Eleven days of moderate exercise and heat exposure induces acclimation without significant HSP70 and apoptosis responses of lymphocytes in college-aged males

Lindsay L. Hom · Elaine Choung-Hee Lee · Jenna M. Apicella · Sean D. Wallace ·
Holly Emmanuel · Jennifer F. Klau · Paula Y. S. Poh · Stefania Marzano ·
Lawrence E. Armstrong · Douglas J. Casa · Carl M. Maresh

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Abstract The purpose of this study was to assess whether a lymphocyte heat shock response and altered heat tolerance to ex vivo heat shock is evident during acclimation. We aimed to use flow cytometry to assess the CD3⁺CD4⁺ T lymphocyte cell subset. We further aimed to induce acclimation using moderately stressful daily exercise-heat exposures to achieve acclimation. Eleven healthy males underwent 11 days of heat acclimation. Subjects walked for 90 min ($50 \pm 8\%$ $\text{VO}_{2\text{max}}$) on a treadmill (3.5 mph, 5% grade), in an environmental chamber (33°C, 30–50% relative humidity). Rectal temperature (°C), heart rate (in beats per minute), rating of perceived exertion, thermal ratings, hydration state, and sweat rate were measured during exercise and recovery. On days 1, 4, 7, 10, and 11, peripheral blood mononuclear cells were isolated from pre- and post-exercise blood samples. Intracellular and surface HSP70 (SPA-820PE, Stressgen, Assay Designs), and annexin V (ab14085, Abcam Inc.), as a marker of early apoptosis, were measured on CD3⁺ and CD4⁺ (sc-70624, sc-70670, Santa Cruz Biotechnology) gated lymphocytes. On day 10,

subjects experienced 28 h of sleep loss. Heat acclimation was verified with decreased post-exercise rectal temperature, heart rate, and increased sweat rate on day 11, versus day 1. Heat acclimation was achieved in the absence of significant changes in intracellular HSP70 mean fluorescence intensity and percent of HSP70⁺ lymphocytes during acclimation. Furthermore, there was no increased cellular heat tolerance during secondary ex vivo heat shock of the lymphocytes acquired from subjects during acclimation. There was no effect of a mild sleep loss on any variable. We conclude that our protocol successfully induced physiological acclimation without induction of cellular heat shock responses in lymphocytes and that added mild sleep loss is not sufficient to induce a heat shock response.

Keywords Exercise-heat stress · Heat acclimation · Heat shock protein · Apoptosis · Cytoprotection · Sleep deprivation

Introduction

Annually, many suffer from heat injury, heat illness, and heatstroke (Armstrong et al. 1996; Casa et al. 2005; Nelson et al. 2011). Risk factors include chronic medical conditions, history of heat illness, and transient medical/situational factors like febrile illness, self-medication, travel fatigue or jet lag, sleep deprivation, and lack of acclimatization (Nunneley and Reardon 2002; Coris et al. 2004; Armstrong et al. 2007; Muldoon et al. 2007). Guidelines emphasize heat acclimation/acclimatization, or gradual adaption to heat stress, as one of the most effective ways to decrease hyperthermia-associated risks (Armstrong et al. 2007; O'Connor et al. 2007; O'Connor et al. 2010). Exploring *how* acclimation confers protection

L. L. Hom · J. M. Apicella · S. D. Wallace · H. Emmanuel ·
J. F. Klau · P. Y. S. Poh · S. Marzano · L. E. Armstrong ·
D. J. Casa · C. M. Maresh
Human Performance Laboratory,
Department of Kinesiology, University of Connecticut,
Storrs, CT 06269, USA

E. C.-H. Lee
Mount Desert Island Biological Laboratory,
Salisbury Cove, ME 04634, USA

E. C.-H. Lee (✉)
PO Box 35, Old Bar Harbor Road,
Salisbury Cove, ME 04672, USA
e-mail: lee.elainec@gmail.com

can provide valuable insight that has implications in stress and disease research.

Chaperone proteins like heat shock protein 72 (HSP72), which preserve protein and cellular function, structure, and resiliency during stress and infection, may mediate acclimation induced benefits (Horowitz and Assadi 2010). Indeed, several cross-sectional studies (Moseley 1997, 1998; Maloyan et al. 1999) have demonstrated that heat shock protein 70 (HSP70) may facilitate cellular and tissue-level adaptations during heat acclimation. Although research supports heat shock proteins as mediators of tolerance, many questions and discrepancies remain, and may depend upon experimental approach. For instance, extracellular (EC) or circulating HSP70 responses to heat stress have been well studied, but are still imprecisely defined. One consideration is that EC HSP70 changes do not reflect intracellular (IC) changes (Asea 2008) and are only representative of the net balance of the HSP70 being released into and removed from the circulation (Whitham and Fortes 2008). Thus, it might be more meaningful to measure IC HSP70 levels when exploring cellular adaptations that occur with heat acclimation. To our knowledge, only a few studies have examined IC HSP70 levels during heat acclimation (Marshall et al. 2007; Yamada et al. 2007; McClung et al. 2008; Watkins et al. 2008; Magalhaes Fde et al. 2010).

Another experimental approach to studying HSPs in acclimation is to investigate the time course of HSP70 changes *during* acclimation and how those changes affect inducibility, or resilience, of “primed” stressed cells. Heat-exposed cells (Boreham et al. 1997; Samali et al. 1999) and perhaps cells from stressed or acclimated subjects, may survive additional *ex vivo* or *in vitro* heat shock by decreasing apoptotic cell death signaling. This heat tolerance can be indirectly measured by examining cell death or apoptosis during stress. During apoptosis, phospholipid binding protein annexin V can be used to probe for the early apoptotic event of phosphatidylserine (PS) translocation from the inner to outer surface of the cell (Homburg et al. 1995; Vermes et al. 1995). To exclude for extracellular PS expression that occurs with necrotic cell death, an exclusionary dye like propidium iodide can be used to stain necrotic cell DNA (Vermes et al. 1995). Using these types of approaches, we aimed to explore not only observational, but cell-level, functional implications of heat shock protein expression during acclimation.

Finally, considering functionality in a whole-body system, one must consider that tolerance or functional cytoprotection following heat shock protein induction may be mechanistically tied to cross-tolerance and adaptation to multiple, concurrent physiological stressors. One such stressor that is often added to heat stress in military and athletic settings is sleep loss or deprivation. Several studies

have shown that mild sleep loss (24–30 h) detrimentally influences exercise time to exhaustion and thermoregulatory responses (Sawka et al. 1984; Born et al. 1997; Zhong et al. 2005; Oliver et al. 2009). Very little is known, however, about the effects of sleep loss on heat shock proteins. One recent study suggests that heat shock protein levels in the brain may increase as a neuroprotective response to the sustained wakefulness of sleep deprivation (Terao et al. 2003). Considering our interest in stress physiology in the context of human athletes, soldiers, and recreationally active individuals, who are inevitably exposed to continuous multiple stressors, we aimed to include a pilot investigation into the effects of mild sleep loss on the progression of heat acclimation.

We proposed three hypotheses. First, that repeated days of heat acclimation would increase basal expression of inducible, stress-responsive HSP70 and annexin in subject peripheral blood mononuclear cells (PBMC) samples. Second, that with *in vitro* shock to these PBMC samples, cells acquired from more acclimated individuals would show greater tolerance and increased survival to secondary heat stress. Finally, that mild sleep loss would cause annexin and HSP70 expression to increase if subjects experienced a stress response to the added sleep loss protocol. We found that physiological adaptation to heat during 11 days of acclimation can be achieved with minimal changes in HSP70, apoptosis, and heat tolerance of lymphocytes

Methods

Subjects

Eleven healthy males (mean $\pm$ SD; 20 ± 1 years, 183.7 ± 8.4 cm, 81.7 ± 12.2 kg, $20 \pm 1\%$ body fat, 53.2 ± 8.8 ml·kg⁻¹·min⁻¹ VO_{2max}) from the University of Connecticut volunteered to participate in this study. All volunteers were between the ages of 19–27 and active, but not trained, as determined by a questionnaire. Additionally, none of the subjects had exposure to high environmental or internal temperatures (i.e., prolonged fever) within the previous 30 days, as this could compromise the complete heat acclimation process. Subjects completed an informed consent form and medical history questionnaire. This research was conducted in compliance with the Institutional Review Board of the University of Connecticut, which approved this study.

Familiarization visits

Each subject reported to the Human Performance Laboratory at the University of Connecticut to complete three

familiarization visits on the 3 days leading up to the first day of testing. Every subject arrived at the same time he was scheduled to begin each experimental testing day. On each of these familiarization visits, a morning urine sample was collected and an initial body weight was taken in order to establish proper hydration prior to testing. Subjects were introduced to perceptual scales that would be used during testing: Borg's Rating of Perceived Exertion (6–20 point scale; Borg and Kaijser 2006), Thirst Sensation Scale and Thermal Sensation Scale.

Each subject's height was measured to determine body mass index during the first familiarization visit (mean $\pm$ SD; 24 $\pm$ 4). Subjects were instructed to self-insert a rectal temperature probe 10 cm past the anal sphincter to measure internal body temperature. A cannula was placed into the antecubital arm vein to familiarize the subjects with the blood sampling process.

During the second familiarization visit, a seven-site skinfold test (triceps, chest, midaxillary, subscapular, suprailliac, abdominal, thigh) was used to determine body fatness according to Jackson et al. (1978). A $\text{VO}_{2\text{max}}$ test was also given. Subjects completed a 4-min warm-up on a motorized treadmill at 4.5–6.0 mph. The grade was then increased to 4% for 2 min and continued to increase 2% every 2 min until the subject reached exhaustion. Oxygen uptake, minute ventilation, and respiratory exchange ratio were measured every 30 s via indirect calorimetry (metabolic cart, ParyoMedics, True One 2400, Sandy, UT). Heart rate was measured using a chest cardiometer (heart rate monitor, Polar Electro Inc., Woodbury, NY). The attainment of $\text{VO}_{2\text{max}}$ was verified when subjects met two of the following criteria: (a) an increase of oxygen consumption $<150 \text{ ml}\cdot\text{min}^{-1}$ despite an increase in grade, (b) heart rate $>90\%$ of predicted maximum (220 bpm minus chronologic age), and (c) respiratory exchange ratio ($V_{\text{CO}_2}/V_{\text{O}_2}$) >1.10 .

Experimental protocol

On each of the 11 testing days, subjects arrived at the Human Performance Laboratory between 0600 and 1000 hours and provided a morning urine sample, which was analyzed for urine specific gravity (USG) and urine color to verify that subjects were euhydrated (USG <1.025 , color ≤ 4). Subjects then placed their rectal probe and heart rate monitor. Initial body weight was taken wearing only shorts, the rectal probe and the heart rate monitor. The subjects were asked to rate their sleep from the previous night, and asked several compliance questions (i.e., hydration practices, abstinence from caffeine, alcohol, drugs, and exercise). Each was given a standardized breakfast of 1 or 2 Clif® bars (decided by the subject during familiarization visits) plus 300 mL of water.

All 11 exercise sessions were performed in an environmental chamber (Model 2000, Minus-Eleven, Inc., Malden, MA) which was held at 33°C and 30–50% relative humidity. No fluid was consumed during the exercise protocol. Subjects walked for 90 min at $50\pm 8\% \text{VO}_{2\text{max}}$ on a motorized treadmill at 3.5 mph, 5% grade. Rectal temperature and heart rate were measured and recorded every 10 min. Environmental measurements (dry bulb temperature, relative humidity, barometric pressure) were recorded every 20 min. Rating of perceived exertion (RPE) and thirst and thermal sensation were recorded every 30 min. Upon completion of the 90-min exercise protocol, subjects returned to a cool 23°C environment where their post-exercise body weight was taken (shorts, rectal probe, and heart rate monitor only) and they were given water to drink ad libitum.

On days 1, 4, 7, 10, and 11, a sterile catheter (Excel Safelet, Exelint, Culver City, CA) was inserted into each subject's antecubital arm vein. The subject then entered the chamber and stood for 15 min in order to allow body fluids to equilibrate. Immediately prior to the start of exercise, as well as immediately after exercise, 10 mL of blood was collected from the indwelling catheter into a clean, sterile syringe and immediately transferred to a chilled lithium heparin tube to be processed immediately for intracellular/surface hsp70. The 90-min exercise protocol followed the same format as outlined above in the general "Experimental protocol" section.

Mild sleep loss (28 h)

Upon completion of exercise on day 10, subjects were allowed to return home to collect their belongings in preparation for 28 h of sleep loss. They were instructed to report back to the Human Performance Laboratory within 3 h where they were then monitored by graduate student investigators and faculty who rotated over the 28-h period. Subjects stayed awake by engaging in activities such as video games, card games, movies, and music. They were allowed snacks and non-caffeinated beverages throughout the night and were instructed to record their food and fluid intake. After 28 h without sleep, subjects completed the day 11 exercise-heat protocol. As a safety precaution, a responsible student investigator then escorted them home.

Urine analyses

Using the morning void sample, urine osmolality was measured by freezing-point depression osmometry. Urine color assessed using the urine color scale (Armstrong et al. 1994). Both of these urinary measures were examined to confirm that the subject was euhydrated before beginning the exercise protocol each day.

Blood analyses

Peripheral blood mononuclear cell isolation

For PBMC isolation, 10 mL of whole blood was gently inverted eight to ten times in the lithium heparin tube. It was then diluted with 35 mL $1\times$ Dulbecco's phosphate buffered saline (PBS) and inverted gently to mix. The diluted blood was layered over 10 mL of Ficoll-Paque (GE Healthcare Biosciences Corp., Piscataway, NJ) and PBMC isolation was carried out according to the manufacturer's suggestions. After the final wash, the PBMC pellet was resuspended in enough $1\times$ Dulbecco's PBS to obtain a final concentration of 1×10^7 cells/mL. Trypan blue staining (1:1) in 10 μ L of cell suspension was used to exclude dead cells and counted on a Hy-Lite hemocytometer to verify cell concentration. Heat-shocked samples were incubated for 1 h at 42°C, and allowed to recover in CO₂ independent T cell media (500 mL RPMI 1640, 50 mL HI FBS, 5 mL Pen Strep, 5 mL L-glutamine, penicillin/streptomycin) for 24 h prior to staining.

Antibody staining

All steps were carried out with $10\times$ dilution. Cells (10 μ L; 1×10^5) were added to 90 μ L PBS and the cell membranes were treated with 100 μ L of permeabilization buffer (sc-3628, Santa Cruz Biotechnology, Santa Cruz, CA). After two washes (centrifugation at 2,500 rpm, 5 min), cells were stained with 10 μ L of pre-diluted PE-conjugated HSP70 monoclonal antibody (SPA-820PE, Stressgen, Assay Designs, Ann Arbor, MI) (1 μ L+9 μ L PBS) and allowed to incubate on ice in the dark for 30 min. Cells were centrifuged (2,500 rpm, 10 min) and washed with 1 mL of wash buffer (sc-3624, Santa Cruz Biotechnology, Santa Cruz, CA) two times. The pellet was then fixed with 16% paraformaldehyde and incubated for 15 min. After a final wash (centrifugation at 2,500 rpm, 10 min) the pellet was resuspended in 1% paraformaldehyde and kept on ice until read on the flow cytometer.

For annexin staining/PI exclusion, 1×10^6 cells (100 μ L) were immediately resuspended in 1 mL Gibco CO₂-independent medium (Lot 1378012, Invitrogen, Carlsbad, CA) to maintain cell integrity until processing. Cells were centrifuged and excess media was decanted when processing began. Cells were stained with annexin V-FITC-conjugated antibody and propidium iodide from the Annexin V-FITC apoptosis detection kit (ab14085, Abcam Incorporated, Cambridge, MA) according to the manufacturer's instructions. Cells were resuspended in 4% paraformaldehyde and read on the flow cytometer within 40 min of fixation.

For unstained controls, all samples were compared to a 1×10^6 unstained PBMC cell sample that was prepared identically to the HSP70-stained samples without the permeabilization, staining and 30-min incubation steps.

All samples were read on the flow cytometer within 40–60 min after fixation. CD3⁺ and CD4⁺ gates were made prior to the experiment using CD3 FITC mouse monoclonal antibody (sc-70624, Santa Cruz Biotechnology, Santa Cruz, CA) and CD4 PE mouse monoclonal antibody (sc-70670, Santa Cruz Biotechnology, Santa Cruz, CA). Isotype controls for CD3, CD4, HSP70, and annexin V antibodies were performed to compensate for non-specific binding. Ten thousand events were collected on a CD3⁺/CD4⁺ lymphocyte gate and evaluated using FlowJo software (Treestar Incorporated, Ashland, OR) for mean fluorescence intensity (MFI) and percent positive for FL2 (HSP70) and FL1–FL2 (Annexin, with PI exclusion). The CD3 and CD4 surface markers were used to select T helper cell subsets, to assess acclimation effects on this lymphocyte subset specifically.

Statistical analysis

To verify that heat acclimation occurred, Analyses of Variance ANOVA were performed for rectal temperature and heart rate data with time as a 5-level factor (days 1, 4, 7, 10) for both pre- and post-exercise measurements on Statistica™ software. (Statistica version 99, Statsoft Inc., Tulsa, OK). Analyses also examined the effect of exercise by comparing pre- and post-exercise values. Another ANOVA was run comparing only day 1 versus day 10. Separate ANOVA were run for the rectal temperature and heart rate data from days 10 and 11 in order to determine the effect of sleep loss on these measures.

For MFI and percent positive for HSP70 and annexin, as well as MFI of the total live CD3⁺/CD4⁺ lymphocyte population, repeated measures ANOVA were performed on SPSS statistics software (SPSS Statistics 18.0, SPSS Inc., Chicago, IL). Simple (first) contrasts were executed to determine significant differences from baseline measurements. Repeated pairwise comparisons were performed to determine significant differences between consecutive days. A dependent *t* test was conducted on day 1 MFI values for both HSP70⁺ lymphocytes and annexin + lymphocytes to determine effects of baseline heat shock. Bonferroni post hoc comparisons were carried out where warranted. A significance level of $p<0.05$ was used in all statistical procedures. Effect size correlations were also calculated and are indicated as *r* values where nonsignificant *p* values may indicate physiologically important trends. Trends and *p* values approaching significance are reported. Values are presented as means $\pm$ SE.

Results

Physiological responses to heat acclimation

Physiological data support the achievement of heat acclimation Baseline (pre-exercise) rectal temperatures were not significantly different on any day. Post-exercise rectal temperatures were significantly lower on day 4 ($38.11 \pm 0.08^\circ\text{C}$, $p=0.003$), day 7 ($38.00 \pm 0.09^\circ\text{C}$, $p=0.003$), and day 10 ($38.18 \pm 0.09^\circ\text{C}$, $p=0.006$) compared to day 1 ($38.48 \pm 0.14^\circ\text{C}$; Fig. 1a). Days 4, 7, and 10 did not significantly differ from each other. Exercise significantly affected rectal temperature, as post-exercise rectal temperatures were significantly higher ($p=0.001$) than pre-exercise rectal temperatures. Interestingly, rectal temperature was lower post-exercise after sleep deprivation, as day 11 post-exercise rectal temperature ($37.97 \pm 0.07^\circ\text{C}$) was significantly lower ($p=0.047$) than day 10 post-exercise rectal temperature ($38.18 \pm 0.09^\circ\text{C}$).

Further confirming heat acclimation, sweat rates rose throughout the 11 days of heat acclimation from 0.7 ± 0.06 kg body weight loss-exercise time⁻¹ on day 1, to 0.89 ± 0.07 by day 11 (Fig. 1b). Additionally, post-exercise heart rates were all significantly lower on day 4 (143 ± 4 bpm, $p=0.002$), day 7 (137 ± 4 bpm, $p=0.003$), and day 10 (140 ± 4 bpm, $p=0.001$) compared to day 1 (158 ± 3 bpm; Fig. 1c), confirming that heat acclimation had occurred. Days 4, 7, and 10 did not significantly differ from each other. Again, exercise effected heart rate as post-exercise heart rates were significantly elevated ($p=0.001$) from pre-exercise heart rates. Similar to rectal temperature, day 11 post-exercise heart rate (135 ± 11 bpm) was significantly lower ($p=0.001$) than before sleep deprivation on day 10 (140 ± 13 bpm). RPE did not seem to be affected by the acclimation, as there were no significant differences between days or pre- and post-exercise data (data not shown).

HSP70 and annexin

HSP70 and annexin expression on circulating lymphocytes did not significantly increase through 11 days of heat acclimation, though trends for increase are present on both variables. Mild sleep loss does not affect HSP70 or apoptosis in these circulating lymphocytes Mean lymphocyte HSP70 expression did not change consistently during 11 days of heat acclimation. The percent of HSP70⁺ cells decreased from days 1 to 4 (Fig. 2b), though MFI (Fig. 2a) did not change in that time period. On days 7, 10, and 11 of heat acclimation, the percentage of HSP70⁺ cells (Fig. 2b) were not significantly different from each or from day 1 values. Likewise, HSP70 MFI was not significantly

Fig. 1 Physiological verification of heat acclimation. **a** Rectal temperature (degrees Celsius) following exercise on each of the four heat tolerance test days; **b** sweat rate (change in body weight during exercise time) on each of the four heat tolerance test days; **c** heart rate (in beats per-minute, measured with polar heart rate monitor) following exercise on each of the four heat tolerance test days. *Significant difference for Day 1 values, $p < 0.05$

different. However, HSP70 MFI on day 10 did statistically trend towards an increase from day 1 ($p=0.09$, $r=0.60$; Fig. 2a). A representative tracking of flow plots and histograms for one particular subject presents evidence for increases in an HSP70⁺ population of cells that appears and persists throughout the 11 days of acclimation, despite the variability and lack of statistical significance among our participants on average.

Fig. 2 Intracellular HSP70 during acclimation. **a** MFI of HSP70⁺ lymphocytes, normalized to control, day 1; **b** percent HSP70⁺ cells out of total live lymphocytes, normalized to control day 1; **c** representative flow plots of each day of acclimation for one sample subject. Top two panels depict the same cell populations, gating HSP70⁺ cells in regions

marked “2”. Histograms depict the clear separation of HSP70 negative and HSP70 positive populations. Error bars are $\pm$ 2SE. *Significant difference from Day 1 baseline values, $p < 0.05$. Where p values are indicated, values are approaching significance, $p < 0.13$

Annexin, as a marker of early apoptosis, did not significantly change during heat acclimation. However, worthy of note is a consistent increasing trend in both the intensity of annexin staining (Fig. 3a) and percent of annexin⁺ cells (Fig. 3b) through the 11 days of heat acclimation. These visually apparent trends increase are supported by p values indicating statistical trends. There is evidence that MFI of annexin stained cells on day 11 ($p = 0.09$, $r = 0.69$; Fig. 3a) and percent of annexin⁺ cells on day 10 ($p = 0.10$, $r = 0.79$; Fig. 3b) may be higher than on day 1.

A pairwise comparison revealed no differences between days 10 and 11 for HSP70⁺ or annexin⁺. It appears that mild sleep loss does not have an effect on HSP70 expression or early apoptosis in circulating lymphocytes.

Heat tolerance

HSP70 and annexin expression on heat-shocked lymphocytes did not statistically support the evidence of greater cellular heat tolerance acquired during 11 days of heat acclimation. Mild sleep loss does not affect heat tolerance from 1 day to the next Heat tolerance would be evidenced by decreased HSP70 inducibility with decreases in apoptotic cells. There

were no statistically significant changes in heat tolerance during 11 days of heat acclimation (Figs. 4 and 5). There were no pairwise differences between days 10 and 11 with regard to the percent of HSP70⁺ and annexin⁺ cells, nor were there trends or qualitatively apparent evidence that heat tolerance is affected by a mild sleep loss.

Discussion

We aimed to study intracellular and surface HSP70 expression, early apoptosis, and heat tolerance of lymphocytes during 11 days of whole-body heat acclimation. We conclude that our subjects achieved physiological acclimation without evident heat shock responses in isolated lymphocytes. Furthermore, a mild sleep loss stress during heat acclimation did not affect lymphocyte HSP70 or heat tolerance during acclimation. Cellular effects of whole-body heat acclimation may be apparent in some individuals, but not consistently so among our sampling of tested individuals. We attribute this to the conditions of our daily exercise-heat stress and the cell type we assessed. This is the first assessment of HSP70 and annexin responses during acclimation using flow cytometry in

Fig. 3 Apoptosis during acclimation. **a** MFI of early apoptotic marker, annexin V on annexin V⁺ cells (with PI exclusion), normalized to control, day 1; **b** Percent of annexin V⁺ cells out of total live lymphocytes, normalized to control, day 1

whole cells that have not been lysed to quantify total protein or mRNA expression. This is also the first presentation of an interpretation that physiological acclimation can be achieved independently of lymphocyte heat shock responses over 11 days.

We sought to answer research questions about HSP70 and apoptosis during acclimation and secondary ex vivo heat shock of lymphocytes from acclimated subjects. Although the specific role of PBMCs and lymphocytes in acclimation is yet unclear, it is known that PBMCs and lymphocytes represent a circulating population of cells that may provide gene and protein expression patterns similar to target tissue (Connolly et al. 2004). This provides valuable information beyond measures of circulating mediators in blood plasma or serum samples. Before evaluating any cellular variables, we needed to first verify that the adaptations that take place during acclimation had occurred in our subjects. As seen in other studies that quantified acclimation, we found that our acclimated subjects exhibited lower core temperatures during exercise (Buono et al. 1998), increased sweat rates (Patterson et al. 2004; Machado-Moreira et al. 2005), and decreased heart rates during exertion (Nielsen et al. 1993; Nielsen 1998). Our physiological results coincide with the well-established notion that heat acclimation reduces physiological strain, improves exercise endurance, and reduces the risk

of heat illness (Armstrong et al. 1996; Casa et al. 2005; Armstrong et al. 2007).

HSP70 and apoptosis during acclimation

Though the heat shock response has been typically associated with thermotolerance following acute single heat exposures (Beckham et al. 2010), there is also evidence that it may be involved in the long-term adaptations seen with acclimation (Yamada et al. 2007; McClung et al. 2008; Sandström et al. 2008; Magalhaes Fde et al. 2010; Kuennen et al. 2011) as Moseley et al. proposed 15 years ago (Moseley 1997). Among the five or so studies that have examined intracellular HSP70 during acclimation, two show evidence to the contrary in intracellular studies of HSP70 during acclimation (Marshall et al. 2007; Watkins et al. 2008). Our results apparently contribute to the evidence that basal intracellular HSP70 does not reflect whole-body acclimation. Furthermore, with our daily exercise-heat protocol, though subjects achieved physiological acclimation, isolated lymphocytes did not exhibit significantly increased heat tolerance to secondary ex vivo shock.

However, based on the differences in exercise intensity, ambient temperature during acclimating exercise, tissue studied, and method of HSP70 quantification among the

Fig. 4 HSP70 expression during ex vivo heat shock of lymphocytes. **a** MFI of HSP70⁺ lymphocytes, normalized to control, day 1; **b** Percent HSP70⁺ cells out of total live lymphocytes, normalized to control, day 1. Error bars represent ± 2 SE

Fig. 5 Apoptosis during ex vivo heat shock of lymphocytes. **a** MFI of annexin V⁺ lymphocytes; **b** percent of annexin V⁺ cells out of total live lymphocytes. Error bars represent $\pm$ 2SE. *

current literature, we conclude that our results do not necessarily contradict the idea that there are notable HSP70 responses and roles during acclimation, but rather attest to the sensitivity of HSP70 to variables other than just achievement of acclimation as assessed by physiological measures. The qualitatively observable trends toward statistical significance in our protocol support this interpretation.

Other researchers have also addressed the question of whether HSP72 is indeed involved in acclimation. Different phases of acclimation may involve different HSP70 kinetics and roles and this may account for inconstancy in the literature. Horowitz (Horowitz 2001) proposes in the early phase (days 1–5) of heat acclimation, or short-term heat acclimation (STHA), accelerated sympathetic excitability stimulates HSP72. In a study of heat-acclimating rats, Maloyan et al. (Horowitz et al. 1997) found that intracellular HSP70 in the heart was lower 48 h after heat stress versus control. In their follow-up measurements of HSP70, they found that after the STHA, during long-term heat acclimation (LTHA; >3 weeks), intracellular HSP70 was upregulated. The authors hypothesized that HSP accumulation during LTHA could contribute to increased heat tolerance, widening the thermoregulatory range. Although we did not see statistically significant evidence that HSP70 is required for acclimation, our protocol did not address the possibility presented in a 2010 review (Horowitz and Kodesh 2010). They present a model whereby sympathetic signaling activates HSP70 mRNA transcription during STHA. Subsequently augmented HSP70 reserves during LTHA contribute to the HSP70 mediated acclimation benefits (Horowitz and Kodesh 2010).

Other variables in acclimation protocols may affect the interpretation of HSP70 roles in heat adaptation. For example, in this study, core temperatures reached a mean maximum of 38.48°C (post-exercise, day 1) and heart rates reached a mean maximum of 158 bpm (post-exercise, day 1). Although these means overlap with ranges of core temperatures and heart rates observed post-exercise in

studies that showed between 17% and fourfold increases in intracellular resting HSP70 (Yamada et al. 2007; McClung et al. 2008; Magalhaes Fde et al. 2010), the means and maximums in these studies are slightly greater than ours. Relatedly, lower core temperatures and heart rate maximums were seen in the acclimation study of Marshall et al. (2007), which found no change in intracellular HSP70 during early acclimation. Although Watkins et al. (2008) also saw no change in intracellular HSP70 with observations of heart rates that reached between 165 and 185 bpm, their observed maximum core temperature was approximately 38.8°C and they measured HSP70 of muscle tissue lysates. If we attribute to possible differences in cell type or tissue response of HSP70, as has been shown (reviewed in Yamada et al. 2008), it is possible that in order to perceive HSP70 responses in circulating cells, acclimating subjects must experience extreme hyperthermia and cardiovascular strain during daily exercise-heat exposures.

Further supporting this notion that degree of hyperthermia during acclimation is related to whether or not HSP70 is expressed intracellularly, is that Yamada et al. (2007), McClung et al. (2008), and Magalhaes Fde et al. (2010) used ambient temperatures of 42.5°C, 27.95% relative humidity (RH), 49°C, 20% RH, and 40°C, 45.1% RH, respectively, in their daily acclimation exercise. Considering the combination of environmental temperature and humidity, the greatest combination of humidity and ambient temperatures above 40°C, resulted in the greatest, almost fourfold increase in resting, intracellular HSP70 from pre- to post-acclimation (Magalhaes Fde et al. 2010). The lesser degree of intracellular HSP70 changes seen in the study of McClung et al. (2008) versus the study of Yamada et al. (2007), may be ascribed to the greater humidity in Yamada et al.'s protocol, or to the greater intensity of exercise. Among studies that found no change in intracellular HSP70 during acclimation, Marshall et al. used 38°C, 60% RH environmental conditions (Marshall et al. 2007), Watkins et al. used 39.5°C,

27% RH (Watkins et al. 2008), and our protocol was in an environmental chamber set at 33°C, 30–50% RH. Less stressful environmental temperatures and lower humidity seem to be associated with lower intracellular HSP70 responses to acclimation among the few human studies.

Exercise intensity or variability in exercise intensity also contribute to the degree of hyperthermia (Harri et al. 1982; Mora-Rodriguez et al. 2008); exercise intensity alone has been associated with increased intracellular HSP70 responses (Liu et al. 2000; Milne and Noble 2002; Liu et al. 2004). Though extracellular HSP70 responses to exercise in rats may require concomitant hyperthermia (Ogura et al. 2008), Whitham et al. showed that with a thermal clamp, exercise alone was sufficient to induce hyperthermia-independent eHSP70 release (Whitham et al. 2007). These observations can also be applied to the discrepancies in intracellular HSP70 responses among human acclimation studies. Our daily exercise protocol consisted of a 90-min walk at 3.5 miles per hour (mph), 5% grade. Marshall et al. used a daily 2-h cycling protocol at 38% of maximal oxygen consumption (Marshall et al. 2007), and Watkins et al. used a 30 min cycling protocol at 75% peak oxygen consumption (Watkins et al. 2008). These studies and our present study saw no intracellular HSP70 response, and had lower exercise intensity than the daily running protocols of acclimation studies that found intracellular HSP70 changes (McClung et al. 2008; Yamada et al. 2008; Magalhaes Fde et al. 2010). Whether the exercise intensity disparities led to increased heat storage and greater hyperthermia, or greater hyperthermia-independent mechanisms of HSP70 induction, it is important to consider the effects of this variable on cellular responses to acclimation.

Perhaps due to the less strenuous nature of our daily exercise protocol compared to those of other studies, we found no statistical significance or consistent HSP70 and apoptosis response among our study participants. Likewise, the trends we observed in lymphocyte heat tolerance to secondary ex vivo heat shock reflect the lack of a heat shock response, although our participants achieved physiological benefits of acclimation. The intracellular HSP70 during secondary heat shock, or inducibility of these cells in boosting a heat shock response, was highly variable and similar to that of cells acquired from unacclimatized first day samples. Expression of early apoptotic markers was variable as well, but may have trended towards decrease through acclimation. The ex vivo heat shock of PBMCs in the study by McClung et al. (2008) resulted in a characteristic, decreased inducibility of HSP72 associated with acclimation. Maloyan et al. showed a similar result in rats (Maloyan et al. 1999) and from this we can conclude that we did not see the acclimation induced heat tolerance benefits seen in those studies.

Methodologically, our HSP70-specific antibody did not distinguish between inducible heat shock protein HSP72

and constitutively expressed heat shock protein 73, HSC73. The 73-kDa member of the 70 kDa HSP family, HSC73, is constitutively expressed in all cells and does not readily respond to heat shock. This indicates a stress-independent chaperone function of this protein. It is the 72 kDa family member, HSP72 that specifically is induced during heat shock and serves as a stress-responsive chaperone. Because our antibody is not specific for HSP72, there is a possibility that differences in our data are muted by a notable presence of constitutive HSC73 in our HSP70 positive cell counts. Nevertheless, the differences in HSP72 were not drastic enough to overcome any existing pool of HSC73 expressing cells, and do not thus indicate an extreme change in HSP during our acclimation protocol. Future research should exploit more specific antibodies available for HSP72.

Our pilot exploration into the effects of sleep loss during acclimation, on heat shock protein responses indicated that a mild stress between days 10 and 11 of acclimation do not prevent the physiological attainment of acclimation, nor does it alter the heat shock response. We utilized a mildly strenuous daily exercise-heat protocol to induce acclimation, one that did not induce a significant HSP70 response, apoptosis changes, or heat tolerance in lymphocytes. It seems that in this context, the added stress of a mild sleep loss does not alter this response. It may be that added stressors during more strenuous acclimation protocols affects cellular responses, but that requires further study.

In conclusion, we present the perspective that during our 11-day, mildly strenuous exercise-heat acclimation, our subjects achieved classic physiological benefits of acclimation, without exhibiting HSP70 responses of lymphocytes. Although lymphocytes are exposed to hyperthermic, core temperatures, and display stress responses (Ryan et al. 1991; Sonna et al. 2002), they may not reflect induction of acclimation if acclimation is induced with more moderate daily exercise protocols. Further study is warranted to determine the specific effects of environmental temperature, humidity, exercise intensity, and other variables on hyperthermia, acclimation, and cellular protection during whole-body stress adaptation.

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