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Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans

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Yamada PM, Amorim FT, Moseley P, Robergs R, Schneider SM. Effect of heat acclimation on heat shock protein 72 and interleukin-10 in humans. *J Appl Physiol* 103: 1196–1204, 2007. First published July 5, 2007; doi:10.1152/japplphysiol.00242.2007.—Heat acclimation (HA) results in whole body adaptations that increase heat tolerance, and in addition, HA may also result in protective cellular adaptations. We hypothesized that, after HA, basal intracellular heat shock protein (HSP) 72 and extracellular IL-10 levels would increase, while extracellular HSP72 levels decrease. Ten male and two female subjects completed a 10-day exercise/HA protocol (100-min exercise bout at 56% of maximum O_2 uptake in a 42.5°C DB, 27.9% RH environment); subjects exhibited classic adaptations that accompany HA. Peripheral blood mononuclear cells (PBMCs) were isolated before and after each acclimation session on *days 1*, *6*, and *10*; plasma and serum were collected before and after exercise on the 1st and 10th day of HA. SDS-PAGE was used to determine PBMC HSP72 levels during HA, and ELISA was used to measure plasma IL-10 and serum HSP72 concentrations. The increase in PBMC HSP72 from pre- to postexercise on the 1st day of HA was not significant (mean ± SD, 1.0 ± 0 vs. 1.6 ± 0.6 density units). Preexercise HSP72 levels on *day 1* were significantly lower compared with the pre- and postexercise samples on *days 6* and *10* (mean ± SD, *day 6*: 2.1 ± 1.0 and 2.2 ± 1.0, *day 10*: 2.0 ± 1.3 and 2.2 ± 1.0 density units, respectively, $P < 0.05$). There were no differences in plasma IL-10 and serum HSP72 postexercise or after 10 days of HA. The sustained elevation of HSP72 from *days 6* to *10* may be evidence of a cellular adaptation to HA that contributes to improved heat tolerance and reduced heat illness risk.

exercise; peripheral blood mononuclear cell; serum; plasma; in vivo

INTRACELLULAR (IC) HEAT SHOCK protein (HSP) 72 are highly conserved proteins, which are involved in maintaining cellular protein conformation and homeostasis during stress (33). Examples of such stressors are hyperthermia, hypoxia, hyperoxia, cytokines, inflammation, and injury, increases in metabolism and IC calcium, and a decrease in glycogen and ATP (13), acidosis, stress hormones, and ischemia-reperfusion (32). Extracellular (EC) HSP72 is thought to stimulate innate immunity (31), act as a danger signal resulting in increased immune responses, and facilitate host defense to pathogenic challenges (15). If a pathogenic challenge does not occur, then EC HSP72 will have little impact on innate immune cell production of proinflammatory cytokines. However, if a pathogenic challenge ensues, then EC HSP72 could help to eliminate bacterial pathogens (15).

Studies have shown that marathon runners can tolerate core body temperatures (T_c) as high as 41.5°C without exhibiting clinical symptoms of heat illness (3, 20, 29). These observations challenge current guidelines, which state that heatstroke will typically occur as T_c approaches 40.6–41.3°C (39). Furthermore, runners who have collapsed during a triathlon were not more hyperthermic than other noncollapsed runners (34), so it appears that some athletes are more "protected" from heat illness. Some athletes may acquire heat acclimatization and cellular preconditioning during training, enabling them to withstand a severe, subsequent stress. IC HSP72 may be involved in improving cellular and animal thermotolerance. Dokladny et al. (7) showed that HSP72 expression plays a role in protecting the intestinal epithelial tight junction barrier in Caco-2 cells when exposed to modest increases in temperature (37–41°C). The preservation of tight junctions prevents large amounts of endotoxin release into the portal vein, activation of macrophages, and the subsequent proinflammatory cytokine cascade (24, 27). In extreme cases, this inflammation can lead to sepsis, suggesting that proinflammatory cytokines play a role in heatstroke pathophysiology. Therefore, we sought to determine whether heat acclimation (HA) could be used to "precondition" humans and improve thermotolerance as manifest by an increase in HSP72 and a decrease in proinflammatory cytokine response. Since plasma TNF-α levels were undetectable across HA (data not shown), it was unrealistic to determine whether TNF-α decreased during HA and therefore, IL-10, an anti-inflammatory cytokine was measured. Our first purpose was to assess the changes in IC HSP72 in response to an acute bout of exercise in the heat. Since HSP72 is involved with increased cellular protection and tolerance, we sampled peripheral blood mononuclear cells (PBMCs) because they are directly related to immunity. We hypothesized that IC HSP72 would increase from pre- to postexercise because earlier studies showed that IC HSP72 increases in response to exercise (11, 40, 43). Our second purpose was to determine whether resting IC HSP72 levels changed across 10 days of HA. We hypothesized that resting levels would progressively increase throughout HA, as previously shown (31a). Our third purpose was to determine whether basal serum (EC) HSP72 would decrease from pre- to post-HA. Since Kresfelder et al. (23) showed that basal serum HSP72 levels decreased in 14 subjects who successfully completed 5 days of HA, we hypothesized that basal serum HSP72 levels would decrease after 10 days of HA. We also hypothesized that serum HSP72 will increase from pre- to postexercise (after an acute exercise bout), as other studies have shown (9, 12, 30). In vitro experiments show that IC HSP72 suppresses mRNA transcription of proinflam-

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matory cytokines IL-1 and TNF-α (22). Since intense endurance exercise suppresses T-helper-1 immunity (41) and the production of anti-inflammatory cytokines balances proinflammatory cytokines (8), we postulated that the anti-inflammatory cytokines (i.e., IL-4, IL-10, IL-1ra) would be upregulated. Therefore, to test this hypothesis, our fourth purpose was to determine plasma IL-10 levels from pre- to postexercise on the 1st and 10th day of HA. We hypothesized that IL-10 would be upregulated from pre- to postexercise and from the 1st to 10th day of HA.

MATERIALS AND METHODS

Twelve (10 men, 2 women) healthy, nonsmoking subjects [mean ± SD, age: 24 ± 4 yr, maximum O_2 uptake ($\dot{V}O_{2\,max}$): 54.5 ± 9.1 $ml \cdot kg^{-1} \cdot min^{-1}$, body fat: 13.6 ± 7.6%] participated in this study. Before testing, the study was approved by the Institutional Review Board. All testing took place at an altitude of 1,572 m (barometric pressure = 635 mmHg) and during February through April, when the average maximal outdoor temperature in April was 23.3°C (74°F). All subjects had less than two positive cardiovascular risk factors, as outlined by the American College of Sports Medicine (1). Subjects were excluded from the study if they had a history of heat illness or gastrointestinal ulcers, known diseases or viral infections, or were taking vitamin supplements or other medications that would affect HSP72, exercise, or thermoregulatory responses. They were also excluded if they traveled to a warm climate or had visited a sauna or hot tub the month before testing.

Baseline measurements. After the initial screening, body density was determined using the sum of three sites and the Jackson and Pollack equation (1); ethnic-specific equations were used to calculate percentage of body fat from body density (1). All subjects completed a graded treadmill $\dot{V}O_{2\,max}$ test in a thermoneutral environment. The $\dot{V}O_{2\,max}$ test consisted of 1-min stages with incremental increases in speed and grade, and it was terminated at volitional fatigue. $\dot{V}O_{2\,max}$ was defined as the highest 30-s average, and the maximal heart rate (HR_{max}) attained was recorded using a telemetric heart rate (HR) transmitter strap and watch (S810i series, Polar). Breath-by-breath O_2 uptake was measured with a fast-response turbine flow transducer (K. L. Engineering model S-430, Van Nuys, CA) and custom-developed software (LabVIEW, National Instruments, Austin, TX) with O_2 and CO_2 electronic gas analyzers (AEI Technologies, model S-3A and model CD-3H, Pittsburgh, PA).

HA protocol. No sooner than 2 days after the $\dot{V}O_{2\,max}$ test, subjects completed a 10-day HA protocol (HAP), where they exercised in the heat during each session. Subjects were instructed to eat a high-carbohydrate meal (they were given a list of foods high in carbohydrate). They were instructed to avoid exercise, caffeine, alcohol, and diuretics 24 h before testing. All trials were completed at the same time of day and within 14 days. Each night before the HA session, subjects were instructed to swallow a telemetric temperature sensor capsule (Jonah Ingestible Core Temperature Capsule, Mini Mitter, Bend, OR) that was used to measure T_c. Ingesting the capsule the previous night ensured that it would be emptied from the stomach and be unaffected by water ingestion during the HAP. Before ingestion, each capsule was calibrated against a Fisherbrand factory-calibrated thermometer (National Institute of Standards and Technology traceable, Fisher Scientific, 15-041A).

In the morning of the session, subjects were instructed to drink 500 ml of water before reporting to the laboratory. Subjects were asked to collect a urine sample and if a subject was deemed dehydrated (specific gravity >1.030, or color >6) (2), they ingested an additional 200 ml of water. Baseline clothed and nude body weights were measured, and subjects dressed in a t-shirt, shorts, socks, and athletic shoes.

Blood samples were obtained on *days 1*, *2*, *4*, *6*, *8*, and *10* through venipuncture of the antecubital vein. Subjects sat for 20 min before the initial blood draw, and during this period they were instrumented with a HR transmitter strap (S810i series, Polar); skin thermistors (Grant, UK, EUS-U-VL5-0) attached to the right side of the body: chest (~5 in. below midclavicle), lateral arm (midpoint between olecranon and acromion processes), lateral thigh (6 in. above the superior patella border), and lateral calf (midpoint between the mallelous and the bottom border of patella). The signal from the temperature capsule was detected with an external data logger (VitalSense, Mini Mitter), and baseline T_c was recorded. A baseline blood sample was drawn in thermoneutral conditions: 5 ml of blood were allowed to clot to determine serum osmolality and serum HSP72; 1 ml of heparinized blood was aliquoted for the measurement of hemoglobin (Hb) and hematocrit (Hct); on *days 1*, *6*, and *10*, an additional 12 ml of heparinized blood were collected for the separation of PBMC. Postexercise, another blood sample was drawn from the antecubital vein and aliquoted as outlined above. Figure 1 outlines the procedures during a HA session.

Subjects then entered the environmental chamber (mean ± SD, 42.5 ± 0.1°C DB, 25.9 ± 0.4°C WB, 30.6 ± 0.3°C WBGT, 41.9 ± 0.6°C globe, 27.9% RH, 0.3 m/s wind speed) and warmed up by walking for 5 min at 3.5 mph, 0% grade, and then exercised at 56% of their $\dot{V}O_{2\,max}$. Depending on their fitness level, subjects either walked or ran for 100 min (two 50-min work bouts), separated by 15 min of rest. Treadmill speed and grade ranged from 3.8 to 6.3 mph and 0 to 4%, respectively. Termination criteria included the following: completion of the protocol, a T_c of 39°C, 95% of HR_{max}, the subject's request to stop, or symptoms of exertional heat illness. During the protocol, subjects were allowed to drink water ad libitum.

During the test, a data logger (Squirrel 2020 series, Grant Instruments, Cambridgeshire, UK) recorded skin temperatures at 15-s intervals. T_c was monitored and recorded by the external data logger (VitalSense, Mini Mitter). HR was recorded every 5 s by the HR watch. Mean skin temperature (MST) was calculated with the following formula: $MST = 0.3 * (T_{chest} + T_{arm}) + 0.2 * (T_{calf} + T_{thigh})$, where T_{chest}, T_{arm}, T_{calf}, and T_{thigh} are skin temperatures at the specified site (37).

Hb, Hct, serum osmolality, and cell count. Heparinized blood was used to measure Hct in triplicate and Hb in duplicate. Microcapillary tubes were filled with heparinized blood and spun in a centrifuge for 5 min (Damon/IEC Division), and then Hct was immediately recorded as a percentage using a Hct reader. For the measurement of Hb, 20 μl of whole blood were added to 5 ml of Drabkins solution, and absorbance was read at 540 nm (Thermospectronic 401, Milton Roy, Louisville, CO). Whole blood was allowed to clot, and the serum was removed. Serum osmolality was measured using freezing point depression in duplicate (model 3D3, Advanced Instruments, Norwood, MA). Plasma volume (PV) expansion from the 1st to 10th day of HA was calculated from preexercise Hct and Hb measurements (6).

PBMC HSP72. PBMC were isolated on *days 1*, *6*, and *10* (Histopaque 1077, Sigma-Aldrich, St. Louis, MO). The cell pellet was

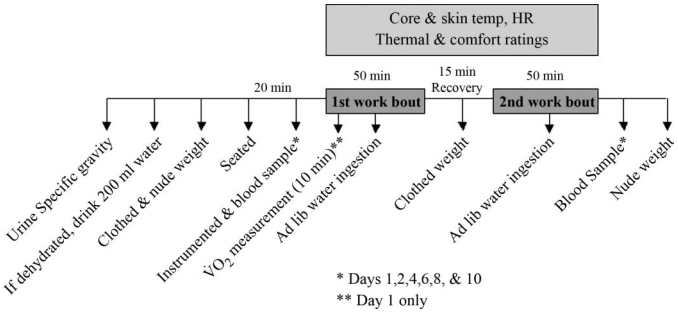

Fig. 1. Procedures for each heat acclimation (HA) session. $\dot{V}O_2$, O_2 uptake; HR, heart rate.

resuspended in warmed RMPI-1640 (Roswell Park Memorial Institute, 37°C) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, Carlsbad, CA) and 50-μl penicillin streptomycin (100 U/ml, GIBCO, Invitrogen). The cells were washed three times and during the second wash, cells were counted on a hemacytometer using 0.4% Trypan blue (Sigma); viability was expressed as a percentage (97%). The pellet was stored at −20°C.

The pellet was homogenized, and the supernatant was used in SDS-PAGE. Total protein concentration of the supernatant was measured using the Bradford assay. Cell lysates were separated on a 12% polyacrylamide gel by electrophoresis. Care was taken to load 14 μg of total protein per lane. Five microliters of a protein standard were used to identify HSP72 (Precision Plus Protein Standard, BioRad, Hercules, CA). Protein was transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, catalog no. RPN303E), and then nonspecific binding sites were blocked with 8% milk. To detect HSP72, the primary antibody (mouse anti-HSP70 SPA810, Stressgen, Assay Designs, Ann Arbor, MI) was applied, and the appropriate secondary antibody was applied (horseradish peroxidase rabbit anti-mouse IgG_1, Invitrogen). The proteins were developed using a chemiluminescent reagent specific for horseradish peroxidase-conjugated secondary antibodies (Western blotting luminol reagent, Santa Cruz Biotechnology). The blot was scanned into the computer and printed (GeneSnap version 6.05, SynGene, Beacon House, Cambridge, UK). The bands were then scanned (HP Precision Scan Pro 2.5) into Adobe Photoshop (version 7.0), and luminosity was measured and then multiplied by the number of pixels (intensity). Ratios were used to express differences in bands, with the first band being the control, or 1. The intensities for subsequent bands were divided by the intensity of the first sample (preexercise, *day 1* control), and expressed as a ratio.

Serum HSP72 and plasma IL-10. A commercially available ELISA (EKS-700A, Assay Designs, Ann Arbor, MI) was used to determine serum HSP72 on the 1st and 10th day of HA. Serum dilutions of 1:2 and a standard curve ranging from 0.78 to 12.5 ng/ml ($r^2 = 0.999$) were used to determine HSP72 concentration. The ELISA sensitivity is reported to be 0.2 ng/ml for HSP72, and intra-assay coefficient of variance was 4.7%. A commercially available ELISA with a sensitivity of 4 pg/ml was used to determine plasma IL-10 concentrations, and intra-assay coefficient of variance was 4.6%. A 1:1 plasma dilution and a standard curve ranging from 2 to 125 pg/ml ($r^2 = 0.993$) were used to determine plasma IL-10 concentration on the 1st and 10th day of HA (BD OptEIA no. 555157, BD Biosciences, San Diego, CA). All samples were analyzed in duplicate, and absorbance was read at 450 nm (Molecular Devices, Versamax Tunable Microplate Reader, Sunnyvale, CA). Preexercise plasma IL-10 and serum HSP72 values on *day 10* were corrected for changes in PV. Data are presented in the uncorrected and corrected form when applicable.

Lactate dehydrogenase. Lactate dehydrogenase (LDH) was measured (CytoTox96 Non-radioactive Cytotoxicity Assay, Promega, Madison, WI) in serum samples obtained preexercise on *days 1*, *2*, and *10* of HA. The assay was conducted as specified by manufacturer's guidelines. The absorbance on *day 1* was set as the control, or 1, and the two remaining samples were expressed as a ratio of the control.

Statistical analyses. Dependent *t*-tests (Statisitica, version 5, Tulsa, OK) were used to determine whether significant differences existed between *days 1* and *10* of HA; dependent variables were duration, peak HR expressed relative to HR_{max}, peak T_c, and peak MST. These methods were used to confirm that subjects were heat acclimated.

One-way repeated-measures ANOVA were used to determine the following: *1*) the change in pre- to postexercise PBMC HSP72 on *days 1*, *6*, and *10*; *2*) the change in basal PBMC HSP72 levels on *days 1*, *6*, and *10* of HA; *3*) changes in plasma IL-10 and serum HSP72 pre- to postexercise on the 1st and 10th days of HA; and *4*) changes in LDH during HA. If there was a significant main effect, Tukey's post hoc test was used. If data were missing, Tukey's honestly significant difference post hoc test for unequal *N* was used. Based on a priori power analyses and similar studies, seven subjects would result in a 99% probability of detecting a difference in PBMC HSP72 (40), incorporating eight subjects would result in a 93% probability of detecting differences in serum HSP72 (42), and 10 subjects would provide sufficient power (100%) to detect a difference in plasma IL-10 levels (35). Dependent *t*-tests were used to compare preexercise serum HSP72 and plasma IL-10 levels on *days 1* and *10*. Lastly, correlation analyses were used to determine the relationship between serum and PBMC HSP72 pre- and postexercise on the 1st and 10th day of HA for each subject (Graphpad Prism V.4, San Diego, CA). Figures and data represent means ± SD; statistical significance was set at $P < 0.05$.

RESULTS

This study initially involved 12 subjects; however, *subject 3* was unable to complete the study due to hip pain. Subject characteristics are outlined in Table 1.

HA. Subjects were heat acclimated, as they were able to exercise longer with lower T_c and HRs on *day 10* compared with *day 1* (see Fig. 2). Furthermore, subjects exhibited a PV expansion of 9.25 ± 3.25%. The PV expansion in the present study was mirrored by a significant decrease in preexercise Hct and Hb concentration from *days 1* to *10* (47.6 ± 2.6 vs. 46.1 ± 2.5%, 15.3 ± 1.0 vs. 14.4 ± 0.9 g/dl, respectively, $P < 0.05$). Subjects were hydrated before each HA session, as urine color and specific gravity were 2 ± 0 and 1.012 ± 0.002, respectively.

PBMC HSP72. SDS-PAGE was used to detect PBMC HSP72, but due to technical difficulties, we only had samples for eight subjects. Figure 3 outlines the individual data and mean changes in PBMC HSP72 during HA. *Subjects 10* (preexercise, *day 10*) and *6* (*day 6*) did not have sufficient sample, and therefore data are not shown. The Western blot did not detect any HSP72 preexercise on *day 1* for *subject 12*. Therefore, the group's mean ratio of pre- to postexercise on *day 1* was applied for this subject, and the theoretical change was used in the ratio calculations.

A significant main effect for HSP72 during HA [$F(5,25) = 4.78$, $P = 0.003$] was found. Tukey's post hoc test (for unequal *N*) revealed that basal HSP72 increased from *days 1* to *6* ($P = 0.009$) and from *days 1* to *10* ($P = 0.043$); there were no differences between basal levels on *days 6* and *10* ($P = 0.343$, Fig. 3*A*). Figure 3 illustrates the blot from *subject 8*, which represents the sample's mean HSP72 response to HA. To summarize, PBMC HSP72 significantly increased from preexercise on *day 1* to preexercise on the 6th day and remained elevated thereafter. HSP72 plateaued from preexercise on *day 6* and did not increase from pre- to postexercise on *day 6* or *10*.

EC HSP72, IL-10, and LDH. Plasma IL-10 was measured in 10 subjects because one plasma sample was missing for *subject 10*. Figures 4 and 5 show that there were no significant differences among serum HSP72 and IL-10 means from pre- to

Table 1. *Subject characteristics*

	Age, yr	$\dot{V}O_{2max}$, ml·kg^{-1}·min^{-1}	Weight, kg	Height, cm	Body Fat, %
Mean	24	54.5	74.1	177.3	13.6
SD	4	9.1	6.1	5.5	7.6

$\dot{V}O_{2max}$, maximum O_2 uptake.

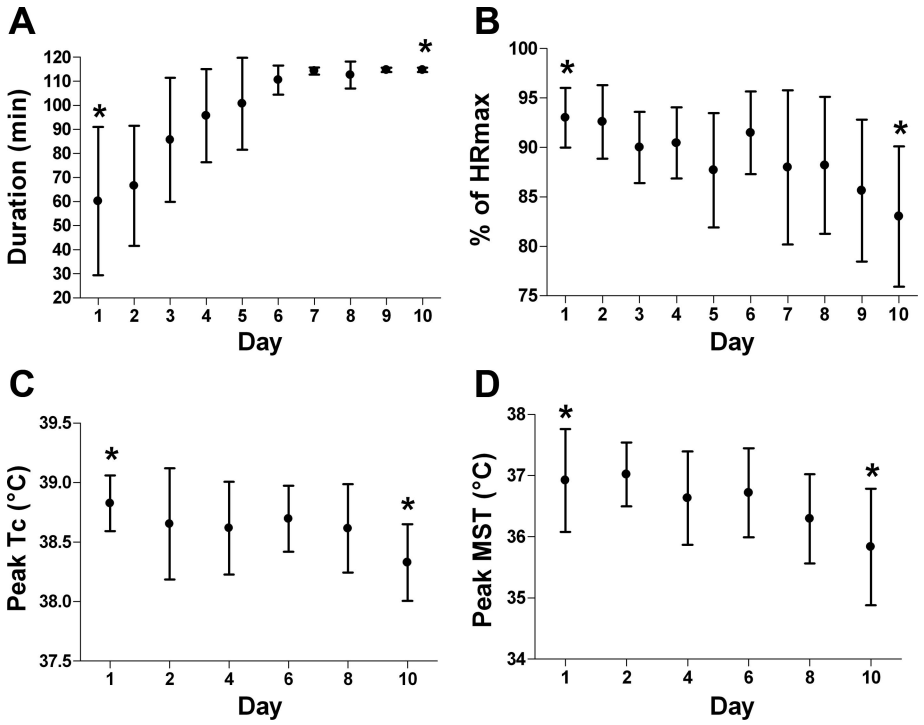

Fig. 2. Physiological measurements during HA. *A*: mean duration; *B*: peak HR expressed as a percentage of maximal HR (HR_{max}); *C*: peak core body temperature (T_c); *D*: peak mean skin temperature (MST). *Significant change between the 1st and 10th day of HA, $P < 0.01$.

postexercise on *days 1* and *10*, even when adjusted for PV expansion. Figure 5, *C* and *D*, illustrates uncorrected and corrected individual serum HSP72 data, respectively.

Correlations were determined for seven subjects, as serum HSP72 was below detection limits for *subject 8*. The Pearson's *r* was 0.64 ± 0.12 and 0.56 ± 0.39 when uncorrected and corrected for changes in PV, respectively (Figs. 6 and 7). Pearson's *r* was >0.95 for *subjects 2* and *12*. There were no mean differences in serum LDH among *days 1*, *2*, and *10* of HA (1 ± 0, 0.97 ± 0.08, 1.00 ± 0.10, respectively).

DISCUSSION

The major finding of this study was that IC HSP72 increased within the first 6 days of HA. The elevated IC HSP72 may confer protection from heat illness by maintaining cellular protein homeostasis (33), or reducing the risk of endotoxemia by preserving epithelial tight junctions of the gut (7). We did not see changes in EC HSP72 or EC IL-10 during HA.

IC HSP72. Since subjects did not perform a separate exercise bout in thermoneutral conditions, it is not possible to separate the effects of heat and exercise on HSP72; only the combined effects of exercise in the heat on HSP72 will be discussed below. Although not significant, there was a tendency ($P = 0.34$) for HSP72 to increase from pre- to postexercise on the 1st day of HA. On average, the exercise duration on the 1st day of HA was 59.3 min. This is in accordance with Shin et al. (40), who showed that leukocyte HSP72 increased in 10 trained and 10 sedentary subjects immediately post- and 30-min postexercise (trained: 127 and 141%; sedentary: 127 and 139% from baseline, respectively). The authors reported that the subjects ran at 70% of HR reserve for 1 h (24°C, 60% relative humidity). The present study used a lower exercise intensity and a higher ambient temperature, while the Shin et al. (40) study used a higher exercise intensity and lower ambient temperature. Although the two studies used different exercise conditions, both provided the necessary stimulus to elicit the HSP72 response, with the present study showing a larger increase.

As subjects became heat acclimated, they exhibited PV expansion and a decrease in peak HR, T_c, and MST, decreasing

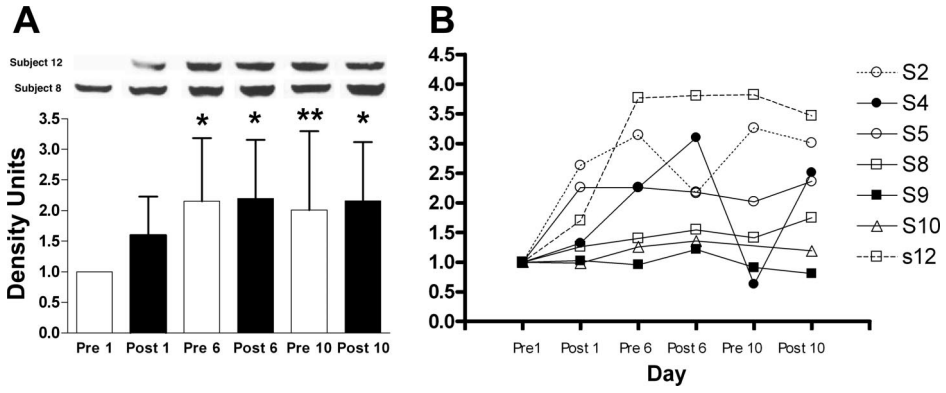

Fig. 3. Peripheral blood mononuclear cell (PBMC) heat shock protein (HSP) 72 during HA ($n = 7$). *A*: pre- and postexercise PBMC HSP72 on *days 1*, *6*, and *10* of HA. Open bars, preexercise; solid bars, postexercise. Ratios are presented in relation to preexercise *day 1*. *B*: individual subject (S) data. Legend identifies subjects. Significant increase compared with preexercise *day 1*: *$P < 0.01$, **$P < 0.05$.

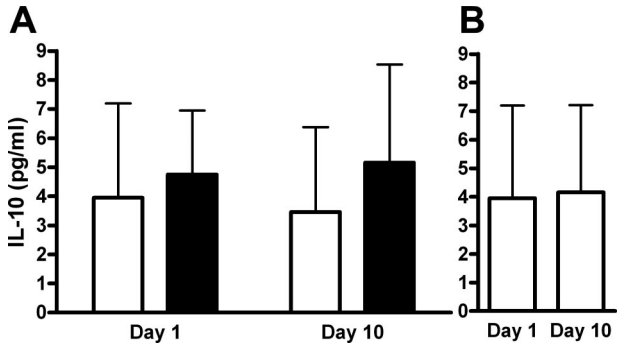

Fig. 4. IL-10 during HA (n = 10). *A*: data uncorrected; no significant differences (ANOVA). *B*: preexercise *day 10* values are corrected for changes in plasma volume (PV); no significant differences (*t*-test). Open bars, preexercise; solid bars, postexercise.

the relative stress over HA. Despite this decrease in relative stress, the level of PBMC HSP72 remained elevated in the group. Furthermore, PBMC HSP72 remained elevated, despite the fact that *1*) exercise durations were not the same for each subject because exercise was terminated when HR or T_c reached critical levels; *2*) not all subjects terminated exercise due to a critical T_c of 39°C, thus levels of "peak heat stress" were different; *3*) the decrease in relative stress during HA probably differed among subjects, causing subjects to receive different levels of stress; *4*) the physiological and psychological response to exercise and heat stress probably differed among subjects; and *5*) although all subjects exercised at 56% of $\dot{V}O_{2\,max}$, some subjects ran while others walked. This is a very interesting observation, as HA is capable of increasing and maintaining PBMC HSP72 levels throughout the entire HAP, despite varying levels of exercise and heat stress among subjects. Since the protocol required subjects to complete 10 days of HA within a 14-day period, each subject had different schedules, which consisted of different consecutive days. Interestingly, *subjects 2*, *4*, *5*, and *12* had at least 48 h of rest before the 6th day of HA. The results show that the HSP72 response was elevated (or maintained), even in the absence of exercise the previous day. These results are consistent with Fehrenbach et al. (14), who found that PBMC HSP72 levels remained elevated 24 h after a 2.5- to 3.5-h marathon in 12 well-trained male athletes.

As HA decreases the set point for onset of sweating, HA may also decrease the stress threshold necessary for HSP72 upregulation, providing enhanced cellular protection. Maloyan et al. (28) reported that HA rats exhibited a shorter time to peak HSP72 mRNA levels compared with control rats. They concluded that HA increased transcription rates in the acclimated state, with HSP72 accumulation being faster with increased levels of stress. HA may also affect posttranscriptional activities; however, this study did not investigate this potential acclimatory phenomenon.

EC HSP72. Even when data were corrected for PV expansion, there was no mean change in serum HSP72 and plasma IL-10 levels. However, four subjects exhibited an increase, while the remaining four showed no change in preexercise serum HSP72 from *day 1* to *10* when corrected for PV expansion. The significance of a postexercise increase in EC HSP72 remains unclear. Pittet et al. (36) reported that higher serum HSP72 levels were correlated with survival in trauma

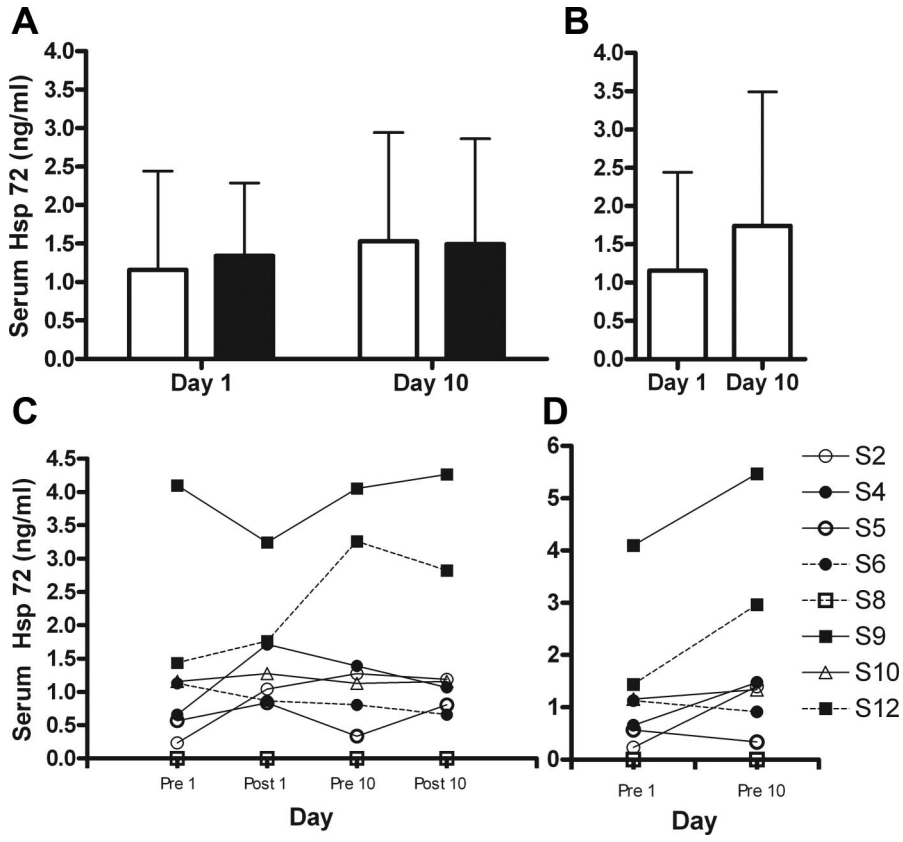

Fig. 5. Serum HSP72 during HA (n = 8). *A*: no differences among means (ANOVA). Data are uncorrected for PV changes. Open bars, preexercise; solid bars, postexercise. *B*: no difference between preexercise means (*t*-test). Data are corrected for PV changes. *C*: individual subject data. *D*: individual subject data. Preexercise *day 10* data are corrected for PV changes.

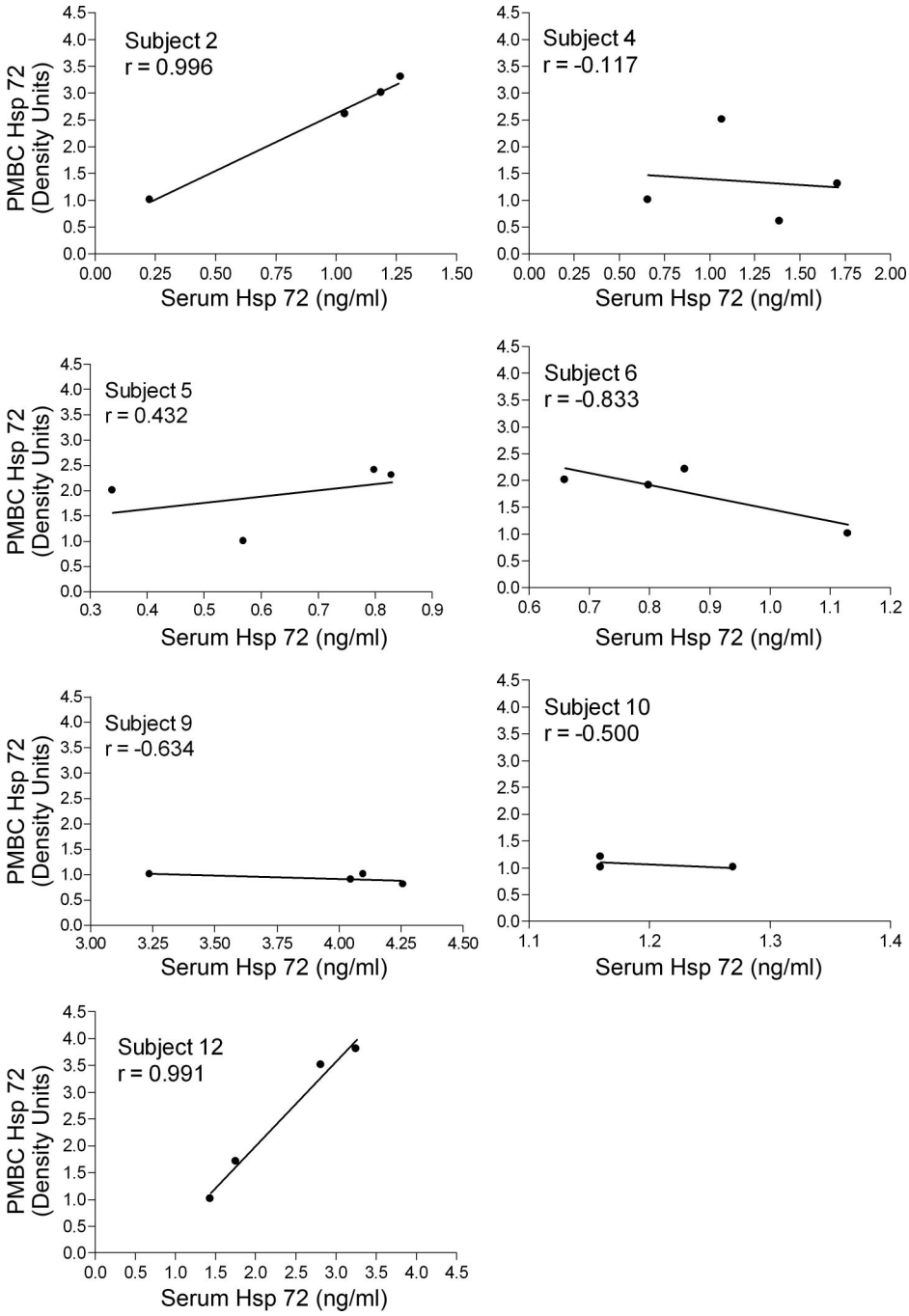

Fig. 6. Correlation between serum and PBMC HSP72 in samples obtained pre- and postexercise on *days 1* and *10* of HA. Data are uncorrected for changes in PV.

patients, whereas da Rocha et al. (5) reported that lower serum HSP72 levels correlated with survival in patients with traumatic brain injury. Increased serum HSP72 may indicate that the subject's immune system was preparing for a pathogenic challenge or initiating an immune response.

It is difficult to explain why only two of seven subjects had strong, positive relationships because little is known about the signals that stimulate the in vivo release of HSP72 into the EC space or the cells that release it (21). However, previous studies have shown in vivo HSP72 release from the human liver (10) and the human brain (26). HSP72 may also be released through exocytosis by glial cells (19), B cells (4), PBMC (25), lipid rafts (2a), or exosomes (4, 25), or released as a result of cell necrosis (17). Furthermore, there is no obvious commonalities between these two subjects, as they were different sexes, have very different fitness levels, and had different changes in T_c throughout exercise (as the female subject started with a higher T_c, resulting in a smaller change in T_c).

Since *subjects 2* and *12* had increased serum HSP72 levels during HA, but no change in serum LDH, a marker of cell damage, this suggests that EC HSP72 release did not occur through muscle damage or cellular necrosis associated with the daily exercise program. Johnson and Fleshner (21) suggest that an increase in EC HSP72 is due to sympathetic nervous system stimulation, which causes norepinephrine to bind to α_1-adrenoreceptors, causing an increase in IC calcium, which results in

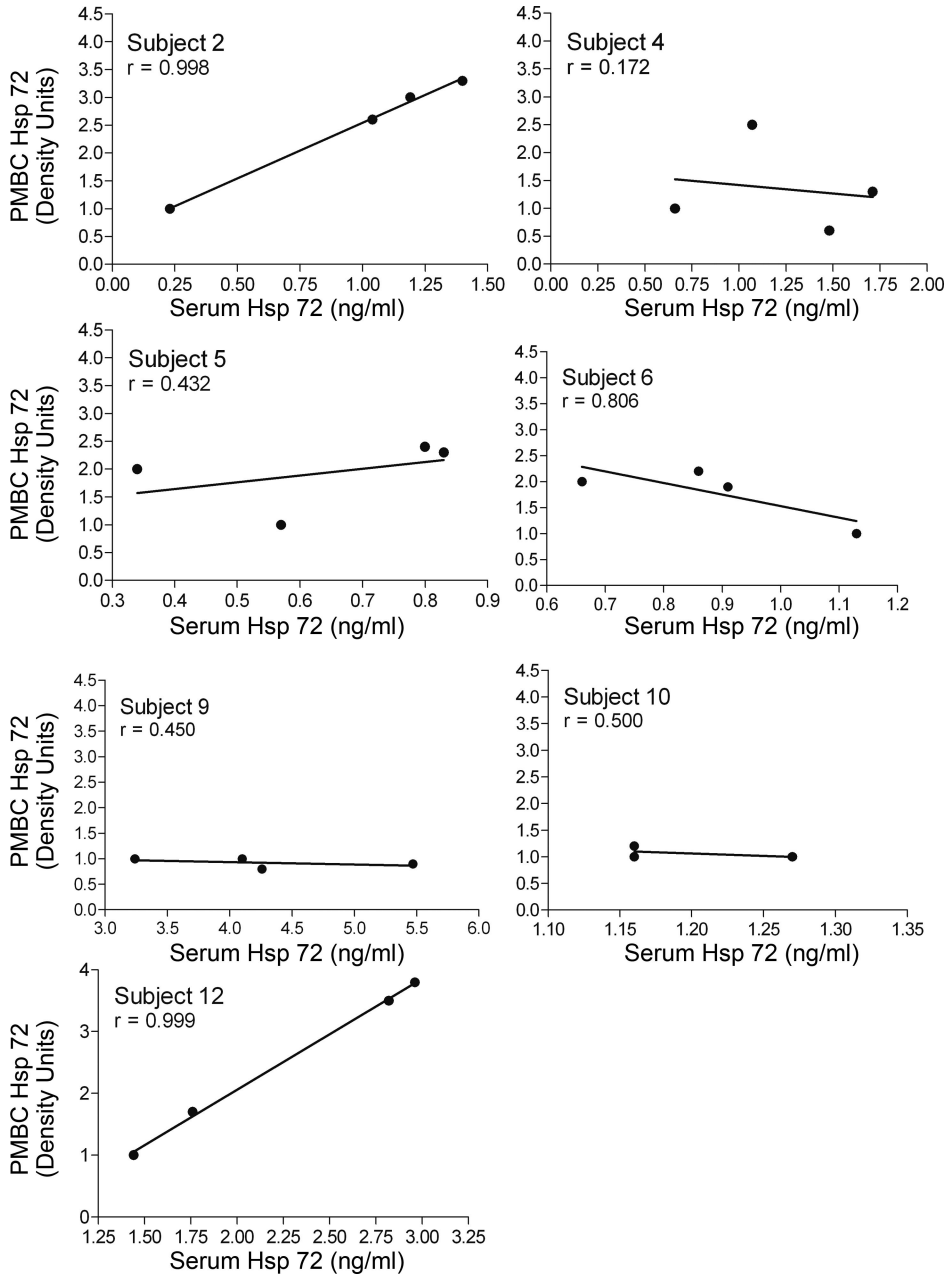

Fig. 7. Correlation between serum and PBMC HSP72 in samples obtained pre- and postexercise on *days 1* and *10* of HA. Preexercise *day 10* data are corrected for changes in PV.

the release of HSP72-filled exosomes. Thus previous studies that observed an increase in serum HSP72 postexercise may have caused sufficient skeletal muscle damage to induce necrosis and release of HSP72 into the EC space or had greater HSP72 release through exocytosis or exosomes.

Resting serum HSP72 values in the present study fall in the relatively large range (0.1–4.0 ng/ml) reported in previous studies (12, 30, 42). The results of previous studies indicate that serum HSP72 levels usually increase after exercise (12, 30, 42); however, we did not show this response. Specifically, Marshall et al. (30) demonstrated an increase in serum HSP72 levels from pre- to postexercise in subjects who cycled on 2 consecutive days and a decrease in basal levels of serum HSP72. Also, Kresfelder et al. (23) showed that basal serum HSP72 levels decreased after 5 days of HA. The reason for this discrepancy is unclear. Since the exercise protocols used in previous studies (12, 30) were similar in intensity as that in the present study, the exercise protocol does not explain the discrepancy. The insensitivity of ELISA may account for the discrepancy. The assay sensitivity is 0.2 ng/ml, which shows that resting values of 0.1 ng/ml are theoretical. Furthermore, postexercise values of previous research fall on the lower 1/10th of the standard curve (12, 30, 42), making the detection of small changes in serum HSP72 values difficult.

Plasma IL-10. The lack of change in IL-10 levels suggests that the HAP did not provide sufficient stress, because previous studies that showed postexercise increases in IL-10 incorporated higher intensities [i.e., running at 85% of $\dot{V}O_{2\,max}$ (35), a long distance triathlon, and a 100-km run (18)]. If the HAP provided greater exercise stress, or cells were stressed in vitro with endotoxins, different IL-10 responses may have been observed. Alternatively, plasma may also have been sampled

too soon, as cytokine levels have been shown to peak 30 min postexercise (38).

Conclusions. In conclusion, this is a unique study, as it is the first to measure PBMC HSP72 during 10 days of HA. Physiological adaptations of HA traditionally include whole body adaptations. This study shows that PBMC HSP72 increases in response to HA, which may illustrate a cellular adaptation of HA in humans. It is also interesting to note that although the relative stress decreased during the 10 days of HA, HSP72 remained elevated, perhaps exhibiting a lower threshold for HSP72 production. Whether PBMC HSP72 is upregulated during HA in response to a lower "stress threshold" or whether the upregulation is due to an alteration of posttranscriptional regulation needs to be elucidated.

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