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Whole Body Stress and Immune Cell Responses to Exercise, Heat and Dehydration in the 2012 Ironman World Championship and Controlled Laboratory Study

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Colleen Muñoz, M.S.

University of Connecticut, 2014

Abstract

Background: Global changes in immune cells and circulating molecules might indicate whole body stress magnitude. Heat shock protein 70kD (HSP70) and plasma HSP70 might protect through different mechanisms. HSP70 transcription factors, Heat Shock Factor 1 (HSF1) and Nuclear Factor of Activated T-cells 5 (NFAT5) respond to heat and dehydration stress, respectively, and might quantify specific stress exposures.

Purpose: Characterize circulating and cellular markers indicating cytoprotective responses following acute stress and recovery in humans.

Methods: Laboratory: 36 healthy males completed: 1) "Baseline" followed by 16h fluid deprivation, 2) "Pre" and "Post" 2h cycling (~38°C, ~40%RH), 3) partial rehydration measured "Post1h", 4) and complete recovery ("Recov") upon 24h. Peripheral Blood Mononuclear Cells (PBMCs) were measured for HSP70, HSF1, and NFAT5, and plasma HSP70. Field: 22 healthy males at the 2012 Ironman World Championship. Analyses included Complete Blood Count (CBC) with differential, plasma cytokines, and plasma HSP70 at "Base", "Post", "Post1d", and "Post2d". Athletes who lost least (n=8; Euhy) and most (n=8; Dehy) body mass during the race examined dehydration influences in all variables.

Results: Laboratory: PBMCs expressed greater HSP70 at all time points above Base, and at Recov above Pre ($p<0.05$), NFAT5 at Post than Base ($p=0.026$), and nearly at Pre and Post1h than Base ($p=0.056$ and $p=0.072$, respectively), with no changes in HSF1. Plasma HSP70 increased at Post beyond Base and Pre ($p<0.01$), and decreased at Recov beyond Post and Post1h ($p<0.01$, and $p=0.001$, respectively). Field: Leukocytes (L) increased at Post above all time points ($p<0.001$), and returned to Base at Post2d (Post1d>Post2d; $p=0.002$). More neutrophils (L; $p=0.018$; %; $p=0.030$) and nearly less lymphocytes (%; $p=0.063$) were observed in Dehy than Euhy. Greater IL-10, IL-6 and IL-8 existed at Post than all time points ($p<0.001$). More TNFα and nearly IL-12p70 resulted in Dehy than Euhy at Post1d than Post2d ($p=0.048$, $p=0.074$, respectively). Plasma HSP70 was greater at Post than all time points ($p<0.05$), with no hydration influence.

Conclusion: Whole body stress stimulated an acute inflammatory state in PBMCs and circulating markers. Cellular versus plasma HSP70 exhibited distinct responses, and NFAT5 might indicate whole body dehydration. Understanding cellular mediators might permit greater stress expsure resistance.

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APPROVAL PAGE

Doctor of Philosophy

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INTRODUCTION

Physiological stress ensues from numerous environmental and behavioral insults. Remarkably, investigators have documented homeostatic responses and adaptations that counterbalance physiological perturbations at the cellular and genetic level. General fascination aside, the study of these stress-induced responses applies to all human beings, and might permit discovery of manipulable outcomes and therapeutic targets for healthy and diseased populations.

Common whole body stressors include exercise, heat exposure, and dehydration, which result both voluntarily and involuntarily. These stressors often occur in combination, which compound homeostatic requirements. Measurement of molecules and cells circulating in the blood provide valuable information for understanding the extent of whole body stress exposure, while gene-level tissue/cellular analysis likely provides more mechanistic information about stress responses and adaptations.

Circulating Indicators of Whole Body Stress

Quantification of circulating blood constituents serves as one tool for measuring physiological stress responses. Considering the blood acts as a transporter, creating means for nutrient delivery, waste removal, communication, etc., this compartment and the cells circulating throughout it receive a plethora of chemical information. Therefore, stress introduction frequently elicits counteractions among circulating cells, permitting examination of these cellular populations and their functionality to describe the extent of stress exposure.

Cellular Populations

Classically, acute exercise induces biphasic thrombocytosis (Lippi and Maffulli, 2009) and leukocytosis from elevations in lymphocytes, neutrophils, and monocytes (Smith, 1997, Okutsu et al., 2008). Observed leukocytosis likely result from shear stress from enhanced cardiac output, and /or cortisol and catecholamine influence (McCarthy and Dale, 1988, Okutsu et al., 2008, Wang, 2006). Lymphocytosis occurs during and immediately after exercise, but this population returns to pre-exercise quantities soon after (Walsh et al., 2011). The extent of lymphocyte dynamics during and immediately post exercise appears primarily relative to exercise intensity but also duration, while increased presence upon recovery is consistent with elapsed time from start of exercise (McCarthy and Dale, 1988). However, several investigations reported nearly opposite patterns of circulating lymphocytes, which appear attributable to exercise intensity and chronic exercise exposure (Ferry et al., 1990, Robson et al., 1999). Consistent reports of exercise-induced neutrophilia exist, illustrating extreme initial elevations in neutrophil presence with an additional smaller increase a few hours later, purportedly due to cortisol release (McCarthy and Dale, 1988). Not surprisingly, the magnitude of neutrophilia corresponds with intensity and duration of acute exercise, as tissue damage induces neutrophil phagocytic activity. Because circulating cellular populations drastically change during exercise-associated stress, quantification of their presence proves informative.

Inflammatory Cytokines

As cellularly derived signaling molecules that and act together with soluble cytokine receptors and inhibiting molecules, inflammatory cytokines connect and regulate humoral and cellular immune responses. Circulating cytokines can indicate an inflammatory response, as well as immune cell recruitment, activation, and proliferation. Inflammatory cytokines play unique roles to either promote or suppress the inflammatory response (pro-inflammatory and anti-inflammatory cytokines, respectively), while most anti-inflammatory cytokines possess pro-inflammatory capabilities to some degree (Opal and DePalo, 2000).

Common pro-inflammatory cytokines include Interleukin (IL) 1, IL-6, IL-8, IL-12 and Tumor Necrosis Factor (TNF) α. The expression of these molecules can indicate cellular stress exposure from, for example, heat shock or osmotic stress, as these stressors and classic inflammatory stimuli act through mitogen-activated protein kinases (MAPK; Dinarello, 2000). Exercise particularly of long duration, consistently results in dramatic increases in circulating IL-6, while research now shows IL-6 release during both eccentric and concentric muscle actions implying poor indication of muscle damage (Pedersen, 2000). Investigations of IL-8 describe pro-inflammatory actions through stimulating neutrophil degranulation and tissue damage (Dinarello, 2000). Further, IL-12 produces pro-inflammatory actions through stimulating interferon gamma production (Chang and Radbruch, 2007), while IL-1 and TNFα respond to infection, trauma and ischemia (Dinarello, 2000). IL-10 serves as a classic anti-inflammatory cytokine; secretion of this cytokine from monocytes and lymphocytes promotes negative feedback in order to mitigate over-production of pro-inflammatory cytokines (Opal and DePalo, 2000). Recent evidence

suggests IL-12p70 plays a meaningful role in IL-10 production upon secondary immune responses *in vitro*, suggestive of an atypical anti-inflammatory role of IL-12p70 (Chang and Radbruch, 2007). In essence, the meticulous balance of pro- and anti-inflammatory cytokines permits an efficacious immune response when faced with physiological insults.

Plasma Heat Shock Protein 70kd (HSP70)

Circulating concentrations of stress-inducible, 70 kD (kiloDalton) heat shock protein (HSP70) appears to signify a protective response. The chaperone protein HSP70 is critical to protein homeostasis and participates in: 1) proper folding of newly synthesized proteins, 2) prevention and/or treatment of pre-existing protein misfolding and aggregation upon stress exposure, 3) folding maintenance as proteins transport out of the cell, 4) degradation promotion of un-renaturable proteins, and 5) apoptosis inhibition (Moseley, 1997, Lanneau et al., 2010). At the turn of the millennium, investigators observed chronically elevated concentrations of plasma HSP70 in diseased populations (such as those with renal disease and hypertension; Wright et al., 2000, Pockley et al., 2002). This finding lead to subsequent investigations demonstrating that stress-induced plasma HSP70 likely belonged in the normal stress response paradigm (Fleshner et al., 2003, Campisi et al., 2003). Additionally, release of plasma HSP70 occurred in the presence and absence of necrotic cell death, and during stressors such as exercise (reviewed in Johnson and Fleshner, 2006, Magalhaes Fde et al., 2010, Suzuki et al., 2006).

Plasma HSP70 also appears to have an immunological role through: 1) suspected release into the circulation from exosomes which also contain antigen presenting and adhesion molecules, 2) profound stimulation of nitric oxide and cytokine release (including IL-6, IL-1β, and TNFα)

from macrophages and neutrophils, and 3) TLR2 and TLR4 surface receptors which facilitate an innate immune system inflammatory signal, although this responses might only exist in CD14+ cells (reviewed in Johnson and Fleshner, 2006). Consistent inclusion of plasma HSP70 measurement in whole body stress research will likely elucidate its precise protective mechanisms.

Cellular Protein and Transcriptional Indicators of Whole Body Stress

Examining intracellular leukocyte responses from whole body stress exposure is useful for a number of reasons: 1) leukocytes systemically circulate and infiltrate tissues, 2) leukocytes are the first responders to stress-induced damage, and 3) leukocytes are easily accessible via venipuncture and basic benchtop techniques.

Cellular HSP70, NFAT5, and HSF1

Numerous proteins exist to counter physiological perturbations, but cellular HSP70 arguably represents the most widely studied cellular protein in stress research. Originally discovered through heat exposure, evidence now describes ubiquitous stress stimulation of HSP70. While cellular HSP70 reduces protein aggregation, protein refolding, and apoptosis, HSP70 also impacts immune function through chaperoning MHC I peptides (Srivastava, 2002), and might also negatively regulate pro-inflammatory cytokines to avoid damage accrued during inflammation (Ianaro et al., 2001). Many investigations describe exercise-induced increases in cellular HSP70 not only immediately post exercise, but also up to two days later (Fehrenbach et al., 2000b, Fehrenbach et al., 2000a).

Traditional transcription factors of HSP70 include members of the Heat Shock Factor (HSF) family. HSF1 acts as the most notable HSF stimulator of HSP70, both constitutively and in a more pronounced manner during environmental stress exposure (i.e., heat and oxidative stress; Cotto et al., 1996). Relatively recent evidence describes HSP70 transcriptional regulation through the osmotic-specific nuclear factor of activated T-cells 5 (NFAT5; also known as tonicity-responsive enhancer binding protein (TonEBP)). Cellular osmolality increases induce a collection of homeostatic perturbations, including: 1) DNA damage and associated cell cycle arrest, 2) inflammatory cytokine production, 3) greater reactive oxygen species (ROS) that can alter DNA bases and carbonylate proteins which then inhibit enzyme activity, 4) protein synthesis inhibition, and 5) mitochondrial perturbation, among others (reviewd in Burg et al., 2007, Neuhofer, 2010). To counteract detrimental consequences of these osmolality-induced cellular changes, NFAT5 transcriptionally promotes HSP70 along with a series of transport proteins and organic osmolytes to reinstate optimal cell volume, limit destruction associated with inorganic osmolytes, and prevent cell death (Burg et al., 2007). The contributions of HSF1 and NFAT5 in HSP70 regulation further support a ubiquitous stress stimulation of the HSP70 protective response.

Peripheral Blood Mononuclear Cell (PBMC) Transcriptomics

Transcription is the first step in stress-specific protein synthesis. Innovatively using technologies that allow transcriptomic study of stress responses and adaptation permit deep exploration of cellular stress survival mechanisms. Many investigations have examined the influence of whole-body stress on circulating molecules, proteins, and cells, while very few have measured transcriptomics. Neutrophil transcriptomics from acute exercise resulted in greater expression of

upregulated than downregulated genes in categories such as immune and defense processes, signal transduction (Radom-Aizik et al., 2008), molecules recognizing bacterial endotoxin release, and negative regulators of pro-inflammatory responses (Neubauer et al., 2013). Examining whole body stress responses at the transcriptional level will permit exploratory analysis that might elucidate poorly understood protective mechanisms and lead to future research directions.

Purpose and Rationale

Recent research from our laboratory found that physiological adaptation (heat acclimation) developed without a detectable cellular stress response (i.e., HSP70; Hom et al., 2012). This suggested chronic cellular stress responses require more pronounced whole body insults to overwhelm homeostatic mechanisms. Our laboratory subsequently investigated the influence of a more severe and compound stress scenario, which included exercise, heat, and dehydration. This study served as a first time human examination of the novel, osmotic-specific HSP70 transcription factor, NFAT5. Trends suggested that NFAT5 could indicate whole body dehydration, but limitations caused requirement for further and broader study (field vs. laboratory setting, wider population variety, and more stress validation).

Currently, no studies examine global changes in cellular responses to concurrent whole body exercise, heat, and dehydration stress. As circulating immune cells represent a cell subset capable of expressing a stress response beyond the body's ability to maintain homeostasis, immune cell responses during stress might elucidate questions regarding immune function during stress that remain unanswered. Therefore, the aim of this dissertation was to quantify and qualitatively

characterize circulating and cellular protein expression changes at the protein and mRNA levels to evaluate cellular responses following acute stress and recovery in humans.

LABORATORY INVESTIGATION

Materials & Methods

Participants

Thirty-six, recreationally active males (age = 23 ± 3, mass = 76.6 ± 13.3 kg, height = 175.5 ± 6.4 cm, body fat = 15.3 ± 5.5) participated in this investigation. Exclusionary criteria included history of heat intolerance, tobacco use, recent illness, musculoskeletal injuries, or disease or use of any medication that might influence exercise in the heat, metabolic, immune, cardiovascular, and fluid homeostasis. All participants read and signed informed consent documents approved by the University's Institutional Review Board.

Experimental Design

This investigation utilized a single group, repeated measures design, requiring three consecutive visits to the laboratory: Visit 1) Baseline (Base), 2) Experimental, and 3) Recovery (Recov).

Baseline Visit

The day before Base, subjects were instructed to 1) consumed their typical diet and to complete an 8h, overnight fast, and 2) practice their typical fluid consumption habits, and additionally consume 500 mL of water within two hours prior to going to bed, and 500 mL upon waking the morning of Base. Testing began between 7:00 and 9:00 am to provide measurements of body mass, height, body fat (via three site skin-fold technique; Lange Skinfold Calliper; Beta

Technology Inc, Houston, TX), urine (to evaluate hydration state (methods described below)), thirst perception (REF), and blood variables (described below). Upon departing the lab, participants were instructed to 1) refrain from consuming all fluids and to avoid consuming foods with high fluid content (examples of these foods were provided) beginning 16 hours (h) prior to the experimental visit, and 2) complete an 8h, overnight fast.

Experimental Visit

Participants arrived at the lab for the experimental visit ± 1 h of their arrival time for Base to limit a potential influence of stress-related hormone diurnal variation. Upon arrival to the lab, participants provided a thirst perception measurement, urine and blood sample (time point "Pre"), and subsequently consumed a standardized breakfast of a Clif Bar and banana (fluid deprivation persisted). Participants inserted a rectal thermistor 10-15 cm beyond the anal sphincter to monitor internal temperature (YSI 401 rectal probe, Yellow Spring, OH), and donned a heart rate monitor (Timex Group USA, Middlebury, CT). Upon obtaining a valid rectal temperature (T_{rec}) and heart rate (HR) measurement, participants entered the heat chamber (Model 2000, Minus Eleven, Inc., Malden, MA) set at 38° C and ~30% relative humidity, and cycled at a moderate intensity (between 60-70% heart rate reserve) for two hours; T_{rec} and HR were collected every 15min.

Following the two hour active fluid loss in the heat, participants provided a thirst perception measurement, and urine and blood samples (time point "Post"). Fluid loss was derived from body mass difference before and after the two hour exercise bout (Pre and Post difference). Investigators then provided participants with a volume of water equivalent to active dehydration fluid loss, which was consumed within one hour. At this time, a final thirst perception

measurement, and urine and blood samples were collected (time point "Post1h"). Prior to departing the lab for the day, participants were instructed to 1) consume ample fluid, 2) monitor their urinary excretions to achieve light yellow colored urine, 3) refrain from dehydrating activities (i.e. exercise and alcohol consumption), 4) maintain their typical eating habits, and 5) complete an 8h, overnight fast.

Recovery Visit

Participants returned to the lab ± 1 h of their arrival time for Base. Investigators collected a final body mass, thirst perception measurement, and urine and blood samples (time point "Recov").

Sample Collection and Measurement

Urine

Urine was collected as mid-stream, non-first morning samples, measured for urine specific gravity (U_{sg}) via light refractometry (Atago Inc., model A300CL, Spartan, Tokyo, Japan), urine osmolality (U_{osm}) via freezing point depression osmometry (Advanced Instruments, Model 3320, Norwood, MA), and urine color (U_{col}) via a validated color chart (i.e., HSP70; Hom et al., 2012).

Blood

Blood was collected from the anticubital fossa in a Mononuclear Cell Preparation tube (BD Vacutainer® CPT™ Tube) with sodium citrate additive, and a gel tube with lithium heparin additive (Greiner Bio-One Vacuette™). Blood processing and analyses from CPT collected blood included Peripheral Blood Mononuclear Cell (PBMC) isolation and antibody staining, and plasma HSP70 via High Sensitivity EIA kits (Enzo Life Sciences, Inc.). Analysis from lithium heparin tubes included plasma osmolality (P_{osm}) via freezing point depression osmometry (Advanced Instruments, Model 3320, Norwood, MA).

PBMC isolation permitted evaluation of a systemic, cellular response. Investigators handled CPT tubes according to manufacturer's suggestions, followed by two washes in 1X PBS with 1% BSA. Cell counting was conducted with trypan blue (1:1) in 15 μL of cell suspension, with a Hy-Lite hemocytometer, and proceeded with a sample volume equivalent to one million cells. Cells were subjected to fixation with paraformaldehyde, washed once, and permeabilized with ice cold 100% methanol. Following two washes, cells were stained with HSP70 (n = 32), NFAT5 (n = 32), and HSF1 (n = 25) primary antibody (ab5439, ab3446, ab61382, respectively), underwent two washes, and were then stained with respective secondary antibodies (ab150119, ab72465, ab150157). Following one final wash, cells were resuspended and read with the BD AccuriC6 Flow Cytometer (BD Biosciences, San Jose, CA). Investigators collected 20,000 events in the pre-determined lymphocyte gate, and analyses were completed with BD Accuri C6 software.

Statistical Analyses

Data analyses were performed with SPSS version 20 (IBM Corporation, Champaign, IL) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Descriptive data (means, standard deviations (SD) and standard error of the mean (SE)) were calculated for all variables. Oneway repeated-measures analysis of variance was administered to quantify differences across time. Where appropriate, Fisher's LSD *Post Hoc* tests examined pairwise differences. Linear regression was used to examine relationships of a continuous nature. An alpha level of $p < 0.05$ defined significance. Unless otherwise stated, data are reported as means ± SD. Figures were generated with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Hydration, Exercise & Heat Stress

All hydration variables (Mass, P_{osm}, U_{osm}, U_{sg}, and U_{col}) changed across time as expected, in response to fluid balance manipulation (Figure 1). Body mass changes equated to -2.0 ± 0.9 % from Base to Pre (16h fluid deprivation) and an additional -2.1 ± 0.5 % from Pre to Post (two hours of heat and exercise-induced fluid loss), resulting in a total of -4.2 ± 0.9 % upon Post. Heart rate, representative of exercise intensity, rose from 0 to 15min, upon which it plateaued ($p < 0.05$). Rectal temperature continued to rise until ~60min of exercise and heat exposure ($p < 0.05$; Figure 2).

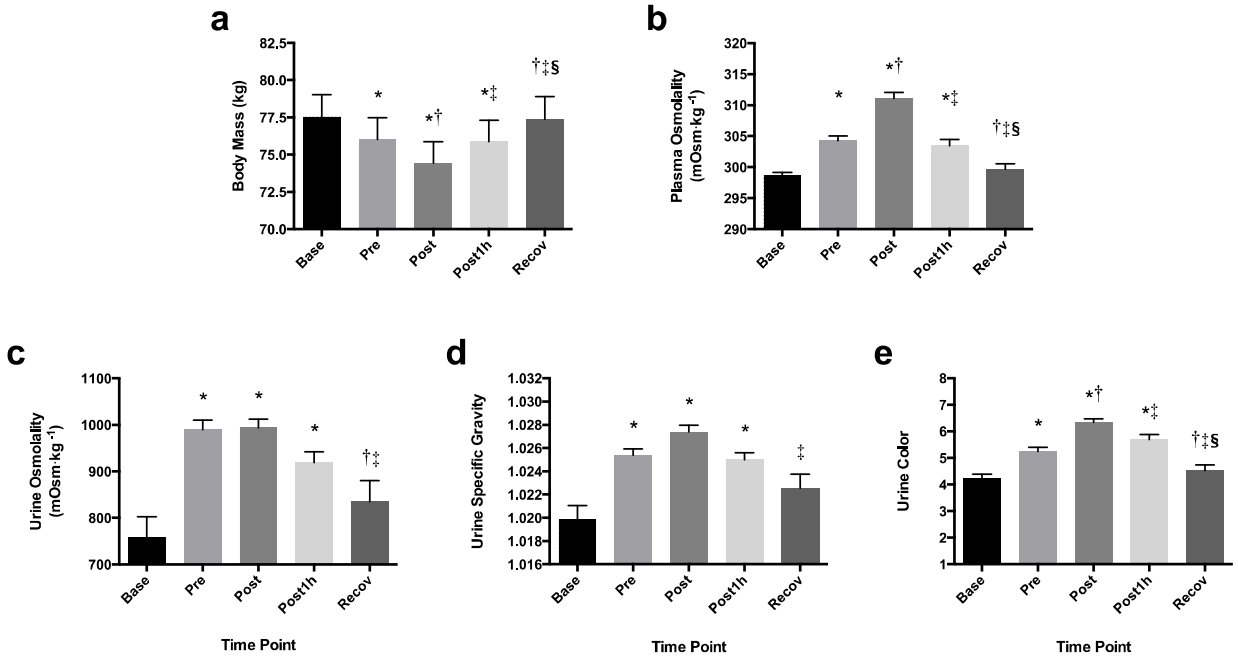

Figure 1. Hydration biomarkers in response to 16h fluid deprivation (Pre), exercise and heat-induced fluid loss (Post), partial rehydration (Post1h), and further rehydration the following day (Recov; means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; † from Pre; ‡ from Post; § from Post1h.

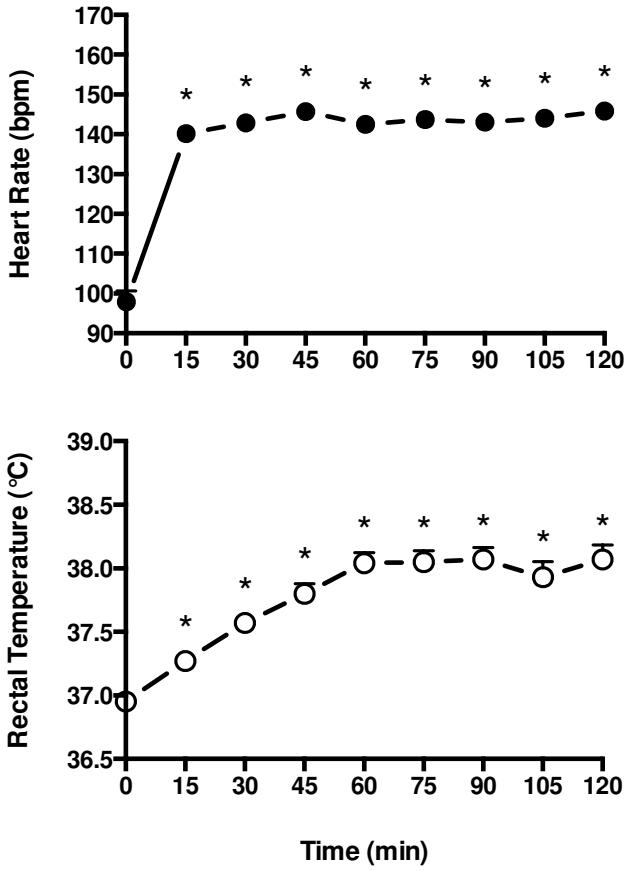

Figure 2. Heart rate and rectal temperature as indicators of exercise and heat stress during two hours of moderate intensity cycling in ~38° C and 40% RH (means ± SE).

HSP70, NFAT5, and HSF1 Expression in PBMCs

Cellular HSP70 expression increased above Base at Post, Post1h, and Recov, and above Pre at Recov ($p < 0.05$; Figure 3a). PBMCs expressed greater NFAT5 at Post compared to Base ($p = 0.026$), and nearly at Pre and Post1h compared to Base ($p = 0.056$ and $p = 0.072$, respectively; Figure 3b). Correlations revealed that only U_{sg} predicted NFAT5 expression ($p = 0.025$; $r^2 = 0.03$). Intriguingly, PBMC NFAT5 expression indicated two diverse response patterns to 16h fluid deprivation, and subsequent stressors (Figure 4), such that one group increased ("positive

responders"; n = 21) and one decreased ("negative responders"; n = 11) from Base to Pre, and maintained opposing patterns for subsequent time points. Once examined individually, P_{osm} ($p = 0.009$; $r^2 = 0.07$), U_{osm} ($p = 0.027$; $r^2 = 0.05$), and U_{sg} ($p = 0.006$; $r^2 = 0.07$) predicted NFAT5 expression in the positive, but not negative responders. HSF1 expression did not change from Base during or following stress exposure (Figure 3c).

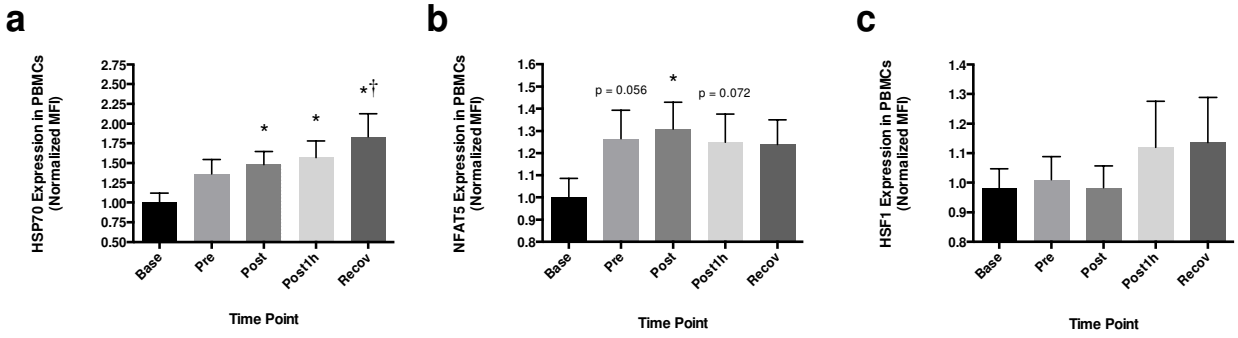

Figure 3. PBMC expression of HSP70 (a), NFAT5 (b), and HSF1 (c) in response to 16h fluid deprivation (Pre), exercise and heat-induced fluid loss (Post), partial rehydration (Post1h), and further rehydration the following day (Recov; means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; and † from Pre.

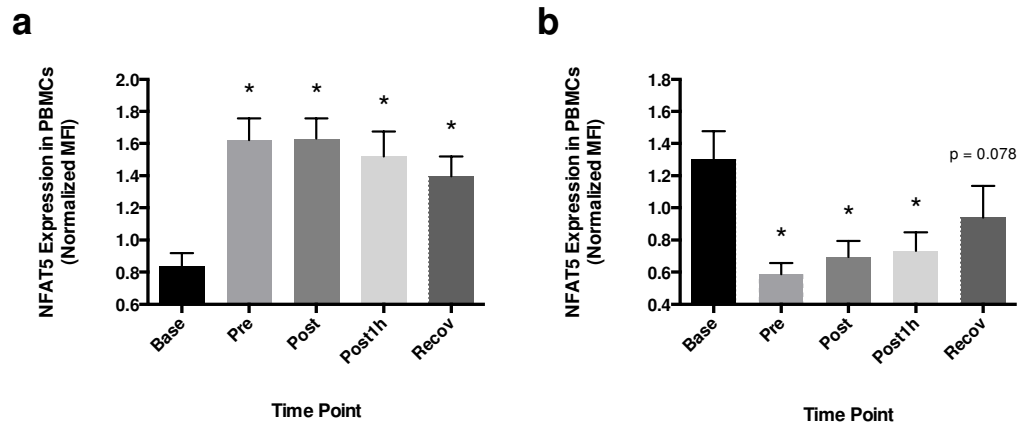

Figure 4. PBMC expression of NFAT5 in those who positively (a; n = 11), and negatively (b; n = 21) responded to 16h fluid deprivation (Pre), and oppositely responded to exercise and heat-induced fluid loss (Post), partial rehydration (Post1h), and further rehydration the following day (Recov; means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; illustrated p value compared to Pre.

HSP70 in Circulating Plasma

Freely circulating, plasma HSP70 increased in concentration at Post beyond Base and Pre (both $p < 0.01$), and reduced in concentration at Recov beyond Post and Post1h ($p < 0.01$, and $p = 0.001$, respectively; Figure 5).

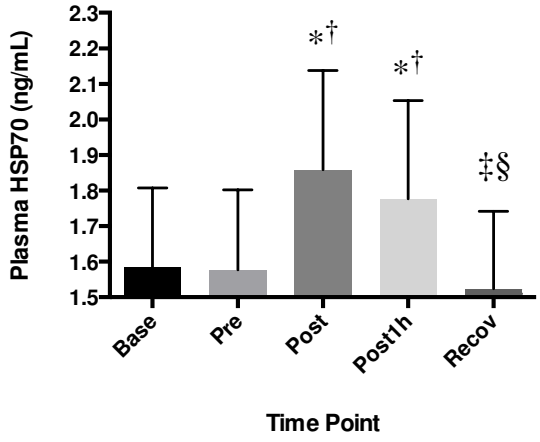

Figure 5. Plasma HSP70 in response to 16h fluid deprivation (Pre), exercise and heat-induced fluid loss (Post), partial rehydration (Post1h), and further rehydration the following day (Recov; means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; † from Pre; ‡ from Post; § from Post1h.

Discussion

This investigation builds upon our laboratory's recent work, showing acute cytoprotective responses to alternating whole body stressors (exercise, heat and dehydration) and recovery periods. Our protocol successfully introduced exercise, heat and dehydration stress in a controlled manner. Similar to our previous work, the current data supports that cellular (PBMC) HSP70 responds acutely to compound stress, as a general cytoprotective mechanism against exercise, heat, and dehydration associated damage. While classically this chaperone protein responds to heat stress, this research supports a universal stress response, as cellular HSP70 expression began to increase following 16h fluid deprivation. Further, cellular HSP70 expression continued to elevate even upon removal of stress stimuli, and did not recover subsequent to stress removal. This continuous rise in cellular HSP70 likely represents preliminary processes for adaptation, as seen in greater basal cellular HSP70 expression in heat acclimated individuals (McClung et al., 2008, Maloyan et al., 1999).

Plasma HSP70 appeared in response to exercise and heat stress, and returned to Base upon 24h recovery as observed in previous literature (Suzuki et al., 2006, Magalhaes Fde et al., 2010). However, unlike cellular HSP70, 16h fluid deprivation did not elicit elevated plasma HSP70. Hydration biomarker research, conducted over the course of several hours to multiple days, illustrates careful regulation of blood composition, as only narrow fluctuations in plasma concentration occur (Munoz et al., 2013, Perrier et al., 2013b, Perrier et al., 2013a). Because fluid deprivation in this study lasted 16h, ample time for plasma osmolality regulation likely reduced the osmotic stress stimulus, and perhaps suggests an osmolality threshold for plasma HSP70 release. Further, circulating cells might contribute water to regulate plasma

hyperosmolality (Lang et al., 1998, McManus et al., 1995), in turn inducing cellular hyperosmolality. This phenomenon would explain greater cellular HSP70 expression (Burg et al., 2007), without a concomitant plasma HSP70 increase. Alternatively, and perhaps more likely, exercise and heat induced sympathetic output might explain increased Post plasma HSP70, as previous research describes norepinephrine mediated exocytotic HSP70 release (Fleshner and Johnson, 2005). Interestingly, plasma norepinephrine increases upon rehydration following 24h fluid deprivation (Geelen et al., 1996), strongly suggesting plasma HSP70 responds to exercise and heat stress more than dehydration.

Our data describe NFAT5 as a potential indicator of whole body dehydration, as NFAT5 expression trended towards identifying 16h fluid deprivation, clearly responded to additional fluid loss at Post, and trended towards identifying partial rehydration. However, only U_{sg} predicted NFAT5, despite direct PBMC contact with the osmotically changing plasma. Literature now describes urinary hydration indices as the best indicators of hydration status across days (Perrier et al., 2013a, Perrier et al., 2013b), and specifically U_{sg} during non-exercise induced passive dehydration (Munoz et al., 2013), implying U_{sg} best represents hydration changes in this examination. Our findings also allude to NFAT5 best representing fluid deprivation than exercise and heat-induced fluid losses noted through magnitude of change, further supporting the ability of U_{sg} to predict NFAT5.

Two distinct responder patterns of NFAT5 emerged, with one third of the participants "negatively" responding, and two thirds "positively" responding to fluid losses. Once examined individually, all hydration indices predicted NFAT5 expression in positive and not negative

responders. No clear rationale for this phenomenon appears among the data, as groups exhibited nearly identical baseline hydration status according to all collected hydration indices, and these participants did not complete testing on the same day which would propose analytical error. Future investigations are required to elucidate a potential physiological explanation.

The classically recognized transcription factor for HSP70, HSF1, did not indicate clear responses to exercise, heat or dehydration stress. Nonetheless, HSP70 did overexpress in response to whole body stress. One potential explanation points to the initial dehydration stress and HSP70 expression transcribed via NFAT5, with subsequently suppressed HSF1 function through the documented negative feedback loop initiated by HSP70 upon its overexpression (Ding et al., 1998). Overall, the data presented here indicate a highly complex cytoprotective system involving a wide range of stress stimuli and response networks.

FIELD INVESTIGATION

Materials & Methods

Participants

Twenty-two, healthy male triathletes (age = 45 ± 10, mass = 73.0 ± 6.7 kg, height = 177 ± 6 cm, body fat = 9.4 ± 3.7) and participated in this investigation in October, at the 2012 Ironman World Championship, Kona, HI. Exclusionary criteria included 1) known chronic health problems, 2) exertional heat stroke in the past three years, and 3) current musculoskeletal injuries. All participants read and signed informed consent documents approved by the University's Institutional Review Board.

Experimental Design

This cohort design investigation observed the physiological impact of the triathlon on the group as a whole (22 male triathletes) and by hydration status. Hydration groups were determined following all data collection, by selecting those eight who lost the least and the most body mass (termed "Euhy" and "Dehy", respectively) during the race (see Table 1). All participants completed five visits to the field laboratory: 1) baseline (BASE), 2) pre race (PRE), 3) post race (POST), 4) one day post race (POST+1d), and 5) two days post race (POST+2d).

Baseline Visit

Participants arrived for BASE between two and four days prior to race day. Investigators collected urine samples (analytical methods described below), followed by measurements of height, body mass and body composition (InBody720 Body Composition Analyzer, Seoul, Korea), and blood samples (methods described below).

Pre Race Visit

Participants visited the field laboratory for PRE in the early morning hours prior to race commencement. Investigators obtained a urine sample and body mass measurement. Blood samples were not collected to avoid interfering with race performance. Participants were asked to pay close attention to all food and fluid consumption for POST dietary recall.

Post Race Visit

Participants arrived to the field laboratory for POST approximately 30min following race completion. Investigators collected a urine sample, followed by body mass, and blood sampling. Participants were interviewed individually by a Registered Dietitian to recall all food and fluid

consumption during the race, which was later quantified via commercial software (Nutritionist Pro™, Axxya Systems, Stafford TX).

1 & 2 day Post Race Visits

Participants arrived to the field laboratory in the late morning and early afternoon hours for POST+1d and POST+2d. Investigators collected a urine sample, followed by body mass, and blood sampling.

Sample Collection and Measurement

Urine

Urine samples were measured for urine specific gravity (U_{sg}) via light refractometry (Atago Inc., model A300CL, Spartan, Tokyo, Japan), and urine color (U_{col}) via a validated color chart (Armstrong et al., 1994).

Blood

A trained phlebotomist collected blood samples from the anticubital fossa while participants were in the supine position with the chest slightly elevated. For every time point, the phlebotomist collected blood directly into a EDTA coated Plasma Preparation tube (BD Vacutainer® PPT™ Tube), which was inverted eight to ten times and centrifuged according to manufacturer's suggestions.

Plasma from EDTA treated tubes was analyzed for: 1) Complete Blood Count (CBC) with differential, 2) inflammatory cytokines (IL-12p70, TNFα, IL-10, IL-6, IL-1β, and IL-8), and 3) circulating HSP70. CBC with differential was completed by Clinical Laboratories of Hawaii, LLP, Kona, HI. Inflammatory cytokines were analyzed via BD Cytometric Bead Array, Human

Inflammatory Cytokine Kits (BD Biosciences, San Jose, CA) and read on the BD FACSCaliber™ flow cytometer (BD Biosciences, San Jose, CA). Plasma HSP70 was analyzed through High Sensitivity EIA kits (Enzo Life Sciences, Inc.).

Statistical Analyses

Data analyses were performed with SPSS version 20 (IBM Corporation, Champaign, IL) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Descriptive data (means, standard deviations (SD) and standard error of the mean (SE)) were calculated for all variables. Oneway repeated-measures analysis of variance was administered to quantify differences across time. Where appropriate, Fisher's LSD *Post Hoc* tests examined pairwise differences. Comparisons were made between groups via oneway analysis of variance (ANOVA) for all dependent and independent variables. An alpha level of $p < 0.05$ defined significance. Unless otherwise stated, data are reported as means ± SD. Figures were generated with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Environmental conditions on race day ranged 24 to 30° C and 53 to 66% relative humidity (RH; data from Kona International Airport weather station). The 22 male triathletes completed the race in 682 ± 98 min (11:22 ± 1:38 h:min). Table 1 displays male triathletes selected for Euhy and Dehy groups.

Table 1. Euhydrated (Euhy; n = 8) and dehydrated (Dehy; n = 8) group demographics (*Baseline*), and race descriptors (*Race*) including hydration biomarkers and dietary intake.

Time Point	Variable	Euhy	Dehy	p
Baseline	Age (y)	44 ± 11	41 ± 4	0.43
	Baseline Mass (kg)	69.2 ± 6.6	77.1 ± 5.4	0.02*
	Height (cm)	175 ± 7	177 ± 6	0.43
	Body Fat (%)	9 ± 4	12 ± 4	0.14
Race	Finish Time (min)	647 ± 62	699 ± 126	0.32
	Mass Loss (%)	-2.0 ± 1.2	-5.9 ± 1.1	<0.01*
	Post Urine Specific Gravity	1.021 ± 0.008	1.027 ± 0.004	0.10
	Urine Specific Gravity Δ	0.012 ± 0.008	0.008 ± 0.006	0.36
	Kilocalories	330 ± 75	720 ± 621	0.10
	Carbohydrate (g)	72 ± 19	170 ± 149	0.09
	Fat (g)	5 ± 4	10 ± 11	0.34
	Protein (g)	7 ± 5	9 ± 20	0.72
	Sugar (g)	33 ± 17	79 ± 82	0.14
	Caffeine (mg)	13 ± 20	34 ± 61	0.38
	Sodium (mg)	8229 ± 21648	27919 ± 75688	0.49

* Indicates group differences ($p < 0.05$)

Inflammatory Markers

Among all male triathletes, CBC with differential resulted in marked changes across time. Within red cell indices, Red Blood Cells (RBCs; L), hemoglobin (Hb; g/dL), and hematocrit (Hct; %) presence in circulation declined on Post1d compared to Base and Post (all $p < 0.01$) and Post2d compared to Base and Post (all $p < 0.01$), while mean corpuscular hemoglobin concentration (MCHC; g/dL) rose above Base on Post1d and Post2d ($p < 0.01$, and $p = 0.013$, respetively) and above Post on Post1d ($p < 0.01$; Figure 6).

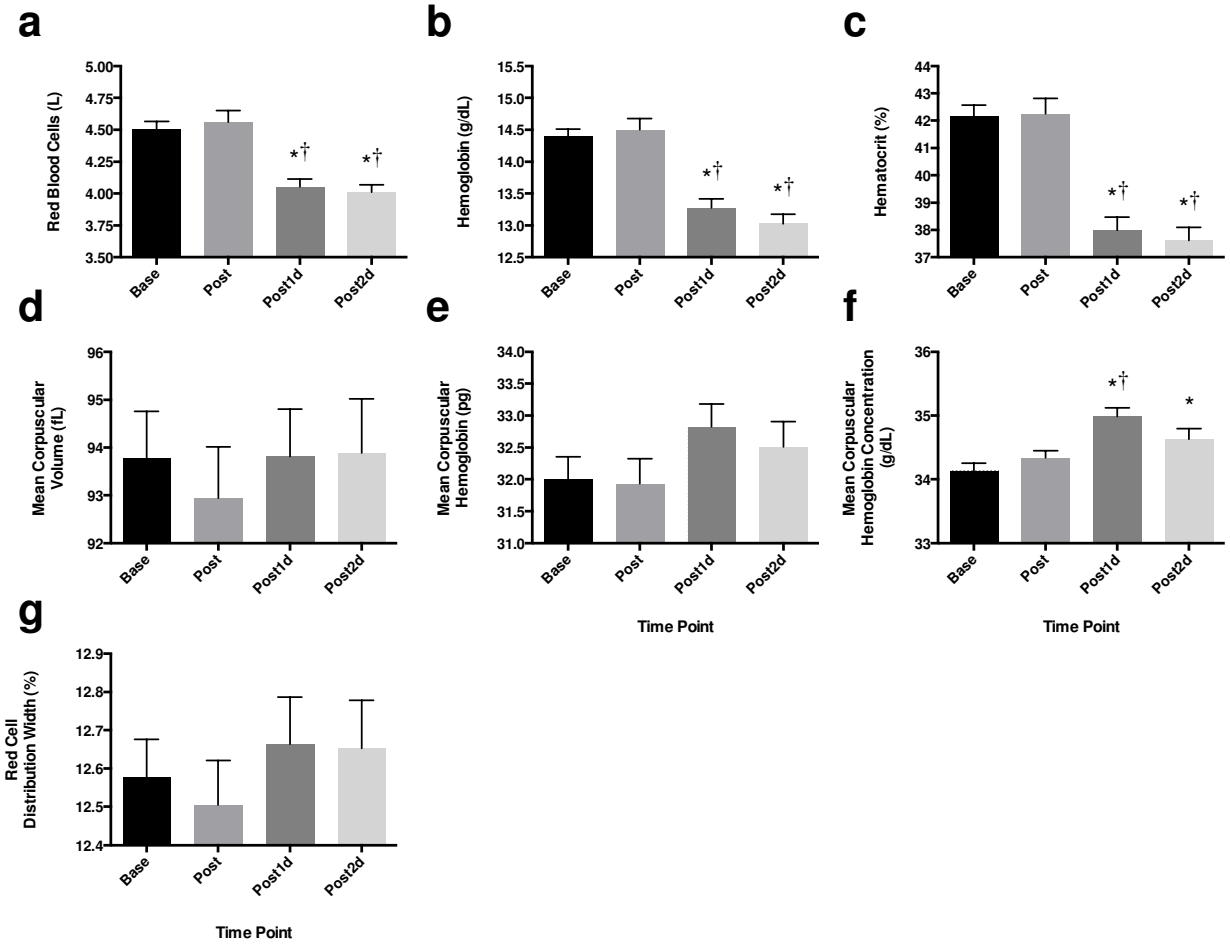

Figure 6. Red cell indices in male triathletes from days prior (Base), ~30min post (Post), one day post (Post1d), and two days post (Post2d) World Ironman Championship triathlon (means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; and † from Post.

As suspected, circulating white cell indices too reponded to the triathlon associated stress (Figure 7). White Blood Cells (WBCs; L) increased at Post above all other time points (all $p < 0.001$), and returned to Base concentrations at Post2d (as Post1d was greater than Post2d; $p = 0.002$). Race stimuli reduced circulating lymphocytes (expressed as L and %) and monocytes (%) compared to Base ($p = 0.003$, $p < 0.001$, and $p = 0.042$, respectively); subsequently, lyphocytes

(L and %) and monocytes (%) rose above Post on Post1d (all $p < 0.001$), while lymphocytes (L) and monocytes (%) more pronounced elevation was also greater than Base on Post1d ($p = 0.005$ and $p = 0.012$, respectively). At Post2d, lymphocytes (L and %) and monocytes (%) remained greater than Post ($p = 0.024$, $p < 0.001$, and $p = 0.001$, respectively), while lymphocytes (L) fell below Post1d concentrations ($p = 0.002$). Monocytes (L) presented a rather different pattern, with greater presence in the circulation at Post and Post1d than Base (both $p < 0.001$), which returned to similar Base concentrations at Post2d (Post2d less than Post and Post1d; $p < 0.001$ and $p = 0.014$, respectively). Lastly, neutrophil (L and %) presence in the cirulation increased beyond Base at Post (both $p < 0.001$), while neutrophils (L) remained above Base at Post1d ($p = 0.029$) and (L and %) diminished below Post at Post1d (both $p < 0.001$). Upon Post2d, neutrophils (L and %) maintained lower circulating presence than Post (both $p < 0.001$) and neutrophils (L) diminished below Post1d ($p = 0.042$).

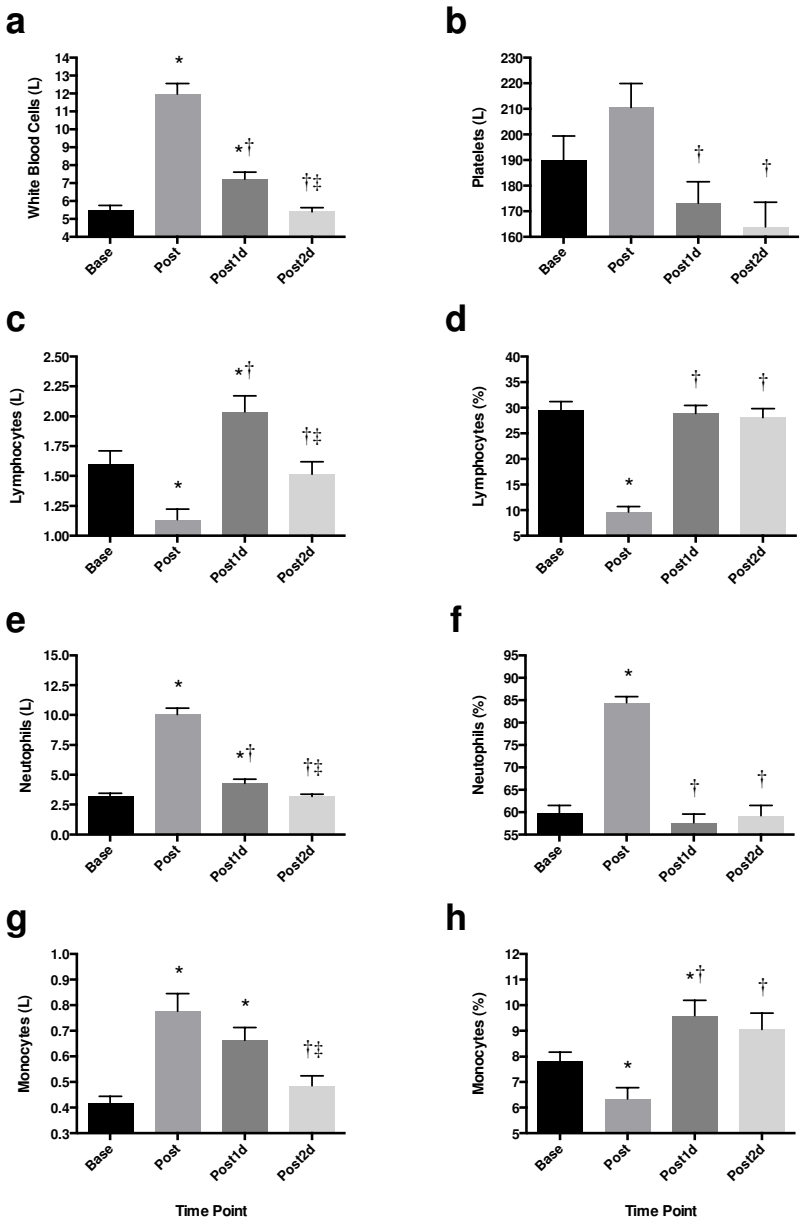

Figure 7. White cell indices in male triathletes from days prior (Base), ~30min post (Post), one day post (Post1d), and two days post (Post2d) World Ironman Championship triathlon (means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; † from Post; and ‡ from Post1d.

Interestingly, hydration status measured via U_{sg} predicted circulating presence of WBCs ($p = 0.020$; $r^2 = 0.07$), neutrophils (L; $p = 0.028$; $r^2 = 0.06$), monocytes (L; $p = 0.033$; $r^2 = 0.06$), and nearly lymphocytes (%; $p = 0.062$; $r^2 = 0.05$). However, hydration status measured via body mass

only nearly predicted circulating presence of lymphocytes (%; $p = 0.064$; $r^2 = 0.05$; L; $p = 0.06$; $r^2 = 0.05$), and neutrophils (L; $p = 0.085$; $r^2 = 0.04$). Examination of hydration groups revealed that Dehy had more circulating neutrophils (L; $p = 0.018$; %; $p = 0.030$) and nearly less lymphocytes (%; $p = 0.063$; Figure 8) than Euhy. No differences in red cell indices were detected between groups.

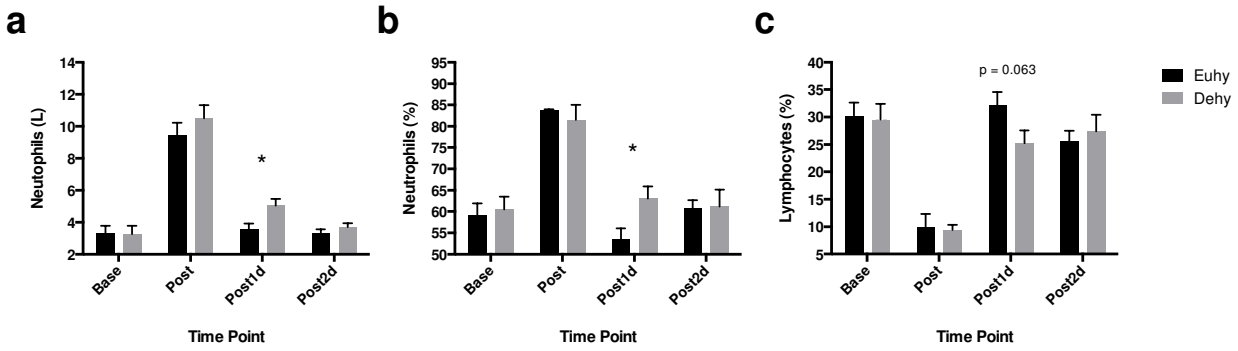

Figure 8. Differences in circulating blood cells between the most euhydrated (Euhy) and dehydrated (Dehy) male triathletes from the World Ironman Championship (means ± SE). * Indicates statistical group difference ($p < 0.05$).

Race associated stress elevated plasma inflammatory cytokines IL-10, IL-6 and IL-8 at Post compared to Pre, Post1d, and Post2d (all $p < 0.001$). No differences were observed in IL-12p70, TNFα, or IL-1β (Figure 9).

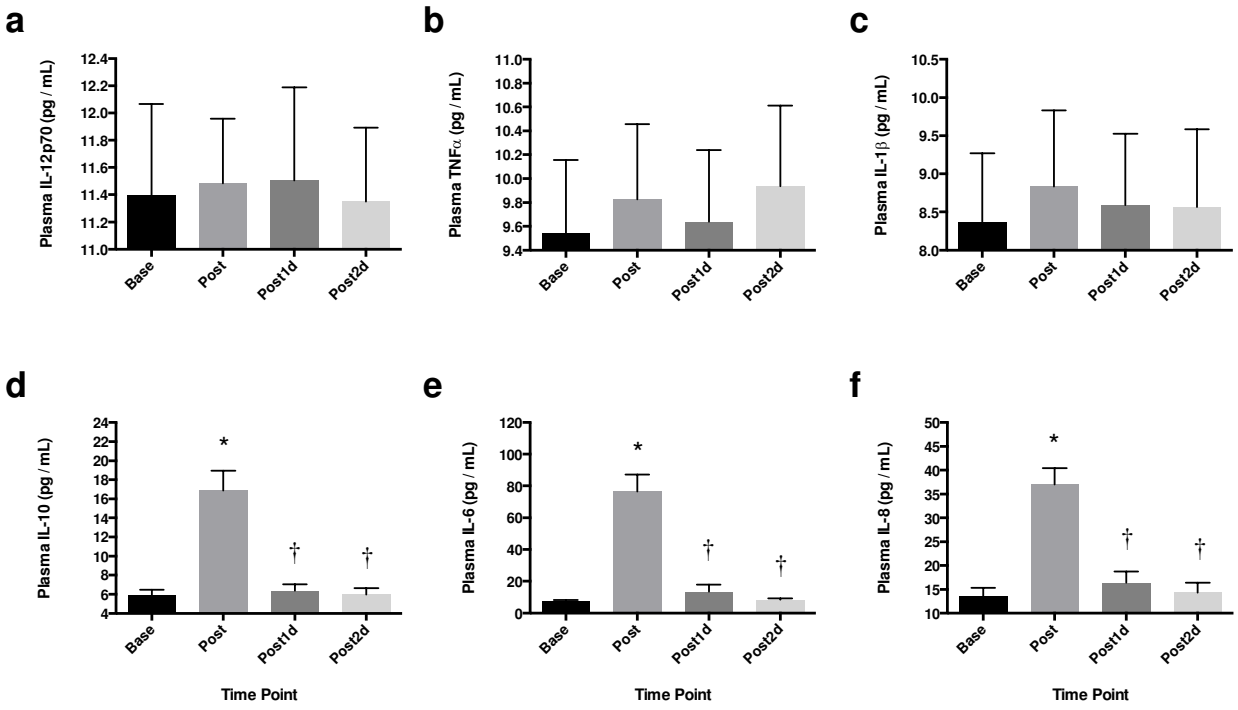

Figure 9. Plasma inflammatory cytokines in male triathletes from days prior (Base), ~30min post (Post), one day post (Post1d), and two days post (Post2d) World Ironman Championship triathlon (means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; † from Post; and ‡ from Post1d.

Hydration status measured via body mass predicted IL-12p70 ($p = 0.005$; $r^2 = 0.12$), TNFα ($p = 0.049$; $r^2 = 0.06$), IL-10 ($p = 0.034$; $r^2 = 0.07$), IL-6 ($p = 0.041$; $r^2 = 0.06$), and IL-8 ($p = 0.033$; $r^2 = 0.07$). However, classifying hydration status through U_{sg} only predicted IL-12p70 ($p = 0.023$; $r^2 = 0.08$). Hydration group examination revealed greater circulating IL-1β in Dehy than Euhy at Post ($p = 0.048$), and trended towards greater IL-12p70 in Dehy than Euhy at Post2d ($p = 0.074$), with no other differences observed (Figure 10).

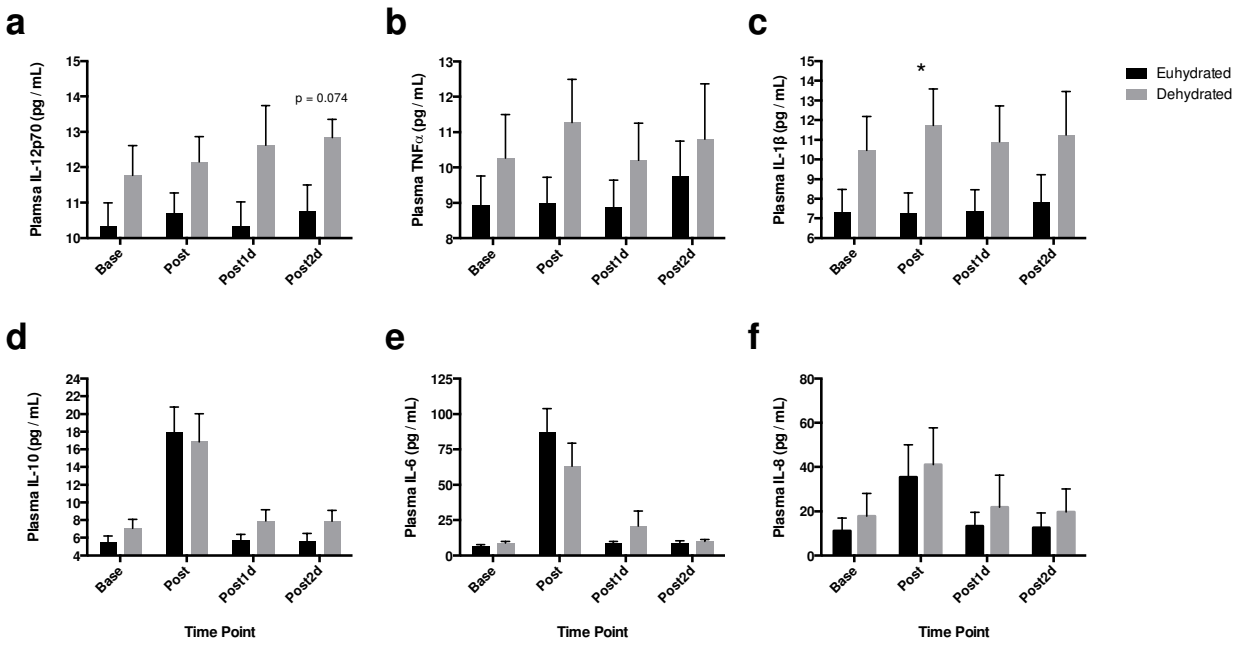

Figure 10. Differences in plasma inflammatory cytokines between the most euhydrated (Euhy) and dehydrated (Dehy) male triathletes from the World Ironman Championship (means ± SE). * Indicates statistical group difference ($p < 0.05$).

Plasma HSP70

Plasma HSP70 resulted in greater concentrations at Post than Base, Post1d, and Post2d ($p = 0.005$, $p = 0.009$, and $p = 0.012$, respectively). Five male control subjects were included for qualitative comparison, and contributed similar concentrations of HSP70 at Base compared to triathletes (no statistical comparison was made do to greatly different sample sizes). No differences in control subject plasma HSP70 existed across time (Figure 11).

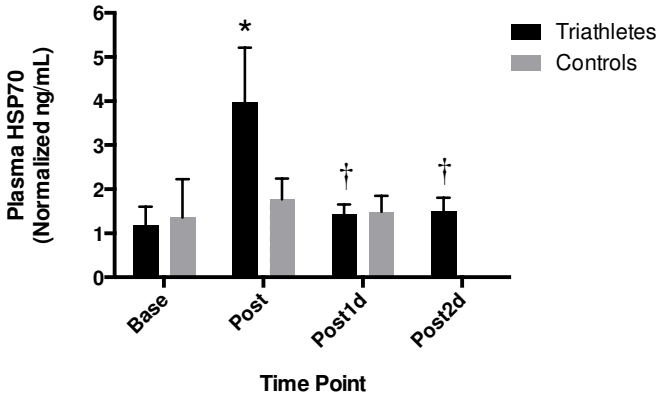

Figure 11. Plasma Heat Shock Protein 70kd (HSP70) in male triathletes from days prior (Base), ~30min post (Post), one day post (Post1d), and two days post (Post2d) World Ironman Championship triathlon (means ± SE). * Indicates statistical difference ($p < 0.05$) from Base; and † from Post.

Hydration status measured via body mass nor U_{sg} resulted in prediction of plasma HSP70.

Further, no hydration group differences were observed in plasma HSP70 (Figure 12).

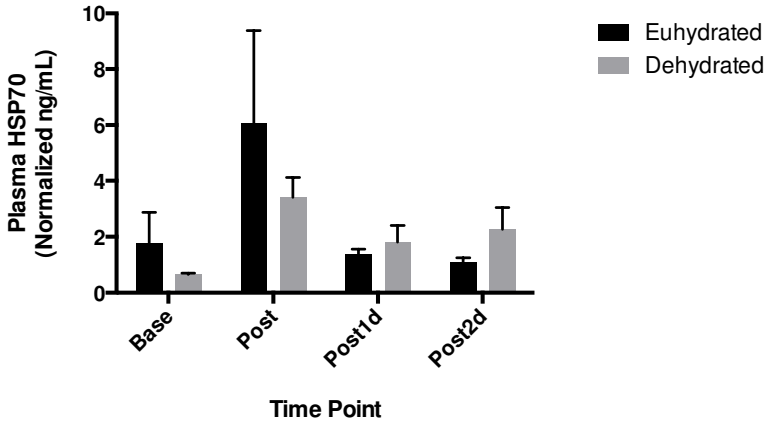

Figure 12. Plasma Heat Shock Protein 70kd (HSP70) in the most euhydrated (Euhy) and dehydrated (Dehy) male triathletes from the World Ironman Championship (means ± SE).

Discussion

This unique investigation examined 22 highly trained, male triathletes undergoing significant exercise stress in moderately hot and humid conditions at the Ironman World Championship. The compound and uncompensable stress experienced by these athletes generated a pronounced protective response.

Quantified circulating cell populations derived from the CBC with differential indicated the extent of physiological stress. Red cell indices displayed a typical and drastic reduction in total red blood cells, hemoglobin, and hematocrit, and an increase in mean corpscular hemoglobin concentration upon one and two days following the race. These findings align with common reports of dramatic post exercise increases in plasma volume frequently termed "sports anemia", likely explained through Aldosterone-mediated sodium and water retention in response to frequent training sessions involving fluid losses (reviewed in Mairbaurl, 2013).

As suspected, the examined athletes exhibited leukocytosis supported by greater total white blood cells, neutrophils (L and %) and monocytes (L), likely attributable to shear stress from increased cardiac output (Smith, 1997, Okutsu et al., 2008). Contrarily, lymphocytes (L and %) greatly decreased from the circulation, potentially due to habitual exercise practices (Ferry et al., 1990, Robson et al., 1999). Our observations of neutrophilia match those of previous literature and likely result from high concentrations of circulating cortisol (McCarthy and Dale, 1988, Walsh et al., 2011). In support of these findings, chronic submaximal exercise (as practiced in triathletes) appears to cause mobilization of neutrophils over lymphocytes during strenuous exercise (Smith, 1997).

Neutrophils perform phagocytosis in response to tissue damage providing rationale for their increased presence during exercise. Acute exercise intensity modulated mobilization of properly functioning neutrophils, and research suggests reduced functionality of neutrophils post exercise (Smith, 1997). The high prevalence and critical contribution of neutrophil function during exercise has lead to new investigations on acute exercise stimulated neutrophil gene expression to better understand their role in health during exercise. Acute exercise stimulated hundreds of upregulated and downregulated neutrophil genes that indicated not just a general stress response, but rather individually calculated responses to specific exercise insults (Radom-Aizik et al., 2008, Neubauer et al., 2013). This study and other data from the same laboratory concluded that exercise appears to not only stimulate greater numbers of circulating immune cells to respond to stress, but also appears to act as a "wake up" call for gene upregulation to prepare for growth, repair, and infection (Radom-Aizik et al., 2008, Radom-Aizik et al., 2009).

Changes in circulating immune cell populations implies a substantial pro-inflammatory environment facilitated by exercise-associated stress, and circulating cytokines corroborate this conclusion. As observed in previous literature, the exercise associated stress in this study lead to greater circulating IL-6, IL-8 and IL-10 (Suzuki et al., 2006, Zaldivar et al., 2006, Neubauer et al., 2013, Peake et al., 2005, Nieman et al., 2004). Unlike previous literature, we did not observe greater circulating TNFα (Zaldivar et al., 2006). We attribute this finding to the intensity and duration of exercise stress, as other triathlon studies too found no change in TNFα (Jeukendrup et al., 2000) potentially explained through release inhibition from the predominating presence of IL-6 (Pedersen, 2000). Further, we and others (Suzuki et al., 2006) observed consistently low

concentrations of IL-1β. However, TNFα and IL-1β classically indicate infection rather than an exercise stress response (Walsh et al., 2011), supporting their lack of presence among our data. Further IL-1β and IL-12p70 contain similar regulatory pathways, and IL-12p70 concentration also did not respond to exercise associated stress in the current or previous investigations (Sugama et al., 2013); research implies the extreme catecholamine and glucocorticoid presence that theoretically existed during the Ironman blunted the production of IL-12p70 (Elenkov et al., 2000).

Plasma HSP70 served as our final measure of circulating whole body stress. As seen in a previous examination of triathlon related stress (Suzuki et al., 2006), plasma HSP70 dramatically increased at Post and returned to Base within 24h. Some researchers speculate that elevated plasma HSP70 might reflect energy crisis, as carbohydrate ingestion during exercise blunted this response (Febbraio et al., 2004). While our participants did consume foods and beverages containing carbohydrates during the race, the amount of carbohydrate ingested was either insufficient or the exercise-associated stress too severe to noticeably attenuate plasma HSP70. Overall, our data supports a pro-inflammatory state surrounding participation in an Ironman event when considering the combined responses of circulating cellular populations, circulating cytokines, and HSP70.

Dehydration and the Stress Response

We uniquely conducted a sub-investigation to explore the potential impact of dehydration on circulating indicators of whole-body stress. Despite evidence for drastic cellular manipulation upon cell swelling and shrinking (reviewed in Burg et al., 2007, Lang et al., 1998), hydration

status predicted only some variables, and few resulted in group differences. However, one persistent complication within hydration research pertains to difficulty in quantifying hydration status, as no gold standard currently exists. In an attempt to mitigate this complication, we implemented two hydration biomarkers in our investigation: body mass and U_{sg}. Hydration measured through U_{sg} better predicted cellular population changes (WBCs, neutrophils (L), and monocytes (L)) than body mass. However, we selected to use body mass to identify the least (Euhy) and most (Dehy) dehydrated participants, as body mass permitted a more clear interpretation even during confounding dietary and exercise scenarios. Once divided into hydration groups, Dehy had greater circulating neutrophils (L) and nearly less lymphocytes (%). While dehydration-induced decreases in plasma volume would potentially explain these findings, no red cell indices resulted in differences between groups. To our knowledge, one other investigation has examined the influence of dehydration on circulating immune cell populations and found a greater percent change in neutrophils following Judo practice in those who lost more body mass attributable to fluid losses (Chishaki et al., 2013). Because neutrophils classically respond to tissue damage, we initially suspected greater indicators of muscle damage in Dehy versus Euhy; however, circulating creatine kinase and myoglobin concentrations did not distinguish these groups, and no differences in finish time substantiated similar quantities of muscle damage indicators. However, we do offer a theoretical explanation: a dehydration initiated increase in plasma Angiotensin II likely persisted at Post1d, despite rehydration practices (Di Nicolantonio and Mendelsohn, 1986). Angiotensin II receptors have been located in neutrophils and recent evidence describes the down regulation of the adhesion molecule CD62L, which would permit neutrophil detachment from the vascular endothelial wall and increase the number of circulating neutrophils (Vega et al., 2010).

We also found that body mass predicted IL-12p70, TNFα, IL-10, IL-6, and IL-8, whereas U_{sg} only predicted IL12p70. Hydration grouping presented greater IL-1β in Dehy than Euhy at Post, and nearly IL-12p70 at Post2d. Evidence supports greater IL-8 synthesis in human PBMCs during NaCl-induced hyperosmotic conditions. However, hyperosmolality with NaCl only increased IL-1β and TNFα mRNA, and required the addition of LPS to NaCl to induce protein synthesis (Shapiro and Dinarello, 1997). These findings substantiate the potential influence of dehydration on IL-8, but suggest mechanisms other than / in addition to dehydration induce greater plasma IL-1β and TNFα such as elevated catecholamines and glucocortacoids (Elenkov et al., 2000). Alternatively, as greater TNFα and IL-1β classically indicate an infection rather than exercise stress response (Walsh et al., 2011) our findings might propose that dehydration contributes to infection risk following exercise, and perhaps in part explains varying results among exercise and infection risk research. To our knowledge, no previous experiments investigate the influence of dehydration on IL-12p70, but our findings implore more study. Further, no literature exists to our knowledge explaining how osmotic stress could stimulate greater IL-6 and IL-10 production, supporting the traditional rationale that skeletal muscle contractions primarily contribute to greater circulating IL-6, and the substantial pro-inflammatory signals requiring negative feedback input of IL-10 (Opal and DePalo, 2000).

Conclusions

Our data suggest that whole body stress can be quantified by examining circulating and cellular responses. We observed distinct responses of cellular and circulating HSP70 (stress inducible

HSP72) which supports our previous work. Our novel mechanistic approach to studying HSP responses to exercise revealed NFAT5 as a potential indicator of whole body dehydration. We observed that post-translationally, HSF1 expression might not change despite a measurable thermal, whole body stress. Since HSF1 traditionally contributes to heat-shock induced HSP expression, the subtleties of HSF1 responses to exercise require further investigation. Great value exsists in understanding not only the responses of specific HSPs characterized as stress adaptation mediators, but also the regulators of those HSPs to understand what cellular mediators might make individuals more resistant to multiple stress exposures over time.

We also observed changes in downstream immune markers of inflammation and global immune health (circulating cell populations). Documenting how cellular stress markers correlate to and perhaps mediate physiological function of specific systems like the immune system defines a true, translational approach to cellular and whole body stress research. We found that exercise and dehydration in stressful environmental conditions stimulates a measurable, acute inflammatory state in both cytokine and PBMC profiles. Whether heat shock proteins drive any of the immune system changes post exercise-stress remains to be determined.

Our future aims include: 1) global transcriptional analysis of PBMCs to determine the full complement of stress-induced gene up- and down-regulation via RNAseq methods 2) folding capacity for a functional analysis of control and experimental blood sera (this will provide information about the global chaperone capacity post-exercise), and (3) exploration into oxidative stress and DNA damage associated with PBMCs in our exercise-stress protocol . Our approach to Aim 1 will include a high-throughput sequencing

approach with isolated total RNA. Aim 2 will use a newly developed fluorescent reporter assay (patented Glow-Fold™ protein, Boston Biochem) to assess how well sera facilitates folding of the Glow-Fold™ protein in a cuvette-based assay. For Aim 3, we will utilize well-defined standard ELISAs and comet assay techniques in cryopreserved PBMCs.

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