpha$  mRNA content as compared to resting samples. Analysis of arterial-venous plasma differences across active force-producing muscle indicated no net release or uptake of TNF- $\alpha$  during exercise. The findings that TNF- $\alpha$  mRNA content is not increased and active muscle is not the source of increased [TNF- $\alpha$ ] circulating is interesting considering that circulating white blood cells do not seem to be the source and skeletal muscle has been shown to express TNF- $\alpha$  in a fiber type specific manner (143). However, Kupffer cells from rats undergoing 20

min of liver occlusion spontaneously released significantly more TNF- $\alpha$  than cells from sham and control animals in conjunction with significantly higher plasma [TNF- $\alpha$ ] in the liver occluded rats (187). Thus it may be possible that the source of exercise-induced TNF- $\alpha$  may be Kupffer cells undergoing ischemia and hypoxia due to blood redistribution caused by elevated work rates.

Rhind et al. (147) found a significant correlation ( $r=0.493$ ,  $p<0.0001$ ) between  $T_c$  and plasma [TNF- $\alpha$ ] in individuals cycling at 65%  $VO_{2peak}$  while immersed in cool (18°C) or hot (39°C) water. In this study plasma [TNF- $\alpha$ ] was significantly increased after 20 min (1.5-fold) and after 40 min (1.7-fold) as compared to resting samples in the Hot trial conditions; no significant increases were found at any sampling time in the cool trial. In a similar study of cycling while immersed in cool (25°C) and warm (38.5°C) water, Hosick et al. (64) reported a greater plasma [TNF- $\alpha$ ] increase after exercise in warm water conditions and observed a positive yet non-significant correlation ( $r = 0.833$ ,  $p = 0.136$ ) between  $T_c$  and [TNF- $\alpha$ ]. Peake et al. (129) observed significantly greater plasma [TNF- $\alpha$ ] concentrations after 90 m of cycling in 32°C water compared to 18°C water.

Starkie et al. (171) directly compared the cytokine response to cycling at 70%  $VO_{2max}$  in 35°C (HEAT) vs. 15°C (CON) and found significantly greater plasma [TNF- $\alpha$ ] after 90 m as compared to rest in HEAT trial but not in CON. In agreement with studies mentioned earlier, this study also revealed that monocytes did not spontaneously produce more TNF- $\alpha$  after exercise in either treatment. Similar results were observed in rats swimming to exhaustion, only rats swimming in 38°C water had significantly elevated plasma [TNF- $\alpha$ ].

Selkirk et al. (161) had trained ( $\text{VO}_{2\text{peak}} > 65 \text{ ml} \cdot \text{kg LBM}^{-1} \cdot \text{min}$ ) and untrained ( $\text{VO}_{2\text{peak}} < 50 \text{ ml} \cdot \text{kg LBM}^{-1} \cdot \text{min}$ ) individuals walk at 4.5 mph and 2% grade for 3 h and 2 h respectively;  $T_c$  and HR were similar in both groups at exercise termination. Blood samples were taken at rest and at every  $0.5^\circ\text{C}$   $T_c$  increase during exercise until  $T_c$  reached  $39.5^\circ\text{C}$ . Untrained individuals had non-significantly higher plasma  $[\text{TNF-}\alpha]$  than trained individuals at all  $T_c$  during exercise.

Hailes et al. (52) studied the changes in exercise-induced plasma  $[\text{TNF-}\alpha]$  after 5 consecutive days of exercise in the heat ( $38^\circ\text{C}$  and 40% RH). Cyclists exercising at 70%  $\text{VO}_{2\text{max}}$  until  $T_c$  reached  $39.5^\circ\text{C}$  had a 7.5-fold increase after exercise as compared to resting samples on Day 1 of exercise but only a 1.3-fold increase after exercise on Day 5 despite significantly longer exercise durations. A key finding of this study was that plasma  $[\text{TNF-}\alpha]$  was 2.5-fold greater in resting samples on Day 5 as compared to Day 1, though these values were found to not be significant. The findings of this study are interesting because: 1) a clear adaptive process, in which the pro-inflammatory response is blunted, has occurred after 5 consecutive days of exercise in the heat; 2) despite the adaptive process individuals had higher resting plasma concentrations of the pro-inflammatory cytokine  $\text{TNF-}\alpha$ . The latter finding is of interest due to the findings of Lim et al. (81), in which rats with a pharmacologically induced pre-existing inflammatory status were less likely to survive a lethal heat challenge than control rats.

Evidence from these studies (52, 81, 119, 121, 129, 143, 147, 161, 169, 171, 172, 175, 184, 187) indicate that: 1) acute bouts of exercise may, but not always, induce elevated levels of plasma  $[\text{TNF}\alpha]$ ; 2) exercise in hot environments may augment this increase; 3) Kupffer cells undergoing ischemia and hypoxia, not active skeletal muscle or circulating blood mononuclear cells, may be the major site of release of  $\text{TNF-}\alpha$  during

exercise; 4) TNF- $\alpha$  increases induced by exercise are related to increases in  $T_c$ ; 5) exercise induced increases are influenced by training status; 6) consecutive days of exercise in hot environments promote an adaptive process that blunts TNF- $\alpha$  response to exercise.

IL-10. IL-10 is readily detected in human plasma/serum following long duration (52, 119, 130, 161) exercise, eccentric contractions (167), and exercise in hot environmental temperatures (52, 161), Peake et al. (130) demonstrated that plasma [IL-10] significantly increased (~6.3 fold) after 60 min of treadmill running at 85%  $VO_{2max}$  but not after 60 min of treadmill running at 60%  $VO_{2max}$  (< 1 fold change) while Ostrowski et al. found (119) a 120 –fold increase (~120 pg/ml) in participants completing a marathon. These studies suggest an intensity and duration dependence for the magnitude of the IL-10 response following an acute bout of exercise.

Whole body hyperthermia induced an inflammatory (~80-fold change in IL-10) response in cancer patients who were passively heated to a  $T_c$  of 41.8 °C. Cycling exercise (90 min; 60%  $VO_{2max}$ ) in hot (32°C) as compared to ambient (18°C) environmental conditions significantly elevated plasma [IL-10] at exercise termination (129). Authors speculated that this is due to a greater catecholamine response associated with exercise in hot environmental conditions in which they found norepinephrine levels were significantly higher in the hot trials. Studies *in vitro* indicate an epinephrine and norepinephrine dose dependent IL-10 release (36) and a decreased IL-10 release when the non-selective  $\beta$ -blocker propranolol was added to the PBMC isolation medium (190). However, it is likely that stimulation of IL-10 release is multi-factorial and catecholamines are not solely responsible for increased IL-10 release with exercise in hot environments.

Selkirk et al. (161) compared the inflammatory response to exercise in the heat between trained ( $\text{VO}_{2\text{peak}} = 70 \text{ ml} \cdot \text{kg}^{-1} \text{LBM} \cdot \text{min}^{-1}$ ) and untrained ( $\text{VO}_{2\text{peak}} = 50 \text{ ml} \cdot \text{kg}^{-1} \text{LBM} \cdot \text{min}^{-1}$ ). Participants walked until exhaustion (3 h for trained vs. 2 h for untrained) at a treadmill velocity 4.5 km/h. Blood samples were taken at every  $0.5^\circ\text{C}$  increase in  $T_c$ . Plasma [IL-10] concentrations were not significantly elevated in any group until a  $T_c$  above  $38.5^\circ\text{C}$  in the trained group ( $\sim 7.0 \text{ pg/ml}$  at exhaustion). The trained group had an average  $T_c$  of  $39.7^\circ\text{C}$  at exhaustion as compared to  $39.1^\circ\text{C}$  in the untrained group and the authors speculated that the anti-inflammatory response, and increased LPS tolerance, seen in the trained group may play a crucial role in increased heat tolerance (i.e. longer exercise bout and higher final  $T_c$ ).

Yamada et al. (193) found that plasma[IL-10] was not significantly altered after exercise, on Day 1 or Day 10, during a 10 day heat acclimation protocol. In contrast, Hailes et al. found (52) plasma [IL-10] to nearly double as compared to resting ( $10.0 \text{ pg/ml}$  vs.  $5.2 \text{ pg/ml}$ ) after cycling at  $70\% \text{ VO}_{2\text{max}}$  in hot environmental temperatures. After four consecutive days of cycling in the hot environment plasma [IL-10] was significantly elevated at rest on Day 5 as compared to at rest on Day 1 ( $6.8 \text{ pg/ml}$  vs.  $5.2 \text{ pg/ml}$ ). However, exercise on Day 5 did not significantly alter plasma [IL-10] despite significantly increased exercise time. The study of Kuennen et al. (72) observed IL-10 was not significantly elevated after exercise on Day 7 of exercise in the heat whereas it was on Day 1 of exercise. Contrary to the results of Hailes et al. (52), resting values of IL-10 were lower on Day 7 compared to Day 1. Differences in exercise intensity and modality used in the studies likely explain the differences in results; the participants Hailes et al. participants cycled at  $70\% \text{ VO}_{2\text{max}}$ ; participants in the study of Kuennen et al. walked at approximately  $23\% \text{ VO}_{2\text{max}}$ ; and participants in the study Yamada et al.(193)

walked 100 min at 56% of  $VO_{2max}$ . The study of Hailes et al. resulted in significantly elevated resting levels of TNF- $\alpha$  on Day 5 as compared to Day 1; this result was not seen in the study Kuennen et al. Thus, the elevated pro-inflammatory cytokine at rest may have resulted in the elevated IL-10 at rest.

In conclusion these studies (36, 52, 72, 119, 130, 161, 190, 193) provide evidence that: 1) plasma [IL-10] increases in the circulation following exercise; 2) exercise induced plasma [IL-10] increases are duration and intensity dependent; 3) exercise in hot environmental conditions result in a significantly greater IL-10 response as compared to exercise in cool environments and; 4) consecutive days of exercise in the heat results in a blunted IL-10 response to an acute exercise bout.

### **Systemic Inflammatory Response Syndrome's Role in Heat Stroke**

In 2002 Bouchama and Knochel (6) redefined the pathophysiology of heat stroke as, “ a form of hyperthermia associated with a systemic inflammatory response leading to a syndrome of multiorgan dysfunction in which encephalopathy predominates.” A combination of animal (rats, mice, rabbits, and baboons) and perspective human (admission to hospital following event) studies have provided evidence for the role of cytokines in heat-induced SIRS. This evidence includes: 1) elevated levels of cytokines in patients admitted to the hospital after heat stroke (5, 8, 12, 57, 85, 186) and in experimental animal models (7, 9, 58, 82, 83) of heat stroke; 2) decreased mortality rates in rats administered cytokine antagonists in heat stroke models (83); 3) pre-existing inflammatory conditions (pharmacologically induced) significantly increased mortality rates during heat stress (81) and; 4) the link between endotoxemia in heat stroke (8, 56, 75, 83) and the known role of cytokines in endotoxic shock (141). At the time of admission to the hospital, heat stroke patients present with increased circulating levels of

multiple cytokines (5, 8). Anecdotal evidence has suggested a link between plasma [IL-6] and the severity of heat stroke, though this observation is likely complicated due to differences in admission time following the event (5).

Inflammation, as a result of the SIR, is the protective cost paid for elimination and neutralization of pathogens, foreign antigens, and tissue damage. However when dysregulation of this natural process occurs, progression toward septic-like symptoms, and septic shock, are commonly seen (23, 146). Dysregulation, termed the Systemic Inflammatory Response Syndrome, occurs when the acute phase response becomes exaggerated and an excessive amount of cytokines, specifically pro-inflammatory cytokines, are released into the circulation. This condition is linked with multiple organ dysfunction syndrome (MODS) and acute respiratory distress syndrome (ARDS) (110). Injection of inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  in high dosage (>100 ng/kg) results in fever, headache, hypotension, sleepiness, and coagulation in humans, which mimics the events of septic shock (31). Accordingly, use of TNF- $\alpha$  neutralizing antibodies prevented septic shock (181) or mitigated mortality rates (3, 90) in animal studies. Numerous studies of experimental heat stroke in animals and in patients recovering from heat stroke have shown high levels of LPS and both pro- and anti-inflammatory cytokines. In these studies septic and shock-like symptoms are often observed. (5, 7, 8, 85). However, utilization of cytokine neutralization in clinical trials of sepsis, similar to that seen in heat stroke mortality, has provided mixed and inconclusive results (26, 115). Furthermore, IL-6 null mice had increased mortality rates following cecal ligation puncture (an established model for septic conditions) whereas TNF- $\alpha$  receptor null mice had increased survival rates. Thus, it is apparent that the SIR plays a

crucial role in the outcome of sepsis and septic like conditions and it is the dynamic regulation and interplay between the components of the SIR that influence this outcome.

### **Conclusion and Purpose**

A significant amount of research (5-9, 78, 79) indicates that the SIRS plays a crucial role in the outcome of heat stroke mortality. The SIRS in heat stroke patients most likely results from increased circulating levels of LPS commonly associated with the lumen of the GI tract. Numerous studies indicate that LPS is a potent inducer of the SIR. The translocation of LPS into the circulation is most likely a consequence of the cardiovascular adjustments associated with thermal regulation. Decreased splanchnic blood flow (150) during heat stress results in an ischemic and hypoxic splanchnic tissue (71, 75). This ischemic and hypoxic environment may result in decreased GI barrier function, thus an increase in bacterial translocation from the GI lumen to the portal circulation. Performance of work (e.g. exercise) in hot environmental conditions may further augment blood flow redistribution and splanchnic hypoxia (151-153) during thermal stress. Subsequently, increased LPS in the systemic circulation occurs after exercise in both cool (1) and hot environmental temperatures (161).

The SIR to exercise has been widely studied using a variety of modalities, intensities, and durations (38, 132-134, 138). In short, IL-6 increase is the greatest response, by comparison, of any cytokine that increase an acute bout of exercise, with the duration of the exercise being the primary determinant of the magnitude of IL-6 increase (38). However when exercise is performed in hot environmental temperatures, pro-inflammatory cytokines increase in the circulation to a greater extent as compared to exercise in cool environmental temperatures (64, 171). Furthermore, consecutive days of

exercise in the has resulted in significantly elevated levels of pro-inflammatory (TNF- $\alpha$ ) cytokines at rest (52).

While a significant amount of research does exist, more complete research into the time course of the cytokine response to exercise in hot environmental temperatures is needed in humans. Furthermore, study of the adaptation of this response to consecutive days of exercise in the heat is severely lacking. Finally, controlled laboratory studies comparing the cytokine response to exercise, in hot or cool environmental temperatures, in relation to changes in plasma bacteria (LPS) are lacking. Thus the purpose of this study was to determine the plasma LPS response and SIR to acute bouts of exercise in the heat and any adaptive response that occurs during consecutive days of exercise in the heat. We hypothesize the LPS response to an acute bout of exercise will be significantly decreased on Day 5 of exercise in the heat as compared to Day 1. Subsequently the SIR to exercise on Day 5 will result in a blunted IL-6 response and a less pro-inflammatory (TNF- $\alpha$ ) response as compared to Day 1.

## CHAPTER III

### JOURNAL MANUSCRIPT

#### ABSTRACT

**PURPOSE:** The purpose of this study was to determine the LPS-mediated SIR to acute bouts of exercise in the heat and any adaptive response that occurs during consecutive days of exercise in the heat. **METHODS:** 8 healthy males (Age =  $24 \pm 3$  yrs,  $VO_{2max} = 55.3 \pm 3.6$  ml\*kg<sup>-1</sup>\*min<sup>-1</sup>) were asked to run (~78%  $VO_{2max}$ ) in a hot environment on 5 consecutive days. Participants ran each day until core temperature ( $T_c$ ) was elevated 2°C above rest or volitional exhaustion. Blood samples were obtained pre-exercise, post-exercise, 1 h post-exercise, and 3 h post-exercise on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day of exercise. **RESULTS:** Resting  $T_c$  ( $P < 0.05$ ) was significantly decreased on Days 4 and 5 as compared to Day 1. Heart rate (resting and exercise) and Physiological Strain Index were not significantly altered by 5 consecutive days of exercise in the heat. Exercise time was not significantly different among days. Plasma [IL-6] was significantly elevated post-exercise each day (14.8 pg/ml vs. 10.6 pg/ml) as compared to resting values. No significant difference in the IL-6 response was detected among days. Plasma [TNF- $\alpha$ ] was not significantly elevated above rest after exercise on any day though mean plasma concentrations were significantly lower on Day 5 at 1h (3.1 pg/ml vs. 4.7 pg/ml;  $P = 0.01$ ) and 3 h (3.5 pg/ml vs. 4.7 pg/ml;  $P = 0.05$ ) post exercise as compared to Day 1. Plasma [IL-10] was significantly (0.9 pg/ml vs 0.5 pg/ml;  $P < .01$ ) elevated from baseline at 1 h post exercise with no difference among days. Plasma LPS activity significantly

increased post-exercise (1.4 EU/ml vs. 0.9 EU/ml;  $P < 0.01$ ) each day as compared to rest. No difference was detected among days for LPS activity. CONCLUSION: These data indicate that consecutive days of exercise in the heat results in a less pro-inflammatory (TNF- $\alpha$ ) response to exercise and this adaptation may be a result of an increasing LPS tolerance.

## INTRODUCTION

In 2002 Bouchama and Knochel (6) redefined the pathophysiology of heat stroke to include the systemic inflammatory response (SIR) along with the combined effects of heat cytotoxicity and coagulation abnormalities. Evidence of significantly elevated levels of pro- and anti-inflammatory cytokines in individuals admitted to the hospital following heat stroke (5, 8, 12, 57, 85, 186) and in experimental models of animal heat stroke (7, 9, 58, 82, 83) support this definition. Furthermore, animal studies utilizing cytokine antagonists showed increased survival rates following heat stroke (83) while pharmacologically inducing a pre-existing inflammatory state significantly decreased survival rates in response to a lethal heat challenge in rats (81). The exaggerated inflammatory response, referred to as systemic inflammatory response syndrome (SIRS), is believed to be driven by leakage of gut-derived bacteria (endotoxin; LPS) normally contained to the gastrointestinal (GI) lumen. Exercise in the heat results in cardiovascular adjustments that promote redistribution of blood to active force-producing skeletal muscle and to the peripheral vasculature to promote heat dissipation (71) at the expense of less metabolically active tissue (i.e. splanchnic) (54). Decreased blood flow to splanchnic tissue results in an ischemic and hypoxic environment (54, 56) and an increase in translocation of LPS into the portal circulation due to decreased GI barrier function (75).

LPS is a component of gram-negative bacteria located on the surface of the GI lumen (88). Injection of LPS intravenously results in cardiovascular, inflammatory, and

hemodynamic responses associated with early stages of septic shock (89). At rest, LPS enters the portal circulation but is successfully removed by reticuloendothelial cells of the liver and by antibodies (41). Increased translocation of LPS into the portal circulation and/or a reduced removal rate by defense mechanisms, which may result due to redistribution of cardiac output during exercise and thermal stress, results in accumulation in the systemic circulation. Consequently, LPS has been shown to be elevated in the circulation after both short (< 1 h) (1) and long (> 1 h) (11, 99, 105) duration exercise in a core temperature ( $T_c$ ) dependent manner (161). Once into circulation LPS complexes with LPS-binding protein (LBP) which facilitates interaction with the surface receptor complex CD14/TLR4/MD2 on immune tissue (89). This interaction results in NF- $\kappa$ B activation which promotes activation of genes associated with inflammatory mediators such as TNF- $\alpha$  and IL-1 $\beta$  (84). Elevated levels of LPS have been demonstrated in humans suffering a heat stroke (6, 8) and in individuals requiring medical attention following exhaustive exercise (11). Furthermore, evidence from animal studies show increased heat tolerance (156) and survival rates (81) if endotoxemia was pharmacologically prevented.

It is well documented that exercise elicits a SIR in which interleukin-6 (IL-6) increases in the circulation more than any other acute phase cytokine (138). During exercise, IL-6 prevents release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), promotes the synthesis and release of the anti-inflammatory cytokine interleukin-10 (IL-10), and the release of the antagonistic soluble receptors for TNF- $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ) (137). Exercise in high environmental temperatures has been shown to elevate circulating levels of IL-6 (106, 129, 171), TNF- $\alpha$  (129, 171), and IL-10 (129) to a greater extent when compared to exercise in a thermoneutral environment. Despite extensive research into the

cytokine response to exercise and exercise in the heat, little is known about the time course of the cytokine response to consecutive days of exercise in the heat. Hailes et al. (52) showed that cycling for 5 consecutive days in the heat resulted in a blunted inflammatory (IL-6, TNF- $\alpha$ , and IL-10) response to exercise and elevated resting levels of TNF- $\alpha$  and IL-10. However, this study sampled prior to and immediately after exercise which does not capture the total inflammatory response. Similar results were found by Kuennen et al. (72) who sampled the inflammatory response on the 1<sup>st</sup> and 7<sup>th</sup> day of a heat acclimation protocol. Results from this study should be interpreted cautiously due to a randomized cross-over design in which participants underwent two separate heat acclimation periods separated by 90 days while supplementing on a placebo or a known antioxidant, quercetin. It is not clear if the initial heat acclimation period or the supplementation, despite the 90 day separation, may have influenced the results of the second acclimation period. More extensive knowledge of the time course, and any adaptative responses, of the cytokine response to consecutive days of exercise in the heat may provide valuable insight into the role of the SIR during a time in which the individual should become more accustomed (e.g. acclimatization) to heat stress.

The purpose of this study was to determine the plasma LPS response and SIR to acute bouts of exercise in the heat and any adaptive response that occurs during consecutive days of exercise in the heat. We hypothesize the LPS response to an acute bout of exercise will be significantly decreased on Day 5 of exercise in the heat as compared to Day 1. Subsequently the SIR to exercise on Day 5 will result in a blunted IL-6 response and a less pro-inflammatory (TNF- $\alpha$ ) response as compared to Day 1.

## METHODS AND PROCEDURES

### *Participants*

Male volunteers meeting the following inclusion criteria were invited to enroll in the study: 1)  $VO_{2\max} \geq 50 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ; 2) healthy, as determined by Medical Par Q Screening (Appendix A); 3) not exposed to ambient temperatures  $> 27^{\circ}\text{C}$  on 10 consecutive days in the past month (self-reported); 4) have no history of heat related illness; 5) currently not taking any antioxidant supplementation, non-steroidal anti-inflammatory drugs (NSAIDS), antibiotics, or probiotics and; 6) have no known gastrointestinal medical condition. Descriptive data is presented in Table 1.

### *Preliminary Procedures and Assessments*

Qualifying volunteers were scheduled for an initial visit to the Auburn University Thermal Lab for further screening. The study was approved by the Institutional Review Board at Auburn University and participants were provided with and asked to read and sign an institutionally-approved informed consent document prior to any screening process. Volunteers meeting all inclusion criteria then continued with anthropometric and preliminary physiological measurements.

### *Anthropometric and Physiological Assessment*

*Session 1.* Anthropometric measurements including height and weight were obtained. Height was determined to the nearest 0.25 inch with a stadiometer and weight measured to the nearest 0.25 kg using a calibrated scale (Michelli Scales, Harahan, LA). Body composition was measured by Dual-emission X-ray absorptiometry (DXA). Each

participant then completed a series of six minute constant treadmill speed running bouts with five minute recovery periods between each bout. The treadmill was held at 0% grade throughout the session while the speed was increased before each successive 6 minute bout. At the end of each 6 minute bout a capillary blood sample was collected via finger puncture and whole blood lactate concentration ( $[La^-]$ ) was measured by a handheld lactate analyzer (Lactate Pro, Arkray, Inc. Kyoto, Japan). Testing was halted when participants reached or exceeded 4 mM  $[La^-]$ . A curvilinear regression of each subject's treadmill speed-blood  $[La^-]$  relationship was used to estimate the treadmill speed associated with 4mM blood  $[La^-]$ . The treadmill speed was then used to set the individual's treadmill speed for all remaining tests and exercise sessions.  $VO_2$  values were plotted against treadmill velocity and a linear regression line was used to calculate %  $VO_{2max}$  at the treadmill speed associated with 4mM blood  $[La^-]$  (Table 2).

*Session 2.* Participants then performed a progressive incremental maximal oxygen uptake ( $VO_{2max}$ ) test on a laboratory treadmill. Treadmill velocity was maintained constant at each participant's individual treadmill speed associated with 4mM  $[La^-]$  throughout the entire test. Treadmill grade was increased 2% every two minutes. The highest observed oxygen uptake average over 1 min was considered  $VO_{2max}$  if the respiratory exchange ratio was  $>1.15$ , heart rate was within 10 beats per minute (bpm) of age predicted maximum, or subject reached volitional exhaustion. Oxygen uptake ( $VO_2$ ) was measured with an automated metabolic testing system (True Max 2400 Metabolic Testing System, Parvo Medics, Salt Lake City, UT).

## **Experimental Procedures**

Participants performed five consecutive days of exercise and heat exposure (40°C, 40% RH) in the Thermal Lab's climate controlled chamber. No fewer than 72

hours following the final exercise session in the heat, participants completed a time matched exercise session in a normothermic environment (22°C-24°C). This trial was time matched to the first day of exercise in the heat. All trials were completed at the same time each day to reduce the effect of diurnal temperature variation. Participants were instructed not to consume any food within 10 h of the beginning of exercise. Upon awakening, participants were provided an energy containing beverage (250 kcal, 22% Fat, 64% carbohydrate (CHO), 14% protein (PRO)) to consume no later than 45 min prior to exercise. On evenings preceding blood sampling trials, participants were provided with standardized take-home meals (280-350 kcal). Participants were asked to refrain from physical activity outside of study parameters during the time course of the study and to continue regular dietary practices when not provided with nutrition.

Upon arrival at the lab, participants provided a urine sample for determination of hydration status. Samples were analyzed for urine specific gravity using a refractometer (Atago Co., Tokyo, Japan), and the participants were cleared for exercise if urine specific gravity was less than 1.020 g/ml. Nude body mass was measured using a calibrated scale. Each participant inserted a reusable temperature probe (Measurement Specialties, Inc., Dayton, OH) with disposable cover 10cm beyond the anal sphincter for measurement of core temperature and placed a heart rate monitor on his chest (Polar Electro Inc., Lake Success, NY). Core Temperature ( $T_c$ ) and heart rate (HR) were monitored continuously throughout each exercise session and recorded every minute. Venous blood samples were taken from the antecubital vein prior to the start of exercise (pre), immediately post-exercise (Post), 1 hour (Post 1-h), and 3 hours (Post 3-h) post exercise. On Days 3 and 5 of exercise in the heat a blood draw was taken at the time of

exercise termination on Day 1 of exercise in the heat. Participants refrained from food consumption until after the 3-h venous blood collection.

### *Exercise Conditions*

Each participant exercised on a laboratory treadmill (Woodway USA Inc., Waukesha, WI) at the treadmill speed associated with 4mM blood  $[La^-]$ . Exercise lasted until volitional exhaustion or until  $T_c$  increased by 2°C. Participants then exited the chamber, immediately towed dry and provided a final nude body mass measurement.

### *Blood Sampling Procedure*

Participants reported to the lab on exercise heat exposure days at least 30 minutes prior to exercise. On the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day of exercise heat exposure, after providing urine samples and measurement of body weight, a 21 gauge flexible catheter (Terumo Medical Corp., Somerset, NJ) was inserted into an antecubital vein and secured for the duration of the day's trial. The catheter was connected to a 0.9 ml male luer lock extension (Baxter, Deerfield, IL) capped with an intermittent injection port (Kawasumi Laboratories, Tampa, FL). Catheter patency was maintained by injecting 1 ml of Na<sup>+</sup>-Heparin (10 USP units/ml) into the extension line to prevent clotting. After insertion of the catheter, participants rested for 10 minutes before the first blood sample was drawn. Venous blood was drawn into K<sup>+</sup>-EDTA tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) at the sample times identified above. Blood samples were immediately spun for 15 min at 6,000 rpm and 0°C; plasma was syphoned and stored at -80°C until analysis. Fresh whole blood was used to determine hematocrit and hemoglobin. These values were used to estimate plasma volume changes using the method of Dill and Costill (29).

### *Analysis of Plasma LPS*

Plasma LPS was determined with the Limulus amoebocyte lysate (LAL) Chromogenic Endpoint Assay (Hycult Biotech Inc., Lymouth Meeting, PA). The sensitivity of the assay is 0.04 EU/ml. Briefly, plasma samples were thawed and brought to room temperature before being incubated at 56°C for 5 min. Samples were then centrifuged at 10,000 rpm for 5 min. 50 µl of samples/standards were added to the appropriate wells before addition of 50 µl of provided LAL reagent. Samples and standards were incubated at room temperature for 30 min before being read at 405 nm using a SpectraMax M2 microplate reader (Molecular Devices LLC., Sunnyvale, CA). If the OD of the two highest standards were within 10% of each other, 50 µl of acetic acid was added to each well. If the OD of the top two standards were not within 10% of each other, samples were incubated for 5 additional minutes before addition of acetic acid. Plasma [LPS] was determined using a line of best fit for known standards.

### *Analysis of Plasma Cytokines*

Plasma cytokines TNF- $\alpha$ , IL-6, and IL-10 were measured in plasma using commercially available quantitative ELISA kits. IL-6 and IL-10 were measured in triplicate while TNF- $\alpha$  was measured in duplicate.

Plasma [IL-6] was determined with the Human IL-6 ELISA Ready-SET-Go! kit (eBioscience Inc., San Diego, CA.) following manufacturer instructions for high sensitivity (2 pg/ml). Briefly, high affinity binding plates (Corning Inc., Tewksbury, MA) were coated with purified anti-human IL-6 antibody and incubated at 4°C overnight. Plates were washed as instructed with wash buffer (10mM PBS + 0.05% Tween) and blocked for 1 hour with provided assay diluent. Plates were then washed again and 100

$\mu\text{l}$  of standard and undiluted samples were added and incubated overnight at  $4^{\circ}\text{C}$ . After washing, biotinylated anti-human IL-6 detection antibody was applied and plates were incubated for 1 hour at room temperature. Plates were then washed and incubated at room temperature for 30 min with Avidin-HRP followed by another wash.  $100\ \mu\text{l}$  of supplied substrate solution was added to each well and incubated for 15 min at room temperature at which time  $50\ \mu\text{l}$  of stop solution ( $1\ \text{M}\ \text{H}_2\text{SO}_4$ ) was added. Absorbance was read at 570 nm and 450 nm; wavelength subtraction ( $\text{OD at } 579\ \text{nm} - \text{OD } 45\ \text{nm}$ ) was applied to analyze data. Plasma [IL-6] was determined via linear regression of known standards.

Plasma [TNF- $\alpha$ ] was determined with the Human TNF- $\alpha$  Ultrasensitive ELISA kit (Invitrogen Corp., Camarillo, CA). The sensitivity of this assay is  $<0.09\ \text{pg/ml}$ . Samples were diluted 1:2 with provided assay diluent in the pre-coated antibody coated plates.  $50\ \mu\text{l}$  of biotinylated TNF- $\alpha$  conjugate was added prior to 2 hour incubation at  $37^{\circ}\text{C}$ . Plates were then washed with provided wash buffer before incubating plates with  $100\ \mu\text{l}$  streptavidin-HRP for 30 min at room temperature. Plates were then washed and  $100\ \mu\text{l}$  of stabilized chromogen was added and plates incubated at room temperature in the dark for 30 min. After incubation,  $100\ \mu\text{L}$  of stop solution was added and absorbance read at 450 nm. Plates were blanked against a control well containing  $100\ \mu\text{L}$  each of stabilized chromogen and stop solution. Plasma [TNF- $\alpha$ ] was determined via linear regression of known standards; values were multiplied by 2 to correct for the 1:2 dilution.

Plasma [IL-10] was determined with the Human IL-10 Ultrasensitive ELISA kit (Invitrogen Corp., Camarillo, CA). The sensitivity of this assay is  $<0.2\ \text{pg/ml}$ . Briefly, unknown samples were diluted 1:2 in the provided antibody coated plates and incubated for 2 hours at  $37^{\circ}\text{C}$ . Plates were then washed with provided wash buffer and

100  $\mu$ l of biotinylated TNF- $\alpha$  conjugate was added and plates incubated for 1 hour at room temperature. Following another wash 100  $\mu$ l of streptavidin-HRP was added and plates were incubated for 30 min at room temperature. Plates were then washed and 100  $\mu$ L of stabilized chromogen was added and plates were incubated in the dark at room temperature for 30 min before adding 100  $\mu$ L of stop solution. Absorbance was read at 450 nm and plates were blanked against a control well containing 100  $\mu$ l each of stabilized chromogen and stop solution. Plasma [IL-10] was determined via linear regression of known standards; values were multiplied by 2 to correct for the 1:2 dilution

### *Statistical Analysis*

This study utilized a within-subject design with each participant serving as his own control. Group characteristics are reported as means  $\pm$  SD. A 5 (day) x 19 (time) two-way ANOVA with repeated measures on both day and time was used to compare  $T_c$ , HR, and PSI during exercise in the heat. A three (day) x four (time) ANOVA with repeated measures on both day and time was used to compare blood plasma IL-6, TNF- $\alpha$ , and IL-10 for exercise in the heat. Planned pairwise comparisons were made between pre- and post-exercise for IL-6. A two (day) x four (time) ANOVA with repeated measures on both day and time was used to compare blood plasma IL-6, TNF- $\alpha$ , and IL-10 on Day 1 of exercise in the heat and the normothermic trial. A three (day) x two (time) ANOVA with repeated measures on both day and time was used to compare blood LPS activity for exercise in the heat. A two (day) x two (time) ANOVA with repeated measures on both day and time was used to compare blood LPS activity on Day 1 of exercise in the heat and the normothermic trial. The significance level for this study was set at  $P < 0.05$ .  $P$  values were evaluated with the Greenhouse-Geisser adjustment to correct for sphericity violations. Significant differences were further explored utilizing a

Tukey post hoc test. Statistics were analyzed with commercially available software (OriginLab Corp., Northampton, MA).

## Results

### *Participants*

In total, nine male volunteers met the study inclusion criteria and agreed to participate in the study. One participant withdrew from the study due to personal reasons; therefore eight participants completed the entire study protocol. Participant baseline physiological characteristics are presented in Table 1.

### *Exercise Conditions*

All participants were able to complete the five days of exercise in the heat and the control exercise trial at ambient room temperature. Environmental temperature and relative humidity were recorded every 5 min during exercise and averaged for each bout. Temperatures and relative humidity were not significantly different among the trials. Exercise conditions are presented in Table 2.

### *Exercise Data*

There was no significant difference among days for exercise times, resting body weight, sweat rate, resting heart rate, and H<sub>2</sub>O consumption during exercise in the heat. Exercise data are presented in Table 2.

### *T<sub>c</sub>, HR, PSI Response to Exercise*

HR and T<sub>c</sub> were monitored throughout each exercise session while PSI was calculated from these values; values were recorded each minute. Exercise termination was set at volitional exhaustion or a 2°C increase in T<sub>c</sub>; thus all data samples were transformed to a percentage of completion time. T<sub>c</sub> and PSI response to exercise were

analyzed with  $n = 7$  due to loss of one subject's Day 2 data.  $T_c$ , HR, and PSI data are presented in Table 2.

$T_c$ . Resting  $T_c$  was significantly lower on Day 5 ( $36.76^\circ\text{C} \pm 0.2$ ;  $P = 0.02$ ) and Day 4 ( $36.76^\circ\text{C} \pm 0.3$ ;  $P < 0.01$ ) as compared to Day 1 ( $37.10^\circ\text{C} \pm 0.2$ ) of exercise in the heat. Final  $T_c$  was also significantly lower on these days. In general  $T_c$  was significantly ( $P < 0.001$ ) elevated above rest by 38% completion each day of exercise in the heat and remained so until exercise termination. Analysis of  $\Delta T_c$  indicated there was no significant difference in the rate of  $T_c$  increase among days of exercise in the heat ( $P = 0.08$ ).  $T_c$  was significantly lower at rest prior to the normothermic control trial ( $36.83^\circ\text{C} \pm 0.3$ ;  $P < 0.001$ ) as compared to Day 1 of exercise in the heat and remained significantly lower throughout.

HR. No significance was found among days for resting HR. Significance ( $P < 0.001$ ) was detected across time; HR was significantly elevated above rest at 5% completion time each day and remained so until exercise termination. HR was significantly ( $P < 0.05$ ) lower on Days 2, 3, 4, and 5 of exercise in the heat at 5% and 10% completion of exercise as compared to Day 1. No significant difference was detected at exercise termination for any day of exercise in the heat. Resting HR was not significantly ( $P = 0.66$ ) different prior to the normothermic trial as compared to Day 1 of exercise in the heat. On both days HR was significantly ( $P < 0.001$ ) elevated from resting values by 5% completion and remained so until exercise termination. At 65% completion time HR was significantly ( $P < 0.01$ ) lower during the normothermic control trial ( $163 \pm 9$  bpm) as compared to Day 1 of exercise in the heat ( $181 \pm 10$  bpm) and remained lower for the remainder of the trial.

*PSI.* There was no significant difference ( $P = 0.07$ ) detected among days of exercise in the heat. PSI was significantly elevated ( $P < 0.001$ ) above rest by 10% of exercise completion and remained so until exercise termination. During the normothermic trial PSI was significantly lower ( $P < 0.001$ ) from 53% completion until exercise termination as compared to Day 1 of exercise in the heat.

#### *Plasma LPS response to exercise*

Exercise in the heat significantly ( $P < 0.001$ ) elevated plasma LPS activity above resting (Day 1: 0.8 EU/ml vs. 1.2 EU/ml, Day 2: 0.6 EU/ml vs. 1.0 EU/ml, Day 3: 0.5 EU/ml vs. 1.0 EU/ml) each day of exercise in the heat. Resting values gradually declined throughout the 5 days of exercise in the heat, however no significant differences ( $P = 0.19$ ) was detected among days at any sampling time. These data would indicate that consecutive days of exercise in the heat did not significantly alter the plasma LPS response to exercise despite lower resting and post-exercise values. Plasma [LPS] data are presented in Figure 1. Plasma [LPS] was significantly ( $P < 0.01$ ) elevated above rest (0.5 EU/ml) post-exercise (1.1 EU/ml) in the normothermic environment. No significant difference was detected between Day 1 of exercise in the heat normothermic environment ( $P = 0.07$ ). These data indicate that Day 1 of exercise in the heat and exercise in a normothermic environment elicited similar plasma [LPS] responses. Plasma LPS data for exercise in a normothermic environment are presented in Figure 2.

#### *Cytokine response to exercise*

IL-6, TNF- $\alpha$ , and IL-10 concentrations were determined in plasma with commercially available kits immediately pre-exercise, immediately post-exercise, 1 h post-exercise, and 3 h post-exercise on Days 1, 3, and 5 of exercise in the heat and during the normothermic trial. Plasma samples were run in triplicate for IL-6 and IL-10 while

TNF- $\alpha$  was run in duplicate. Repeated freeze-thaw cycles of plasma were minimized as much as possible.

*IL-6.* Mean plasma concentrations of IL-6 showed the expected, though not significant ( $P = 0.18$ ), increase post-exercise and then begin to decline and had returned to near resting values by 3 h post exercise. Planned pairwise comparison indicated that mean plasma concentrations were significantly ( $P < 0.01$ ) elevated immediately post-exercise (14.8 pg/ml) as compared to rest (10.6 pg/ml). No significance was detected among days ( $P = 0.62$ ). These data indicate that the mean plasma IL-6 response to exercise in the heat was not altered over 5 consecutive days of exercise. Mean plasma concentrations increased 1.4-fold immediately post-exercise as compared to resting values in the normothermic trial. Significance was detected ( $P = 0.01$ ) between Day 1 of exercise in the heat vs. the normothermic trial while values were not significantly ( $P = 0.08$ ) elevated above rest on either day. Post-hoc analysis indicated IL-6 was significantly higher immediately post exercise (15.3 pg/ml vs. 11.0 pg/ml;  $P = 0.03$ ) on Day 1 of exercise in heat as compared to the normothermic trial. These data indicate that exercise in the heat elicited a greater plasma IL-6 response as compared to exercise the normothermic environment despite equal exercise times. Plasma [IL-6] data are presented in Figure 3 and 4.

*TNF- $\alpha$ .* Mean plasma TNF- $\alpha$  concentration gradually increased from rest to post exercise on Days 1 and 3 and continued to increase to 3-h post exercise. Mean plasma TNF- $\alpha$  concentration on Day 5 of exercise in the heat decreased from rest to post exercise and remained below resting until 3 h post exercise. No statistical difference was detected across time ( $P = 0.13$ ) for mean plasma concentrations while statistical differences were found among days ( $P = 0.04$ ) of exercise in the heat. Post-hoc analysis indicated mean

plasma concentrations were significantly lower 1-h post exercise on Day 5 (3.2 pg/ml) as compared to Day 3 (4.7 pg/ml;  $P = 0.018$ ) and Day 1 (4.5 pg/ml;  $P = 0.004$ ) while 3-h post exercise values were significantly lower on Day 5 as compared to Day 1 (3.6 pg/ml vs. 5.0 pg/ml;  $P = 0.05$ ). These data indicate that the inflammatory response to exercise in the heat was less pro-inflammatory after 5 consecutive days of exercise in the heat. No significant difference between days ( $P = 0.11$ ) or across time ( $P = 0.37$ ) was detected between Day 1 of exercise in the heat vs. the normothermic trial. These data indicate that exercise in a hot environment did not produce a more inflammatory response to exercise in the heat. Plasma [TNF- $\alpha$ ] data are presented in Figure 5 and 6.

IL-10. Exercise in the heat gradually increased mean plasma IL-10 concentration as compared to resting values. Peak plasma [IL-10] occurred 1 h post exercise and was significantly different from resting values (0.9 pg/ml vs. 0.5 pg/ml;  $P < 0.01$ ) and had returned to near resting values by 3-h post exercise. No significant differences ( $P = 0.18$ ) were detected at any sample time between days of exercise in the heat. These data indicate that exercise in the heat resulted in an anti-inflammatory response post exercise in the heat and that this response was not altered after 5 consecutive days of exercise in the heat. Mean plasma IL-10 concentrations stayed at or near resting values after exercise in the normothermic environment. No significance was detected between days ( $P = 0.09$ ) or across time ( $P = 0.40$ ) between Day 1 of exercise in the heat and the normothermic trials. Plasma [IL-10] data are presented in Figure 7 and 8.

## Discussion

This study represents the most detailed analysis of the SIR in conjunction with the LPS response to consecutive days of exercise in hot environmental temperatures. The main findings of this investigation were: 1) each day of exercise in the heat resulted in significantly elevated plasma [LPS]; 2) consecutive days of exercise in the heat resulted in no alteration in the IL-6 response to an acute bout of exercise in the heat; 3) resting values of cytokines were unaltered over the 5 days of exercise in the heat; and 4) a decreased pro-inflammatory (TNF- $\alpha$ ) response post-exercise on Day 5 as compared to Days 1 and 3 of exercise in the heat. A strength of this study was the use of serial sampling pre- and post-exercise on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day of exercise in the heat. Given that individuals who undertake repeated bouts of exercise in hot environmental temperatures become acclimatized to exercise in these conditions, we sought to understand any adaptive response that may occur to the plasma LPS response and SIR (176) during this process. The findings indicate that no change in the plasma LPS response to exercise in the heat occurred over five consecutive days of exercise in the heat. This is surprising when one considers the acclimation processes that take place over this time which include an expanded blood volume (100). The findings also suggest a decreased TNF- $\alpha$  response post exercise on Day 5 as compared to Days 1 and 3. This would indicate that the SIR to exercise in the heat was less pro-inflammatory after 5 consecutive days.

Plasma LPS. Plasma [LPS] significantly ( $P < 0.01$ ) increased above rest after exercise each day of exercise in a hot environment in this study. Acclimatization to exercise in the heat results in, among other things, hypervolemia, increased sweating rates, an earlier onset of sweating during exercise (48, 100, 166). These adaptive responses would serve to mitigate the cardiovascular strain by increasing heat dissipation and maintenance of the central blood volume, thus allowing for greater perfusion of the visceral tissue without compromising cutaneous or force-producing skeletal muscle tissue blood flow. Contrary to our hypothesis, the plasma LPS response was not mitigated over consecutive days of exercise in the heat. The cohort used in this current study consisted of moderately trained ( $VO_{2max} = 55.3 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ; Range 50 – 59  $\text{ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) individuals which may have already exhibited a partial heat acclimated phenotype. The findings by Selkirk et al. (161) support this concept by showing trained individuals had a greater LPS tolerance and heat tolerance than their untrained counterparts. It is likely that the trained individuals were able to maintain a greater percentage of cardiac output to the splanchnic circulation during exercise in the heat, especially at lower  $T_c$ , than their untrained counterparts. Thus, the disturbances to the GI barrier as a result of reduced blood supply would likely be mitigated in this group to an extent. Furthermore, it is likely that trained individuals possess higher levels of natural defense mechanism, such as Anti-LPS IgG, as seen in individuals training for/competing in endurance events (4, 67).

Mechanisms behind decreased barrier function are numerous and include the direct effects of heat, increased radical production, and decreased NO production (54-56, 75). Reductions in visceral blood flow has been demonstrated in individuals during light ( $HR < 90 \text{ bpm}$ ) exercise (117) while gastric ischemia has been reported in individuals

cycling at maximal effort (123) in normothermic environments. Subsequently, Pals et al. (124) showed that small intestinal permeability was increased following 60 min of running at 80% of  $VO_{2max}$ , but not after running at 40% or 60% in normothermic. Endotoxemia has been reported following both short (< 1 h) (1, 72) and long duration (> 1 h) (11, 14, 15, 105, 161) exercise. This study is the first to probe the LPS response to exercise on multiple days during the acclimation process. While the results indicate that no change in this response was detected over 5 days of exercise in the heat a longer time period may be needed to see significant alterations in the LPS response to exercise in the heat. Elucidation of this response in a less trained cohort may also minimize the influence of prior training status on this response.

*Inflammatory Response.* Plasma [IL-6] significantly ( $P = 0.01$ ) increased 1.4-fold post-exercise on Day 1, 3, and 5 of exercise in the heat and returned near baseline 1 and 3 h after exercise each day of exercise in the heat (Figure 3). In circulation, IL-6 interacts with membrane bound gp130 and IL-6 receptor or complexes in circulation with the soluble IL-6 receptor (sIL-6r) which facilitates binding to gp130 on cells without the IL-6 receptor (98). IL-6 signaling has been demonstrated to serve a role in LPS induced fever (107, 155), production of anti-inflammatory mediators (174), activation of hypothalamic-pituitary axis (38), B cell differentiation (59), synthesis of acute phase proteins (18), glucose homeostasis (37), and fat metabolism (139). Our finding of an unaltered IL-6 response to exercise in the heat, while contrary to our expected results, is not surprising considering the similar exercise times on each day as the IL-6 response to a bout of exercise has been found to be highly dependent on the duration of the exercise bout (38). Given the majority of IL-6 released during exercise is from active skeletal muscle, a

blunted IL-6 response to exercise during heat acclimation would likely indicate a decreased IL-6 production from tissue other than contracting skeletal muscle.

The results of this study also indicate that exercise in a normothermic environment resulted in a significantly ( $P = 0.03$ ) lower plasma [IL-6] post-exercise as compared Day 1 of exercise in the heat. Past findings have been mixed with multiple studies showing a significantly lower IL-6 response to exercise in a normothermic environment (147, 171) and multiple studies showing no difference (106, 129). Use of different modalities, durations, and intensities of exercise in these studies may explain the conflicting findings between the studies. Regardless, an explanation regarding the mechanism/cause of a greater IL-6 release during exercise in a hot environment is lacking. It is suggested that a greater catecholamine (e.g. epinephrine) response with exercise in a hot environment stimulates synthesis/release of IL-6 via cAMP activation (10, 147, 171). However, use of epinephrine on skeletal muscle *in vitro* caused no change in IL-6 mRNA synthesis or protein release (63). Interestingly, low muscle glycogen has been shown to alter the pattern of IL-6 release during exercise (86) while exercise in the heat has been shown to increase rate of glycogen utilization/depletion (67). We can only speculate as these variables being plausible explanations for our findings as they were not measured in the current study

Our study resulted in small subclinical ( $< 2$  fold) changes in plasma [TNF- $\alpha$ ] each day of exercise in the heat. Plasma [TNF- $\alpha$ ] was not significantly elevated above rest at any sampling time point throughout the consecutive days of exercise in the heat. However, on Day 5 of exercise in the heat, plasma [TNF- $\alpha$ ] decreased below resting values post exercise and was significantly lower than Days 1 ( $P = 0.02$ ) and 3 ( $P < 0.01$ ) at 1 h post exercise and Day 1 ( $P = 0.05$ ) at 3 h post exercise. A similar pattern was

demonstrated by Kuennen et al. (72) after seven days of walking in the heat. The small increases seen with exercise in our, and others, study is most likely the result of the ever transient TNF- $\alpha$  kinetics and fast removal from the circulation (178). Exercise induced IL-6 increases, as seen in our study, have also been shown to prevent LPS induced TNF- $\alpha$  release in humans (168). It is also possible that exercise in hot environmental temperatures increases the permeability of the blood-brain barrier (188) and inflammatory mediators such as TNF- $\alpha$  and IL-1  $\beta$  are taken up and possibly play a role in exercise fatigue (17). Subsequently no difference in the TNF- $\alpha$  response was detected between Day 1 of exercise in the heat and the normothermic trial. The lack of significant elevations in TNF- $\alpha$  would indicate that significantly elevated levels of LPS are not mediating a more pro-inflammatory response in our study.

Plasma [IL-10] values were significantly elevated ( $P < 0.01$ ) 1 h post exercise as compared to resting and had returned near baseline 3 h post exercise with no significance detected among days during consecutive days of exercise in the heat. The plasma [IL-10] response to exercise in a normothermic environment approached ( $P = 0.09$ ) but was not significantly different from Day 1 of exercise in the heat. Regulation of synthesis and release of IL-10 from activated macrophages is likely multi-factorial but includes IL-6 (174) and adrenergic stimulation (36, 190). While the catecholamine response was not measured in this study past investigations have indicated a greater catecholamine response to exercise in hot environments as compared a normothermic environment (130, 171). IL-10 serves a crucial anti-inflammatory regulatory role in the SIR by decreasing TNF- $\alpha$  receptor expression on membranes (28, 69) and inhibiting inflammatory cytokine production (114). T

*Perspective of Inflammatory Response.* The SIR to infection or major tissue/organ damage results first in marked increases in pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ . This is then followed by the anti-inflammatory response mediated by IL-6 in which IL-1ra, sTNFr, and IL-10 increase in the circulation (138). Contrarily, the SIR to exertion is characterized by an increase in IL-6 to a greater extent than any other cytokine and the subsequent anti-inflammatory cascade. Furthermore, the large pro-inflammatory response to infection and tissues damage is often not seen as a result of exercise (138). However, multiple studies (52, 72, 129, 147, 161, 171) have shown small increases in pro-inflammatory mediators to exertional hyperthermia. Typically the levels of these pro-inflammatory cytokines are not of the magnitude of that seen in cases of heat stroke (5, 8).

*Conclusion.* This study found that consecutive days of exercise in the heat elicited an inflammatory response and an increase in plasma [LPS]. Plasma LPS values were significantly elevated post exercise each sampling day during the consecutive days of exercise in the heat with no significant differences among days despite lower resting and post-exercise values. There was a significant increase in plasma [IL-6] concentration one each day with no significant changes among days, indicating no adaptive response over this time. Plasma [TNF- $\alpha$ ] did not significantly increase above resting at any sampling time point, however post exercise plasma values were significantly lower on Day 5 of exercise in the heat as compared to Days 1 and 3. Plasma [IL-10] was significantly elevated 1 h post exercise with no significant changes among days. Plasma [LPS] significantly increased after exercise in the heat on each sampling day with no significant change among days. There was a noticeable trend for plasma LPS values to be lower pre and post exercise over the consecutive days possibly indicating an increased LPS

tolerance. In conclusion, 5 consecutive days of exercise in the heat elicited similar IL-6 and IL-10 response post-exercise whereas TNF- $\alpha$  values were significantly lower post exercise after 5 days. This study represents the most detailed analysis of the SIR in conjunction with the LPS response to consecutive days of exercise in hot environmental temperatures. Further research into the role of GI barrier function and the natural host defense to GI derived bacteria may provide valuable insight into their role into acquiring heat tolerance and protection against heat related injuries (72, 74, 156). Lastly, more studies analyzing LPS-mediated SIR to exercise in the heat over a longer acclimation time period may provide better time frame for understanding the relationship between heat acclimation and LPS-mediated systemic inflammatory response.

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## Tables

Table 1. *Baseline physiological characteristics*

<b>Variables</b>	<b>Mean <math>\pm</math> SD</b>	<b>Minimum</b>	<b>Maximum</b>
Age, yr	24 $\pm$ 3	20	27
Height, cm	180 $\pm$ 8	165	193
Weight, kg	76.8 $\pm$ 9.1	60.5	91.8
Body fat %	17.9 $\pm$ 5.7	11.2	27.4
VO <sub>2max</sub> , ml·kg <sup>-1</sup> ·min <sup>-1</sup>	55.3 $\pm$ 3.6	50.2	59.0

All values presented as mean  $\pm$  SD and minimum and maximum values. VO<sub>2max</sub>, maximal oxygen uptake.

Table 2. *Exercise data*

<b>Variables</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Day 4</b>	<b>Day 5</b>	<b>Normothermic</b>
Run speed, mph	6.9 ± 1.2					
% VO <sub>2max</sub>	78.0 ± 1.2					
Starting T <sub>c</sub> , °C	37.1 ± 0.2	36.9 ± 0.2	36.9 ± 0.3	36.8 ± 0.3*	36.8 ± 0.2*	36.8 ± 0.2 <sup>§</sup>
Final T <sub>c</sub> , °C	39.0 ± 0.2	38.9 ± 0.2	38.9 ± 0.2	38.7 ± 0.2	38.7 ± 0.2	38.4 ± 0.2
Resting HR, bpm	84 ± 12	90 ± 10	87 ± 8	83 ± 14	83 ± 8	82 ± 9
Final HR, bpm	191 ± 10	192 ± 9	188 ± 10	186 ± 9	179 ± 9	167 ± 11 <sup>§</sup>
Final PSI	9.5 ± 0.4	9.5 ± 0.7	9.2 ± 0.9	8.9 ± 0.4	8.7 ± 0.7	7.2 ± 0.4 <sup>§</sup>
Exercise Tolerance, min	23.6 ± 3.2	23.8 ± 3.2	26.0 ± 4.5	23.8 ± 2.5	25.6 ± 4.2	23.6 ± 3.2
Sweat Rate, L/hr	2.5 ± 1.4	1.9 ± 0.7	2.3 ± 0.8	2.0 ± 0.8	2.0 ± 1.2	1.5 ± 0.7
H <sub>2</sub> O ingestion, ml	224 ± 190	241 ± 206	266 ± 297	175 ± 156	181 ± 241	117 ± 107
Environmental Temperature, °C	41.4 ± 0.5	41.4 ± 0.6	41.3 ± 0.9	41.1 ± 0.7	41.0 ± 0.8	
Relative Humidity, %	41.0 ± 1.1	40.6 ± 0.6	41.3 ± 0.7	40.1 ± 0.5	40.9 ± 0.8	

All values presented as mean ± SD. \* Indicates significant difference from Day 1 in the heat (P < 0.05). § Indicates normothermic trial significantly different (P < 0.05) from Day 1 of exercise in the heat. %VO<sub>2max</sub>, % of VO<sub>2max</sub> elicited during running.

### Figures

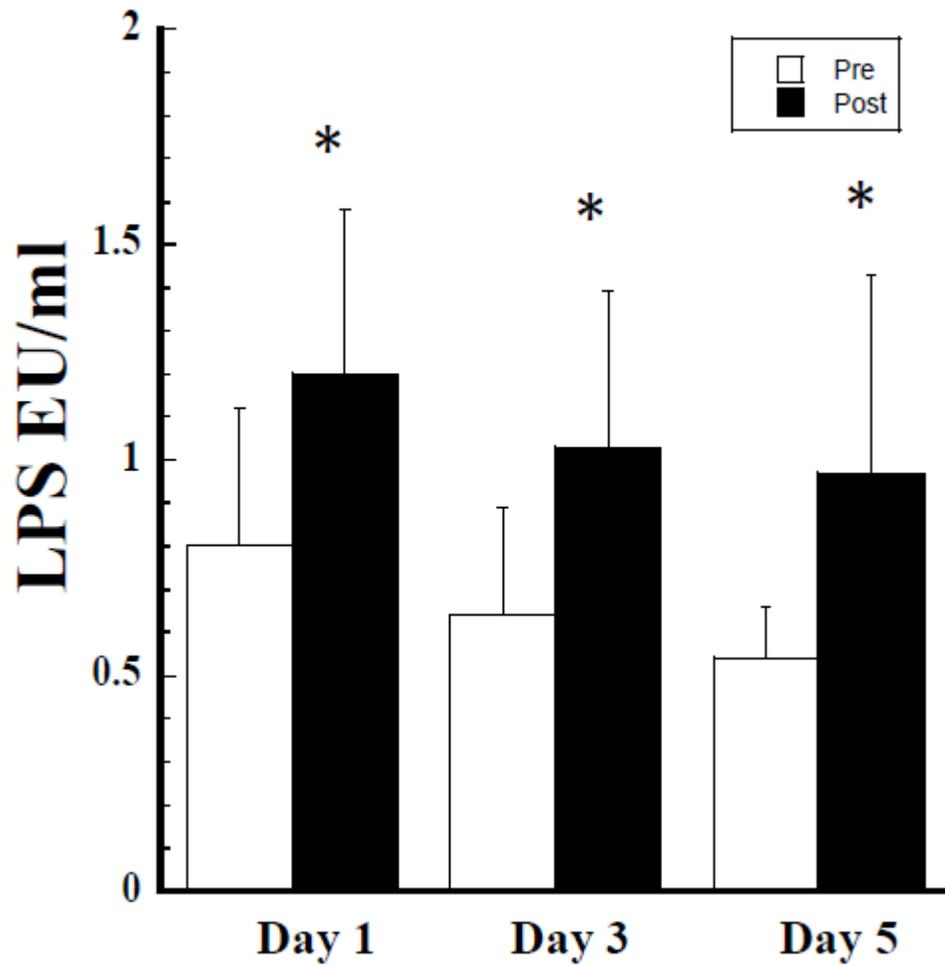


Fig. 1. Plasma LPS before and after exercise on Day 1, 3, and 5 of exercise in the heat. Values are reported as means  $\pm$  SD. \*Significantly elevated from pre-exercise ( $P < 0.05$ ). No significance difference was detected among days.

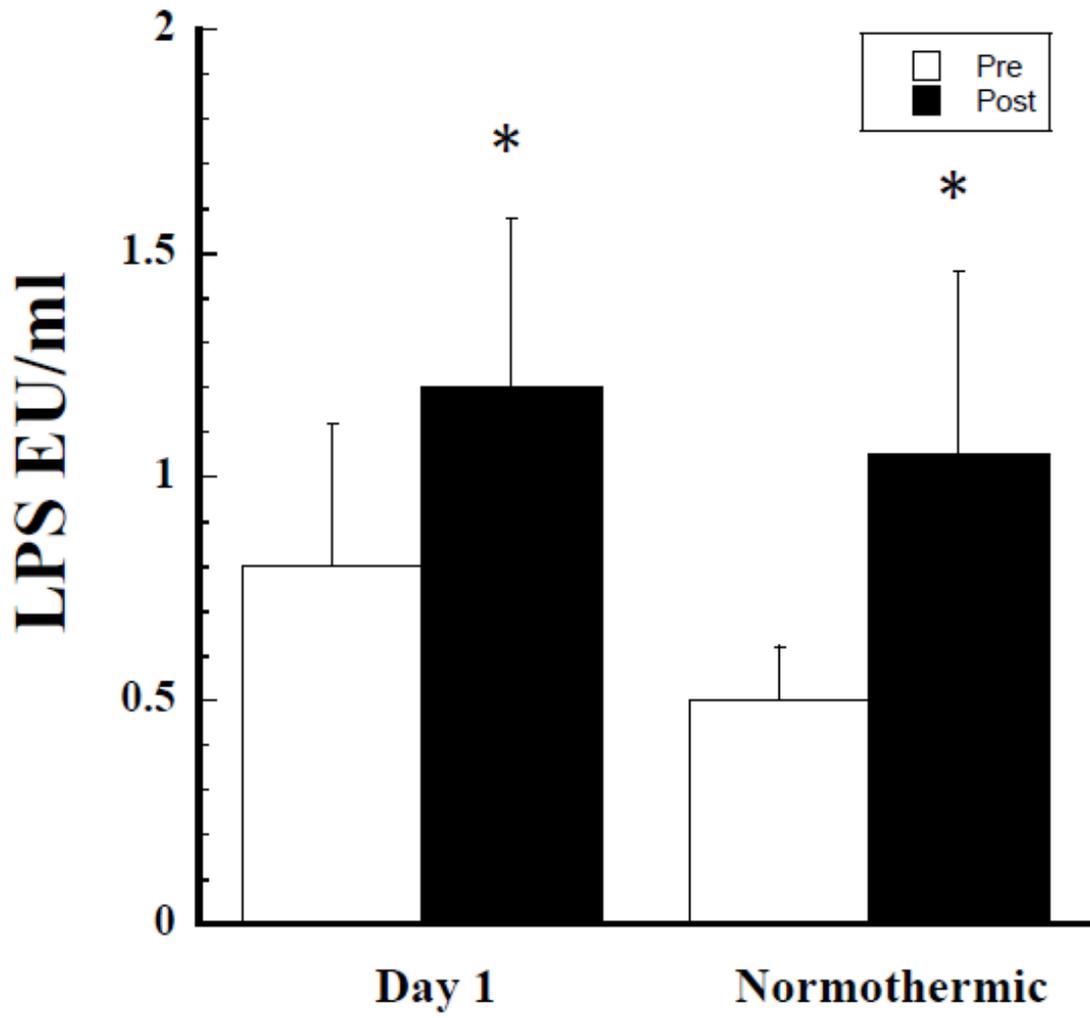


Fig. 2. Plasma LPS before and after exercise on Day 1 of exercise in the heat and in the normothermic environment. Values are reported as means  $\pm$  SD. \* Significantly different ( $P = 0.03$ ) from pre-exercise value. No significant difference was detected between days.

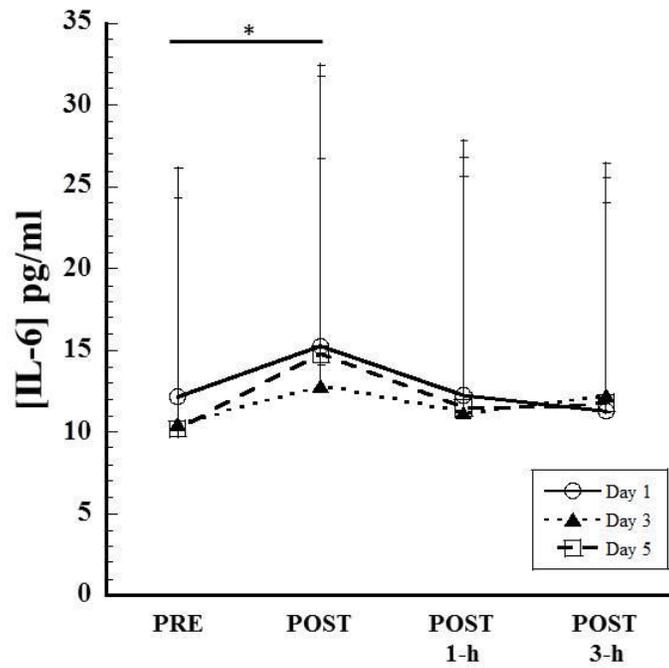


Fig. 3. Plasma [IL-6] before and after exercise on Days 1, 3, and 5 of exercise in the heat. Values are reported as means  $\pm$  SD. \* Significant increase from pre-exercise values. No significance among days was detected.

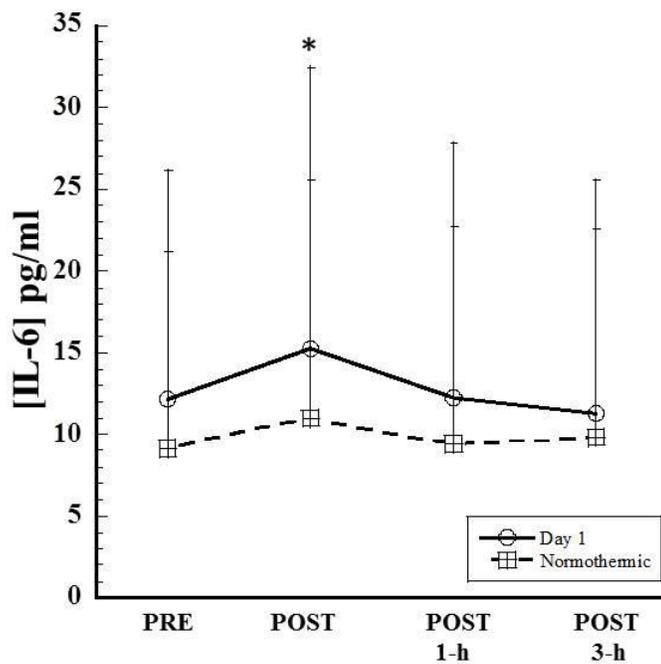


Fig. 4. Plasma [IL-6] before and after exercise on Day 1 of exercise in the heat and in the normothermic environment. Values are reported as means  $\pm$  SD. \* Significant difference between trials. No significance across time was detected. Note that the SD bars indicate variability among subjects.

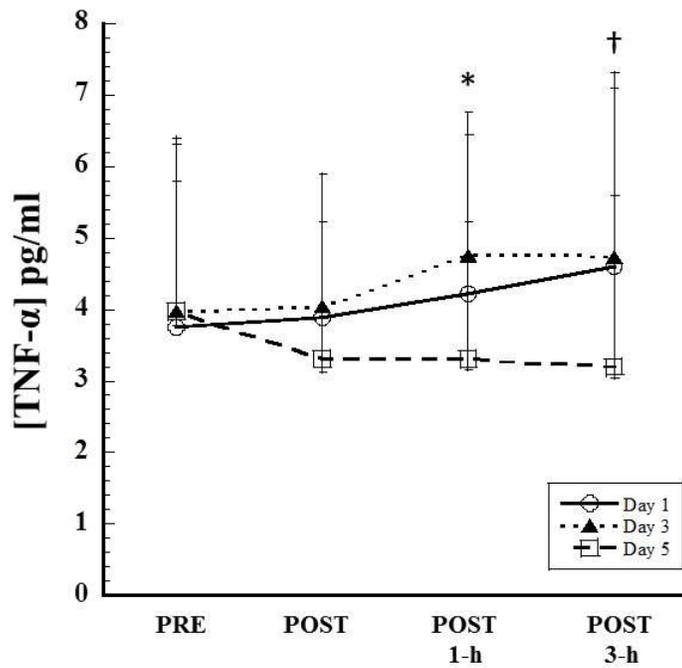


Fig. 5. Plasma [TNF- $\alpha$ ] before and after exercise on Days 1, 3, and 5 of exercise in the heat. Values are reported as means  $\pm$  SD. \* Day 5 significantly less than Day 3 and Day 1 ( $P < 0.05$ ). † Day 5 significantly less than Day 1 ( $P < 0.05$ ). No significant differences were detected across time.

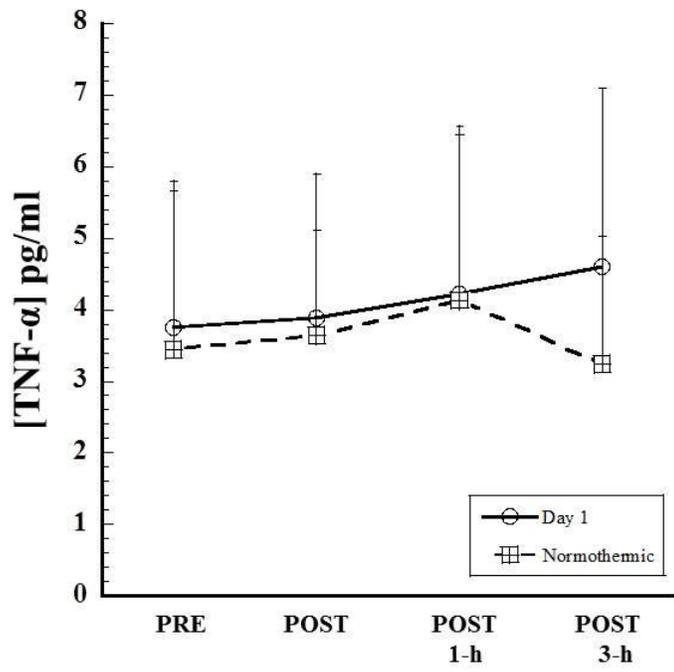


Fig. 6. Plasma [TNF- $\alpha$ ] before and after exercise on Day 1 of exercise in the heat and in the normothermic environment. Values are reported as means  $\pm$  SD. No significant differences among days or across time were detected.

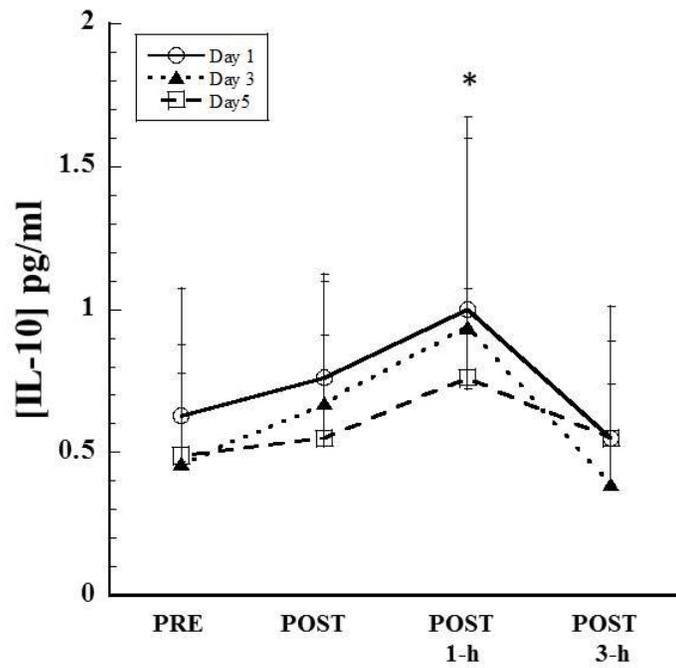


Fig. 7. Plasma [IL-10] before and after exercise on Days 1, 3, and 5 of exercise in the heat. Values are reported as means  $\pm$  SD. \* Significantly elevated from pre-exercise and Post 3-h values ( $P < 0.01$ ). No significant differences were detected among days.

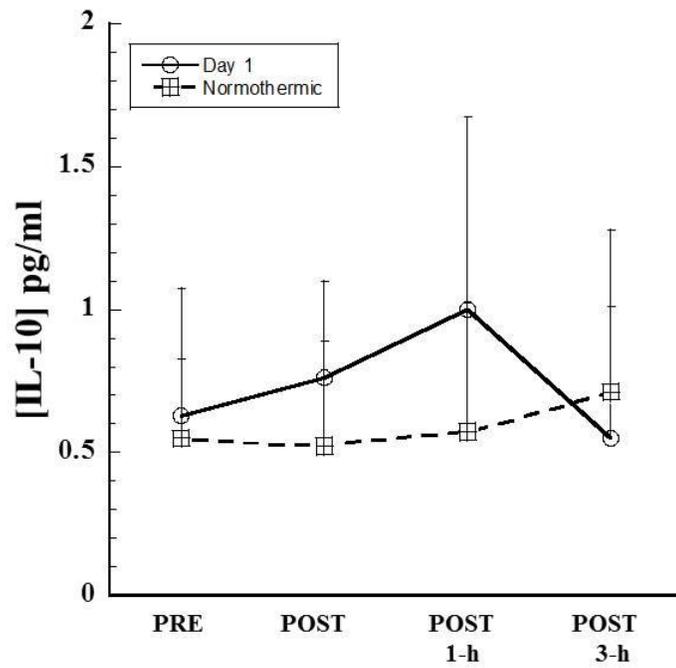


Fig. 8. Plasma [IL-10] before and after exercise on Day 1 of exercise in the heat and in the normothermic environment. Values are reported as means  $\pm$  SD. No significant differences between days or across time were detected.

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## **Appendices**

## Appendix A

### Physical Readiness Activity Questionnaire (Par Q) \*

**Please read each question carefully and answer honestly. If you do not understand the question, please ask the investigator for clarification. Check the appropriate answer.**

No Yes

- \_\_\_\_\_ \_\_\_\_\_ 1. Are you under the age 19 or over the age of 35?
- \_\_\_\_\_ \_\_\_\_\_ 2. Do you presently smoke or have been a regular smoker?
- \_\_\_\_\_ \_\_\_\_\_ 3. Has your doctor ever said you have heart trouble?
- \_\_\_\_\_ \_\_\_\_\_ 4. Do you have a family history of early cardiovascular death before the age of 50?
- \_\_\_\_\_ \_\_\_\_\_ 5. Have you ever had a heart murmur, rheumatic fever or respiratory problems?
- \_\_\_\_\_ \_\_\_\_\_ 6. Have you ever been told that you have a fast resting heart rate?
- \_\_\_\_\_ \_\_\_\_\_ 7. Have you ever been told by your doctor or nurse that your blood pressure is too high?
- \_\_\_\_\_ \_\_\_\_\_ 8. Have you ever been told that your cholesterol is too high?
- \_\_\_\_\_ \_\_\_\_\_ 9. Have you been told that you have a kidney disorder?
- \_\_\_\_\_ \_\_\_\_\_ 10. Have you been told that you have diabetes or that your blood sugar is too high?
- \_\_\_\_\_ \_\_\_\_\_ 11. Have you been told that your electrocardiogram (EKG), 12 lead EKG or stress test is not normal?
- \_\_\_\_\_ \_\_\_\_\_ 12. Have you been hospitalized in the past year or ever hospitalized for heat related illness? (Heat related illnesses consisting of fainting, excessive swelling, severe skin irritation, core body temperatures > 104°F, a lack of cooling by the body, and heat stroke due to high environment temperatures.)
- \_\_\_\_\_ \_\_\_\_\_ 13. Have you ever experienced any of the above heat related illnesses and not received medical treatment/attention for them?
- \_\_\_\_\_ \_\_\_\_\_ 14. Are you taking prescription medicine, NSAIDS, or Probiotics?  
If so, what? \_\_\_\_\_

\_\_\_\_\_ 15. Has your doctor ever told you that you have a muscle, bone, or joint problem such as arthritis that has been aggravated by exercise, or might be made worse by Exercise?

\_\_\_\_\_ 16. Do you have any reason to believe that your participation in this investigation may put your health or well being at risk? If so, please state reason.

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If you have answered “Yes,” to any of these questions you cannot be allowed to participate in this study. You may keep this form for your own records and dispose of it when and how you wish. No record of it will be kept in the Thermal Lab.

Signature of subject \_\_\_\_\_ Date \_\_\_\_\_  
\*Adapted from British Columbia Department of Health and Michigan Heart Association

# “Gastrointestinal Distress and Systemic Inflammatory Response to 5 Consecutive Days of Exercise”

## Appendix B

### Auburn University

Auburn University, Auburn, AL 36849-5323

Department of Kinesiology  
2050 Memorial Coliseum  
Thermal Lab

Telephone: (334) 844-4483  
Fax: (334) 844-1467  
Lab Phone: (334) 844-1479

#### **Informed Consent for a Research Study Entitled**

“Gastrointestinal Distress and Systemic Inflammatory Response to 5 Consecutive Days of Exercise”

**Project Overview:** You are invited to participate in a research study examining the effect of consecutive days of exercise in the heat on gastrointestinal distress and the systemic inflammatory response. Participants will perform 5 consecutive days of strenuous exercise (running) in a hot environment. Blood draws will be taken both before and after completion of exercise on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day and on control trial day.

**Purpose:** The purpose of this investigation is to examine the effect of 5 consecutive days of exercise in the heat on blood bio-markers of gastrointestinal distress and systemic inflammation.

**Participant Requirements:** To be eligible you must be:

1. A male participant of 19 to 35 years of age
2. Maximal oxygen consumption ( $VO_{2max}$ )  $\geq$  50 ml/kg/min, as determined by preliminary testing.
3. Low risk for medical complications as determined by physical activity readiness questionnaire (PAR-Q)
4. Have no history of hospitalization for heat related illness
5. Have not been exposed to ambient temperatures  $> 27^{\circ}C$  ( $80^{\circ}F$ ) on 10 days in the past month.
6. Are not currently taking any antioxidant supplementation, non-steroidal anti-inflammatory drugs (NSAIDS), antibiotics, or probiotics.
7. No known gastrointestinal medical condition.

**In order to participate in this study you must meet all the requirements listed above.**

## **“Gastrointestinal Distress and Systemic Inflammatory Response to 5 Consecutive Days of Exercise”**

### ***Testing Procedures:***

**Participation Requirement Screening:** To be included in this study you must meet all requirements listed above. To screen these requirements the PAR-Q and aMaximal Oxygen Consumption ( $VO_{2max}$ ) test and blood lactate analysis will be employed.

**Visit 1:** On the first visit to the lab, you will complete the PAR-Q and then read and sign the University-approved informed consent form if you have not answered “Yes,” to any questions on the PAR-Q. By not answering “Yes,” you have been determined at “low risk” for exercise complications. Either Matthew Barberio (Doctoral Candidate) or Dr. David D. Pascoe will be present for all informed consent briefings. Participants will be asked to fill out a recent training questionnaire.

Descriptive data will be obtained (age, height, nude body weight, and body fat composition via Dual-emission X-ray absorptiometry (DXA)).

You will then be tested for blood lactate on the laboratory treadmill.

You will be given time to familiarize yourself with laboratory treadmill and properly warm-up for exercise. After warm-up you will then complete four 6 minute trials at different treadmill velocities. At the end of each trial a small finger prick will be administered to collect blood for blood lactate analysis. The results of this test, along with the  $VO_{2max}$ , will be used to help to determine your exercise intensity for exercise in the heat.

**Visit 2:**  $VO_{2max}$  testing will last approximately 10-15 minutes and will consist of incrementally increasing exercise intensities. To be eligible for participation you must meet cardiorespiratory fitness standards described above.

**Estimated time commitment for Visit 1 & Visit 2 to the Thermal Lab is approximately 2 hours.**

**Weigh-ins:** Between Visit 1 and the start of exercise heat exposure, participants will report to the Thermal Lab daily to record nude body weight. All nude body weights will be collected in a private bathroom by you. Once in the private bathroom you will be required to fully disrobe, measure your nude body weight on the scale, and dress before reporting your weight to the researchers.

**Exercise Heat Exposure:** At the end of Visit 1 participants will schedule their return to the thermal lab for the start of 5 consecutive days of exercise in hot environments. The first exercise heat exposure will be no sooner than 5 days after Visit 1 to the Thermal Lab. Participants will be provided with pre-prepared dinners to be consumed no later than 10 hours before the beginning of the next day’s exercise session, and a breakfast shake that is to be consumed 1 hour prior to exercise. It is recommended that participants consume water regularly throughout the duration of the study to maintain adequate hydration. To prevent outside interference during this study, regular exercise routines should be discontinued 48 prior to first exercise heat exposure and should not resume until completion of study.

## **“Gastrointestinal Distress and Systemic Inflammatory Response to 5 Consecutive Days of Exercise”**

Upon arrival to the lab participants will provide a small urine sample for the determination of urine specific gravity (USG) to determine hydration status. Nude body weight of the participant will be measured in the facilities private bathroom as described above. If you are determined to be dehydrated by USG or body weight you will be given a chance to rehydrate before the trial begins, or the trial will be rescheduled.

Participants will be provided with a heart rate monitor to strap to their chest for the monitoring of heart rate and a re-usable rectal thermometer to insert into their rectal cavity. On the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day of heat exposure participants will have a venous catheter placed into a wrist vein; this catheter will remain in place for the remainder of that day’s trial. Participants will remain seated for 10 minutes after the insertion of the catheter before a sample is drawn.

Participants will then enter the environmental chamber in the Auburn University Thermal Lab. The climate of the chamber will be set to 40°C (~104°F) with 40% relative humidity. Participants will then run on the treadmill at velocity determined by blood lactate analysis testing until their core temperature is elevated 2°C above rest. The exercise intensity will be considered moderate (~70-80% of maximal capability). You will be provided water throughout the entire time trial.

Immediately following exercise participants will again measure nude body weight. On blood sampling days participants will remain at the Thermal Lab for blood collections post exercise. During this time participants will be provided water and use of Thermal Lab and Coliseum facilities.

**Control Trial:** No earlier than 3 days after the conclusion of 5 days of exercise in the heat, participants will perform a control exercise trail in cool/dry (22°C and 40% RH) environment. This exercise trial with be identical to the exercise performed on Day 1 of exercise in the heat.

**Blood Sampling:** A venous catheter will be placed into a dorsal wrist vein prior to exercise on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> day of heat exposure and during the control trial. This catheter will remain in place throughout the day’s trial. Blood samples will be taken before exercise, immediately post exercise, 1 hour post exercise, and 3 hours post exercise. On exercise days 3 and 5, an addition blood sample will be drawn during exercise at the time of exercise termination on exercise day 1 in the heat. 8 milliliters (~1.5 teaspoons) of blood will be collected at each sampling time point with a total of 144 milliliters (~30 teaspoons) for the entire study. Blood samples will be used for analysis of gastrointestinal distress and systemic inflammation. Estimated time commitment for Exercise Heat Exposure days 2 & 4 is 1 hour. Estimated time commitment for Exercise Heat Exposure days 1, 3, 5, and Control trial is 4 hours.

**Total Estimated Time Commitment for Study: 20 hours.**

## “Gastrointestinal Distress and Systemic Inflammatory Response to 5 Consecutive Days of Exercise”

### Potential Risk:

1. While performing any exercise there is a chance of muscle strains, sprain, and even death. The American College of Sports Medicine estimates the risk of death at 0.5 per 10,000 individuals.
2. Strenuous exercise in the heat increases the risk of dehydration and heat related illness.
3. With any blood draw procedure there is a risk of infection, bruising, irritation at the injection site, fainting, and/or contact with blood borne pathogens.
4. There is a chance that you could have an allergic reaction to the ingredients in the dinner and breakfast meals. You should notify researchers of any food allergies prior to beginning of supplementation so that they can be checked against ingredients.

*NOTE: It is important for you to realize that you are responsible for any medical costs incurred in the event of an injury.*

### Precautions:

1. Although the exercise for this trial is high intensity, all individuals being recruited and allowed to participate for this study will exceed the 80<sup>th</sup> percentile for age-dependent cardiorespiratory fitness guidelines as determined by  $VO_{2max}$  testing. Risk of death and serious injury/medical issues are reduced in this population. Additionally the use of the PAR-Q will assist in eliminating participants that have potential medical or orthopedic identified risks. Exercise will be continuously monitored by Matthew Barberio, David Elmer, Khalil Lee, and Richard Laird, all of which maintain current CPR certification
2. Heart rate and core temperatures will be continuously monitored throughout all trial sessions by the above mentioned researchers. To prevent issues associated with dehydration you will be given water throughout the duration of all trials and will not be allowed to begin a trial if pre-trial testing indicates you are dehydrated. Exercise will be terminated when core temperatures rises 2°C above resting core temperature which should maintain your body temperature below 39.5°C. If a rise in core temperature of 2°C above resting will elevate core temperature above 39.5°C exercise will be stopped at 39.5°C.
3. Only sterile, aseptic techniques will be used for blood collections. CDC guidelines for blood collection and handling are followed in the Thermal Lab. All blood collections will be performed by trained phlebotomists. All researchers involved in data collection have completed Lab Safety and Blood-Borne Pathogen training.
4. CDC guidelines are followed for sterilization of all equipment utilized in the Thermal Lab. A re-usable rectal thermometer and heart rate monitor will be assigned to participants at the beginning of the study and utilized by only them. Appropriate sterilization procedures will be used after each use.
5. Upon termination of trials you will be monitored until heart rate is <100 beats per minute and core temperature falls to 1°C above resting. If you are unresponsive or need further medical care the EMS will be notified. Emergency medical numbers are listed within easy access of all locations in the Thermal Lab and there is a current emergency action plan in place.

**“Gastrointestinal Distress and Systemic Inflammatory Response to 5 Consecutive Days of Exercise”**

**Benefits:** As part of participation in this study participants will receive assessment of their body composition as measured by DXA. Participants will also receive assessment of their maximal aerobic capacity as measured in preliminary VO<sub>2max</sub> assessment. Any questions about results and their meanings will be explained to the participants

**Your participation in this study is completely voluntary.** If, at any point, you decide against participating any further you can withdraw. If you choose to withdraw your data can be withdrawn as long as it is identifiable. Your decision to withdraw from the study will not jeopardize your future relations with Auburn University, the Department of Kinesiology, or the Thermal Lab.

**Your privacy will be protected.** Any information obtained in connection with this study will remain anonymous.

If you have any questions, we invite you to ask us now or anytime they may occur. You can contact Matthew Barberio or Dr. David Pascoe by calling 334-844-1479 or email [mdb0012@tigermail.auburn.edu](mailto:mdb0012@tigermail.auburn.edu) or [pascodd@auburn.edu](mailto:pascodd@auburn.edu). You will be provided with a copy of this document to keep. For more information regarding you rights as a research participant, you may contact the Auburn University Office of Human Subjects Research of the Institutional Review Board by calling or emailing [hsubjec@auburn.edu](mailto:hsubjec@auburn.edu) or [IRBchair@auburn.edu](mailto:IRBchair@auburn.edu).

**HAVING READ THE INFORMATION PROVIDED IN THIS DOCUMENT, YOU MUS DECIDE WHETHER OR NOT YOU WISH TO PARTICIPATE IN THIS REASEARCH STUDY. YOUR SIGNATURE INDICAES YOUR WILLINGNESS TO PARTICIPATE.**

_____	_____	_____
<b>Participants Signature</b>	<b>Date</b>	<b>Print Name</b>
_____	_____	_____
<b>Investigator obtaining consent</b>	<b>Date</b>	<b>Print Name</b>
_____	_____	_____
<b>Co-Investigator</b>	<b>Date</b>	<b>Print Name</b>